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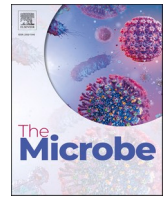
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Growth and phosphorus uptake of micropropagated southern highbush blueberry plants inoculated with ericoid endophytic and mycorrhizal fungi in varied growth substrates

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ABSTRACT

Phosphorus (P) acquisition and use by southern highbush blueberry *Vaccinium corymbosum* L. plants is critical during early stages of development and impair root development, especially for growth substrates with poor nutrient contents. However, inoculation of blueberry plants with ericoid mycorrhizal fungi (ErMF) or dark septate endophytes (DSE) can improve P during the plants acclimation stages and reduce plant mortality, especially in dry northern Mediterranean climate conditions. Herein, we grew southern highbush blueberry micro-cuttings in conditions without inoculation (control) or in inoculated with four strains: two Ericoid mycorrhizal sp. (D01), and (C01), E. endophyte (C31), and *Phialocephala fortinii* Wang & Wilcox in sandy (S), fresh field (FF), and FF+S soil mix substrates for 10 months before harvest. At harvest, root colonization levels, plant height, leaf area, the fresh matter of roots and shoots, root-to-shoot ratio, P content, and P utilization efficiency (PUE) were measured. We found that the root colonization levels were inhibited in the S for the different inoculated F treatments, owing to the elevated carbonate and salt concentrations present. The average P uptake responses from the different F inoculated strains were 52.2 %, 29.6 %, and 22.4 % in the S, FF, and FF+S substrates. Inoculation of blueberry plants with C31 strain exhibited the highest (59.1 %) P uptake average response, inoculation with *P. fortinii* strain showed the lowest (15.8 %) response. The root growth responses were inhibited in the FF+S (-0.2 %), increased in the S (8.4 %), and FF (6.2 %) substrates. Our findings therefore describe responses under controlled nursery conditions with single-strain inoculation and three substrate types. Because blueberry roots in the field are commonly co-colonized by multiple ericoid and endophytic fungi, interactions among partners may amplify or dampen the effects observed here; future work should test co-inoculation consortia and validate performance under field conditions.

1. Introduction

P is the second essential macronutrient required for plant growth and development alongside nitrogen (N) and involve in various metabolic

functions and many living cell regulatory processes are P-dependent (Bechtaoui et al., 2021). For a vegetative propagating plant such as Highbush blueberry, P deficiency impair initiation of lateral adventitious roots formation and critical physiological functions, water and

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nutrient absorption, carbohydrate storage, and subsequently induce plant mortality at their establishment stages (Wang et al., 2017; Wei et al., 2020). However, strategies to increase P access of blueberry micro propagated plants and increase their establishments during transplantation have only limited investigations, in particular for growers in Northern Mediterranean soil conditions of Morocco (Hamim et al., 2020).

Highbush blueberry cultivation is gaining interest among the edible fruits in the Mediterranean regions. Blueberries are known by so many health benefits, such as the high-water soluble antioxidants content, necessary to reduce the risk of cardiovascular disease attacks (Kalt et al., 2020) and low saturated fat, cholesterol with high sodium contents which protect against obesity (Varo et al., 2021). Blueberry production contributes to the regional economy that is favored by a pressing growing global demand consumption and the proximity of market of the North African Mediterranean regions with many European consumers (Fang et al., 2020; Hamim et al., 2017; Mazzoni et al., 2020). Over the last two-decade, global production of highbush blueberry has steadily increased, reaching over 164 % of expanded arable lands for cultivation and over 60 % of fresh fruit consumption (DeVetter et al., 2015; Fang et al., 2020). The blueberries industry is a rapid burgeoning economic sector in many regions of northern Mediterranean Africa, with a steady increase in fruit production (FAOSTAT, 2021). However, the actual fresh fruit yield production remains low compared to many other world regions, only at 45 tons/hectare below 67 tons/hectare observed in US production systems (Fang et al., 2020; FAOSTAT, 2021).

Blueberry roots associate with diverse beneficial microbes to promote their uptake of nutrients in environmental conditions. The root association with ErMF and DSE with blueberry represent one of the well-studied beneficial plant-microbe interactions (Leopold, 2016; Lukešová et al., 2015; Vohník et al., 2005; Vohník and Albrechtová, 2011). Enhanced root colonization by the ErM and DSE fungi improve plants' access to soluble inorganic or organic N and P and promotes growth (Wei et al., 2022). However, root colonization has only been sporadic and low in nursery conditions (Lukešová et al., 2015; Wei et al., 2020). Several biotic and abiotic factors impair the colonization levels in the growing conditions, method of plant propagation, species or cultivars, low availability of fungal propagules, poor compatibility with hosts, the inherent substrate composition and the nursery practices that can also affect root colonization by endophytic and mycorrhizal fungi (Gomes, 2011; Wei et al., 2020).

There are well-documented studies on the beneficial effects of ErM and DSE fungi on the growth and uptake of highbush blueberry (Li et al., 2021), but the interaction between the inoculated fungi and the substrate growth has been poorly studied (Markovic et al., 2020; Ochmian et al., 2019; Pascual et al., 2018; Sales et al., 2020). A low-quality substrate because of its very low organic matter content (< 0.3 %), minimal nutrient reserves, and limited water-holding capacity, it can inhibit root colonization and reduces the benefit from the inoculated microbes. On the other hand, a suitable substrate is critical to achieving a high fruit production in nursery (Paal et al., 2011; Sales et al., 2020). In a recent study, the use of sand amended soils with biochar enhanced plant growth and mycorrhizal root colonization levels of blueberry plants (Sales et al., 2020), owing to better protection from predation and drying of inoculated microbes. Peat is the popular growth substrate to grow micro-cuttings of blueberry plant because of their chemical and physical properties. However, the sources, the increasing environmental concerns, and extra costs of importation to Morocco could make them not always become profitable (Hamim et al., 2020; Pascual et al., 2018). Alternatives to peat substrate such as compost, fertile fresh field, sand soils, or their mixtures or amended with biochar can be used (ARAÚJO et al., 2019; de Sousa Antunes et al., 2021). Furthermore, the incorporation of plant growth-promoting rhizobacteria or biological control agents can induce advantages. Previous studies demonstrated a better and quicker nutrient solubilization, suppression of diseases, and enhancement of nutrient availability due to incorporated microbes

(Narisawa, 2017; Newsham, 2011; Wei et al., 2020). The stimulation of plant hormonal functions at the primary stages of root initiation and development is an added advantage of microbial incorporation (Wang et al., 2017). However, there are minimal investigations about the interactions between the substrate composition and the inoculated microbial endophytes fungi for root colonization, growth, and uptake of nutrients in blueberry plants in Moroccan growing conditions (Hamim et al., 2020, 2017).

