



Effect of *Glomus fasciculatum* On Nutrient Uptake and Growth of a Medicinal Plant, *Coleus forskohlii*

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/EJMP/2020/v31i430227

Editor(s):

- (1) Patrizia Diana, University of Palermo, Italy.
(2) Marcello Iriti, University of Milan, Italy.

Reviewers:

- (1) Sanjeet Kumar, Ambika Prasad Research Foundation, India.
(2) Manal Hassan Shabana, National Research Centre, Egypt.
(3) Schirley Costalonga, Universidade Federal do Espírito Santo, Brazil.
Complete Peer review History: <http://www.sdiarticle4.com/review-history/55062>

Received 16 November 2019

Accepted 21 January 2020

Published 22 January 2020

Original Research Article

ABSTRACT

Aims: *Coleus forskohlii* Briq. (syn. *Coleus barbatus* Benth.) belonging to the family Lamiaceae is a well known plant throughout the country and known as 'pashanbhedi' in Sanskrit and 'pathatchur' in Hindi is one of the most potential medicinal plants of the future, as its pharmacoproperties have been discovered only recently. Its tuberous roots are found to be rich source of forskolin which is being used as a remedy for hypertension, glaucoma, asthma, congestive heart failures and certain types of cancers and also being use as vegetables. In addition, forskolin is reported to have used in the preparation of medicines for controlling body weight, preventing hair greying and restoring normal colour of grey hairs. Its foliage is also employed in treating intestinal disorders and used as a condiment since a long time. Keeping the above views in mind, the present investigation was undertaken to study the influence of an arbuscular mycorrhizal (AM) fungus, *G. fasciculatum* on phosphorus uptake and growth of *Coleus forskohlii*.

Study Design: Various tests or experiments were done in this study. Total leaf tissue phosphorus content was determined following the Microkjeldhal method of Jackson (1973) and chlorophyll estimation was done following the method of Mahadevan and Sridhar (1982). Root colonization of *C. forskohlii* by *G. fasciculatum* was observed by the 'Rapid clearing and staining technique' (Phillips & Hayman, 1970). Forskolin content in *C. forskohlii* tubers was estimated by High performance liquid chromatography (HPLC).

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Place and Duration of Study: Sample: The experiment was carried out in the Department of Botany, Burdwan University. A soil culture of *Glomus fasciculatum* was propagated as endomycorrhizal species in *Zea mays* as suitable host in pot culture using sterile sand and soil (pH 5.6; organic carbon 0.6%, P content 45 ppm) in 1:1 ratio. 400 gm of rhizosphere soil with AM-inoculated root fragments of *Zea mays* was introduced in the dry, loamy, fine-textured soil (pH 5.8; organic carbon 0.6%, P content 45 ppm, total nitrogen 0.03%) of pot culture (2.0 kg of soil per pot) at a depth of 2 inches below the *Coleus* cutting (12-16 cm long comprising 3-4 pairs of leaves) being planted, between June 2011 and July 2012.

Results: Responses of an arbuscular mycorrhizal (AM) fungus, *Glomus fasciculatum* on phosphorus uptake by the plant at different developmental phases have been studied. The result indicates that symbiotic association of mycorrhizal fungus amounts to greater uptake of phosphorus and increased chlorophyll content in AM treated plants than in non mycorrhizal plants which gradually happened to be maintained throughout their developmental stages. Different growth parameters like plant height, root length, no. of roots, no. of leaves, no. of branches, fresh weight of shoot, roots, tubers and forskolin content were significantly higher in AM treated plants than in respective controls.

Conclusion: The better growth of mycorrhizal plants was attributed to improved nutrient uptake, especially phosphorus. The result suggested that *G. fasciculatum* is one of the most potentially effective bio-fertilizer of *Coleus forskohlii*.

Keywords: *Coleus forskohlii*; *Glomus fasciculatum*; nutrient uptake; growth.

