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Effect of arbuscular mycorrhizae (AM) inoculation on growth performance and bioactive compound production by *Andrographis paniculata* (Burm.f.) Nees

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Abstract

The present study investigates the effects of arbuscular mycorrhizal (AM) fungi, *Glomus mosseae* and *Glomus fasciculatum* on the growth and physical parameters of *Andrographis paniculata* (Burm.f.) Nees (Kalmegh). The AM fungi were inoculated individually in pure culture and in mixed culture to check the combinational effect of AM on the sample plants. The plants treated with AM fungi and respective control plants were harvested at 30, 60 and 90 days of inoculation. Growth parameters (root, shoot, dry weight, wet weight, stem girth and leaf area); physical parameters (alkaloids, flavonoids, saponins, tannins, phenols, proteins, cardiac glycosides, terpenoids and carbohydrate); microbiological parameters (Percentage of mycorrhizal infection) and biological activity (antioxidant, antimicrobial activity) were determined. The results revealed that the AM fungal inoculated plants had significantly higher biomass and growth and new phytochemicals were produced in the treated plant. The average antimicrobial and antioxidant activities were seen to increase in the AM-treated plant samples. The bioactive compounds produced in treated and control plants were characterized by High-performance liquid chromatography, Preparative high-performance liquid chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear magnetic resonance (NMR) techniques. The treated plant extract was constituted of Azulene at a higher concentration and was responsible for the bioactivity. In the control plants, Glucopyranoside was produced at higher concentration 4. This study proves that a mixture of AM fungi is more beneficial to the host plant in growth and bioactive compound production than the pure culture.

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Introduction

Traditionally medicinal plants have played a significant role in the rural and tribal people of India (Chao and Lin, 2010). Many plants having ethno pharmaceutical significance are exploited for their therapeutic properties (Akbar, 2011). *Andrographis paniculata* (Burm.f.) Nees is one such medicinal plant used in many countries (Mishra *et al*., 2007). It belongs to the Acanthaceae family which includes about 40 different plant species among which *A. paniculata* is excessively used in traditional medicine (Singha *et al*., 2003). It is an annual plant generally known as Kalmegh or Chirayetah which means "King of bitters" or the Creat (Rao *et al*., 2004). The bitterness is attributed to the presence of kalmegin and another compound called andrographolide (Puri *et al*., 1993). It is used in the Unani and Ayurveda because of its ability to purify blood and cure skin abnormalities like skin eruption, boils, scabies etc. (Kumar *et al*., 2004). Traditional usage of the plant is in the form of infusion, powder or decoction along with other medicinal plants (Rajagopal *et al*., 2003).

The clinical trial has been performed for the commercial production of the drugs from the plant. It is used as an antidote for snake bites and some insects (Dai *et al*., 2019). It is used in the treatment of influenza, dysentery, malaria dyspepsia, respiratory infection, anti-inflammatory, latent heat-clearing and detoxicant (Handa and Sharma 1990). It is also used as an antiphlogistic and analgesic agent for treating acute infections of the respiratory, urinary system and gastrointestinal tract (Li *et al*., 2007). Its occurrence is common in the plains of India, Srilanka and China (Thamlikitkul *et al*., 1991). It is commonly seen growing in the hedgerows throughout the country and sometimes it is also cultivated in the backyard owing to its medicinal properties (Jayakumar *et al*., 2013). The reports prove that the plant is a rich source of flavonoids, diterpenoids, flavonoids, and polyphenols (Coon and Ernst, 2004). The bioactivity of the plant is credited to the presence of the major compound andrographolide (Zhang and Tan, 1997). The 14-deoxy-11, 12-didehydroandrographolide (DDA), neoandrographolide an analogue of andrographolide reported to be anti-atherosclerotic, immunostimulatory and immunomodulatory (Zhang and Tan, 2000). The andrograpanin a comparatively less abundant compound in the *A. paniculata* is proved to be anti-inflammatory, anti-infective and tumour suppressive (Jarukamjorn and Nemoto, 2008). The cultivation of such medicinally important plants has a greater significance due to their tremendous potential for treating diseases with very less side effects (Kuroyanagi *et al*., 1987). Hence, they are used as raw materials for pharmaceutical products.

