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Exploration and identification of endomycorrhiza in chrome-contaminated areas as active ingredients of biofertilizer applied to *Canavalia ensiformis*

Muh. Akhsan Akib[1*], Tutik Kuswinanti[2], Syatrianti Andi Syaiful[3], Sarjiya Antonius[4], Amir Mahmud[5]

[1] Department of Agrotechnology, Faculty of Agriculture, Animal Husbandry and Fishery, Muhammadiyah University of Parepare, Jl. Jend Ahmad Yani KM.6, Parepare, South Sulawesi, 91131, Indonesia
[2] Department of Plant Pest and Disease, Faculty of Agriculture, Hasanuddin University, Jl. Perintis Kemerdekaan KM.10, Makassar, South Sulawesi, 90245, Indonesia
[3] Department of Agrotechnology, Faculty of Agriculture, Hasanuddin University, Jl. Perintis Kemerdekaan KM.10, Makassar, South Sulawesi, 90245, Indonesia
[4] Center for Applied Microbiology Research (BRIN). Jl. Raya Jakarta-Bogor km 46, Cibinong, West Java, 16911, Indonesia
[5] Departement of Agricultural Socioeconomics, Faculty of Agriculture, Muhammadiyah University of Kendari Jl. Ahmad Dahlan No. 10, Kendari 93117, Indonesia
* Corresponding autho's e-mail: akhsanbagus@yahoo.co.id, akhsanakib@gmail.com

ABSTRACT

Jack bean (*Canavalia ensiformis*) is a legume and a rich source of nutrition. However, in its cultivation, biofertilizer endomycorrhizal or arbuscular mycorrhizal (AM) fungi has not been optimally utilized as an environmentally friendly production facility. This study aims to (1) identify endomycorrhizal spores in chromium-contaminated areas as active biofertilizer ingredients and (2) determine the effect of their application on *Canavalia ensiformis*. Study was conducted in three stages, namely (1) exploration and identification of endomycorrhizal spores; (2) production of endomycorrhizal spores as biofertilizer; and (3) application of endomycorrhizal spores as biofertilizer on *Canavalia ensiformis*. A randomized block design used with various combinations of endomycorrhizal as treatments, namely: without endomycorrhizal (A0); a mixture of Acaulospora and Glomus endomycorrhizal (A1); a mixture of Acaulospora and Gigaspora endomycorrhizal (A2), and a mixture of Glomus and Gigaspora endomycorrhizal (A3). The research results can be concluded that the first and second stages identified three types of endomycorrhizal spores, namely Acaulospora, Glomus, and Gigaspora, and had the potential to be active biofertilizer ingredients. However, the species of the three endomycorrhizal species were not identified at this stage. In the third stage of the study, it was shown that a mixture of Acaulospora and Glomus endomycorrhizae could infect the root tissue of *Canavalia ensiformis* by up to 66%. In addition, this combination of endomycorrhizae increased root volume (7 ml^{-3}), leaf area index (<1), and *Canavalia ensiformis* biomass (60 grams). However, this study could not identify which endomycorrhizal genus had a complete structure in *Canavalia ensiformis* root tissue. The use of endomycorrhizal spores as a biofertilizer offers significant practical value because they will continue to develop in plant roots and are environmentally friendly. The use of endomycorrhizal spores isolated from chromium-contaminated areas is a unique feature of this study, which had a significant effect on the test plants.

Keywords: assimilation, heavy metals, infection, photosynthesis, symbiosis.

INTRODUCTION

Nuts are a potential source of nutrients such as protein, minerals, vitamin B, complex carbohydrates, and dietary fiber (Bonku and Yu, 2020; Markiewicz-Zukowska et al., 2022). Furthermore, nuts contain several phenolic and polyphenolic compounds, which have antioxidant effects and are highly beneficial for health (Goncalves et al., 2023; Wojdyło et al., 2022). Nuts' health

benefits include maintaining heart health, controlling blood sugar, improving digestion, weight loss, bone health, preventing anemia, and improving eye and brain health (Gervasi et al., 2021; Gonçalves et al., 2023).

