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Production of Arbuscular Mycorrhizal Fungi using *In vitro* Root Organ Culture and Phenolic Compounds

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Abstract

Arbuscular mycorrhizal fungi form obligate symbiotic associations with most of plant families. This limits their *in vitro* culturing and large-scale production. *In vitro* root organ culture is very useful for studying these symbiotic relationships and for mass production of arbuscular mycorrhizal fungal inoculants. This research aimed to study the effect of different phenolic compounds on the growth of the arbuscular mycorrhizal fungus *Gigaspora gigantea* using *in vitro* tomato root organ culture. Eight phenolic compounds were used against control without phenolic compounds. The phenolic compounds used in this research were cinnamic acid, catechin anhydrous, protocatechuic acid, ferulic acid, tannic acid, coumarin, esculetin and catechol. The experiments were done at two different pHs (5.7 and 6.5) in both solid and liquid media. Phenolic compounds exhibited different effects including stimulatory, inhibitory or no effects. Catechin anhydrous (which is a flavonoid compound) showed the most significant increase in both mycorrhizal root colonization and arbuscular abundance with moderate growth of root hairs at pH 6.5. Tannic acid inhibited the growth of root hairs, mycorrhizal colonization and formation of arbuscules at pH 5.7. Solid media were superior to liquid media in both mycorrhizal colonization and arbuscular formation.

Keywords: Arbuscular mycorrhizal fungi, *Gigaspora gigantea*, Phenolic compounds, *In vitro* root organ culture, Symbiosis, Transformation.

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are a very important component occurring in the rhizosphere[1]. They form an obligate symbiotic association with the roots of 90% of the terrestrial plants species[2,3]. This means that each step of their life cycle requires the association with a living plant.

AMF can stimulate plant growth by increasing nutrients uptake (particularly immobile elements like P, Zn and Cu), enhancing growth regulating materials, improving osmotic adjustment under drought and salinity stresses, stimulating photosynthesis and increasing plant resistance against pathogens[4-8]. The extensive extra radical mycelial networks of mycorrhizal fungi aid plants to obtain nutrients and water from soils which plant roots cannot reach[2]. AMF also reduce the applied quantities of fertilizers to soils[9].

The pot culture is the common method of vesicular-arbuscular mycorrhizal (VAM) fungal inoculum production. There are many problems with pot culture. Regulation of some conditions such as pH and mineral levels is cost and hard. These things can inhibit VAM colonization or sporulation reducing the quality of an inoculum[10].

The obligatory symbiotic nature of AMF limits the large-scale production of mycorrhizal inoculums to satisfy the farmer needs which force us to make another strategy for more aseptic production of arbuscular mycorrhizal propagules[11].

The *in vitro* root organ culture establishment has large potential in understanding the symbiosis of AMF and the production of monoxenic inoculum[12]. Cultivation of VAM fungi in transgenic hairy roots is a powerful tool to study its biology and this method can be used for the mass production of VAM fungal monoxenic inocula[13].

The use of excised roots as host partner in AMF symbiosis was first introduced by Mosse and Hepper[14]. They established monoxenic culture of *Glomus mosseae* in hairy roots initiated through inoculation with *Agrobacterium rhizogenes*. Hairy roots, which are biochemically and genetically stable, are able to grow in hormone-free media. Transformation of roots by the soil-borne bacterium *Agrobacterium rhizogenes* provided a novel way for mass production of roots in a very short time with using relative poor substrates[11]. *Agrobacterium rhizogenes* carries a large plasmid containing transfer DNA (T-DNA). When contact with host plant, it inserts copies of T-DNA using its own DNA delivery mechanism[15]. The inserted DNA fragment carries genes for growth hormones and the transformed roots have a rapid, strong and homogenous root growth. Another important feature of the transformed hairy roots is the negative geotropism which facilities contacts with AMF hyphae[16,17].

