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NOTE

Effect of arbuscular mycorrhiza on germination and initial growth of *Cinchona officinalis* L. (Rubiaceae)

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ABSTRACT

Cinchona officinalis, known locally as cascarilla or cinchona, is a plant species native to South America. It was used as a source of quinine to combat malaria in the 17th century. The species is threatened by various anthropogenic activities. Further, the propagation of the species depends on seed dispersal and its germination capacity. Therefore, it is necessary to conserve and propagate this species. Because *C. officinalis* seeds have a low germination capacity, we determined the effect of arbuscular mycorrhizae (AM) on their germination and growth. A randomized design was employed with two treatments, one treated with mycorrhizae (CM) and another without mycorrhizae (SM). For each treatment, three replicates of 100 seeds were used. Germination, growth, and fungal characteristics were evaluated. In germination parameters, the CM treatment showed better performance, but the improvement was statistically insignificant. However, the application of AM significantly improved seedling height (cm), root length (cm), leaf area (cm^2), and root number by 53.52, 28.72, 29.73, and 61.66%, respectively. Likewise, mycorrhization intensity (%), mycorrhization frequency (%), and extraradical mycelium length (cm) in the CM treatment were 37.13, 3.44, and 174.97% higher compared to the SM treatment, respectively. Therefore, the use of AM fungi proves to be advantageous in the propagation of *C. officinalis*, and these results provide a basis for the largescale and sustainable propagation of this species.

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Arbuscular mycorrhiza; cinchona tree; forest nursery; forest seedlings; seed propagation

Introduction

Peru is home to diverse cultures, ecosystems, and flora and fauna (Fajardo et al. 2014), including very important medicinal and food plants (De-la-Cruz et al. 2007). *Cinchona* is a genus of medicinally valuable plants, such as *Cinchona officinalis*, *Cinchona pubescens*, and *Cinchona calisaya*, the barks of which contain alkaloids, such as quinine, which provided the only treatment against malaria for over 300 years (Cóndor et al. 2009; Canales et al. 2020).

Several studies have reported that *C. officinalis* needs specific conditions to grow and its distribution ranges are limited (Armijos-González and Pérez-Ruiz 2016). In Peru *C. officinalis* is found in small pockets of Andean forest, particularly in the Cajamarca and Piura regions (Huamán et al. 2019). This species is threatened by urbanization, migratory agriculture, cattle ranching, and widespread selective logging (Arbizu et al. 2021), which has led to the prioritization of its conservation and recovery in Peru (Albán-Castillo et al. 2020). The restoration and preservation of this iconic species require the generation of knowledge related to its propagation (Sánchez-Santillan et al. 2021). The survival of *C. officinalis* in natural environments depends on seed dispersal; however, the species has a low germination capacity (De-la-Cruz et al. 2007; Valdiviezo et al. 2018), which is affected by factors, such as seed quality, humidity, temperature, and microbial activity (Santos et al. 2010).

Arbuscular mycorrhizae (AM) are a group of obligate symbionts involved in diverse ecological processes (van der Heijden et al. 2015). AM are symbiotically related to more than 85% of terrestrial plants (Brundrett and Tedersoo 2018; Dey and Ghosh 2022). This relationship provides diverse benefits to the host plant; for example, it helps mitigate environmental stress (Hosseyni Moghaddam et al. 2021), enhances the uptake of P (Grümberg et al. 2015) and other less mobile nutrients (Lehmann and Rillig 2015; Garg and Singh 2018), facilitates low hydraulic gradient water uptake (Augé et al. 2015), provides protection from pathogen attack, slows nitrification and nitrogen leaching, and accelerates organic matter degradation (Veresoglou and Rillig 2012; Leifheit et al. 2014; Powell and Rillig 2018; Veresoglou et al. 2019). In addition, AM provides nutrients that are of great

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importance for seed germination and subsequent plant establishment (Dearnaley 2007). At the laboratory level, AM has been reported to positively affect seed germination in forest species (Ballina et al. 2017). Under natural conditions, AM hyphal networks may positively affect both germination and seedling establishment (Varga 2015).

In the present study, we sought to determine whether AM affects the germination and initial growth of *C. officinalis* as there is little information on the use of these biofertilizers in the propagation of this important medicinal species. In the future, AM could be used as a biofertilizer to accelerate the growth of *C. officinalis*. Furthermore, the results of this study could be used to implement recovery plans and programs for this species.