1. INTRODUCTION

Coleus forskohlii (Willd.) Briq. [synonym *C. barbatus* (Andr.) Benth.] is a member of the mint family Lamiaceae. It is indigenous to India [1] and is recorded in Ayurvedic Materia Medica under the Sanskrit name 'Makandi' and 'Mayani' [2]. The common names of *Coleus* are Pashan Bhedi in Sanskrit, Pathatchur in Hindi, Makandiberu in Kannada, *Coleus* in English, Garmalu in Gujarati, Maimnul in Marathi. The swollen primary roots (tubers) are found to be rich source of forskolin, a labdane diterpene (7 β -Acetoxy-8, 13-epoxy-1 α , 6 β , 9 α -trihydroxy-labd-14-ene-11-one) which is being used as a remedy for hypertension [3], glaucoma [4], asthma [5], heart diseases [6], diabetes [7], obesity [8] and cancer [9]. It also inhibits platelet activating factor [10] (Nourshargh and Hoult, 1986), increases the rate of sensory nerve regeneration in freeze-lesioned sciatic nerves [11]. In addition, forskolin is reported to have used in the preparation of medicines for controlling body weight, preventing hair greying and restoring normal colour of grey hairs. Its foliage is also employed in treating intestinal disorders and used as a condiment since long.

Mycorrhiza is a reciprocal association between soil-borne fungi and the roots of higher plants. This association is characterized by a bi-directional transfer of nutrients between plants and the concerned fungi. In this mutual relationship usually plants provide sugar

molecules to the fungi and in lieu of that the benefitted fungi help plants to uptake of minerals for their nutrition from the soil [12]. This beneficial interaction between arbuscular mycorrhizal (AM) fungi and horticultural crops has been well documented by different researchers [13]. It is one of the most important beneficial microorganisms in the rhizospheric soils and has potential use to medicinal plants for promoting growth and productivity [14-17]. Arbuscular mycorrhizal (AM) fungi colonize the majority of herbaceous plants roots in natural ecosystems all over the world [18]. Generally these fungi use some of the root exudates and modify their root physiology by altering the microbial equilibrium on the root surface [19]. AM fungi play a dominant role for increasing phosphorus solubilisation and uptake of Potassium (K), Sodium (Na), Calcium (Ca), Sulphur (S), Magnesium (Mg), Manganese (Mn) and Chloride (Cl) by plants [20,21]. AM actually helps in the plants by increasing the availability of soil surface by growing through soil pores and paces and affect phosphorus absorption beyond the depleted zone.

Normally AM fungi are known to improve the nutritional status, increase photosynthetic rates, and enhance levels of growth by regulating substances and altered patterns of root exudation due to changes in membrane permeability. *G. fasciculatum* is also known to protect plants against root pathogens [22,23] and offer resistance during drought [24] and salinity

[25]. AM fungi are now-a-days are well recognized as biofertilizer due to their manifold advantages supplying to the host plant besides to increase the uptake of water and nutrient [26]. Several studies have been shown that arbuscular mycorrhizal fungi stimulate plant growth and nutrient uptake, especially of phosphorus [27]. Moreover, Diouf [28] has been shown that phosphorus is the key element for higher yield of sesame. Keeping the above views in mind, the present investigation is undertaken to study the influence of an AM fungus, *G. fasciculatum* on phosphorus uptake and growth of *Coleus forskohlii*.

2. MATERIALS AND METHODS

2.1 Preparation of Inoculum of VAM

The experiment was carried out in the Department of Botany, Burdwan University. The inoculum of an arbuscular mycorrhizal (AM) fungus, *Glomus fasciculatum* was obtained from Department of Forestry, Vidyasagar University, West Bengal, India. The multiplication of inoculum of AM fungus was done on *Zea mays* roots in pots having sterile sand and soil (pH 5.6, organic carbon 0.6%, P content 45 ppm) in 1:1 ratio. A thin layer of mycorrhizal inoculum was placed 4 cm below the grains in pot soil in which maize grains were sown. After 30 days of sowing, the roots of the maize seedlings were chopped off and mixed with pot soil, in which a second crop was raised. Finally, the inocula consisting of both the spores in rhizosphere soil and the colonized root fragment was used for further experiment.