The growth of plants is influenced by various environmental factors in the soil (Rajani *et al*., 2000). Many different approaches have been used for the management of the soil environment for the enhancement of plant growth, yields and increased production of the biological compound (Sanjutha *et al*., 2008). One such approach to achieve improved plant growth is the application of rhizospheric microbial populations, especially Arbuscular Mycorrhizal (AM) fungi (Amroyan *et al*., 1999). They play a major role in maintaining root health, and nutrients thereby increasing the crop yield (Sheeja *et al*., 2007).

The Arbuscular Mycorrhizal (AM) fungi interact either directly with other soil microorganisms or they may indirectly influence these microorganisms that could change root morphology, physiology and patterns of exudation into the mycorrhizosphere (Chanda *et al*., 2014). They form symbiotic relationship with more than 80% of plants which includes many important medicinal plants and forest tree species (Sharma *et al*., 2008). Endomycorrhizal fungi can be found both inter and intracellular and penetrate the root cortical cells and arbuscule and vesicles known as Vesicular-arbuscular Mycorrhiza (VAM). In some cases, no vesicles are formed and they are simply known as Arbuscular Mycorrhizae (AM) (Hemashenpagam and Selvaraj, 2011). Reports showed that AM fungi play an important role in the conservation of some valuable medicinal plants (Zeng *et al*., 2013). Many studies prove that the *Glomus* sp. is widely used for the increased yield of essential medicinal plants (Quiroga *et al*., 2001). Therefore, the medicinal plant *A. paniculata* was selected as the host, inoculated with AM fungi and studied their

interaction. This work elucidates the influence of AM fungi interaction on the growth and antioxidant properties of the selected medicinal plants and antimicrobial activities of the medicinal plants extract against the test organisms.

Materials and methods

Collection of AM culture

The Arbuscular Mycorrhizae (AM) culture namely *Glomus mosseae* and *Glomus fasciculatum* were collected from the Centre for Natural Biological Resources and Community Development, Bengaluru.

Collection of host

The host medicinal plant sample *A. paniculata* (Burm.f.) Nees (15-day-old plants) were collected from the Indian Institute of Horticultural Research (IIHR), Bengaluru.

Preparation of potting mixture and polyhouse experiment

Pots were filled with 600g of sterilized soil and 200g of sterilized farmyard manure (cattle dung) in 3:1 ratio (w/w), after mixing thoroughly, the mixture was filled into pots. The AM fungi were treated in both pure culture and mixed culture (1:1) (w/w) conditions. About 10g of the inoculum was added to the substrate mixture (10g fresh weight per pot containing approximately 1000-1200 spores) and was placed in the pot at 15cm depth, immediately before planting the host plants. Each treatment was performed in triplicates and a control setup was kept for comparative studies. Plants were grown in polyhouse conditions and were watered regularly for a predetermined period of 30, 60 and 90 days (Mathew *et al.*, 2018).

Physiological parameters

The plants treated with AM fungi and the respective control plants were carefully uprooted after a predetermined period of 30, 60 and 90 days of inoculation. Different growth parameters were measured and calculated. The length of the individual shoot and root and the weight of the plants were recorded for each plant and each treatment. Shoots and roots were excised and dried. The following parameters were determined on 15 plants per treatment: average shoot and internodal length, stem girth and leaf number. The dry weight was determined after shade drying and grinding (Beltrano *et al.*, 2013).

Mycorrhizal colonization

About five root parts were randomly selected to evaluate the level of AM fungal colonization from each treatment. The frequency of roots colonized (F%), the intensity of AM fungal colonization in a root system (M%), and the abundance of arbuscules (A%) were determined according to Trouvelot *et al.*, (1986) after staining with 1% methyl blue in lactic acid (Maltz and Treseder, 2015).