ErMF form mutualistic associations with the hair roots of Ericaceae plants, enhancing nutrient acquisition, particularly under nutrient-poor and acidic conditions (Vohník, 2020). In some cases, they may also display parasitic traits, forming intracellular coils within rhizodermal and occasionally outer cortex cells (Leopold, 2016). Increasing evidence indicates that these fungi improve root development and growth by altering their physiology and rapid access to nutrients within and out of rhizosphere (Fehrer et al., 2019; Leopold, 2016; Wei et al., 2020). Besides, they degrade soil organic molecules and promote a better P uptake and utilization efficiency by host plants, therefore positively influence the growth and survival of host species (Hobbie and Hobbie, 2006; Vohník et al., 2012a). Concerning the endophyte DSE fungi, they are broadly classified as conidial or sterile septate fungal endophytes and form melanized structures such as inter- and intracellular hyphae and microsclerotia in the plant roots present in the rhizosphere (He et al., 2020; Jumpponen and Trappe, 1998). Among the endophyte's fungi species, *Phialocephala fortinii* (Narisawa, 2017; Wang and Wilcox, 1985) is the most widely tested, and plant responses may vary from negative (Lukešová et al., 2015) to positive effects (Newsham, 2011; Ridout et al., 2019). In clonally propagation methods by cuttings, the highest benefits from beneficial microbiota symbionts are expected to be pronounced at the initial stage of root initiations and development (Leopold, 2016; Wei et al., 2020). Taxonomic revisions have consolidated key ericoid lineages under *Hyaloscypha* (syn. *Rhizoscyphus*/Meliomyces), refining how isolate identities are interpreted in Ericaceae symbioses (Fehrer et al., 2019).

Despite extensive research in temperate ecosystems, data remain scarce for Mediterranean soils and nursery systems, where substrate composition, temperature, and water availability differ markedly from northern environments. Moreover, low and inconsistent fungal colonization during the acclimation stage has been repeatedly reported, compromising plant establishment and growth. The influence of substrate type on fungal colonization and P-use efficiency in blueberry nurseries remains particularly underexplored. The successful acclimation of micropropagated plantlets remains a major challenge, largely because of their limited root development and nutrient uptake efficiency.

This study therefore aimed to evaluate how different ericoid and endophytic fungi interact with contrasting substrates (peat, agricultural soil, sand) to affect colonization intensity, P uptake, and (PUE) in micropropagated blueberry plants under Moroccan nursery conditions. PUE is the measure of how effectively the plant uses the phosphorus that has been absorbed to produce biomass or yield, and it can be calculated as total biomass divided by total phosphorus uptake, or grain yield divided by total phosphorus uptake. We hypothesized that (i) colonization and nutrient responses depend on the fungal isolate and substrate, and (ii) peat-based media favor both symbiosis and P efficiency compared to mineral substrates.

2. Materials and methods

2.1. Biological materials

Vigorous and uniform microcuttings of southern highbush blueberry (*Vaccinium corymbosum* L.) regenerated from mother plants that are free of ErMF colonization, through tissue culture were used in this experiment. Prior to transplanting, plantlets were maintained in-vitro until a well-developed root system was formed, ensuring uniformity at

the acclimation stage.

Young plants of the variety “Star” were obtained from the laboratory of Multibaie nursery, Angers, France. Four fungal isolates belonging to the order Helotiales were used as inocula: two ErMF (D01 and C01) originally isolated from *Woollsia pungens* (Cav.) F. Muell roots in Australia, one ericoid endophyte (C31) from *Calluna vulgaris* (L.) Hull roots collected in the United Kingdom, and *Phialocephala fortinii* from *Rhododendron fortunei* Lindl. in China. These isolates have been molecularly characterized (GenBank accessions AF072301.1, AF072296.1, AF252845.1, EU888625.1) and were obtained from previously established culture collections under collaborative agreement. The D01, C31, and *P. fortinii* strains were isolated from the roots of Scots heather (*Calluna vulgaris* (L.) Hull) plants and the C01 strain from common heather (*Erica umbellata* Loeff. ex L.) roots (Hamim et al., 2017). A modified Melin Norkrans Agar media (MMN) was used to maintain the ericoid ascomycete mycorrhizal isolates in a 9 cm diameter Erlenmeyer flask and incubated at 28°C in the dark on an orbital shaker for 2 months (Marx and Bryan, 1971).

The molecular identification of fungal isolates was confirmed through ITS sequencing and BLASTn analyses against the UNITE and NCBI databases. Sequences showed $\geq 99\%$ similarity for *Phialocephala fortinii* (EU888625.1) and 94–96 % for the other isolates (AF072301.1, AF072296.1, AF252845.1) with Helotiales taxa (Hyaloscypha, Meliniomyces, Rhizoscyphus aggregates). Because these similarities fall below the threshold for confident species-level identification, the isolates were designated Ericoid mycorrhizal sp. 1, Ericoid mycorrhizal sp. 2, and Ericoid endophyte sp., consistent with prior works (Hamim et al., 2019, 2017). Nomenclature follows recent revisions of ericoid taxa (Fehrer et al., 2019), and functional expectations were framed per Vohník (2020). Their inclusion in this work aimed to test functionally diverse and genetically distinct ericoid-associated fungi for their capacity to promote blueberry acclimation under Moroccan nursery conditions, rather than to assess the native fungal community composition.

2.1.1. Growth substrate sampling and preparation

The sampled field soil was from the Northern region of Morocco, Larache area (35°17'12. 79" N, 6°02'55. 48" W), belonging to the Atlantic pediment of western Rif (Ibrahim and Alessandro, 2016). The climate is a Mediterranean sub-humid with two main seasons. The type of soil is Haplic Arenosols and Vertisols according to USDA soil taxonomy, annual rainfall of 695.5 mm (Ibrahim and Alessandro, 2016). The chosen site was one-year-old fallow land covered with natural grasses species at the sampled sites. Five kilograms of fresh topsoil (0–20 cm) samples were collected in late spring 2018 from several delimited subplots of 8 × 4 m, bulked, and transferred to the laboratory. The air-dried soil was sieved to pass a 4-mm sieve and used for the experiment. Ten kg of fresh sand from beach area around the Larache region was collected and transferred to the laboratory for chemical analyses.

2.2. Soil analyses

The three substrates were selected to represent distinct physico-chemical environments. Peat corresponds to the standard commercial medium widely used for nursery production of blueberries in Morocco. Fresh agricultural soil reflects a transitional substrate similar to that used in acclimation or preliminary plantation stages. In contrast, the sandy substrate, although not employed in commercial cultivation, was included to create a low-organic, low-nutrient benchmark for assessing the effect of substrate fertility and texture on fungal colonization and plant nutrient responses.

The following chemical characteristics were measured: pH, electrical conductivity (EC), soil carbon content (C), available phosphate (Pi), and total N concentrations. The pH was measured in aqueous soil suspension (1:2.5, v: v) using the Corning 125 pH meter (Corning Life Sciences, Amsterdam, Netherlands) after shaking the samples for 16 h. The EC was measured in a diluted soil sample (1:2.5, v: v) using an electrical

conductivity meter (EC-3M, HM Digital, Manhattan, USA). We applied the method of Walkley-Black (Walkley and Black, 1934) using a chromic acid wet oxidation to measure the SOM concentration in the FF substrate. The available Pi was determined using the Olsen chemical extraction method (Olsen, 1954). Succinctly, 30 mL of Olsen extractant was added to 3 g air-dried soil, and the mixture (1:10, soil: solution ratio) was shaken for 5 min, filtered, and the concentration of inorganic Pi of the extract colorimetrically measured (Murphy and Riley, 1962). The N concentration was measured using the method described by Novozamsky et al. (1983). The field soil had the following chemical characteristics: pH (H₂O), 5.5 ± 0.02; EC (dS/cm), 0.2 ± 0.02; C (%), 2.8 ± 0.08 %; available Pi (mg P kg⁻¹), 30.7 ± 0.36, total N (%), 0.1 ± 0.05, and CaCO₃ (%), 0.15 ± 0.09. The chemical properties of the sand substrate were pH (H₂O), 5.3 ± 0.16; EC, 0.8 ± 0.03 dS/cm; C, 0.6 ± 0.018 %; available Pi (mg P kg⁻¹), 0.6 ± 0.004; total N (%), 0.1 ± 0.05, and CaCO₃ (%), 2.04 ± 0.20.