Phenolic compounds are a big class of plant secondary metabolites, displaying a variety of structures, from simple structures (such as phenolic acids) through polyphenols (as flavonoids which comprise many groups) to polymeric compounds (depend on these different classes)[18]. Phenolic compounds, particularly flavonoids, enhance spore germination, mycelial growth and ramification of numerous AMF[19,20], in addition to the stimulation of colonization of roots[21].

The formation of the arbuscular mycorrhizal symbiosis is a result of a complex signals exchange between AMF and hosts. Flavonoids are secondary compounds present in root exudates. They have been reported as important signalling compounds for plant microbe interactions in the soil. These interactions such as plant interactions with nitrogen fixing rhizobia and arbuscular mycorrhizal fungi[22]. An obvious effect of some flavonoids on the pre symbiotic AMF development has been stated[23-25]. The flavonol morin and the flavones luteolin and chrysin display a stimulatory effect on the pre symbiotic hyphal growth of numerous *Gigaspora* and *Glomus* species, while the flavonols isorhamnetin, rutin, and kaempferol enhance hyphal growth of *Gigaspora* but not *Glomus* species[26].

The main purpose of this study was to provide a simple, inexpensive, contamination-free, continuous and effective method for the *in vitro* mass production of *G. gigantea* propagules (spores and hyphae). This was by using *in vitro* tomato root organ culture with exogenous application of phenolic compounds.

MATERIALS AND METHODS

Establishment of pot culture

Gigaspora gigantea (T.H. Nicolson & Gerd.) Gerd. & Trappe was isolated from soil,

identified morphologically and propagated in greenhouse pot culture with tomato as host. Tomato plant (*Solanum lycopersicum* L.) were cultivated in plastic pots containing 1L of a sterelized mixture of sand and clay soils (1:1 v/v), with pH 4.8–5.2, modified with 5.5 g/Kg of rock phosphate and fertilized occasionally with 1/10 strength nutrient solution without P[27]. After growth for 4 month, the contents of the pot were desiccated and roots were detached. The pot culture was kept at refrigerator till demanded[28].

Isolation of spores from root-soil mixtures

Spores were isolated from pot culture by wet sieving and decanting method[29].

Spore sterilization

Isolated spores were surface sterilized by addition of ethanol (98%) for 10 minutes, then rinse with sterile distilled water. After that, calcium hypochlorite (2%) was added for 2 minutes, then rinse with sterile distilled water 3 times. Chloramin T (2%) and tween 20 (2 drops) were then added for 10 minutes followed by rinsing with sterile distilled water. Antibiotic solutions were then added in two steps. Streptomycin sulphate (0.02%) was added firstly then gentamicin sulphate (0.01%) by using syringe filter. Spores were put in fresh sterile petriplate with fresh antibiotics. After surface sterilization, spores were transferred with a micropipette to modified Strullu-Romand (MSR) plates. Medium was solidified with 5 g/L phytagel and was used as a growing medium (pH 5.5). Plates were incubated at 27°C in the dark[30].

Establishment of *in vitro* aseptic cultures of tomato plants

Tomato seeds were surface sterilized by treatment with a solution of 70% (v/v) ethanol for 3 minutes, followed by 20% (v/v) commercial Clorox solution for 10 minutes. The sterilized seeds were rinsed several times with sterile distilled water. By using a Laminar Flow Cabinet sterilized by UV lamp, the sterilized seeds were aseptically transferred and germinated on Murashige and Skooge (MS) basal solid medium[31] which consists of 4.5 g/L MS salts supplemented with 30 g/L sucrose. Cultured seeds were incubated in a growth chamber at 25°C and 16 hours photoperiod (white fluorescent light). Leaves of 1-1.5 month old of tomato seedlings were used as a source of explants for transformation.