2.2 Preparation of Cuttings and Growth Conditions

A soil culture of *Glomus fasciculatum* was propagated as endomycorrhizal species in *Zea mays* as suitable host in pot culture using sterile sand and soil (pH 5.6; organic carbon 0.6%, P content 45 ppm) in 1:1 ratio. 400 gm of rhizosphere soil with AM-infected root fragments of *Zea mays* was introduced in the dry, loamy, fine-textured soil (pH 5.8; organic carbon 0.6%, P content 45 ppm, total nitrogen 0.03%) of pot culture (2.0 kg of soil per pot) at a depth of 2 inches below the *Coleus* cutting (12-16 cm long comprising 3-4 pairs of leaves) being planted. After two weeks, the cuttings (both AM infected and non AM infected) were transplanted.

2.3 Mycorrhization in Roots

Root samples from 30 days old plants were collected and were washed with sterilized distilled water. Staining of root samples were carried out following the Phillips and Hayman [29]. After treating with 10% KOH solution in hot water bath, the samples were again washed with sterilized distilled water and 5% NaCl solution repeatedly upto reaching neutrality. Then the root samples were stained with 0.05% trypan blue in lactophenol. Photographs were taken the method of Phillips and Hayman [29] using trypan blue in lactophenol as stain. Softened bits of stained root samples were mounted in 50% glycerol for examination under a bright field microscope (Lieca DFC295, version V3, Germany) and photographs. Photographs on mycorrhization were placed on Fig. 4.

2.4 Estimation of Phosphate in Host Tissue

Microkjeldhal method of Jackson [30] was employed for determination of total tissue (leaf) phosphate. To estimate the phosphorus, 60% perchloric acid, a combined reagent, H₂O₂ and distilled water were taken. Combined reagent was prepared by mixing the following reagents: 5 (N) H₂SO₄, potassium antimony tartarate (0.275 g in 100 ml distilled water), ammonium molybdate (4 g in 100 ml distilled water), ascorbic acid (1.76 g ascorbic acid in 100 ml distilled water). This combined reagent is bluish or colourless and transparent and is prepared just before use.

Fresh leaf tissue (25 mg) was taken in large Kjeldhal flask. Then 10 ml of 60% perchloric acid was added, mixed thoroughly to digest at 1300C until solution becomes colourless (1-2 ml of H₂O₂ was added if the solution did not decolorized). After digestion for 1-2 hours, the Kjeldhal flask was removed from heater followed by cooling at room temperature. The transparent liquid, thus obtained, was transferred to a graduated tube and care should be taken to leave behind as much as possible. Similarly, 3-4 times washing with distilled water and transferring quantitatively to graduated tube was done and the stock solution made up to 10 ml. Now 5 ml sample was taken and 0.8 ml of freshly prepared combined reagent was added to it. The percentage of phosphate was measured by taking optical density value at 660 nm in a UV- Vis

spectrophotometer (Systronics 117). The result was presented in the Table 1.

2.5 Estimation of Chlorophyll in Host Tissue

In order to determine the amount of chlorophyll in host tissue, method of Mahadevan and Sridhar [31] was adopted. Fresh leaves were collected and after washing with distilled water, dried in air. Now 1 g of leaf sample was cut into small pieces. The leaf tissue was crushed in a mortar and pestle with acetone (80%) and a small pinch of neutral sand was added to facilitate crushing. The homogenized mixture was then decanted and supernatant was filtered through a Buchner funnel using Whatman No. 42 filter paper. Extraction process was repeated with the addition of sufficient quantity of (80%) acetone. After that content was transferred from the mortar to the Buchner funnel and washed with acetone until colourless. The filtrates were taken and the volume made up to 100 ml in a volumetric flask. 5 ml of extract was transferred into a 50 ml volumetric flask and diluted it by making up the volume with acetone (80%). The data were recorded in terms of O.D. value at 660 nm in a UV- Vis spectrophotometer (Systronics 117) and presented in the Table 2.

2.6 Improvement of Growth and Yield of Host Plant

Plant growth responses in *Coleus* plants inoculated with *Glomus fasciculatum* were recorded (Tables 3 and 4) by measuring plant height, root length, number of roots, number of leaves, number of branches and fresh weight of shoot, roots and tubers at 15, 30, 45, 60 and 180 days after inoculation.