Jack bean or *Canavalia ensiformis* is a tropical legume whose seeds are rich in protein (23.8–30.9%), carbohydrates (44.2–56.9%), and fat (1.0–4.09%) (Darini, 2021; Khamidah et al., 2025). This plant is easy to cultivate, can grow in less fertile land, is drought-resistant, and has been developed in Indonesia (Kanetro et al., 2021; Purwandari et al., 2023). *Canavalia ensiformis* has a productivity of up to 3.9-6.0 tons per hectare (Darini, 2021; Rizal et al., 2024). Furthermore, *Canavalia ensiformis* are adaptable, allowing them to be grown on a wide range of soils. However, in its development, farmers have not yet optimally utilized biofertilizers or organic fertilizers to increase the efficiency of water and nutrient absorption for plant growth.

Biofertilizer is a fertilizer that uses microorganisms as active ingredients or main ingredients that play an essential role in increasing the yield and quality of plant production, because microorganisms, as inoculants, can help provide specific nutrients for plants (dos Reis et al., 2024; Yap and Al-Mutairi, 2023). Several researchers have revealed that the application of biofertilizers can increase the growth and production of *Oryza sativa* L (Mthiyane et al., 2024; Sakpirom et al., 2021), *Zea mays* (Gao et al., 2020; Wang et al., 2025), *Glycine max* (Amante et al., 2024; Sadafzadeh et al., 2023), *Brassica juncea* (Bibi et al., 2024; Geremew et al., 2021), and *Spinacia oleracea* (Safdar et al., 2022; Tshikalange et al., 2022). The use of biofertilizers in today's era needs to be introduced and encouraged to farmers based on the awareness that biofertilizers have an essential role in maintaining the environment (Abdo et al., 2022; Wang et al., 2025), changing the composition of microorganisms in the soil (Samantaray et al., 2024; Yang et al., 2022), improving soil quality (Du et al., 2022; Kumar et al., 2022), and increasing the number of microorganisms that are beneficial to plants (Khosravi et al., 2024; Miranda et al., 2024), such as endomycorrhizal fungi. However, these researchers have not yet revealed the role of biofertilizer in the growth of *Canavalia ensiformis*, which may exhibit a different phenomenon from other plants, making it interesting from a physiological perspective.

Endomycorrhizal fungi or arbuscular mycorrhizal (AM) fungi are a group of microorganisms from the Glomeromycota phylum that can form a mutualistic symbiosis with almost 90% of plant roots (Rodino et al., 2025; Zhu et al., 2022), so that they can act as biological fertilizers. AM biofertilizers can help in the process of fertilization efficiency (Navarro and Morte, 2024; Perez-Bernal et al., 2025), intensify root function (Kakouridis et al., 2022; Shen et al., 2023), and increase the efficiency of nutrient absorption (Farhaoui et al., 2025; Han et al., 2025). AM can increase the growth and production of *Zea mays* (Rustikawati et al., 2022; Sun et al., 2022), *Phaseolus vulgaris* (Razakatiana et al., 2020; Rodiño et al., 2025), *Ananas comosus* (Moreira et al., 2019; Trejo et al., 2021), *Aloe vera* (Khajeeyan et al., 2022, 2024), *Amaranthus* sp (Li et al., 2024; Malhi et al., 2021), *Eleutherine palmifolia* (Atikah and Purwanti, 2023), and *Allium cepa* (El-Sherbeny et al., 2022; Shafiq et al., 2023). Wahab et al. (2023), Chowdhary and Songachan, (2025), and Xu et al. (2024), explain that mycorrhizal spores infect plant roots using long fibers called hyphae to absorb water and nutrients for the plant, thereby enhancing the growth and development of the host plant.