Bacterial inoculum

A4 and R1000 strains of *Agrobacterium rhizogenes* were used for transformation. To screen and select the required colony, bacteria from glycerol stock culture were grown on Luria-Bertani (LB) solid medium [yeast extract (5g/L), peptone (10g/L), NaCl (10g/L); pH 7.0] contains 50mg/L kanamycin. One colony was incubated in 20 ml LB medium on rotary shaker (130 rpm) for 12-16 h at 28°C[17]. Centrifugation was done at 4°C, 4200 rpm for 15 min to harvest bacterial cells. Pellets were resuspended in 20 ml liquid MS medium and were used to infect tomato explants.

Transformation and establishment of hairy root cultures

Explants of 1cm^2 pieces were cut aseptically from leaves of 1-1.5 month old tomato seedlings and were immersed for 30 minutes in 20 ml bacterial suspension. Explants were dried with sterile filter paper and transferred on MS plates without hormones. Plates were incubated in complete darkness at 25°C. Hairy roots (4-6 cm in length) were removed from explants and transferred onto fresh MS plates[11].

Effect of phenolic compounds on production of AM fungal propagules

G. gigantea spore suspension was used to inoculate transformed roots of tomato in MSR medium to test their ability to produce new colonies and spores. Eight treatments were used consisting of filter sterilized phenolic compounds at concentration of 10 µM in methanol, added in both liquid and solid media then mixed well, against a control free from phenolic compounds. The phenolic compounds used in these treatments were cinnamic acid, catechin anhydrous, protocatechuic acid, ferulic acid, tannic acid, coumarin, esculetin and catechol. Each experiment was divided into two subsets with two different pHs, 5.7 and 6.5 (that denoted in some literatures the best ones to spore differentiation). Each treatment was contained three replicates. After 3 weeks of incubation at 27°C, the spore numbers were recorded by taking a 0.5 ml of liquid media and counting the spores from each treatment.

Staining of roots by trypan blue

The staining was done according modified Philips and Hayman method[32]. Roots were collected, carefully washed, and macerated in 10%

KOH. Then they were rinsed in water. The roots were then acidified in 5% lactic acid for 24 hours. Roots were then stained with 0.03% trypan blue in lactic acid: glycerol: H_2O (1:1:1 v/v/v). Roots were cut into pieces of 1 cm. They were covered with mixture of lactic acid: glycerol (1:5 v/v)[33].

Estimation of AMF colonization

The microscopic estimation of mycorrhizal colonization of roots was done according to Trouvelot *et al.* method[34]. Thirty root segments were chosen randomly from the stained samples, put on two microscopic slides (15 segments/slide), and carefully crushed with the cover glass. These segments were observed under stereomicroscope for the presence or absence of AMF functional structures (mycelium and arbuscules). The degree of mycorrhizal colonization of each root segment and the abundance of arbuscules were estimated.

The results of the mycorrhizal colonization and abundance of arbuscules were used to calculate the following parameters: F% (mycorrhizal frequency), M% (relative mycorrhizal intensity; for the whole sample), m% (absolute mycorrhizal intensity; for the segments in which there were indication of colonization by mycorrhizal fungi), a% (absolute abundance of arbuscules; for the segments in which arbuscules were present) and A% (relative abundance of arbuscules; for the whole sample) using the 'Mycocalc' software (http://www.dijon.inra.fr/mychintec/Mycocal prg/download.html). The previous parameters were calculated using the following equations:

$$F\% = (l_m / l_t) * 100 \quad ...(1)$$

$$M\% = (95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1) / l_t \quad ...(2)$$

$$m\% = M\% * l_t / l_m = M\% * 100 / F \quad ...(3)$$

$$a\% = (100 * m\%A3 + 50 * m\%A2 + 10 * m\%A1) /100 \quad ....(4)$$

$$A\% = a\% * 0.01\ M\% \quad ...(5)$$

Where:

l_m is the total number of root segments in which mycelium were found.

l_t is the total number of the examined segments.

n_5–n_1 is the total number of root segments in which the degree of colonization by mycorrhizal structures was 5-1.

m%A3, m%A2, m%A1 are the% of m, rated A3, A2, A1, respectively, with $m\%A3 = (95*n_5A3 + 70*n_4A3 + 30*n_3A3 + 5*n_2A3 + n_1A3) / l_m * 100 / m\%$ and the same for A2 and A1.