2.7 Estimation of Forskolin

Forskolin content in *C. forskohlii* tubers was estimated by High performance liquid chromatography (HPLC). The modular High Pressure Liquid Chromatograph (515-Waters Associates) consisted of a constant flow pumps (Waters Associates), a valve type injector, a detector, a column C18 (3.9 x30 cm) obtained with silica and a microsyringe (25 µl, Hamilton). Powdered dried roots (1 g) were weighed and transferred into a conical flask (25 mL) to which 10 mL acetonitrile was added. The samples were sonicated four times for 15 min each. The combined extractions were concentrated and diluted to the final volume of 10 mL with

acetonitrile. Samples were filtered (0.45-µm nylon filter membrane). Mobile phase acetonitrile and water were mixed in the proportion of 80:20 (v/v) and degassed before use. The mobile phase was pumped at the rate of 1 ml/min. The injection volume was 20 µl in the analytical work. Detection was made at 210 nm.

2.8 Statistical Analysis

Data were expressed as mean±standard error. Significant differences among the means were determined by Fisher's least-significant difference test after one-way analysis of variance. Significance of between-treatment means was tested at the 0.05 level of probability using Stat Plus Version 4.8, 2007 software.

3. RESULTS AND DISCUSSION

It is evident from the result (Table 1) that the phosphorus content of leaf tissue remains higher in mycorrhizal *Coleus* plants as compared to non- mycorrhizal ones and there was an increase in the amount of phosphorus with increase in age of the plant. Similar observations pertaining to the increased phosphorus uptake by AM treated plants have been reported by earlier workers [32-34]. The external AM hyphae reach beyond the depletion zone around the root hairs, absorb soil phosphorus and translocate it, perhaps in the form of polyphosphate granules, to the arbuscules where phosphorus is transferred to the plant cell in exchange of carbon [35]. Phosphorous being a constituent of phosphonucleotides which tend to increase cell division [36] might increase the plant growth. The increased 'P' content may be attributed to increase in uptake of P facilitated due to AM colonization through various mechanisms. Hattingh et al. [37] suggested that faster movement of P into mycorrhizal hyphae and solubilization of soil P could be some of the means for increasing P content in mycorrhizal plants. Faster movement of P into the mycorrhizal hyphae is achieved due to increased affinity of P ions and thereby decreasing the threshold concentration required for absorption of P. Further solubilization of soil P is achieved by the release of organic acids and phosphates [38].

From the result of Table 2, it is clear that *Glomus fasciculatum* inoculated *C. forskohlii* showed greater amount of chlorophyll than uninoculated control plants and there existed a positive correlation between the chlorophyll content and the age of the plants. At sixty day after cuttings

planted, the mycorrhizal plants recorded 9.120 mg of chlorophyll per gram of leaf tissue in contrast to 6.108 mg in leaf of non-mycorrhizal plants. Increased chlorophyll accumulation was observed in all AM inoculated plants. Higher P levels in tissues as a result of root colonization by the AM can be expected to increase the chlorophyll content, as P is one of the important components of chlorophyll. Increase in chlorophyll content due to AM symbiosis has been also reported by Adivappar [39], Richmond & Lang [40] and Shivaputra et al. [41].

It is also clear (Tables 3 & 4) that AM inoculation significantly increases the number of leaves of *Coleus* plants. Similar results were found in the case of corn [42], lettuce [43] and maize [44]. The results was also indicated that inoculation with mycorrhizal fungi significantly increases leaf number of *Sesamum* [45]. The aerial biomass increased with the inoculation of AM treated plant. These results are in agreement with certain works which show that the AM inoculation increases the dry biomass of cowpea [46], date palm [47] and sesame [48]. The results also state that AM symbiosis influences significantly the rooting area. The importance of the root-system was due to the presence of a larger number of rootlets, supporting the notion that AM fungi can increase the rooting zone. Our data also suggest that the positive effects on root volume could be correlated to improvement of *Coleus* nutrition since similar results were often recorded in poor fertile soils. Indeed, like certain filamentous fungi species, AM fungi secretes in the rhizosphere some phosphatases [49] and organic acids such as the oxalic acid, catalysing the hydrolysis of the connections phosphoesters [50] and thus placing phosphorus at the disposal of the plants.