Materials and methods

Study area

The study was conducted from 20 November 2021 to 20 April 2022 in the La Cascarilla community (5° 40′ 21.12″ S and 78° 53′ 55.65″ W), district of Jaen, Peru, at 1810 m asl. The annual precipitation is 1730 mm, and the mean annual minimum and maximum temperatures are 13.0 and 20.5 °C, respectively (Fernandez et al. 2021; Fernandez and Huaccha 2022).

Plant material

We used the seeds of *C. officinalis* that were collected in October 2021 from a tree in the San Luis community, Cajamarca region, Peru (6° 22′ 6.68″ S and 79° 3′ 29.50″ W) at 2489 m asl. We collected 0.5 kg of mature capsules (brown to dark brown color) in cloth bags and carried them to the La Cascarilla community, located 100 km from the collection site, and the capsules were stored under shade. Twenty days later, seeds without visible cracks, fungi, and/or nematodes were selected and used for the study. Storage of seeds was avoided as the seeds of *C. officinalis* are recalcitrant, which makes them lose their germination capacity very quickly (Caraguay et al. 2016).

Microbiological inoculation

We used MycoGrow®-Complex (Grow More, Gardena, CA, USA), which contains the AM *Glomus intraradices*, *Glomus mosseae*, *and Glomus aggregatum* as inoculum. The product data sheet recommended using 6 kg of MycoGrow for each cubic meter of the substrate. Thus, considering that the volume of the experimental units was 7260 cm^3, we incorporated 43.56 g of MycoGrow into the substrate of each unit before sowing the seeds.

Substrate

The substrate used for the germination of *C. officinalis* consisted of 100% sand, which was sterilized in an autoclave at 105 °C for 1 h; this process was repeated for 3 consecutive days. The physicochemical characteristics of the substrate were: sandy texture; pH, 7.7; electrical conductivity (dS m^{-1}), 457.33; organic matter, 1.82%; total nitrogen, 0.8%; and phosphorus, 3.21 ppm.

Experimental design and set-up

A randomized design with two treatments and three replicates per treatment was used; 100 seeds of *C. officinalis* were used for each replicate, and 600 seeds were used for the whole trial.

A sub-irrigation chamber as described by Fernandez et al. (2021) was used in the study. The substrate containing mycorrhizae (CM) was introduced to three experimental units, and the substrate without mycorrhizae (SM) was introduced to another three experimental units (Figure 1). The substrate was moistened to field capacity, which was confirmed by touch. The seed viability was determined by the flotation test, wherein the seeds that remained floating on the water were discarded. The entire sub-irrigation chamber was manually irrigated every day with 50 mL of water. The sub-irrigation chamber was covered with Raschel mesh of 85% shade to reduce the direct incidence of solar radiation.