Statistical analysis

The results were displayed as mean ± standard deviations (mean±SD). Data were evaluated by one-way analysis of variance (ANOVAs) using SPSS statistical software. The differences between mean values were evaluated with Duncan's test at $p \leq 0.05$.

Table 1. Scoring of external appearance of root hairs growth in liquid media

Treatment	pH 6.5	pH 5.7
Cinnamic acid	4	1
Catechin anhydrous	3	3
Protocatechuic acid	2	4
Ferulic acid	3	5
Tannic acid	3	0
Coumarin	5	3
Esculetin	2	2
Catechol	3	4
Control	3	3

0: no root hairs, 1: very few root hairs, 2: few root hairs, 3: moderate root hairs, 4: more root hairs, 5: dense root hairs.

Table 2. Number of spores (spore density per ml culture) in liquid media

Treatment	pH 6.5	pH 5.7
Cinnamic acid	150^{e}±2.00	150^{e}±6.23
Catechin anhydrous	100^{d}±2.65	150^{e}±5.00
Protocatechuic acid	105^{d}±5.00	21^{a}±1.00
Ferulic acid	45^{c}±3.00	100^{d}±4.59
Tannic acid	22^{a}±2.00	33^{b}±2.65
Coumarin	34^{b}±2.65	100^{d}±5.20
Esculetin	32^{b}±2.65	55^{c}±3.61
Catechol	50^{c}±2.00	250^{f}±5.00
Control	31^{b}±4.36	31^{b}±2.65

Values are means of triplicate readings±standard deviation (mean±SD). Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

RESULTS

Effect of phenolic compounds on production of *G. gigantea* propagules and root hairs growth in liquid media

These experiments were done to test the effect of different phenolic compounds on the production of *G. gigantea* propagules and root hairs growth in liquid media under two different pHs, 5.7 and 6.7.

The data presented in Table (1) indicated that all phenolic compounds used at pH 6.5 activated root hairs growth. Coumarin was the most effective phenolic compound followed by cinnamic acid. At pH 5.7, ferulic acid activated root hairs growth with the highest rate followed by protocatechuic acid and catechol. No root hairs are formed by using tannic acid. Moderate appearance of root hairs in control treatments

As shown in Table (2), the using of different phenolic compounds at pH 5.7 resulted in higher spore density than using them at pH 6.5 except when using protocatechuic acid. Catechol was the most significant phenolic compound in increasing spores number at pH 5.7, while cinnamic acid was the most significant one at pH 6.5.

The data in Table (3) displayed the effect of different phenolic compounds on mycorrhizal colonization of tomato roots using microscopic examination of roots according to Trouvelot *et al.* method[34]. It was obvious that the effects of phenolic compounds on mycorrhizal colonization were pH-dependent, where pH 6.5 was more significant than pH 5.7. At pH 5.7, few hyphae were observed at all treatments except by using tannic acid where, no colonization was observed. At pH

Fig. 1. Treatment with catechin anhydrous at pH6.5

Fig. 2. Treatment with protocatechuic acid at pH 6.5

Table 3. Scoring of mycorrhizal colonization (0-5) in liquid media

Treatment	pH 6.5	pH 5.7
Cinnamic acid	5	1
Catechin anhydrous	5	1
Protocatechuic acid	4	1
Ferulic acid	5	1
Tannic acid	2	0
Coumarin	4	1
Esculetin	2	1
Catechol	1	1
Control	1	1

0: 0%, 1: <1%, 2: <10%, 3: <50%, 4: >50%, 5: >90%.