The study also indicates that all the growth parameters under consideration like height of the plants, (Fig. 1) length of the roots (Fig. 2), fresh weight of shoot, roots, tubers (Fig. 3) and forskolin content (Fig. 5B) were recorded to be significantly higher in AM treated plants than the control sets. High performance liquid chromatographic profile has shown accurate quantification of forskolin where in the RT is 3.6 minutes (Fig. 5B) in *G. fasciculatum* treated tuber. Thus it is evidenced from the result that arbuscular mycorrhizal association (Fig. 4) imparts some beneficial effects on plant growth. The mycorrhizal association is found to be beneficial to the plants in terms of better nutrient uptake and better water potential which lead the plants to become more healthy and productive than the non-mycorrhizal plants [51]. Vanith et al. [52] reported significant increase in plant height, number of leaves and number of branches, fresh weight and dry weight in *Ocimum kilimandscharicum* on inoculation with *Glomus fasciculatum* compared to non mycorrhizal plants. Ganngge et al. [53] are of the opinion that benefits from AM seem to be confined to particular growth periods namely the seedling stage. Significant increase in chlorophyll content, shoot and root length and total biomass of different plants was observed following inoculation with *Glomus* sp. [54-56]. Thus mycorrhizal fungi offer an environmentally sound biological alternative to chemical fertilizers and pesticides for maintaining plant quality and productivity in agriculture [13] (Menge, 1983), horticulture and forestry [57,58]. *Glomus fasciculatum* and *Glomus* species (mixed) increased shoot and root dry weight was observed by Banni and Faituri [59]. *Coleus aromaticus* inoculated with *G. fasciculatum* and

Table 1. Effect of arbuscular-mycorrhizal inoculation on phosphorus content (%) of leaf tissue of *Coleus forskohlii**

Treatment	Phosphorus content (%) days after cuttings planted			
	15	30	45	60
Non-mycorrhizal plant	1.208 ± 0.004	1.414 ± 0.005	1.612 ± 0.004	2.026 ± 0.005
Mycorrhizal plant	5.808 ± 0.004	10.280 ± 0.037	20.038 ± 0.010	35.062 ± 0.006

* Data are the mean ± standard error of five replicates

Table 2. Effect of arbuscular -mycorrhizal inoculation on chlorophyll content (mg/100 mg dry wt.) of leaf tissue of *Coleus forskohlii**

Treatment	Chlorophyll content (mg/100 mg dry wt.) days after cuttings planted			
	15	30	45	60
Non-mycorrhizal plant	4.504 ± 0.002	5.258 ± 0.004	5.834 ± 0.010	6.108 ± 0.004
Mycorrhizal plant	5.630 ± 0.009	6.426 ± 0.005	7.510 ± 0.007	9.120 ± 0.007

* Data are the mean ± standard error of five replicates

Table 3. Effect of non-mycorrhizal inoculation on growth of *Coleus forskohlii**

Treatment	Plant growth parameters	Days after cuttings planted				
		15	30	45	60	180
Non-mycorrhizal plant	Plant height (cm)	10.420 ± 0.107	16.400 ± 0.130	18.400 ± 0.063	22.000 ± 0.707	
	Root length (cm)	4.400 ± 0.118	7.340 ± 0.103	11.620 ± 0.058	12.600 ± 0.245	
	No of roots per plant	12.400 ± 0.245	16.000 ± 0.548	22.400 ± 1.122	31.800 ± 0.917	
	No of leaves per plant	7.400 ± 0.245	8.400 ± 0.245	23.000 ± 1.225	30.400 ± 0.245	
	No of branch per plant	0.600 ± 0.245	1.200 ± 0.374	1.800 ± 0.374	5.600 ± 0.400	
	Fresh weight of Shoot (gm/plant)	1.956 ± 0.017	2.258 ± 0.005	4.654 ± 0.010	7.920 ± 0.008	
	Fresh weight of Roots (gm/plant)	0.610 ± 0.004	0.802 ± 0.005	1.660 ± 0.019	2.140 ± 0.019	
	Fresh weight of Tubers (gm/plant)	0	0	0	0	6.600 ± 0.245
	Forskolin content (mg/plant)	0	0	0	0	1±0