The AM spores can be found in almost all types of soil (Mahulette et al., 2021; Sefrila et al., 2021), including soil contaminated with heavy metals such as lead (Pb) and chromium (Cr), where plants undergo growth processes that are utilized by mycorrhizal spores as host plants (García-Sanchez et al., 2021; Hu et al., 2024; Ren et al., 2022). Several studies have explored AM spores in the rhizosphere of *Elaeis guineensis* (Rini et al., 2021), *Saccharum officinarum* (Juntahum et al., 2022) and *Oryza sativa* (Martins and Rodrigues, 2019) in uncontaminated land. The use of AM that have adapted to heavy-metal-contaminated environments is a biological asset of higher quality that encourages AM to expand their adaptive capabilities in marginal areas. So, a study is needed to explore and identify endomycorrhizal spores in chrome-contaminated areas as an active ingredient in a biofertilizer applied to *Canavalia ensiformis*. The important point of this research is that biofertilizer containing AM spores resistant to heavy metals possibly acting as a filter for plants, preventing the accumulation of heavy metals in the aboveground parts of plants and preventing interference with metabolic processes.

RESEARCH METHODS

This research was conducted in three stages at three different research sites. The first stage includes rhizosphere sampling in the chrome metal contaminated area at coordinates 2°53'20"S 121°37'31"E (Figure 1a). The second stage exploration, identification, isolation and culture of AM single spore at the Microbiology Laboratory of the Makassar Environmental and Forestry Research and Development Center (now the Makassar Environmental and Forestry Instrument Standards Implementation Center) at coordinates 5°08'09.5"S 119°29'09.6"E (Figure 1b). The third stage involved the application of biofertilizer containing arbuscular mycorrhizal active ingredients in the Nursery area of PT. Vale Indonesia Tbk, Sorowako Village, East Luwu Regency, South Sulawesi, at coordinates 2°34'05.1"S 121°22'46.6" E (Figure 1c). Based on the Koppen-Geiger classification, Sorowako's climate is Af (tropical rainforest climate) with an average annual temperature of 24.5 °C and an average yearly rainfall of 2019 mm.

Rhizosphere soil sampling

Rhizosphere soil sampling, was carried out using a survey method with a purposive sampling technique on 5 types of host plants that grow naturally in chromium-contaminated areas, namely: *Polypodium glycyrrhiza, Morus alba, Cyperus rotundus, Spathoglottis plicata,* and *Wedwlia trilobutu*. Rhizosphere soil was taken from the entire soil surface of each host plant to a depth of ± 10–20 cm with a distance of ± 10–15 cm from the base of the host plant, as much as 3–5 kg. After that, the soil was put into plastic and labeled. (Figure 2a).

Trapping culture

Trapping culture. The medium used was sterile fine black sand. This medium was placed up to 600–700 g (7–10 cm high) in plastic cups with a diameter of 9.5 cm and a height of 15 cm as the first layer. Next, 200–250 g of rhizosphere soil samples from the field were placed on top of the first layer (1–1.5 cm high) as the second layer. After that, 1–2 soaked host plant seeds (*Zea mays*) were planted on the rhizosphere soil and covered again with 200–250 g of sterile black sand (1–1.5 cm high) as the third layer. Trapping culture was carried out for 3 months in the maintenance room. Each type of host plant from the field was replicated 10 times, so that the number of plastic cups used was 50 units. For plant maintenance, nitrogen fertilizer (urea) at a concentration of 2 g per liter is applied weekly, 20–25 ml per plastic pot from week 2 to week 6 after planting. NPK fertilizer at a dose of 0.3–0.5 g per plastic pot is applied when the plants are 6 weeks old. Additionally, the plants are watered daily with sterile water (Figure 2b).

Isolation, identification, and density of AM spores

Isolation and identification of AM spores. After the trapping process, AM spores were isolated using the wet pour-sieve method (Walker et al., 1982), to Paciono, 1992) using a graduated sieve and then centrifuged (Mahulette et al., 2021; Sherashiya and Sahay, 2025). AM spores were then observed using a digital microscope, and spores in good condition (round, intact, and fresh) were selected. AM identification was carried out based on spore characteristics such as color, shape, the presence or absence of spore ornamentation (bulbosa, saccule, additional cells, germination shield)

Figure 1. Location of rhizosphere soil taking of host plants on area contaminated chrome (a); room of microbiology laboratory of the makassar environmental and forestry research and development center (b) and nursery area of pt. vale indonesia tbk, sorowako village, east luwu regency, south sulawesi (c)