2.3. Experimental design

Approximately 3 kg of each substrate composition were filled into 3.5 L cuvettes and 4 replicates for each inoculation treatment and transplanted with *V. corymbosum* micro-cutting. The experimental design was a factorial arrangement with the following factors tested: (i) factor 1: growth substrate, at three (3) levels [sandy (S) soil, fresh field (FF) soil, and FF+S mix on 9/1 fresh soil and sand proportion], (ii) factor 2: Fungal (F) inoculants at five levels: control (non-inoculated), Ericoid mycorrhizal sp 1 (D01), Ericoid mycorrhizal sp 2 (C01), ericoid endophyte sp 1 (C31), and *P. fortinii* strains. One micro-cutting plant was transplanted in each cuvette and placed in the nursery with the following growing conditions: 21°C/18°C Day/night temperature, 70 ± 5 % relative humidity, and regularly watered. Plants received distilled water daily to maintain 70 % of their water holding capacity (WHC). Highbush blueberry plants were grown for ten months and harvested for plant heights, leaf area, a fresh matter of roots and shoots, tissue-P concentration, and shoot-P contents measured. One month after transplanting of blueberry, after initiation of the first lateral roots, inoculation with isolates of the fungal inoculants 1 × 10⁶ colony-forming units (CFU) mL⁻¹ was performed in the respective cuvettes. In brief, 10 mL of concentrated mycelium culture washed-off of residual nutrients was homogenized for 15 s and injected in highbush blueberry rhizosphere.

2.3.1. Measurements

2.3.1.1. Fungal root colonization assessment. The level of root colonization rate by ErMF and DSE was measured using a procedure modified by (Phillips and Hayman, 1970) and the microscopic observation was made following the study made by (Brundrett et al., 1984). Root fragments were carefully washed in tap water, cleared in 10 % (w/v) KOH at 90°C for 1 h, then neutralized with 1 % HCl for 20 min at room temperature were stained with Trypan and Methylene Blue (0.05 % each in lactoglycerol) at 90°C for 15 min, and de-stained in water overnight. Approximately 10 % of the total root system from each plant was sampled for microscopic observation, while the remaining roots were reserved for fresh- and dry-mass determination to avoid bias in biomass data. Regarding microscopic observation, root segments were mounted onto slides and the frequency and intensity of colonization by mycorrhizal fungi were observed under a microscope at 200 × magnification using an Olympus AX70 microscope (Olympus-Japan). The level of fungal colonization (FC) was determined using the following formula of (Trouvelot, 1986) (Eq. 1):

$$FC = [(mycorrhization\ index \times number\ of\ total\ root\ fragments) / number\ of\ root\ colonized\ fragments] \times 100.$$

2.3.1.2. Leaf area index, plant height, fresh matter of shoots and roots. At harvest, leaf area measurement was carried out using a leaf area meter (LI-Cor 3100 Li-Cor, Lincoln, Nebraska USA). The plant height was measured with a commercial decimeter instrument. Then, the shoot from each sample was cut at 5 cm above the cuvette, the fresh matter was measured. The root was freed of substrates, washed, and the fresh mass (FM) was taken using a standard level precision balance (Mettler-Toledo GmbH, Giessen, Germany). Fresh weight (FW) was recorded immediately after harvest for all plants to prevent desiccation and ensure comparability across treatments. After recording fresh weight, shoot samples were oven-dried at 70 °C until constant weight to determine dry matter (DM). Approximately 0.5 g of finely ground dry tissue (< 1 mm mesh) was digested in concentrated H₂SO₄ at 500 °C. P concentrations were determined colorimetrically following [Murphy and Riley \(1962\)](#). The total P accumulated in shoots was calculated by multiplying the P concentration by the corresponding shoot DM.

2.4. Calculation and statistics

Elemental concentrations in the biomass were used to calculate the contents of the P element in the shoots, and these were then used to calculate the contents of elements in the plants. Root and shoot growth and P uptake responses to fungal inoculants were calculated ([Lendenmann et al., 2011](#)). The ratio between the shoot DM and the shoot-P content was computed as the PUE in the shoot, expressed in g DM/mg P per plant ([Rose and Wissuwa, 2012](#)). The Shapiro-Wilk and Levene's tests using R software (R version 4.0.2, 2020) were used to test the data's normal distribution and homogeneity of variance before the statistical analysis. To test the main factor effects, i.e., the Fungal (F) inoculants and substrate composition and interaction on the measured parameters using a one or two-way analysis of variance (ANOVA). Following significant Fischer (*f*)-value from MANOVA, a student-Newman-Keuls significant difference test was computed to compare pair-of treatment means. Data are means ± s.e (standard error). The data of the level of root colonization was arcsine-square root transformed before running the two-way ANOVA conducted.

3. Results

3.1. Substrate composition

The pH of the (FF) substrate was slightly higher than that of the (S) substrate. C, total N, and available P contents were markedly higher in FF than in S, indicating that the fresh soil provided nutrient levels adequate for blueberry plant growth, whereas the sand substrate was nutritionally poor, particularly in P and organic matter. Conversely, the electrical conductivity (EC) and CaCO₃ concentrations were higher in S than in FF. These conditions classify the sandy substrate as low-quality and nutrient-deficient, with limited capacity to support vigorous growth or fungal colonization, while the peat-based medium maintained nutrient levels within the optimal range for blueberry cultivation ([Table 1](#)).

3.2. Root colonization, P content and PUE

The root colonization intensity was always lower in the non-inoculated than in the inoculated plants of highbush blueberry in the three tested substrates ([Table 1](#)). Furthermore, colonization intensities were significantly higher in the FF than in the S substrate in the different F inoculant treatments ([Table 1](#)). Blueberry plants inoculated with the C31 strain exhibited the highest colonization intensity in the FF substrate ([Table 1](#)). In the S substrate, the highest colonization intensity was in plants inoculated with C01 strain. The overall fungal inoculants and substrate compositions, and their interactions on the root colonization intensity were highly significant at *p* < 0.05 ([Table 1](#)).

Microscopic observations of roots inoculated with ErMF and DSE revealed dense trypan blue-stained hyphal coils within rhizodermal cells. In particular, the D01 strain formed well-developed intracellular coils in the rhizodermis of plants grown in the fresh soil (FF) substrate ([Fig. 1a](#)). A dense intracellular coil formed by hyaline-thick hyphae turning blue after staining from inoculated C01 strain, implying a typically ErMF was observed in the FF substrate ([Fig. 1b-d](#)). An intracellularly colonized root segments of highbush blueberry plants with loose to dense trypan-blue-stained coils formed in the outermost primary cells layer of the root's characteristic from C31 strain development

Table 1

Effects of ericoid mycorrhizal and non-mycorrhizal endophytic fungal inoculation on growth parameters, P content, and P use efficiency of blueberry plantlets.