Solvent extraction

The AM fungal treated and control plants were subjected to phytochemical analysis following the standard protocol. The uprooted plants were shade dried and powdered using a grinder. The powder was cold extracted using methanol in a 1:10 (w/v) ratio. The extract was reduced using a rotary evaporator Shimadzu Rotation evaporator QR 2005-S and concentrated to 100mg/ml. The above procedure was repeated for the plants harvested at 60 and 90 days (Yadav and Agarwala, 2011).

Qualitative phytochemical analysis

Qualitative phytochemical analysis for the methanol extract of the treated and control *A. paniculata* was carried out to check the presence of major phytochemical constituents namely alkaloids, flavonoids, saponins, tannins, phenols, proteins, cardiac glycosides, terpenoids and carbohydrates using the standard protocols (Santhi and Sengottuvel, 2016).

Alkaloids (Wagner's reagent)

About 2mL of the sample was treated with 4-5 drops of Wagner's reagent (1.27g of iodine in 100mL of distilled water) and observed for the formation of reddish-brown precipitate (Maatalah *et al.*, 2012).

Carbohydrates (Molisch's test)

About 2ml of the sample extract was treated with 5-6 drops of Molisch's reagent, followed by the addition of 2ml of conc. H_2SO_4 on the sides of the test tube.

The mixture was allowed to stand for 2-3 min. The formation of red or dull violet colour at the interphase of the two layers indicates positive results (Alalor *et al*., 2014).

Cardiac glycosides (Keller Kelliani's test)

About 5mL of the sample extract was treated with 2mL of glacial acetic acid in a test tube, to this a drop of ferric chloride solution was added. Further 1ml of conc. H_2SO_4 was added and checked for the presence of a brown ring at the interface (Rajsekhar *et al*., 2012).

Flavonoids (Alkaline reagent test)

About 2ml of the sample extract was treated with 4-5 drops of 20% sodium hydroxide solution. The formation of an intense yellow colour, which becomes colourless with the addition of dilute hydrochloric acid, indicates the presence of flavonoids (Bhandary *et al*., 2012).

Phenols (Ferric chloride test)

About 2ml of the plant sample extract was treated with 5% aqueous ferric chloride and observed for the formation of deep blue or black colour indicating positive results (Tamilselvi *et al*., 2012).

Proteins (Ninhydrin test)

About 2ml of the sample extract was treated with 4-5 drops of 1% ninhydrin solution (in Acetone) placed in a boiling water bath for 1-2 minutes and observed for the formation of purple colour (Nayuni *et al*., 2013).

Saponins (Foam test)

About 2mL of the plant extract was taken in a test tube to which 6mL of water was added. The mixture was shaken vigorously and observed for the formation of foam that is persistent for a few minutes which indicates the presence of saponins (El Aziz *et al*., 2019).

Tannins (Braymer's test)

About 2ml of the plant extract was treated with 10% alcoholic ferric chloride solution and observed for the formation of blue or greenish colour formation (Yadav *et al*., 2014).

Terpenoids (Salkowki's test)

About 1mL of chloroform was added to 2mL of each plant extract followed by a few drops of conc. H_2SO_4. A reddish brown precipitate produced immediately indicated the presence of terpenoids (Malik *et al*., 2017).

Quinones

About 2ml of the sample extract was treated with 5-6 drops of conc. HCl and observed for the formation of a yellow precipitate which indicates the presence of quinones (Malik *et al*., 2017).

Biological activity

Antioxidant activity by DPPH radical scavenging assay (1, 1-Diphenyl- 2- picryl hydrazyl)

The free radical scavenging activity of the plant extract was analysed by using DPPH (Maryam *et al*., 2012). The methanol extract from plant samples treated with AM for 30, 60 and 90 days and the respective uninoculated control plants were taken in triplicates in the following concentrations (100, 200, 400, 600, 800 and 1000µl) in each test tube and made up to 1ml using methanol. To each of the samples, 3ml of 0.1mM DPPH was added. The mixture was shaken well and incubated in dark for 30mins and the absorbance was measured at 517nm using an Anatech UV-V spectrophotometer. The free radical scavenging activity was calculated by the formula.

$$\text{Scavenging activity (\%)} = \{(Ac - As)/Ac\} \times 100,$$

Where, Ac is the absorbance of the control, As is the absorbance of the sample.