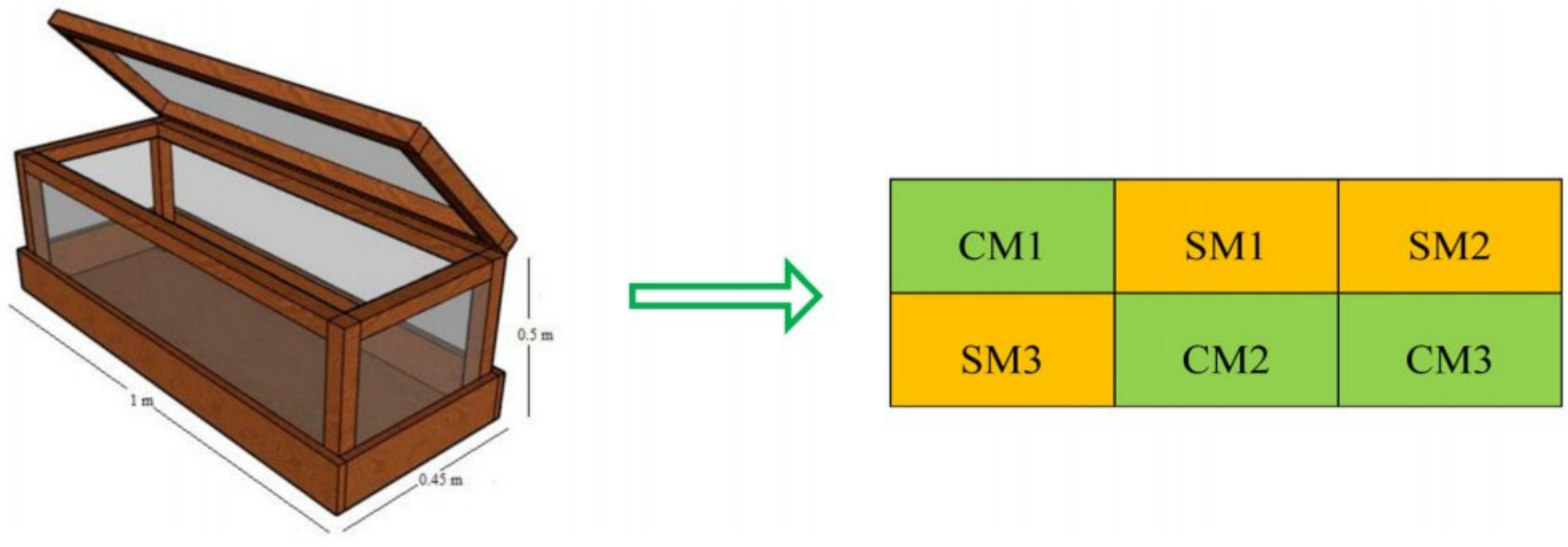

Figure 1. Sub-irrigation chamber and randomized design were used in the trial.

Data collection and evaluation

Germination parameters

Germination percentage was calculated using the following equation:

$$Germination\ (\%) = \frac{Number\ of\ seeds\ that\ germinated}{Number\ of\ seeds\ sown} \times 100$$

Germination rate coefficient (RC) was calculated using the following equation:

$$RC = \frac{\sum n_i}{\sum (n_i t_i)} \times 100$$

Average germination time (T) was calculated using the following equation:

$$T = \frac{1}{RC} \times 100$$

Germination speed (GS) was calculated using the following equation:

$$GS = \sum \left(\frac{n_i}{t}\right)$$

where n_i is the number of seeds that managed to germinate per day i, t_i is the number of days after sowing, and t is the germination time from sowing to the germination of the last seed.

We also calculated several germination parameters, such as germination energy (GE), energy period (EP), germination capacity (GC), and maximum germination value (MGV) according to Czabator (1962) and González et al. (2008). GE is the daily cumulative germination percentage, obtained when germination reaches its maximum; EP is the number of days required to achieve the maximum germination; GC is the percentage of seeds that germinated during the study, along with the healthy seeds that failed to germinate; MGV is the final mean germination, which was calculated by dividing the cumulative germination percentage at the end of the trial by the number of days of the trial; and MV is the maximum daily average germination recorded during the trial.

Growth parameters

Root length, seedling height, leaf area, and the number of roots per seedling were measured 120 days (or on the 120th day) after sowing *C. officinalis* seeds to evaluate the potential influence of AM on the initial growth of seedlings. The seedlings were photographed against a white background (20 × 12 cm cardboard) with a 2 cm reference line drawn next to the leaves for scale in image processing. To extend the leaves, they were covered with 20 × 12 × 0.3 cm transparent glass. The photographs were taken using a smartphone (Huawei P30 Lite, 24-megapixel MAR-LX3A camera). The images were then processed using ImageJ software, according to the following processes: (1) File > Open > Image > Line Width > Analyze > Set Scale > Line Width (to measure the stem and root length); and (2) File > Open > Line Width > Analyze > Set Scale > Polygon Selections > Analyze > Measure (Baker et al. 1996).

Fungal characteristics

To determine mycorrhizal colonization (MC), a root staining process was performed according to the methods of Phillips and Hayman (1970) with minor modifications; the roots were treated with vinegar and hydrogen peroxide, subjected to a water bath, and stained with trypan blue. After staining, they were cut into thirty fragments of 1 cm sections. Each fragment was placed on a slide and then observed under a microscope at 100× objective magnification.

The percentage of internal hyphal colonization, or mycorrhizal frequency (MF), was determined using the formula of Sieverding et al. (1991) given below:

$$MF = \frac{Number\ of\ roots\ colonized}{Number\ of\ roots\ observed} \times 100$$

The mycorrhizal intensity (IM) was determined using the formula of Trouvelot et al. (1986) given below.

$$IM = \frac{(n1 + 5 \times n2 + 30 \times n3 + 70 \times n4 + 95 \times n5)}{N}$$

where N is the total number of roots that were evaluated, and n is the number of classified fragments.