	Root colonization level			P concentration		
	[%]			[%]		
Fungal strains	Sand (S) soil	Fresh field (F) soil	F +S mixture	Sand (S) soil	Fresh field (F) soil	F +S mixture
Non-inoculated	0.0 ± 0.0 cB	10.2 ± 0.5 dA	11.4 ± 0.7 cA	0.08 ± 0.004 dA	0.08 ± 0.002 cA	0.08 ± 0.004 dA
D01	28.7 ± 0.6 aC	50.7 ± 0.7 bA	44.0 ± 0.8 aB	0.08 ± 0.003 cC	0.10 ± 0.001 bA	0.09 ± 0.001 cdB
C01	29.9 ± 0.3 aC	53.9 ± 1.4 bA	37.4 ± 0.5 bB	0.10 ± 0.003 bB	0.13 ± 0.004 aA	0.11 ± 0.006 abB
C31	29.2 ± 0.4 aC	57.7 ± 1.9 aA	47.4 ± 2.3 aB	0.11 ± 0.003 aB	0.13 ± 0.003 aA	0.12 ± 0.005 aA
P. fortinii	26.8 ± 0.7 bB	32.5 ± 0.6 cA	34.6 ± 1.1 bA	0.07 ± 0.002 cB	0.10 ± 0.005 bA	0.10 ± 0.003 bcA
Source of variation	<i>F</i> value			<i>F</i> value		
Fungal (F) inoculants	604.8 (***)			39.2 (***)		
Substrate (S)	352.3 (***)			20.6 (***)		
F x S	39.2 (***)			8.3 (***)		
cv (%)	45.7			21.1		
Fungal strains	P content			P utilization efficiency		
	[mg P g ⁻¹]			[g DM mg P ⁻¹]		
Non-inoculated	1.07 ± 0.10 bA	1.22 ± 0.07 bA	1.34 ± 0.08 cA	1.27 ± 0.06 bA	1.22 ± 0.02 aA	1.20 ± 0.08 aA
D01	1.22 ± 0.10 bB	1.61 ± 0.10 bA	1.40 ± 0.11 bcAB	1.25 ± 0.05 bA	0.97 ± 0.01 bB	1.13 ± 0.02 bA
C01	1.51 ± 0.07 aB	2.11 ± 0.10 aA	1.72 ± 0.06 abAB	1.01 ± 0.03 cA	0.76 ± 0.04 cB	0.91 ± 0.05 cdA
C31	1.72 ± 0.13 aB	2.07 ± 0.02 aA	1.90 ± 0.05 aAB	0.91 ± 0.01 cA	0.77 ± 0.02 cB	0.83 d ± 0.03 dB
P. fortinii	1.07 ± 0.05 bB	1.60 ± 0.14 bA	1.56 ± 0.06 abcA	1.42 ± 0.05 aA	1.0 0 ± 0.06 bB	1.00 ± 0.03 cB
Source of variation	<i>F</i> value			<i>F</i> value		
Fungal (F) inoculants	15.8 (***)			65.0 (***)		
Substrate (S)	10.9 (***)			42.7 (***)		
F x S	2.4 (*)			4.7 (**)		
cv (%)	22.7			19.2		

Different small letters indicate significant differences among the fungal strains within each as substrate tested, respectively (Fisher's test; *p* < 0.05). Different capital letters indicate significant differences among the different substrate tested within for each fungal inoculation treatment, respectively (Fisher's test; *p* < 0.05).

p* < 0.05; *p* < 0.01; ****p* < 0.001; ns, not significant, respectively.

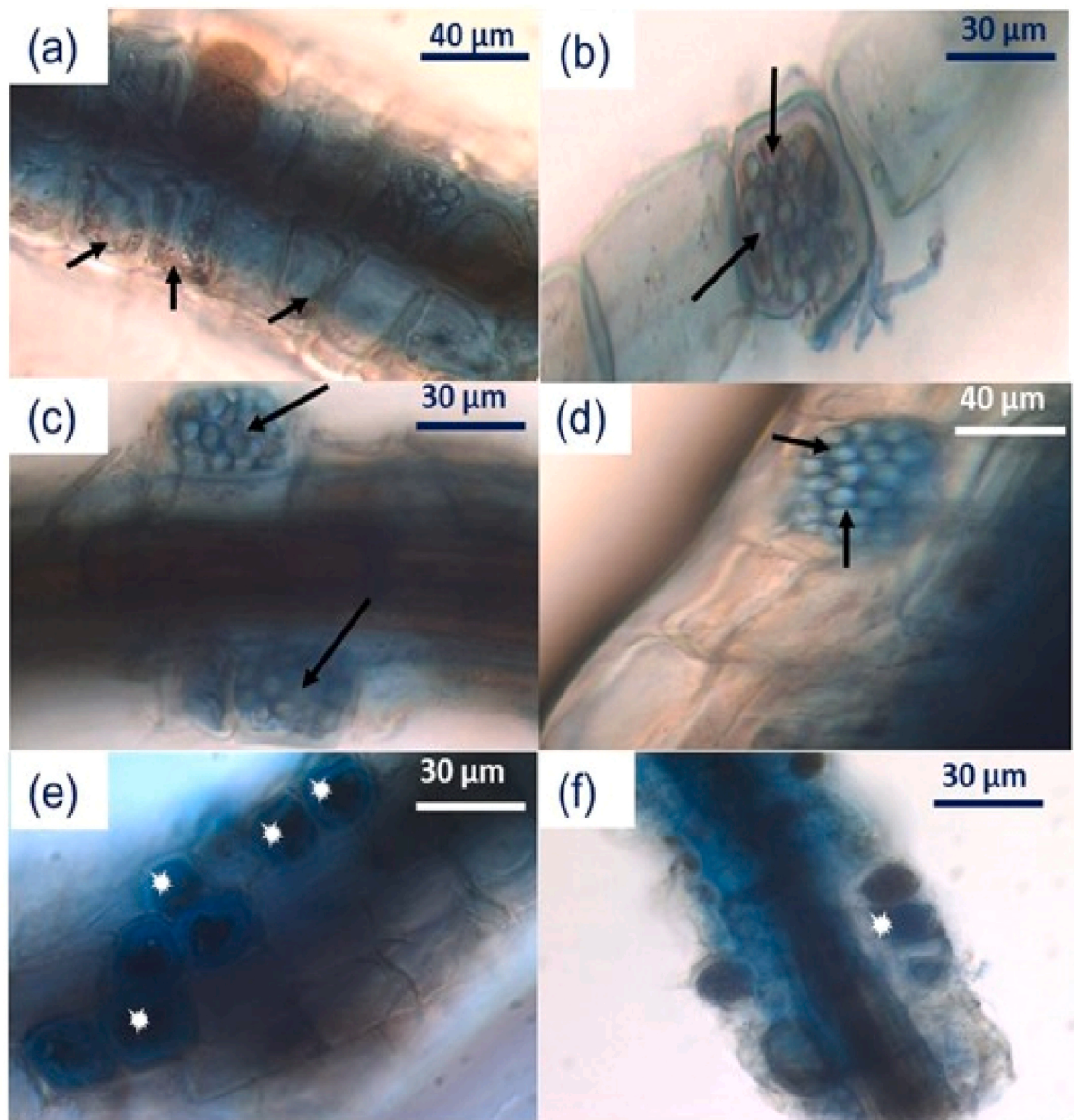


Fig. 1. Colonized rhizodermal cells from (a) Ericoid mycorrhizal sp (D01), (b, c, d) Ericoid mycorrhizal sp (C01) with dense intracellular coils, (e) Ericoid endophyte (C31) with colonized intracellularly the roots by dark septate hyphae, and from (f) *Phialocephala fortinii* strains with intracellularly microsclerotia consisting of melanized or hyaline hyphae by thick dark brown coloration of southern highbush blueberry variety “star” grown analyzed in the fresh field soil substrate. Similar but much sparser hyphae were occasionally observed in control roots; therefore, the micrographs depict characteristic colonization forms rather than confirmed presence of the inoculated isolates.

was present in the FF substrate samples (Fig. 1e). Intra-cellularly microsclerotia with dark to hyaline-thick septate hyphal to brown color inside the root hairs of highbush blueberry plants inoculated with inoculated *P. fortinii* isolate corroborated with their presences in the FF substrate (Fig. 1f).