Antimicrobial activity by agar well diffusion method

Antimicrobial activity of the AM treated and the control plant extracts were determined by agar well diffusion method on Muller Hinton Agar (MHA) plates against the following test pathogens procured from MTCC, Chandigarh (Chaman *et al*., 2013). The pathogens namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Candida albicans*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Streptococcus mutans*, *Salmonella typhi*, *Escherichia coli* and *Enterococcus faecalis* were used as test organisms. The MHA agar plates were prepared and to each of the sterile agar media 500µL of 24hr old culture was inoculated upon which 6mm wells were punched into the agar using a sterile cork borer. To each well 80µL of the sample was added along with methanol and sterile distilled water as a positive and negative control.

The sample plates were incubated at 37°C for 24 hrs. The activity was determined by measuring the inhibition zone inmm.

Statistical analysis

The experiments were performed in triplicates under the same environmental conditions and data is presented as mean value ± standard error. Statistical analysis (analysis of variance, ANOVA) was calculated at P=0.05.

Results

Physiological parameters of AM inoculated plants

The plants treated with the AM fungi in pure and mixed culture showed several notable differences in growth parameters. When compared with the other treatments, the mixed culture of *G. mosseae* and *G. fasciculatum* brought about a notable increase in shoot length, the number of leaves, leaf area, and total biomass in *A. paniculata* (Table 1). From the following table it is noted that, AM inoculated plants showed better growth than the control plants in all the different treatments. Among the pure culture treatment, *G. mosseae* treated samples showed more branches in the root system and more shoot length than *G. fasciculatum*. After 90 days of treatment, the ratio between root and shoot weight was significantly higher in AM-treated plants compared to the control plants (Fig. 1). Root systems of mycorrhizal plants were significantly more branched than those of control plants thus proving that AM colonization significantly increased biomass, root branching and length (Fig. 2).

Mycorrhizal colonization

The inoculated plant roots were found to be colonized by AM fungi however the frequency and the intensity of the mycorrhizal colonization, as well as arbuscule abundance, varied significantly.

The mycorrhizal colonization was found higher in plants treated with the mixed culture of AM fungal inoculum than in pure culture. No AM colonization was detected in roots of control plants. An increase in the arbuscule density (% A) was noted with increase in treatment time (Table 2).

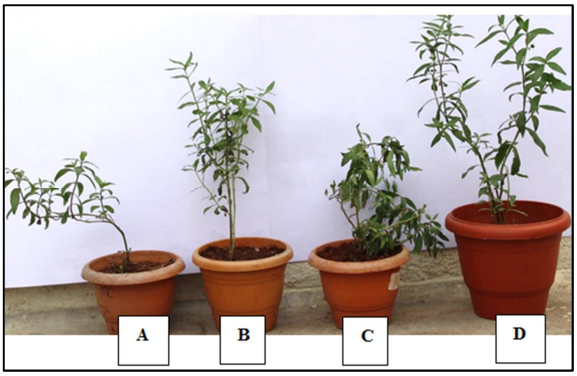

Fig. 1. Physiological parameters of A. paniculata treated with AM fungi for 90 days: **A**-Control, **B**-*G. mosseae*, **C**-*G. fasciculatum*, **D**-Mixed culture.

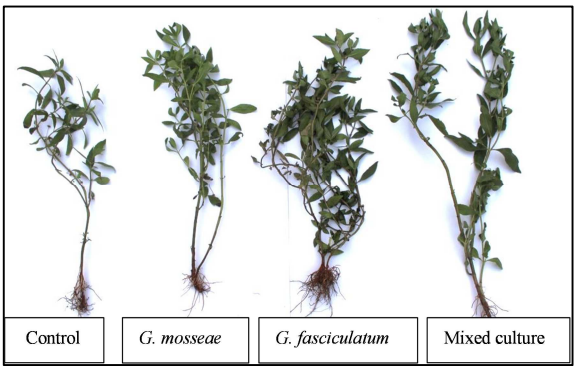

Fig. 2. Uprooted plants after 90 days of AM treatment.