* Data are the mean ± standard error of five replicates

Table 4. Effect of mycorrhizal inoculation on growth of *Coleus forskohlii**

Treatment	Plant growth parameters	Days after cuttings planted				
		15	30	45	60	180
Mycorrhizal plant	Plant height (cm)	13.620 ± 0.049	24.400 ± 0.600	30.240 ± 0.112	45.600 ± 0.980	
	Root length (cm)	10.520 ± 0.092	11.700 ± 0.095	15.340 ± 0.121	22.300 ± 0.122	
	No of roots per plant	30.800 ± 0.490	41.000 ± 0.447	60.800 ± 0.490	67.000 ± 1.225	
	No of leaves per plant	13.800 ± 0.735	21.400 ± 0.600	42.000 ± 0.949	92.400 ± 1.122	
	No of branch per plant	2.600 ± 0.245	5.800 ± 0.735	15.600 ± 0.245	21.000 ± 0.548	
	Fresh weight of Shoot (gm/plant)	5.528 ± 0.010	91.400 ± 1.860	157.41 ± 3.12	230.038 ± 0.016	
	Fresh weight of Roots (gm/plant)	1.322 ± 0.008	6.000 ± 0.548	24.410 ± 0.404	35.480 ± 0.128	
	Fresh weight of Tuber (gm/plant)	0	0	0	0	71.600 ± 3.400
	Forskolin content (mg/plant)	0	0	0	0	6±0

* Data are the mean ± standard error of five replicates

other beneficial bacteria increased the growth and yield [60]. Inoculation with *G. fasciculatum* significantly increasing the growth, plant biomass, phosphorous uptake and yield of tomato compared to control plants [61]. In *G. fasciculatum* inoculated garlic plant showed significant increase in plant growth parameters like plant height, total biomass and bulb diameters, bulb weight, and yield than the control ones [62]. There was 21.10% increment in yield of *Allium* bulb as compared to non mycorrhizal inoculated garlic plant. Almost all the studied AMF strains showed increase in plant growth, biomass and nutrient content (N & P) over the control, while retarded growth response was observed with the inoculation of 6 different AMF species. Considering the shoot length, total biomass, nutrient content, chlorophyll content and root infection, pre-inoculation with 6 AMF species viz; *Glomus fasciculatum*, *G. versiforme*, *G. clarum*, *Glomus* sp., *G. mosseae* and *G. etunicatum* appeared to be the most promising AM fungi for inoculating *Piper longum* medicinal plant [63,64]. The increased absorption surface area offered by the extended soil network of fungal hyphae external to roots [65] might have increased P supply and promoted plant growth z to give high tuber yields. Iqbal and Qureshi [66]

reported 85% increase in height of sunflower plants inoculated with AM fungi compared to uninoculated controls under field conditions. Solubilization of unavailable forms of P through soil acidification by root exudates of mycorrhizal plants is also believed to enhance P uptake [67]. Since P is relatively immobile in soil and transfer mainly occurs by diffusion to the root surfaces, AM fungi could greatly enhance nutrient uptake [68,69]. The AM-inoculated garlic plants had higher fresh bulb yields than uninoculated plants grown in field conditions [62]. Inoculation of mycorrhiza significantly increases the plant height, number of branches, biomass and Phosphorus content of patchouli plants [70]. AM fungi efficiently increase the content of total phenolic compounds in the bark of the medicinal plants *Libidibia ferrea* stem [71,72].

The forskolin content of tuber of *C. forskohlii* was significantly increased by the inoculation treatments of *G. fasciculatum* [73]. Enhanced uptake of phosphorus and increased forskolin content due to AM fungal inoculation, observed in the present study, is in conformity with the observation made by several earlier workers [74-79].