(Al-Hinai et al., 2025; Firdu and Dida, 2024; Mausse-Sitoe and Dames, 2024), and reaction to Melzer's solution using a digital microscope at 4 × 10 magnification. Identified spores were grouped according to their morphology (https://invam.ku.edu) (Figure 2c). Spore density is known based on the number of spores in 100 g of soil, which is calculated based on the following formula:

$$Spore\ density\ (\%) = \frac{Number\ of\ spores\ (\text{unit})}{Weight\ of\ soil\ analised\ (\text{g})} \times 100 \quad (1)$$

Making single spore cultures

Single spores cultures are made using small culture pots in the form of clear plastic cups. Then, the plastic pots are filled with zeolite, sand, and biochar (1:1:1) until full and sufficiently dense. Previously, the zeolite, sand, and biochar were sterilized by autoclaving to kill any pathogens or nematodes that could damage the culture. The isolated MA spores from the trapping culture are collected in a watch glass and separated by genus. The imbibed corn seeds are placed on white paper or tissue paper, and then the spores are removed with tweezers and placed on the seeds. Each seed is inoculated with only one spore. The seeds inoculated with AM spores are transferred to plastic pots and then labeled. The pots are then placed in the maintenance room. The cultures are maintained for 6 months, depending on the sporulation process. The development of sporulation in each culture is observed weekly, starting in the second week after culture creation. If sufficient spores have formed, subcultures are carried out into larger pots (Figure 2d).

Spore multiplication from a single spore culture

Single spore cultures that have produced sufficient spores are immediately sub-cultured to increase the number of spores formed. The subculture technique is carried out by directly planting propagoul from single spore plastic pots into a plastic culture tank that has been filled with a mixture of zeolite, sand, and biochar (1:1:1) as much as one-third of the volume of the plastic tank (layer 1), then insert the propagules from the single spore culture and place corn seeds on top (layer 2), finally cover with the media mixture until full (layer 3). These cultures are maintained in the maintenance room for 3–4 months. The maintenance process involves watering and applying NPK fertilizer at 1–2 g per plastic culture tank every week. The results of the culture harvest are used for testing the combination of AM spore types in subsequent experiments. (Figure 2e).

This research is a quantitative study conducted as a field experiment using polybags. The experimental design used was a randomized block design with arbuscular mycorrhizal treatments, namely:

- A0: Without arbuscular mycorrhizae (WM).
- A1: Mixed mycorrhizae of *Acaulospora* sp (M Aca) and *Glomus* sp (M Glo).
- A2: Mixed mycorrhizae of *Acaulospora* sp (M Aca) and *Gigaspora* sp (M Gig).
- A3: Mixed mycorrhizae of *Glomus* sp (M Glo) and *Gigaspora* sp (M Gig).

The parameters observed included the abundance of AM spores, AM spore morphotype, percentage of roots infected with AM, nutrient content in plant tissue, and plant dry weight.

Mycorrhizal root infections

Canavalia ensiformis roots approximately 50 cm long were stained using the Rajapakse and Miller method (Delpiano et al., 2024; Lin et al., 2021; Thind et al., 2022). The roots were soaked in 10% (w/v) KOH solution for 24 hours, then the roots were washed under running water to remove any remaining KOH. The roots were then placed in 1% (v/v) HCl solution for 10 minutes. The roots were then soaked in trypan blue solution for 24 hours. Root preparations were made by arranging 10 pieces of 1 cm roots on the preparation and covering with a cover glass, then observed using a microscope. Calculation of mycorrhizal root infection was done by counting the number of mycorrhizal infected roots (there is one sign of hyphae/mycelia/spores) in 1 preparation. Mycorrhizal root infection was calculated using the formula:

$$Root\ infection\ (\%) = \frac{Number\ of\ infected\ roots}{Total\ number\ of\ roots} \times 100 \quad (2)$$

Root volume

Root volume (Va) is measured using the water displacement method (Chakari et al., 2024; Rejeth et al., 2020). Root volume measurements were taken at the end of the observation period. Root volume was measured by thoroughly washing the roots, cutting them, placing them in a measuring