Similarly, to root colonization intensity, the plant-P concentration, P content, and PUE significantly differed between the fungal inoculation treatments in the three used substrates, resulting in significant $F \times S$ interaction values (Table 1). In the FF+S mix substrate, the highest P concentration and P content were in plants inoculated with C31 isolates (Table 1). In the FF substrate, inoculation with C01 strain exhibited the highest P content than in the control, D01, and C31, and *P. fortinii* treatments that were also consistent with the P uptake responses (Table 1; Fig. 2a).

Irrespectively of the substrate composition, the average P uptake responses to inoculation were 17.0 %, 46.9 %, 59.1 %, and 15.8 % in

D01, C01, C31, and *P. fortinii* treatments (Fig. 2a). Average P content responses to inoculation were 29.6 %, 52.1 %, and 22.4 %, respectively in the S, FF, and FF+S mix substrate (Fig. 2a). Furthermore, the PUEs were the lowest in southern blueberry plants treated with C01 and C31 fungal isolates in the FF substrate, implying a better adjustment for root growth and development for enhanced P uptake (Table 1). The PUE of blueberry plants decreased in the different fungi inoculation treatments and growth substrates tested, except with *P. fortinii* inoculation in the sand soil growth substrate (Fig. 2b).

3.3. Fresh weights of root and shoot, and root-to-shoot ratio

We reported significant *f*-value testing of the *F* inoculants effect on the fresh root mass of blueberry plants due to the identity microbial strain tested (Table 2). The highest mass of fresh root (22.01 ± 0.81 g plant⁻¹) was reported in C01 inoculation in the FF substrate and the

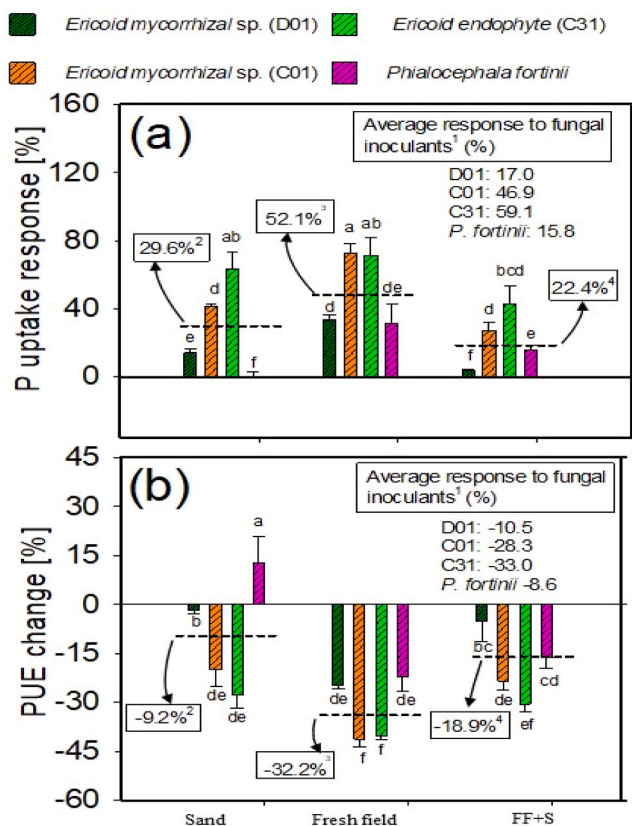


Fig. 2. P uptake (a) responses and P utilization efficiency change (b) of southern highbush plants inoculated with endophytes fungi (ErMF and DSE strains) in three different growth substrates, sand (S) soil, fresh field (FF) soils, and FF+S mix of southern. ¹: Average responses to inoculation under *Ericoid mycorrhizal* sp. (D01), *Ericoid mycorrhizal* sp. (C01), *Ericoid endophyte* (C31), and *Phialocephala fortinii*, irrespective of substrate compositions. ²: Average response to inoculation in the S substrate; ³: Average response to inoculations in the F substrate; ⁴: Average response inoculation in the F+S mix substrate.

lowest ($19.8 \pm 0.23 \text{ g plant}^{-1}$) in the non-inoculated control in the S substrate (Table 2).

The root growth responses (Fig. 3a) of blueberries plants inoculated with the fungi strains in the FF+S mix substrate were always the lowest than in the FF and S substrates (Fig. 3a). The average root growth responses to inoculation were only 3.6 %, 5.0 %, 5.7 %, and 4.8 %, irrespective of the substrate composition in the D01, C01, C31, and *P. fortinii* stains (Fig. 3a). Regardless of the inoculation treatment, average responses to inoculation were 8.4 %, 6.2 %, and -0.2 % in the S, FF, FF+S mix substrates, respectively (Fig. 3a). *In-vivo* observation of root mass development of the inoculated with C01 strain and non-inoculated plants in the S substrate was visible (Fig. 3b).

The fresh matter of shoots was always higher in the FF substrate than in the S and FF+S mix substrate in the F inoculant treatments, except in the D01 strains in the FF+S substrate (Table 2). The overall effect of the Substrate composition on the fresh shoot biomass was significant, while the F inoculants and the $F \times S$ interaction were not (Table 2). The shoot growth responses were similar across the F inoculated strains in the three tested substrates composition, except for inoculation with *P. fortinii* in the FF that unexpectedly displayed a significantly higher than other treatments (Fig. 3c). Average shoot growth responses to inoculation by 14.8 %, 12.0 %, and 2.0 % were reported in the S, FF, and FF+S mix, respectively (Fig. 3c). Similarly, we reported average shoot growth responses were 9.3 %, 3.4 %, 10.1 %, and 15.5 % in the D01, C01, C31, and *P. fortinii* treated plants, irrespective of the substrate composition (Fig. 3c). Furthermore, there was no significant difference in the growth development of the blueberry aerial part between the inoculated and non-inoculated blueberry plants in the fresh field soil substrate (Fig. 2d). The root-to-shoot ratio (Table 2) significantly differed between the used growth substrates and the non-inoculated treatments in the S substrate showed the highest value ($7.22 \pm 0.16 \text{ FM root/FM shoot}^{-1}$), and the inoculation with C31 strain in the FF substrate disclosed the lowest value ($5.42 \pm 0.20 \text{ FM root/FM shoot}^{-1}$) of the root-to-shoot ratio.

3.4. Leaf area index and plant height

Both F inoculated treatments and the S composition had strong and highly significant effects on leaf area index that increased on average responses by 5.5 %, 9.7 %, and 1.4 %, respectively in the S, F, and FF+S substrates (Table 3; Fig. 3e). The interaction between $F \times S$ was also

Table 2

Root, shoot dry fresh biomass, and root-to shoot ratio of inoculated blueberry in different substrate.