To estimate the length of extraradical mycelium (LMER), one gram of soil was weighed and stained according to the methods of Carballar (2010) with some modifications. The stained soil was placed in Petri dishes with 0.5 cm^2 quadrats at the base. The hyphae were observed at the line intersections under a stereoscope at 3× and 4.5× magnification (Carballar 2010). The LMER was calculated using the equation given by Newman (1966).

$$R = \frac{\pi A N}{2H}$$

where

R is mycelium length per unit soil; A is area of the plate, N is the number of intersections, and H is the total length of the lines of the plate (cm).

Data analysis

An independent sample t-test was used to compare the means of the replicates of each treatment ($p = 0.05$) after confirmation of the normality of data using the Shapiro–Wilk test. All statistical analyses were performed using StatGraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA).

Results

In both treatment groups, *C. officinalis* seed germination was characterized by a sigmoid curve and the highest percentage of germination between days 19 and 31 (Figure 2(A)). Notably, the germination curve of the CM treatment group was slightly above that of the SM treatment group. Although a higher cumulative germination percentage was observed in the CM treatment, there were no significant differences between the two groups (Figure 2(B)). Figure 3 and Table 1 shows

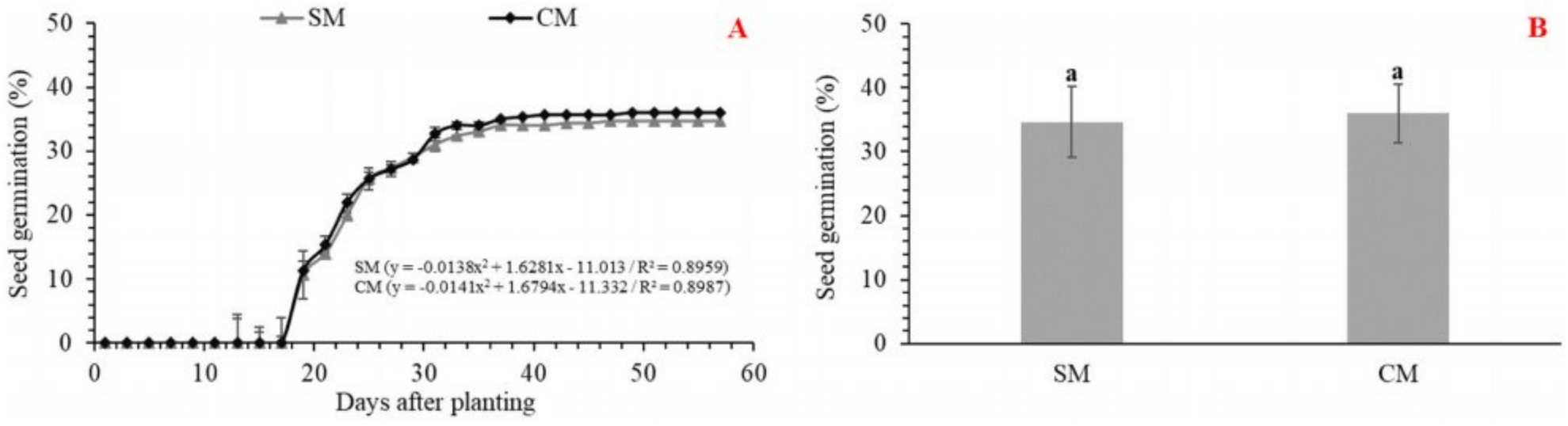

Figure 2. (A) Cumulative germination percentage of *C. officinalis* in substrate containing mycorrhiza (CM) and without mycorrhiza (SM). (B) Percentage germination of *C. officinalis* reached at the end of the trial in each treatment. Bars with the same lowercase letters indicate no significant differences ($p > 0.05$).