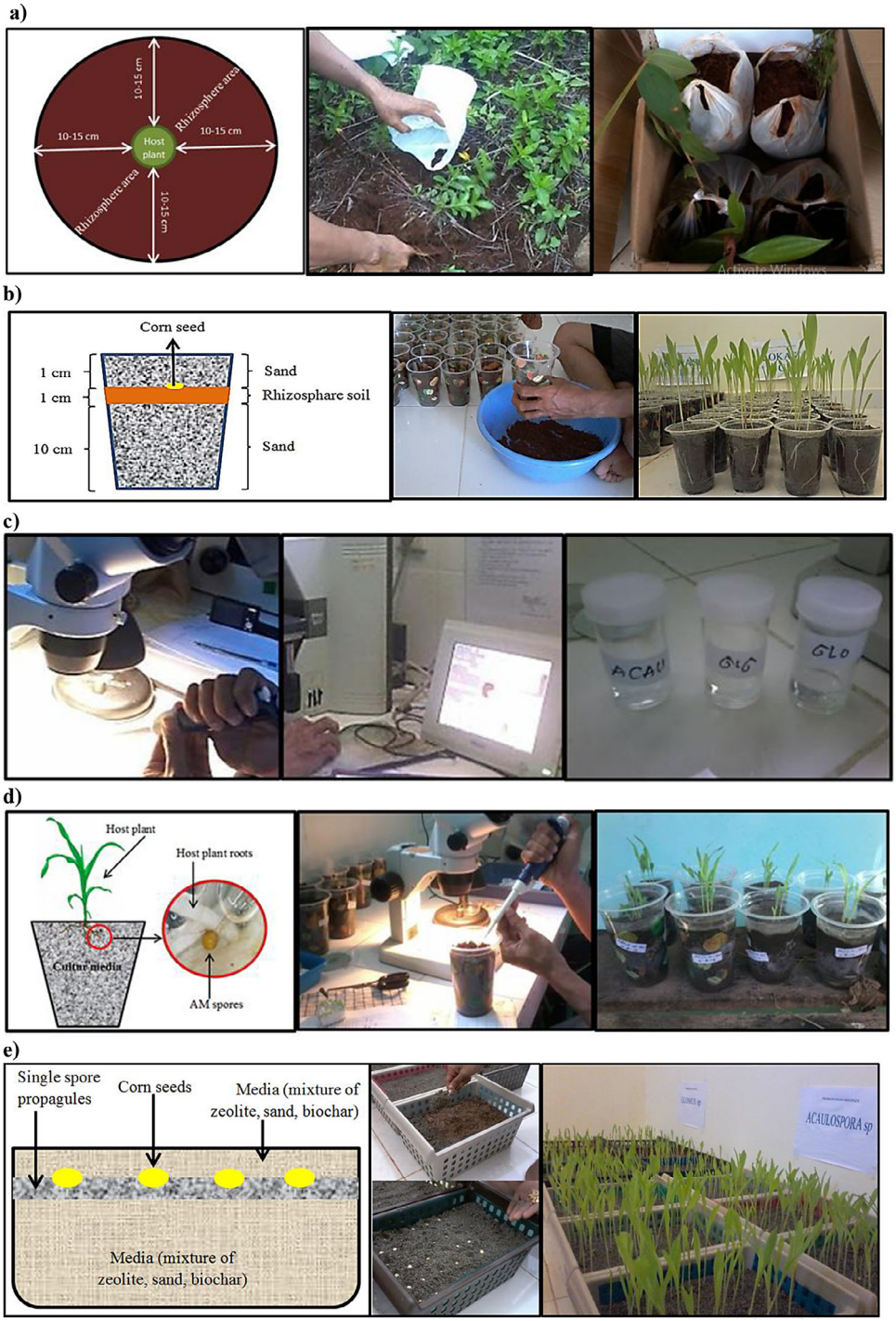

Figure 2. Layout of rhizosphere sampling (a), arrangement of AM trapping culture media (b), isolation and identification of AM spores (c), culture of AM single spore on the host plant (*Zea mays*), and (d) Spore multiplication from a single spore culture

cup, and observing the difference in water volume between the initial water volume and the root volume and can be calculated using the formula:

$$Va = V2 - V1 \quad (3)$$

where: $V2$ is the volume of water after the roots are inserted, and $V1$ is the volume of water before the roots are inserted.