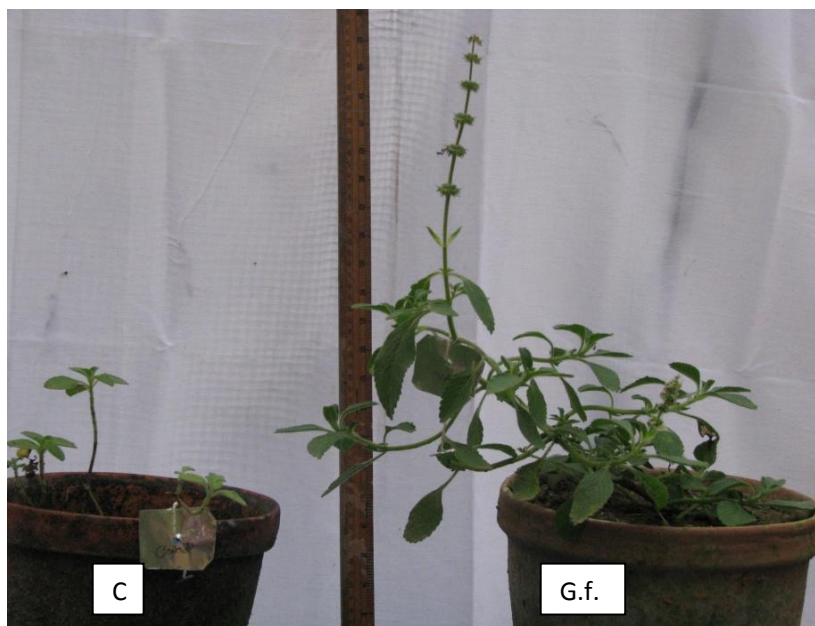


Fig.1. Photographs showing effect of AM fungus (*G. fasciculatum*) on growth parameters of *C. forskohlii* like increased plant height, no. of leaves, no. of branches in inoculated plant than control: (a) Control [C], (b) *G. fasciculatum* [G.f.] treated plant

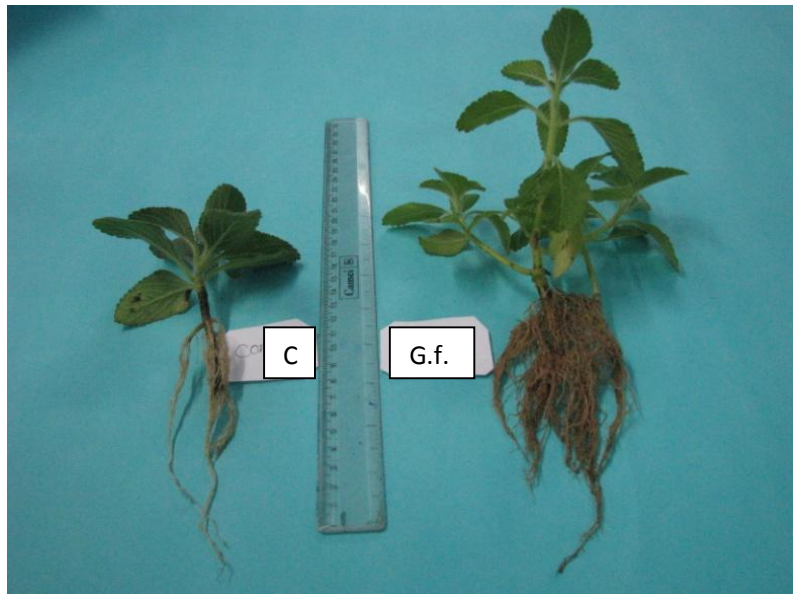


Fig. 2. Photographs showing effect of AM fungus (*G. fasciculatum*) on root biomass production of *C. forskohlii*: (a) Control [C], (b) *G. fasciculatum* [G.f.] treated plant



Fig. 3. Photographs showing the effect of VAM fungus, *G. fasciculatum* on tuber yield of *Coleus* plants: (a) Control [C], (b) *G. fasciculatum* [G.f.] treated tuber