Table 4. Scoring of arbuscular abundance in liquid media

Traetment	pH 6.5	pH 5.7
Cinnamic acid	A1	A1
Catechin anhydrous	A3	A2
Protocatechuic acid	A2	A3
Ferulic acid	A2	A2
Tannic acid	A0	A0
Coumarin	A2	A2
Esculetin	A0	A3
Catechol	A2	A2
Control	A1	A2

A0: None, A1: few (less than 50%), A2: moderate (50-75%), A3: Abundant (75-100%)

6.5, cinnamic acid, catechin anhydrous and ferulic acid caused significant activation of hyphal growth followed by protocatechuic acid and coumarin.

As demonstrated in Table (4), abundant arbuscules were achieved by using protocatechuic acid, esculetin (at pH 5.7) and catechin anhydrous (at pH 6.5). Moderate arbuscular abundance was attained by most of phenolic compounds treatments. Few arbuscular abundance was observed by using cinnamic acid. Inhibition of arbuscular formation was observed by using tannic acid (at both pHs) and esculetin (at pH 6.5).

From the previous data, it was noticeable that the effects of phenolic compounds differed with different pHs. At pH 6.5, catechin anhydrous (a flavonoid compound) showed the most significant increase in both mycorrhizal root colonization and abundance of arbuscules with moderate root hairs growth. Tannic acid inhibited root hairs growth, mycorrhizal colonization and arbuscules formation at pH 5.7.

At pH 6.5, the mean values of mycorrhizal frequency (F%) were ranged from 13.33% to 93.33% which significantly differed by the type of phenolic compounds (Table 5). The highest significant value was detected using catechin anhydrous. At pH 5.7, the mycorrhizal frequency values were ranged significantly from 0.00% to 73.33%. The highest significant values noticed by using catechin anhydrous, protocatechuic acid and esculetin. In the case of relative mycorrhizal intensity (M%), the mean values were ranged with significant differences from 0.67% to 63.67% by using different phenolic compounds at the two tested pHs. The highest significant values were observed by using catechin anhydrous at pH 6.5 and esculetin at pH 5.7.

The absolute mycorrhizal intensity values (m%) at pH 6.5 were significantly lower than control for both cinnamic acid and esculetin. While, the other tested phenolic compounds showed no significant differences than control. At pH 5.7, the highest significant value was 91.88% with ferulic acid. The absolute abundance of arbuscules values (a%) were significantly different by using different phenolic compounds, where catechin anhydrous (at pH 6.5) and coumarin (at pHs 6.5 and 5.7) showed the highest significant values.

The mean values of relative abundance of arbuscules (A%) were ranged from 0.00% to 43.83% at pH 6.5, while were ranged from 0.00% to 60.33% at pH 5.7 which differed significantly by using different phenolic compounds. Catechin anhydrous and esculetin displayed the highest significant values at pHs 6.5 and 5.7, respectively.

At a brief description of the results shown in Table (5), it was observed that all mycorrhizal

Table 5. Parameters of mycorrhizal colonization and abundance of arbuscules in tomato roots grown in liquid media