Leaf area index

Leaf area index (LAI) is measured using the gravimetric method (Brant et al., 2025; Panigrahi and Das, 2021). The following are the steps of the gravimetric methods – use leaf patterns (leaf replicas) drawn on plain paper. Weigh the leaf

replicas using an analytical balance. Cut a 10 × 10 cm strip of paper and weigh it; calculate the leaf area using the formula:

$$LAI = Leaf\ area / Ground\ area \quad (4)$$

where: *LAI* (leaf area index) is the leaf area index. *Leaf area* – is the total green leaf surface area of one side (e.g., in square meters or square centimeters). *Ground area* – is the area of ground covered by the plant (e.g., in square meters or square centimeters).

Plant biomass

Plant biomass is measured using destructive methods (cutting and weighing) (Schettini et al., 2022; Zhao et al., 2024). Dry the sample in an oven at a specific temperature (e.g., 70 °C or 85 °C) until the weight is constant (dry weight). Weigh the dry weight of each plant part.

RESULTS AND DISCUSSION

The AM spore exploration in this study was conducted on five host plants that predominantly grow naturally in lead-contaminated areas (Table 1), and three genera of AM spores were found in varying numbers (Table 2). The most abundant spores, respectively, were *Acaulospora* sp (M Aca), *Glomus* sp (M Glo), and *Gigaspora* sp (M Gig). This phenomenon shows that M Aca. may have a wide level of adaptation, so that heavy metal contamination does not affect its life cycle. According to Marzban and Tesei, (2025), Vimal et al. (2024), and Kochhar et al. (2022), organisms that have broad adaptability enable these organisms to survive and develop well. This study also shows that the three AM genera have different spore morphotypes in addition to the number of spores (Figure 3). In the M Glo, subtending hyphae (SH) ornaments were found and have two layers of spore cell walls

Table 1. Chemical properties of soil contaminated with Chrome (Cr).

No	Chemical properties	Point	Rating
1	pH	5.69	Slightly acidic*
2	OM (%)	1.88	Low*
3	CEC (cmol (+)kg^{-1})	9.63	Low*
4	Ca (cmol (+)kg^{-1})	2.26	Low*
5	Mg (cmol (+)kg^{-1})	3.96	High*
6	K (cmol (+)kg^{-1})	0.27	Low*
7	Na (cmol (+)kg^{-1})	0.12	Low*
8	KB (%)	69	High*
9	Cr (ppm)	26.458	Polluted**

Note: *Soil and Fertilizer Instrument Standard Testing Center, Indonesia, 2023; **Ministry of State for Population and Environment Republic of Indonesia and Dalhousie University Canada. 1992.

(CW) (Figure 3a), likewise in the M Gig, subtending hyphae ornaments and two layers of spore cell walls were also found (Figure 3b). However, in the M Aca, subtending hyphae ornaments were not found, but three layers of spore cell walls and a saccule (SC) were found (Figure 3c). According to Iqbal et al. (2023), Chung et al. (2025), and Lensch et al., (2024), the morphotype or morphology of an organism is largely determined by genetic factors and supported by environmental factors.

Root infection

The mycorrhizal mixture had a significant effect on Canavalia root infection, and each mycorrhizal mixture showed different levels of infection (Figure 4a). The level of Canavalia root infection by the M Aca and M Glo mixture reached 66% at 15 DAP, according to O'Connor et al. (2001) and Tian et al. (2006) this infection rate (66%) is classified as high. The AM structures in Canavalia root tissue include internal hyphae, arbuscules, vesicles, and spores (Figure 4b). Arbuscules play an essential role in the symbiosis between mycorrhizae and