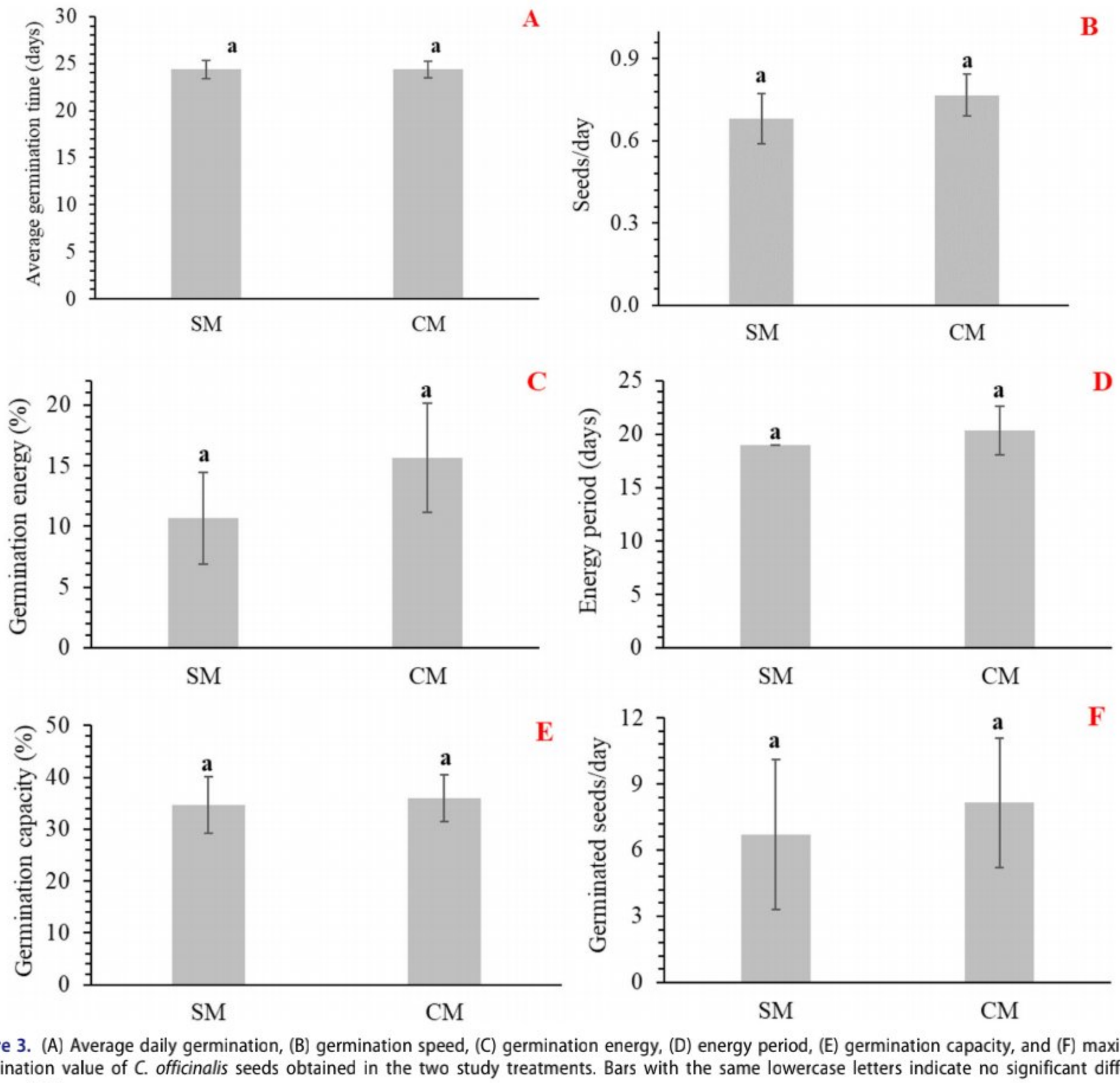

Figure 3. (A) Average daily germination, (B) germination speed, (C) germination energy, (D) energy period, (E) germination capacity, and (F) maximum germination value of *C. officinalis* seeds obtained in the two study treatments. Bars with the same lowercase letters indicate no significant differences ($p > 0.05$).

Table 1. Germination parameters evaluated in *C. officinalis*.

Germination parameters	CM	SM
Average germination time (days)	24.41 ± 0.88	24.39 ± 0.96
Germination speed (seeds/day)	0.76 ± 0.10	0.68 ± 0.11
Germination energy (%)	15.67 ± 4.51	10.67 ± 3.79
Energy period (days)	20.33 ± 2.31	19 ± 0.00
Germination capacity (%)	36 ± 4.58	34.67 ± 5.51
Maximum germination (germinated seeds/day)	8.15 ± 2.93	6.71 ± 3.39