Qualitative phytochemical analysis

The results of qualitative phytochemical analysis of the AM treated and control *A. paniculata* plants proved that phytochemicals like cardiac glycosides, carbohydrates, proteins, tannins and flavonoids were produced in both treated and control plants samples. The metabolites like alkaloids, quinones and phenols were absent in control plants but was produced in the treated plants. Saponins were absent only in *G. fasciculatum* treated plants. Quinones were detected only in the plants treated with the mixed culture and alkaloids was produced only in *G. mosseae* and mixed culture treated plant samples.

Biological activity

Antioxidant activity by DPPH radical scavenging assay (1, 1-Diphenyl- 2- picryl hydrazyl)

The methanol extract from AM-treated plant samples and the respective uninoculated control plants were tested for the free radical scavenging activity by using DPPH.

The results revealed that extract of AM treatment after 90 days with mixed inoculum had the highest IC_{50} value at 522.95µg/mL followed by *G. fasciculatum* with IC_{50} value at 501.96µg/mL and *G. mosseae* at 500.86 and the least at uninoculated control by IC_{50} value as 519.08µg/mL. The scavenging activity was found to increase proportionately with the increase in the concentration of the extract (Fig. 3). *Antimicrobial activity by agar well diffusion method*

The results of antimicrobial activity of methanolic extracts from control and AM treated *A. paniculata* plants are shown in Table 4. The mean inhibition zones for test pathogens treated with AM inoculated plant extracts were higher than the extracts of control plants. The activity increased from an average of 10 to 25mm when the AM inoculation period increased from 30 to 90 days.

The maximum inhibition was observed in extracts from plants treated with mixed culture for 90 days as compared to other test treatments. *B. subtilis* was found most susceptible to the highest inhibition zone.

Table 1.A,B. Effects of inoculation with AM fungi on *A. paniculata* shoot length, root length, shoot diameter, wet weight, dry weight, stem girth, leaf area, internode length and leaf number for 30, 60 and 90 days of treatment.

SL	Treatment	Shoot Length (cm)			Root Length (cm)			Wet Weight (gm)			Dry Weight (gm)		
	DAYS	30	60	90	30	60	90	30	60	90	30	60	90
1	Control	15.12±0.02^{a,b}	21.5±0.02^{a,b}	35.3±0.02^{b}	6.5±0.01^{a}	8.5±0.01^{a}	9.4±0.02^{a,b}	3.8±0.2^{a,b}	5.2±0.01^{a}	6.5±0.2^{a,b}	2.52±0.01^{a}	4.83±0.01^{a}	3.91±0.01^{a}
2	*G. mosseae*	15.73±0.02^{a,b}	25.4±0.01^{a}	38.2±0.01^{a}	14.2±0.2^{a,b}	14.9±0.2^{a,b}	16.2±0.01^{a}	4.2±0.01^{a}	5.31±0.01^{a}	7.43±0.01^{a}	2.9±0.01^{a}	4.91±0.01^{a}	4.89±0.2^{a,b}
3	G. *fasciculatum*	15.35±0.01^{a}	26.2±0.01^{a}	37.3±0.01^{a}	11.5±0.01^{a}	12.8±0.01^{a}	13.4±0.01^{a}	5.21±0.01^{a}	6.78±0.01^{a}	8.21±0.01^{a}	3.84±0.01^{a}	4.25±0.01^{a}	4.27±0.01^{a}
4	Mixed culture	16.27±0.01^{a}	35.2±0.01^{a}	39.1±0.01^{a}	18.5±0.2^{a,b}	19.1±0.01^{c}	20.5±0.01^{c}	7.92±0.01^{a}	9.21±0.2^{a,b}	10.35±0.2^{a,b}	4.41±0.01^{a}	4.75±0.01^{a}	4.56±0.01^{a}
5	Control	1.2±0.02^{a,b}	2.5±0.02^{a,b}	3.3±0.02^{b}	6.5±0.01^{a}	8±0.01^{a}	9±0.02^{a,b}	3.8±0.2^{a,b}	4.2±0.01^{a}	4.5±0.2^{a,b}	15±0.01^{a}	18±0.01^{a}	19±0.01^{a}
6	*G. mosseae*	1.3±0.02^{a,b}	2.4±0.01^{a}	3.2±0.01^{a}	4.2±0.2^{a,b}	9±0.2^{a,b}	8±0.01^{a}	3.2±0.01^{a}	4.31±0.01^{a}	4.43±0.01^{a}	19±0.01^{a}	19±0.01^{a}	19±0.2^{a,b}
7	G. *fasciculatum*	1.5±0.01^{a}	2.2±0.01^{a}	3.3±0.01^{a}	5±0.01^{b}	8±0.01^{a}	7±0.01^{a}	3.21±0.01^{c}	5.78±0.01^{a}	4.21±0.01^{b}	18±0.01^{a}	16±0.01^{c}	17±0.01^{a}
8	Mixed culture	1.7±0.01^{a}	3.2±0.01^{a}	3.1±0.01^{a}	5±0.2^{a,b}	9±0.01^{b,c}	9±0.01^{c}	3.92±0.01^{a}	4.21±0.2^{a,b}	4.35±0.2^{a,b}	19±0.01^{a}	18±0.01^{a}	18±0.01^{a,b}