Table 2. Abundance of AM spores in chrome (Cr) contaminated areas

No	Host plant	Number of AM spora (%)		
		M Glo	M Gig	M Aca
1	*Polypodium glycyrrhiza*	18.18	4.55	77.27
2	*Morul alba*	12.50	16.67	70.83
3	*Cyperus rotundus*	19.05	9.52	71.43
4	*Spathoglottis plicata*	9.09	27.27	63.64
5	*Wedwlia trilobutu*	11.11	44.44	44.44

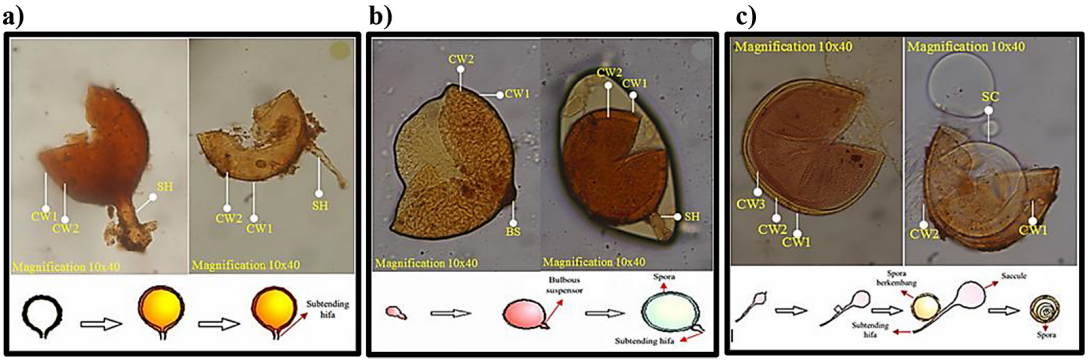

Figure 3. Morphotypes of mycorrhizae *Glomus* sp. (a), *Gigaspora* sp. (b) and *Acaulospora* sp. (c) (SH is subtending hypha, CW is spore cell wall, SC is saccule)

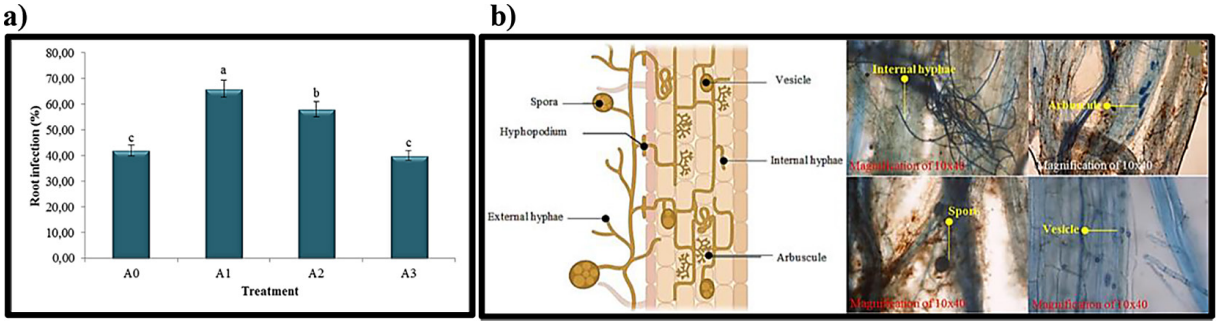

Figure 4. Infection level (a) and MA structure (b) in the root tissue of *Canavalia ensiformis* at 15 DAP (A0; without mycorrhiza, A1; M Aca and M Glo, A2; M Aca and M Gig; A4; M Glo and M Gig). The same letter above the bar indicates that the treatments are not significantly different at the 5% level

plants. According to Farhaoui et al. (2025), Wang et al. (2023), and Khan et al. (2025), arbuscules play a role in energy exchange between mycorrhizae and plants. Farhaoui et al. (2025), Wang et al. (2024), Kuila & Ghosh, (2022), Hawkins et al. (2023), and Rosita et al. (2024) added that in addition to arbuscules, other structures support the development of AM spores, namely, hyphae play a role in the absorption of water and mineral nutrients, vesicles play a role in the accumulation of carbohydrates, proteins, and fats, and spores play a role in the survival of the AM species.