Fungal strains	Root fresh weight [g plant ⁻¹]			Shoot dry weight [g plant ⁻¹]			Root to shoot ratio [FW root/FW shoot-1]		
	Sand (S)	Fresh field (F)	F + S mixture	Sand (S)	Fresh field	F + S mixture	Sand (S)	Fresh field (F)	F + S mixture
Non-inoculated	19.8 ± 0.23 cC	20.6 ± 0.12 bB	21.72 ± 0.15 aA	2.83 ± 0.03 aC	3.73 ± 0.05 aA	3.49 ± 0.04 aB	7.22 ± 0.16 aA	5.42 ± 0.06 aB	6.22 ± 0.09 aC
D01	20.97 ± 0.10 bA	21.67 ± 0.67 abA	21.73 ± 0.70 aA	3.33 ± 0.27 aA	3.74 ± 0.09 aA	3.83 ± 0.10 aA	6.37 ± 0.54 aA	5.81 ± 0.30 aA	5.67 ± 0.09 aA
C01	21.54 ± 0.43 abA	22.35 ± 0.75 aA	21.25 ± 0.41 aA	3.10 ± 0.23 aA	3.70 ± 0.40 aA	3.54 ± 0.52 aA	7.04 ± 0.67 aA	6.24 ± 0.94 aA	6.53 ± 0.87 aA
C31	21.69 ± 0.04 aA	21.92 ± 0.02 bA	22.01 ± 0.81 aA	3.24 ± 0.28 aB	4.05 ± 0.18 aA	3.75 ± 0.23 aAB	6.80 ± 0.59 aA	5.42 ± 0.20 aA	5.92 ± 0.49 aB
<i>P. fortinii</i>	21.73 ± 0.04 aA	21.62 ± 0.30 abA	21.73 ± 0.25 aA	3.33 ± 0.05 aB	3.95 ± 0.12 aA	3.33 ± 0.07 aB	6.52 ± 0.09 aA	5.5 ± 0.23 aA	6.53 ± 0.16 aA
Source of variation	<i>F value</i>			<i>F value</i>			<i>F value</i>		
Fungal (F) inoculants	3.3 (*)			1.06 (ns)			0.61 (ns)		
Substrate (S)	1.1 (ns)			11.25 (***)			7.2 (**)		
F x S	1.4(ns)			0.55 (ns)			0.50 (ns)		
cv (%)	4.0			12.8			13.8		

Different small letters indicate significant differences among the fungal strains within each as substrate tested, respectively (Fisher's test; $p < 0.05$). Different capital letters indicate significant differences among the different substrate tested within for each fungal inoculation treatment, respectively (Fisher's test; $p < 0.05$).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant, respectively.

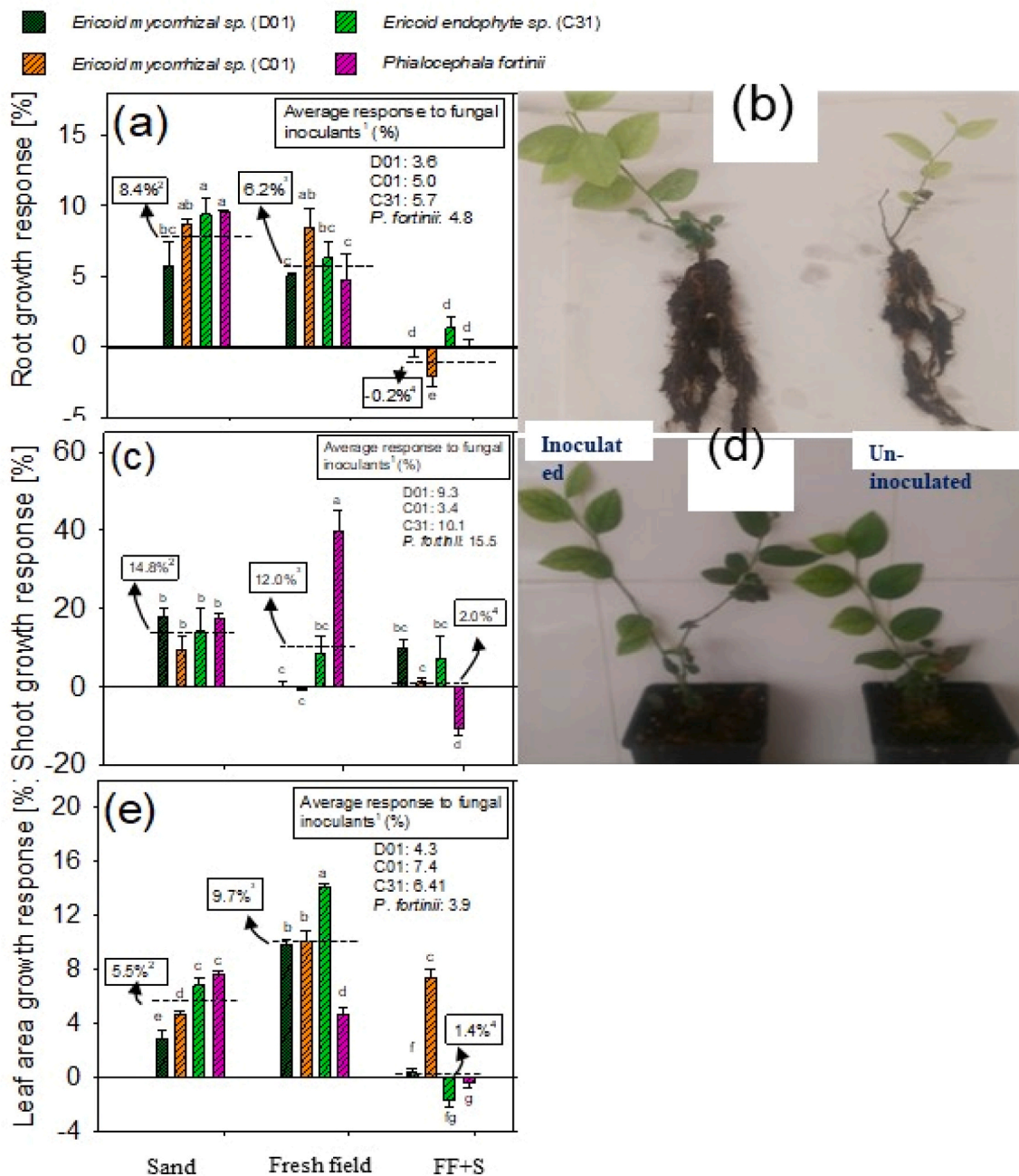


Fig. 3. Root growth response (a), root development (b) (scale = 2 cm), shoot growth response (scale = 5 cm) (c), and shoot development (d) of southern highbush plants inoculated with endophytes fungi (ErMF and DSE strains) in three different growth substrates (scale = 10 cm), sand (S) soil, fresh field (FF) soils, and FF+S mix of southern. The root and shoot development photos were taken in the sand substrate. ¹: Average responses to inoculation under *Ericoid mycorrhizal* sp. (D01), *Ericoid mycorrhizal* sp. (C01), *Ericoid endophyte* (C31), and *Phialocephala fortinii*, irrespective of substrate compositions. ²: Average response to inoculation in the S substrate; ³: Average response to inoculations in the F substrate; ⁴: Average response inoculation in the F+S mix substrate.

highly significant on the leaf area index. Leaf area average responses due to the different inoculated fungi strains were 4.3 %, 7.4 %, 6.4 %, and 3.9 %, respectively in the D01, C01, C31, and *P. fortinii* inoculated blueberry plants (Fig. 3e). The highest and positive inoculation response was in the C31 strain of the FF substrate, while decreased responses were observed in plants treated with the C31 and *P. fortinii* in the FF+S mix substrate (Fig. 3e). The inoculated fungal had a significant effect ($p < 0.05$) on the plant height of blueberry plants, while the substrate composition and the $F \times S$ were not (Table 3).