Values (mean±standard deviation) in each column designated with the same letters are not significantly different (P ≤0.05) according to Fisher's least significant test

Table 2. Frequency of mycorrhiza (F%), AM colonization intensity (M%), and arbuscule abundance (A%) in the root systems of *A. paniculata* at three harvests

Treatment	F%			M%			A%		
	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days
Control	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0	00.0
G. mosseae	64±0.02^{a}	70±0.05^{a,b}	95±0.04^{a}	45±0.06^{c}	54±0.03^{a,b}	68±0.01^{a}	60±0.02^{c}	60±0.05^{a,b}	95±0.04^{c}
G. fasciculatum	65±0.04^{b}	87±0.02^{a,c}	99±0.03^{a,c}	37±0.02^{a,b}	53±0.05^{a}	65±0.03^{a}	61±0.04^{a,c}	67±0.02^{c}	99±0.03^{a,b}
Mixed culture	87±0.02^{c}	93±0.06^{a}	105±0.03^{b}	63±0.05^{c}	76±0.03^{a}	89±0.04^{a}	80±0.02^{a}	73±0.06^{a}	105±0.03^{c}

Values (mean±standard deviation) in each column designated with the same letters are not significantly different (P ≤0.05) according to Fisher's least significant test

Table 3. Qualitative phytochemical analysis of AM fungi treated and control *A. paniculata*.

Phytochemicals	Control	*G. mosseae*	*G. fasciculatum*	Mixed culture
Cardiac Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Quinones	-	-	-	+
Proteins	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	-	+
Tannins	+	+	+	+
Carbohydrates	+	+	+	+
Phenol	-	-	-	-
Alkaloids	-	+	-	+