Parameter	F%		M%		m%		a%		A%	
Treatment	pH6.5	pH5.7	pH6.5	pH5.7	pH6.5	pH5.7	pH6.5	pH5.7	pH6.5	pH5.7
Cinnamic acid	20.00^{b}	33.33^{b}	6.33^{b}	16.47^{b}	31.65^{b}	36.40^{b}	47.70^{b}	58.50^{b}	12.00^{c}	11.40^{b}
Catechin anhydrous	93.33^{h}	73.33^{e}	51.00^{f}	53.07^{ef}	54.64^{c}	72.36^{ef}	99.06^{f}	91.72^{e}	43.83^{f}	48.67^{d}
Protocatechuic acid	73.33^{g}	73.33^{e}	40.67^{e}	57.67^{f}	55.45^{c}	78.64^{fg}	85.95^{e}	93.93^{ef}	32.47^{e}	54.17^{e}
Ferulic acid	13.33^{a}	53.33^{c}	6.40^{b}	49.00^{e}	48.00^{c}	91.88^{h}	85.95^{e}	97.24^{gh}	6.34^{b}	46.67^{d}
Tannic acid	33.33^{d}	0.00^{a}	17.47^{c}	0.00^{a}	52.40^{c}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Coumarin	46.67^{e}	53.33^{c}	25.53^{d}	32.40^{c}	54.71^{c}	60.75^{d}	99.30^{f}	98.79^{h}	25.35^{d}	32.01^{c}
Esculetin	13.33^{a}	73.33^{e}	0.67^{a}	63.67^{g}	5.00^{a}	86.82^{gh}	0.00^{a}	94.76^{fg}	0.00^{a}	60.33^{f}
Catechol	53.33^{f}	60.00^{d}	26.67^{d}	38.73^{d}	50.00^{c}	64.56^{de}	61.38^{c}	82.63^{d}	16.38^{c}	32.01^{c}
Control	26.67^{c}	33.33^{b}	12.80^{c}	31.60^{c}	48.00^{c}	45.00^{c}	69.60^{d}	77.06^{c}	12.68^{c}	13.33^{b}

Values are means of triplicate readings. Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range tests.

F% (mycorrhizal frequency), M% (relative mycorrhizal intensity), m% (absolute mycorrhizal intensity), a% (absolute abundance of arbuscules) and A% (relative abundance of arbuscules).

Table 6. Parameters of mycorrhizal colonization and abundance of arbuscules in tomato roots grown on solid media at pH 6.5

Treatment	F%	M%	m%	a%	A%
Cinnamic acid	73.33^{b}	57.10^{c}	81.57^{c}	99.84^{bc}	57.01^{d}
Catechin anhydrous	53.33^{a}	48.10^{b}	72.36^{ab}	98.29^{a}	47.07^{c}
Protocatechuic acid	100.00^{c}	66.80^{d}	66.80^{a}	99.66^{bc}	66.57^{e}
Ferulic acid	80.00^{b}	57.60^{c}	72.00^{ab}	99.41^{bc}	57.26^{d}
Tannic acid	73.33^{b}	48.10^{b}	68.71^{a}	98.29^{a}	47.76^{c}
Coumarin	53.33^{a}	38.50^{a}	77.00^{bc}	99.35^{b}	38.25^{a}
Esculetin	80.00^{b}	76.60^{e}	95.00^{d}	100.00^{c}	76.00^{f}
Catechol	100.00^{c}	76.60^{e}	76.60^{bc}	99.56^{bc}	76.26^{f}
Control	53.33^{a}	38.50^{a}	71.50^{ab}	98.29^{a}	39.01^{b}

Values are means of triplicate readings. Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.
F% (mycorrhizal frequency), M% (relative mycorrhizal intensity), m% (absolute mycorrhizal intensity), a% (absolute abundance of arbuscules) and A% (relative abundance of arbuscules).

parameters (F%, M%, m%, a% and A%) obtained by using catechin anhydrous at pH 6.5 (Fig. 1) were the most significant values followed by protocatechuic acid (Fig. 2), while all these parameters showed the least significant values by using tannic acid at pH 5.7. This confirmed our previous results.

Effect of phenolic compounds on production of *G. gigantea* propagules on solid media

We observed that plates with treatments at pH 5.7 have small and weak roots, so we couldn't take them for examination. But they were more obvious with treatments at pH 6.5.

As exhibited in Table (6), the mycorrhizal frequency values (F%) varied significantly from 53.33% to 100.00% with different phenolic compounds. Protocatechuic acid and catechol achieved the highest significant values.

Similarly, the values of the other calculated parameters were significantly different by using different phenolic compounds. Esculetin and catechol attained the highest significant M% and A% values, while esculetin reached the most significant m% and a% values.

From Tables (5 and 6), it was observed, in general, that the values of the parameters of mycorrhizal colonization and the abundance of arbuscules in tomato roots grown on solid media were higher than grown in liquid media.