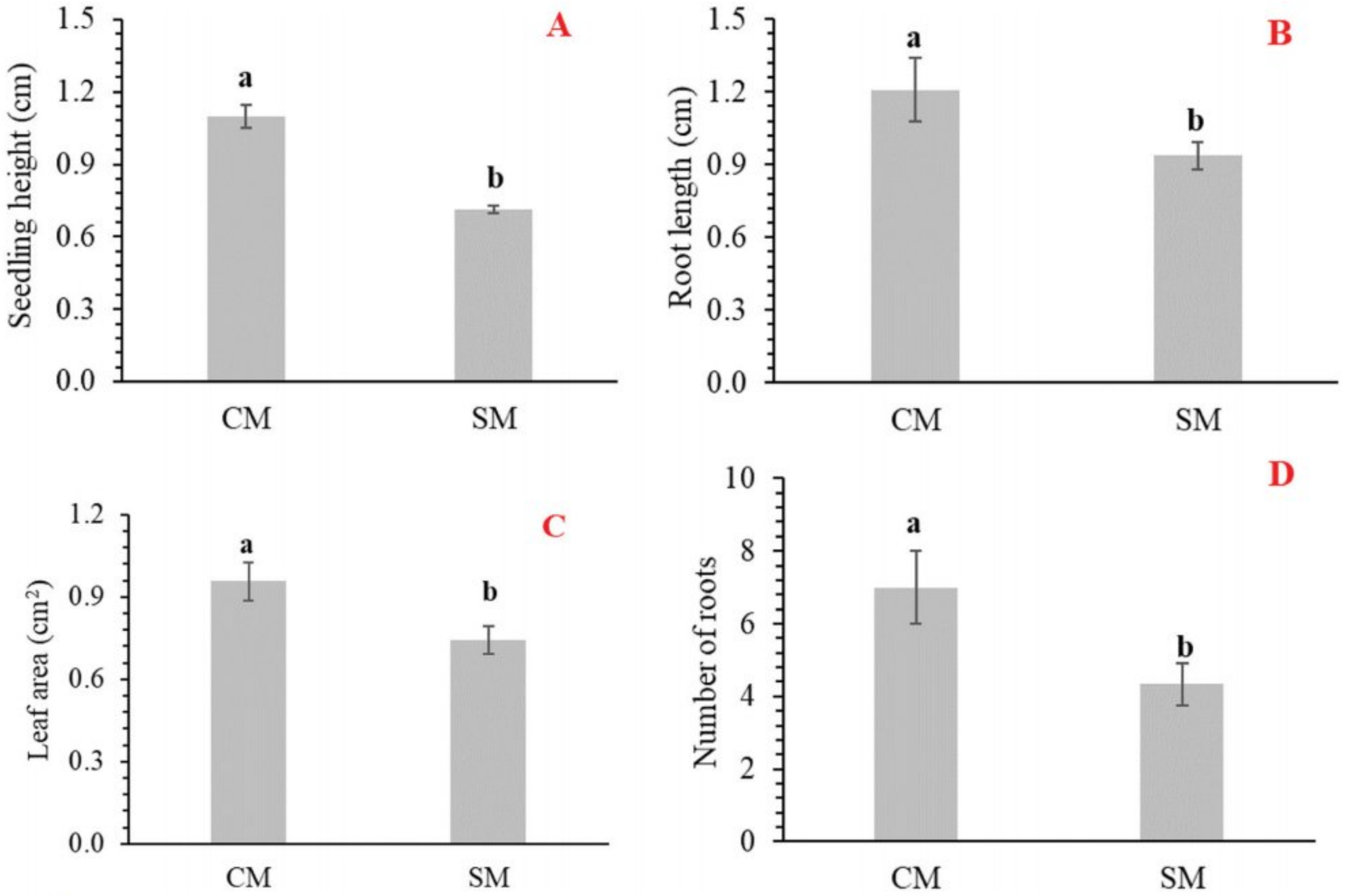

Figure 4. Growth parameters were evaluated 120 days after sowing *C. officinalis* seeds. (A) average seedling height, (B) average root length of seedlings, (C) estimated leaf area, and (D) average number of roots per seedling of *C. officinalis*. Different lowercase letters indicate significant differences ($p < 0.05$).

Table 2. Growth parameters were measured 120 days after sowing seeds of *C. officinalis*.

Growth parameters	CM	SM
Average seedling height (cm)	1.09 ± 0.05	0.71 ± 0.02
Average root length of seedlings (cm)	1.21 ± 0.13	0.94 ± 0.06
Estimated leaf area (cm^2)	0.96 ± 0.07	0.74 ± 0.05
Average number of roots	7.00 ± 1.00	4.33 ± 0.58

the results of the germination parameters evaluated in the study. The CM treatment enhanced the performance of all evaluated parameters; however, no significant differences were found between the treatments.