Root volume, leaf area index, and plant biomass

The AM mixtures significantly affected the root volume of the *Canavalia ensiformis*. At 15 days after planting (DAP), the effects of the M Aca and M Glo mixtures were superior to those of the MA mixtures alone and the other AM mixtures. However, at 30 and 45 days after planting (DAP), all AM mixtures positively affected the root volume of the *Canavalia ensiformis* (Figure 5a). This suggests that all AM spores had

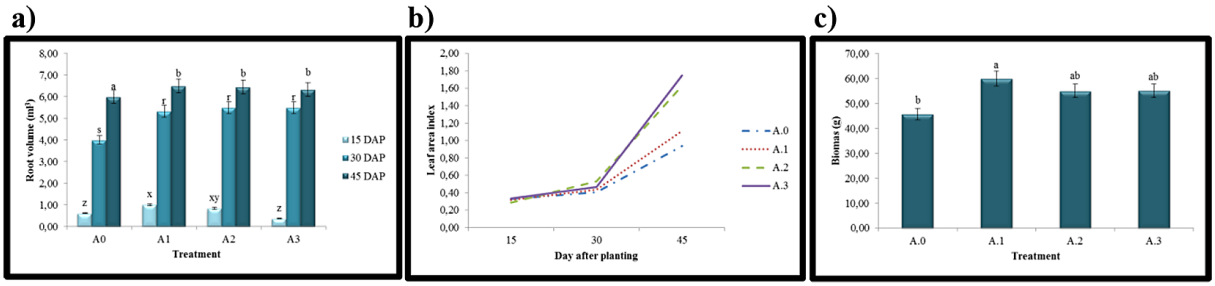

Figure 5. Root volume (a), leaf area index (b), and biomass (c) *Canavalia ensiformis* in MA combination treatments (A0; without mycorrhiza, A1; M Aca and M Glo, A2; M Aca and M Gig; A4; M Glo and M Gig). The same letter above the bar indicates that the treatments are not significantly different at the 5% level

adapted and established a mutualistic symbiosis with *Canavalia ensiformis*, the new host plant. According to Mausse-Sitoe and Dames, (2024), Ghorui et al. (2024), and Khaliq et al. (2022), AM spores can form symbiotic relationships with different plants. Therefore, if a plant is infected with a new host plant, the AM spores will need time to associate and develop on that host.

Leaf area development at 15 and 30 DAP was relatively similar. Still, at 45 DAP, all AM combinations encouraged *Canavalia ensiformis* leaf growth to achieve a leaf area index (LAI) of high (>1), and this suggests that plants with a LAI>1 will cause their leaves to shade each other (Figure 5b). According to Sales et al. (2023), Nowak et al. (2024), and Zhang et al. (2022), plants with a large LAI with a horizontal leaf position will cause the leaves to be ineffective in photosynthesis due to the shading between the leaves. This is likely related to the large root volume at 45 DAP, which causes the plant's water and nutrient absorption to be too active. The amount of *Canavalia ensiformis* biomass applied with the AM mixture showed a significant effect. JB receiving a mixture of M Aca and M Glo produced heavier biomass, followed by those without AM and receiving other AM mixtures (Figure 5c). This is likely related to the phenomenon of shading between *Canavalia ensiformis* leaves. Shaded leaves will become unproductive in producing assimilates due to inhibited photosynthesis, so that shaded leaves become parasitic to the plant itself, causing reduced assimilate accumulation and consequently low biomass. According to Collison et al. (2020), Eskandarzade et al. (2023), and Yang et al., (2020) leaves become unproductive in photosynthesis due to the occurrence of shading.

CONCLUSIONS

Acaulospora sp. spores were mostly found in chromium-contaminated areas. The application of *Acaulospora* sp. and *Glomus* sp. endomycorrhizae positively affected root volume, leaf area index, and plant biomass due to their infectious activity in the root tissue of *Canavalia ensiformis*. Morphological and molecular identification of the three endomycorrhizal genera found is urgently needed to increase endomycorrhizal diversity, as an interesting project for future research.

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