(+) Present (-) Not detected

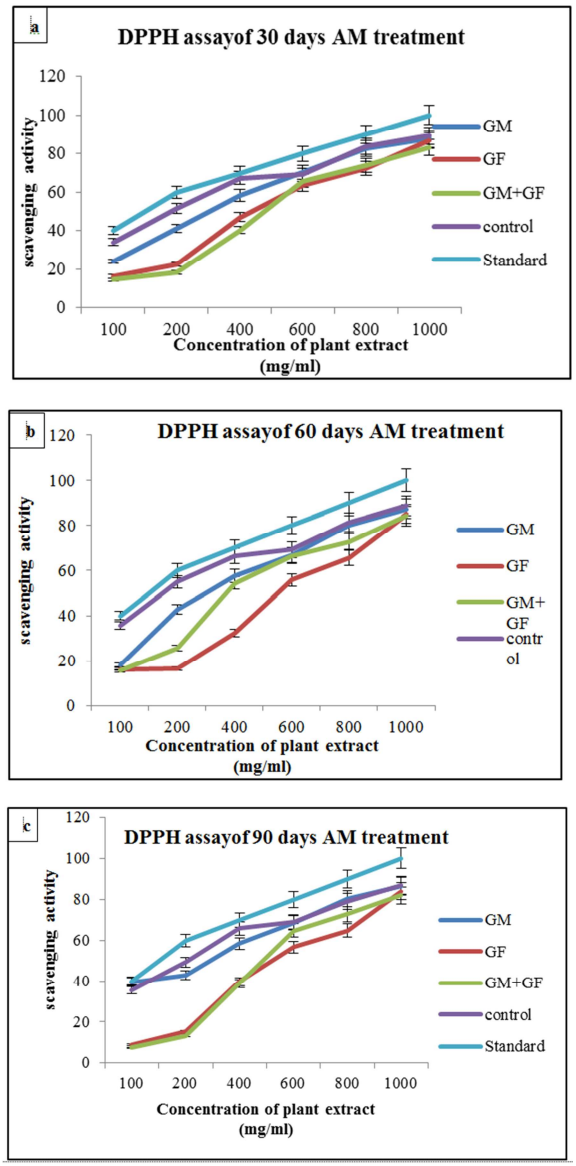

Fig. 3. Antioxidant activity of AM inoculated and control *A. paniculata* extracts. **a-**30 days treatment, **b-** 60 days treatment, **c-** 90 days treatment followed by *S.aureus* and *E.coli*. In the pure culture condition evaluated, the *G. mosseae* and *G. fasciculatum* had a similar inhibitory effect for 30, 60 and 90 days.

Discussion

The present work evaluates the effects induced by AM fungal inoculum *G. mosseae* and *G. fasciculatum* in pure culture and combination on the growth of *A. paniculata* (Burm.f.) Nees plant samples in different durations (30, 60 and 90 days). Plant samples inoculated with the pure culture and mixed culture for 90 days has proved to increase the growth of the plant when compared to control plants. The results also showed that the AM fungi inoculated to the plants affected the phytochemicals production. Phytochemical analysis of the treated plant extracts showed the presence of compounds such as tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids, especially in 90 days of treatment and is in agreement with the the results obtained by Yadav *et al.*, (2011) who proved increase in protein content in wheat grains of AMF inoculated plants (Yadav *et al.*, 2022). Tobacco plants inoculated with AM, alone or in combination with organic amendment, increased plant dry weights and improved phosphorous nutrition considerably (Manoharan *et al.*, 2010). The interaction between mycorrhizal inoculation and environmental CO_2 (ECO_2) in the onion plant induced the increased accumulation of sugars, proteins and proline in leaves, and also increased the shoot growth (Bettoni *et al.*, 2014). The total root length and specific root length of AM inoculated plants were significantly greater than those of untreated plants (Wicaksono *et al.*, 2018). The basil plant was inoculated with *G. fasciculatum* which significantly increased essential oil content and yield. GC-MS analysis of essential oil showed that linalool was the main compound and methyl chavicol profile was considerably increased with AM fungi inoculation (Sun and Tang, 2013). In the present work, the notable finding is the bioactive metabolite Azulene an antibacterial compound which was produced as the major compound in plants treated with mixed culture. These data on *A. paniculata* with enhanced bioactive compound production in AM treated plants prove that AM influences plant growth and compound production efficiently.

Conclusion

The Arbuscular Mycorrhizal (AM) fungi interact directly with organisms present in the soil or indirectly by acting on the physiology of the host which affects the morphology, physiology and growth of the host plant. AM fungi form symbiotic relationships with more than 80% of land plant roots which includes many important medicinal plants and forest tree species. Inoculation of such AM fungi during an early stage of plant growth has proved to be an effective method for improving plant growth. In the present study AM fungal association has not only proved to

enhance the growth of the host plant but also produced many different bioactive compounds. Hence, AM fungi can be used efficiently for improved production of drugs produced from medicinal plants in a comparatively shorter period and at a much lesser cost.

Conflict of interest

The authors declare no conflict of interest

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