4. Discussion

The present research work is among the first studies to examine the interactions between the endophyte's fungal microbes and three substrate compositions using blueberry as a host crop in the Mediterranean climate region of Morocco. The fungal isolates tested here originate from ericaceous hosts in geographically distant regions (Australia, United Kingdom, China). While their use provided well-characterized functional models of ericoid symbiosis, it may not fully represent the diversity or adaptive traits of native Moroccan ErMF. The primary causes might be the nutrient composition between the two substrates, with the available-Pi, C, and total N contents higher in the FF than in the S

Table 3
Leaf area index and plant height of mycorrhizal inoculated blueberry plant.

Fungal strains	Root fresh weight [g plant ⁻¹]			Shoot dry weight [g plant ⁻¹]			Root to shoot ratio [FW root/FW shoot ⁻¹]		
	Sand (S)	Fresh field (F)	F + S mixture	Sand (S)	Fresh field (F)	F + S mixture	Sand (S)	Fresh field (F)	F + S mixture
Non-inoculated	19.8 ± 0.23 cC	20.6 ± 0.12 bB	21.72 ± 0.15 aA	2.83 ± 0.03 aC	3.73 ± 0.05 aA	3.49 ± 0.04 aB	7.22 ± 0.16 aA	5.42 ± 0.06 aB	6.22 ± 0.09 aC
D01	20.97 ± 0.10 bA	21.67 ± 0.67 abA	21.73 ± 0.70 aA	3.33 ± 0.27 aA	3.74 ± 0.09 aA	3.83 ± 0.10 aA	6.37 ± 0.54 aA	5.81 ± 0.30 aA	5.67 ± 0.09 aA
C01	21.54 ± 0.43 abA	22.35 ± 0.75 aA	21.25 ± 0.41 aA	3.10 ± 0.23 aA	3.70 ± 0.40 aA	3.54 ± 0.52 aA	7.04 ± 0.67 aA	6.24 ± 0.94 aA	6.53 ± 0.87 aA
C31	21.69 ± 0.04 aA	21.92 ± 0.02 bA	22.01 ± 0.81 aA	3.24 ± 0.28 aB	4.05 ± 0.18 aA	3.75 ± 0.23 aAB	6.80 ± 0.59 aA	5.42 ± 0.20 aA	5.92 ± 0.49 aB
<i>P. fortinii</i>	21.73 ± 0.04 aA	21.62 ± 0.30 abA	21.73 ± 0.25 aA	3.33 ± 0.05 aB	3.95 ± 0.12 aA	3.33 ± 0.07 aB	6.52 ± 0.09 aA	5.5 ± 0.23 aA	6.53 ± 0.16 aA
Source of variation	<i>F value</i>			<i>F value</i>			<i>F value</i>		
Fungal (F)	3.3 (*)			1.06 (ns)			0.61 (ns)		
Substrate (S)	1.1 (ns)			11.25 (***)			7.2 (**)		
F x S	1.4(ns)			0.55 (ns)			0.50 (ns)		
cv (%)	4.0			12.8			13.8		

Different small letters indicate significant differences among the fungal strains within each as substrate tested, respectively (Fisher's test; $p < 0.05$). Different capital letters indicate significant differences among the different substrate tested within for each fungal inoculation treatment, respectively (Fisher's test; $p < 0.05$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant, respectively.

substrate that the blueberry plants differently benefited. Our results showed that the EC and Ca₃Ca were higher in the S than in the FF substrate. Possibly, due to the origin of sampled sand substrates, the presence of inhibiting agents, particularly the salt concentration and the sources of carbonate impaired fungal establishment inside in roots and reduce their colonization intensity. The salt's adverse effect to inhibit the beneficial microbes in the plant was previously studied (Fakhech et al., 2021). The markedly lower colonization observed in the sandy substrate is more plausibly attributed to its low organic matter (OM) content and limited moisture retention, rather than to its calcium carbonate concentration (Jehlička et al., 2024). Reduced water-holding capacity of sandy substrate and poor nutrient availability can be a reason why generally they exhibit a reduced microbial activity, these are conditions that can restrict the establishment and proliferation of ErMF hyphae it can be because of (Musei et al., 2024). In such substrates, hyphal expansion is constrained by rapid desiccation, which can decrease the likelihood of root–fungus contact and successful colonization (Vohník, 2020).

On the other hand, the beneficial effects are possibly due to the formation of mycelial by the endophytes to access P from labile and non-labile sources to promote their growth and development (Leopold, 2016; Mikheev et al., 2022; Narisawa, 2017; Newsham, 2011). A low level of hyphal colonization was also observed in the non-inoculated controls. Such background colonization is consistent with previous reports showing that ericoid-type endophytes rapidly recolonize roots of micropropagated Ericaceae once transferred to non-axenic substrates (Neves et al., 2025; Vohník et al., 2012b). These fungi are widespread in soils, peat, and even in nursery air dust, and their presence does not necessarily indicate cross-contamination. Importantly, inoculated plants exhibited significantly higher colonization frequency and density, indicating that the introduced fungi successfully established additional symbiotic structures. Nonetheless, the presence of endogenous or adventitious ericoid fungi in controls highlights a limitation of non-axenic inoculation systems. In contrast to ecto-and arbuscular mycorrhizal fungi, the extraradical mycelium of ErMF is generally limited in extent and density, forming only fine hyphae in the immediate rhizosphere (Smith and Read, 2010). These mycelial structures act as a "nutrient highway," enabling mycorrhizal fungi to access both labile and non-labile P sources. Labile P, such as soluble orthophosphate ions, is directly absorbed by hyphae, but its availability in soil is often limited. Non-labile P, which includes organic compounds like phytate and

mineral-bound inorganic P like iron- or aluminum-bound phosphates, requires enzymatic or chemical mobilization. The contribution of this phase to nutrient uptake stays modest and primarily localized. For non-mycorrhizal endophytes, such as *Phialocephala fortinii*, the extra-radical phase is not yet well resolved, and its ecological role in nutrient acquisition remains uncertain (Vohník, 2020).