The analysis of growth parameters after 120 days of sowing the *C. officinalis* seeds showed that plant height in the CM treatment was 53.7% higher than that in the SM treatment (Figure 4(A)). The root length was 29.1% higher in the CM treatment than that in the SM treatment (Figure 4(B)). The leaf area in the CM treatment was 28.7% higher than that in the SM treatment (Figure 4(C)). The number of roots in the CM treatment was 28.7% higher than that of the SM treatment (Figure 4(D)). For all the growth parameters evaluated, significant differences were observed between CM and SM treatments, demonstrating that AM positively influenced the initial growth of seedlings during germination (Table 2).

Mycorrhizal frequency was significantly higher (33.8%) in the CM treatment than in the SM treatment (24.7%) (Figure 5(A)). The mycorrhizal intensity was 100 and 96.7% in the CM and SM treatment groups, respectively, and although the CM treatment group showed a higher frequency, it was insignificant (Figure 5(B)). The length of the extraradical mycelium was significantly higher in the CM treatment (115.7 cm) than in the SM treatment (42.1 cm) (Table 3).

Discussion

In tree species, there are studies that show the positive effects of AM on cumulative germination due to the protective-coating action of AM on seeds, preventing infection by pathogens (Dalling et al. 2011; Huante et al. 2012; Ballina et al. 2017). The same effects occur in various species of orchids that depend on mycorrhizae to supply them with nutrients and water owing to the absence of endosperm in their seeds (Smith and Read 2008; Yuan et al. 2016; Huang et al. 2018; Shao et al. 2020; Figura et al. 2021). However, there are reports that AM can also suppress germination owing to the exudates they release (Louarn et al. 2012; Wu et al. 2014; Varga 2015; Maighal et al. 2016; Ballina et al. 2017). In this study, better germination parameter results were observed when AM fungi were inoculated. However, these results were not significant, presumably because of two factors (1) the high mycorrhizal specificity of some plant species (Zi et al. 2014; Durán-López et al. 2019; Meng et al. 2019; Fuji et al. 2020; Shao et al. 2020); or (2) that *C. officinalis* is not AM-dependent to initiate or increase its germination. Therefore, propose that future research should identify