DISCUSSION

The moderate appearance of root hairs in control treatments might be because the mycorrhization of tomato plants had a synergetic effect on the root development. In relation to our results, several strains of *Glomus* stimulated the longitudinal growth in roots[35]. AMF modify the morphology, growth and roots number in maize, leading to more efficient root system in the uptake of minerals and water from the soil[36].

Arbuscules are brushy branched ends of AMF hyphae which grow in the mycorrhized plant root cells and mediate the metabolic exchange between the host plant and the symbiotic fungus[37,38].

As observed in results section, catechin anhydrous, which is a flavonoid compound, caused the most significant increase in both mycorrhizal root colonization and abundance of arbuscules. Strengthen this observation; Soares *et al.*[39] reported that several flavonoids enhance germination of spores, mycelia growth and colonization of roots by AMF. Quercetin increased spore germination, hyphal elongation, and hyphal branching in *Glomus etunicatum*. 4',7-dihydroxyflavone and 4',7-dihydroxyflavanone improved *Glomus etunicatum* spore germination[40]. It was suggested that flavonoids affect the

arbuscular mycorrhizal root colonization mostly through effects on entry points formation. A nearby relationship between entry points numbers and colonization degree was described. An improved number of entry points resulted in a greater degree of root colonization was detected after application of flavonoids (crysin, luteolin, morin, and rutin) to tomato plants inoculated with *Glomus* or *Gigaspora* species[41,42].

At pH 5.7, Tannic acid inhibited root hairs growth, mycorrhizal colonization and arbuscules formation. In relation to our results, Johnson *et al.*[43] informed that phenolic compounds could decrease the AMF colonization. Becard *et al.*[44] reported that both kind and concentration of phenolic compounds can affect AM fungi positively or negatively. Kaempferol, myricetin and quercetin enhanced *in vitro* spore germination and hyphal growth at a concentration 10 µM. Apigenin, biochanin-A, chrysin and hesperetin at concentrations more than 10µM inhibited *in vitro* hyphal growth in AMF spores. Fries *et al.*[45] discussed that *p*-coumaric acid, *p*-hydroxybenzoic acid and quercetin at concentrations more than 1 mM inhibited the growth and AMF colonization in clover and sorghum plants. Caffeic, ferulic and vanillic acid at 250 mg kg^{-1} soil decreased AMF colonization in roots from *Sorghum sudanese* plants[46].

The values of mycorrhizal colonization and arbuscular abundance parameters of tomato roots grown on solid media were higher than grown in liquid media. This might be because the immobilization of spores on solid media enhanced the production of spores and hyphae. Also poor aeration in liquid media might hinder AMF development.

Plant roots colonization by AMF in soilless cultures was presented in previous studies. Kowalska *et al.*[33] found that the mycorrhizal frequencies in tomato roots inoculated with AMF were 35.79 and 50.82% at 82 and 112 days after transplanting, respectively. Maboko *et al.*[47] attained 78.2% colonized tomato roots growing in coconut and 77.7% growing in sawdust. Cwala *et al.*[48] found that the colonization level of tomato roots was ranged from 14 to 25% in hydroponic cultivation.

The pH of the soil affected growth, mycorrhizal colonization, and uptake of nutrients in *Lygodium microphyllum* significantly. Mycorrhizal colonization degree in *L. microphyllum* roots was significantly low in extremely alkaline and acidic soils. Roots of soil pH ranging from 5.5 to 7.5 recorded high mycorrhizal colonization degree[49].

CONCLUSION

Application of phenolic compounds in culture media containing arbuscular mycorrhizal fungi was beneficial. The advantages of *in vitro* cultures that they can be subcultured, time after time, and yield sufficient aseptic spores. The spores are necessary inocula to serve in beneficial symbiosis in the agriculture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION

All authors have made substantial contribution to the work and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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