Both arbuscular mycorrhizal (AMF) and ErMF contribute to P mobilization, but through different mechanisms. While AMF typically enhance uptake via extensive extraradical hyphal networks that access orthophosphate, ErM and DSE fungi secrete organic acids, phosphatases, and chelating compounds that solubilize otherwise unavailable inorganic phosphates and mineralize organic P forms (Guo et al., 2023; Mikheev et al., 2022). Therefore, the improved P acquisition observed in inoculated blueberry plantlets in this study likely reflects the biochemical capacity of ErM and DSE fungi to mobilize P within the rhizosphere rather than any structural advantage typical of AMF (Alguacil et al., 2016; Kahiluoto et al., 2012). Other benefits from endophytes fungi are their capability to degrade organic molecules and make N and Pi available for root uptake (Narisawa, 2017). The PUE in the shoot was significantly reduced by the substrate composition and the identity of inoculated fungal, and the C01 and C31 strains lowered their PUE in the different growth substrates to permit the highbush blueberry plants to invest in more P acquisition and utilization. However, PUE is a very complex plant trait, and precautions to consider during their interpretations (Heuer et al., 2017). Many studies erroneously equate high P uptake with high PUE. For example, a plant may accumulate P in roots without translocating it to shoots, inflating tissue P content but not biomass (Han et al., 2022). However, the use efficiency of any nutrient depends upon the availability of other nutrients. Low nutrient use efficiency could be an artifact of limitations of other nutrients. Plants under P limitation often increase root biomass allocation to enhance soil exploration. While this boosts P uptake, it doesn't translate to productivity gains. In *Leymus chinensis* (Trin.) Tzvelev, P fertilization increased belowground biomass by 30.6 kg ha⁻¹ but showed diminishing returns for shoot growth (Li et al., 2023). Similarly, *Arabidopsis* root hair mutants accumulated comparable root P to wild types but produced 18–33 % less shoot biomass under low-P conditions (Bates and Lynch, 2000).

Among the tested fungi, C01 promoted higher root FM and development compared to other tested strains and the control without inoculation of the fresh field soil growth substrate (Table 2 and Fig. 3a, b).

Previous investigations using different plant species such as rhododendron (*Rhododendron fortunei* Lindl.), velvetleaf blueberry (*Vaccinium myrtilloides* Michx.), and Labrador tea (*Rhododendron groenlandicum* (Oeder) Kron & Judd) observed the beneficial effects of ErMF inoculation to promote root growth, in particular the formation of adventitious roots (Fadaei et al., 2021; Wei et al., 2020). Fadaei et al., (2021) pointed out the importance of ErM for the growth of ericaceous plants, even under favorable environmental conditions and adequate fertilization. Under *in vitro* conditions, inoculation with an ErMF strain Om19 of clonally propagated rhododendron plants increased the plant hormonal concentrations and precursors involved in the biosynthesis of indole-3-acetic acid (IAA) (Wei et al., 2020). Furthermore, ErM strains induced the synthesis of large concentrations of tryptophan and indole-3-pyruvate and favored the upregulation of genes concomitantly involved in the biosynthesis of IAA, N, and P uptake metabolisms to accelerate the development of adventitious roots (Wei et al., 2020). Also, ErM produce extracellular enzymes like phosphatases, proteases that break down organic matter in recalcitrant substrates, releasing bound N and P for plant uptake (Wei et al., 2022), this nutrient access stimulates root growth, particularly fine root proliferation, which increases the plant's surface area for nutrient absorption. Special example, in *Vaccinium* species, ErM colonization increases root branching and hair formation, enabling efficient foraging in organic horizons (Cairney and Meharg, 2003). Genomic analyses have shown that ErM fungi possess gene repertoires for lipases, polysaccharide-degrading enzymes, proteases, and other enzymes involved in secondary metabolism, at levels comparable to saprotrophic and pathogenic fungi and higher than those found in ectomycorrhizal fungi (Wei et al., 2022). However, dark septate endophytes are a polyphyletic group of ascomycete fungi characterized by melanized, septate hyphae that colonize plant roots across diverse ecosystems (Huertas et al., 2024; Lu et al., 2024). Unlike ERM fungi, DSE associate with a broader range of hosts, including grasses, cereals, and woody plants. DSE promote root development through phytohormone production and nutrient facilitation (Huertas et al., 2024). For instance, *Phialocephala fortinii*, a well-studied DSE, synthesizes auxins (e.g., indole-3-acetic acid) that stimulate lateral root formation and root hair elongation in *Arabidopsis* and maize (Hidayat, 2019; Sukumar et al., 2013). We lack comparative data for the blueberry root growth development in the Morocco testing conditions. It is worth pointing out that this study is among the only ones investigating the contributions of ErM and DSE to promote root development and PUE of blueberry plants using the rhizosphere soil substrate compositions.

Leaf area highly correlates with the photosynthesis process in plants (Mora et al., 2016). In the present study, the fungal inoculation effect on the leaf area was highly significant (Table 3, Fig. 3e). Previous studies report the beneficial effects of fungi inoculations on plant leaves and photosynthetic responses (Lin et al., 2017; Mathur et al., 2019). Moreover, a higher chlorophyll content highly correlates with increased photosynthesis and subsequent better N use efficiency and leaf area expansion (Mora et al., 2016).

In this study, plant growth was evaluated based on fresh weight and nutrient analyses. While this metric reflects relative growth differences among treatments, it can vary with plant water status and is therefore less stable than dry weight (Bashan and de-Bashan, 2005; Huang et al., 2017). Future experiments should include dry biomass measurements to refine estimates of root-to-shoot allocation and validate the present findings under larger-scale or field conditions (Vohník et al., 2012a).

Regarding molecular characterization, although ITS sequencing provided sufficient resolution to confirm all isolates as Helotiales members, the three unidentified strains displayed < 97 % identity to any described species. This result suggests they could represent novel or poorly represented ericoid lineages. Broader taxonomic frameworks using multi-locus sequencing and phylogenetic inference are warranted to clarify their evolutionary placement.

The present study deliberately applied single-strain inoculations in a nursery setting to disentangle fungus- and substrate-specific effects on

colonization, P uptake, and early growth of micro-propagated plants. However, blueberry roots *in-situ* are typically co-colonized by multiple ErMF and endophytic taxa, and their intra-root interactions (priority effects, competition, facilitation) can shift nutrient-foraging, P use efficiency, and growth outcomes relative to single-partner assays. Consequently, our conclusions should be interpreted as proof-of-concept under controlled nursery conditions. To translate to production systems, co-inoculation trials (e.g., ErM + DSE consortia), sequencing-based tracking of partner abundances, and multi-season field validations across substrate regimes are needed to quantify interaction effects on P acquisition and plant performance. Future studies will therefore prioritize the isolation and comparison of local fungal strains from Moroccan soils to assess potential differences in compatibility, colonization efficiency, and nutrient acquisition.

5. Conclusion

This study demonstrated that ErMF and endophytic fungi from the order Helotiales can enhance P acquisition and utilization efficiency of micropropagated blueberry plantlets during the nursery acclimation stage. The magnitude of this response depended on both the fungal isolate and the substrate, with peat providing the most favorable conditions for colonization and nutrient uptake. In contrast, sandy substrate limited fungal establishment and plant performance, likely due to its low organic matter and poor moisture retention. While these findings confirm the functional potential of ericoid symbioses to improve early blueberry growth under controlled conditions, they represent a nursery-scale proof of concept. Future studies should incorporate local fungal isolates, multi-strain consortia, and field trials to assess the persistence and agronomic relevance of these symbioses under commercial Moroccan condition

CRedit authorship contribution statement

Martin Jemo: Writing – original draft, Data curation, Conceptualization. **Lucie Miché:** Conceptualization. **Mohammed Taoussi:** Writing – review & editing, Supervision. **Robin Duponnois:** Writing – original draft, Validation, Supervision, Conceptualization. **Rachid Mrabet:** Supervision, Conceptualization. **Mohamed Hafidi:** Supervision, Conceptualization. **Rachid Lahlali:** Validation, Supervision. **Ahlam Hamim:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization.

Consent to participate and ethics approval

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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