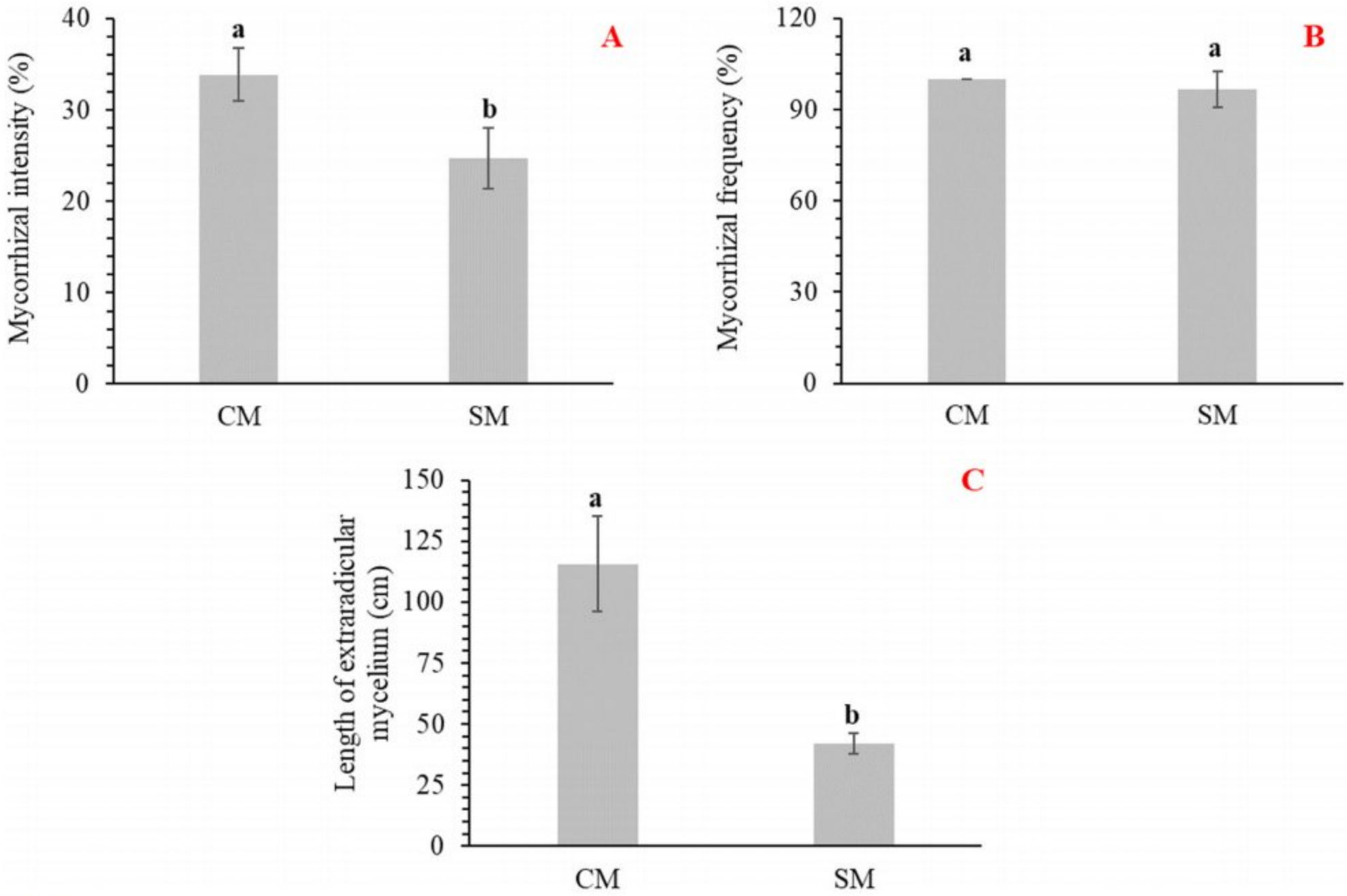

Figure 5. Mycorrhizal parameters were evaluated in the study. (A) percentage of mycorrhizal colonization, (B) frequency of mycorrhizal colonization, and (C) length of extraradical mycelium. Bars with different lowercase letters indicate significant differences ($p < 0.05$).

Table 3. Fungal characteristics were determined in the root system of *C. officinalis* seedlings at the end of the trial.

Fungal characteristics	CM	SM
Mycorrhizal intensity (%)	33.83 ± 2.90	24.67 ± 3.30
Mycorrhizal frequency (%)	100.00 ± 0.00	96.67 ± 5.77
Length of extraradicular mycelium (cm)	115.68 ± 65.70	42.07 ± 18.22

AM species specific to *C. officinalis*, or confirm the results obtained in this study.

However, the AM in this study showed a significant positive influence on the initial growth of *C. officinalis*. This may be attributed to the colonization of the seed radicle by mycorrhizal hyphae, which provide protection and nutrition to seedlings and accelerate their growth (Pankaj et al. 2021). In addition, AM significantly improved the length and number of roots in *C. officinalis* seedlings as observed in different studies (Dovana et al. 2015; Khalediyan et al. 2021; Hagh-Doust et al. 2022). Some authors claim that mycorrhizae can increase root growth by up to 200% (Falcón et al. 2021). This further improves plant growth through increased access to soil nutrients (Weisany et al. 2015; Weisany et al. 2016; Khalediyan et al. 2021), as AM can increase nutrient uptake by 7–250 times depending on the crop (Naranjo et al. 2011).

Finally, the positive effects of AM on leaf area are attributed to the percentage of AM colonization on seedling roots (Yadav and Aggarwal 2015; Palacios et al. 2021) and the LMER which was higher in the mycorrhizal treatment. Colonization of AM can lead to increased water and nutrient uptake by increasing the surface area of uptake through the mycelium into the soil, allowing the plant to have access to more soil; this can lead to enhanced photosynthesis, improved plant growth, and thus an increase in leaf area (Smith et al. 2003; Huang et al. 2018; Khalediyan et al. 2021).

Conclusion

The results of this study suggest that AM are beneficial biofertilizers for the propagation of *C. officinalis* as they significantly improve seedling growth; therefore, AM could be used for the sustainable mass propagation of this important plant species. In addition, the results showed that *G. intraradices*, *G. mosseae*, and *G. aggregatum* can have different effects on the germination process (neutral effect) and growth (positive effect) of *C. officinalis*. Because our study did not evaluate the effects of a specific mycorrhiza on germination or growth, it was not possible to determine the individual effects of the mycorrhizal species under study. Thus, we suggest further research is necessary to determine whether *C. officinalis* has a tendency to associate with particular or diverse mycorrhizal species. From there, it may be possible to identify a mycorrhiza that promotes the germination of *C. officinalis*. In addition, it would be interesting to evaluate the effects of AM for a period longer than 120 days, to analyze the persistence or cessation of the effects observed in this study.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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