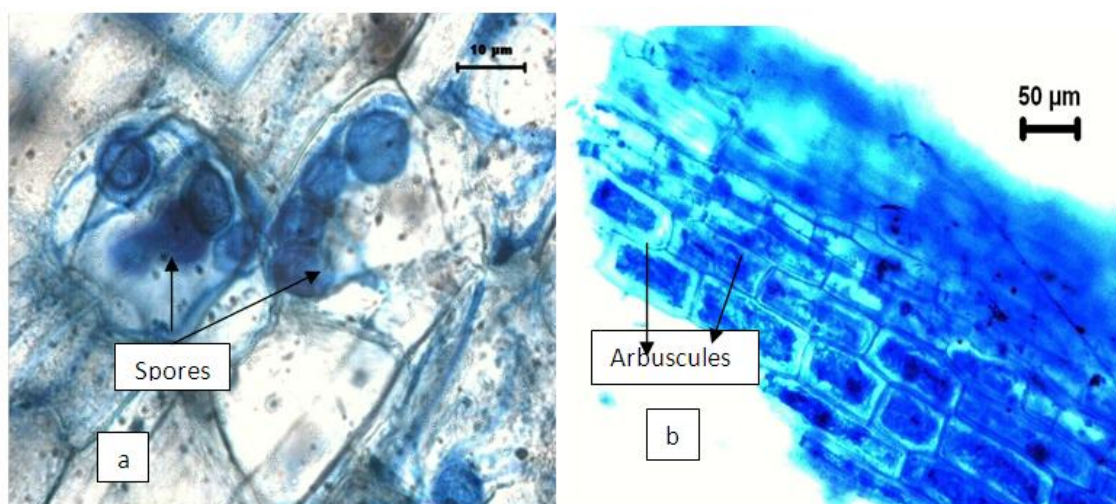


Fig. 4. Photographs showing mycorrhizal root colonization of the *Coleus* plant (a) spores present within the cortex cells of the root, and (b) arbuscules present within the cortex cells of the root

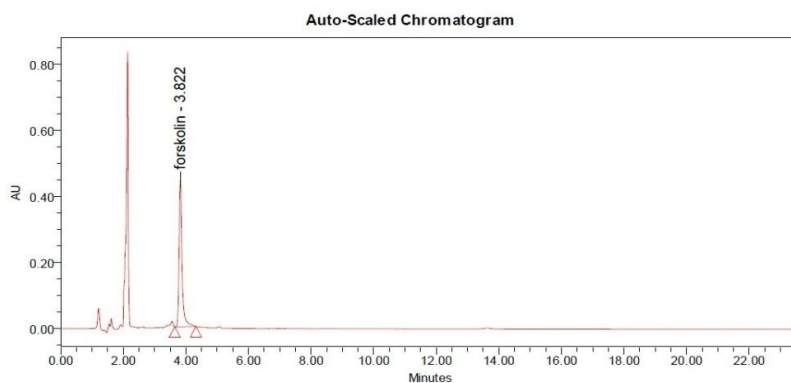


Fig. 5A. Quantitative estimation of forskolin by HPLC: chromatogram showing forskolin standard

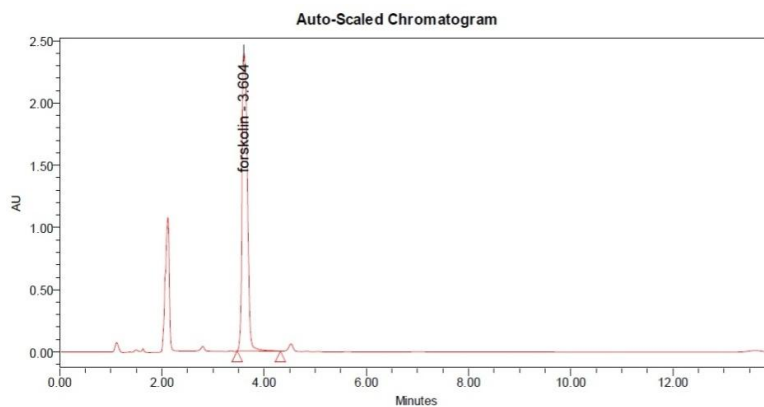


Fig. 5B. Quantitative estimation of forskolin by HPLC: Chromatogram showing forskolin of *Coleus* plant sample in *Coleus* (*G. fasciculatum* treated) tubers

4. CONCLUSION

Our results show that *Glomus fasciculatum* colonization can contribute substantially to the growth, nutrient uptake and help to increase the yield of *Coleus* plants. The improved growth may be because of earlier expression of developmental regulated genes, enhanced uptake of nutrients especially phosphorus. So more investigations are needed to correlate the exact action and mechanism of AM fungi on *Coleus* plants.

DISCLAIMER

This manuscript was previously presented and uploaded in the following conference:

5th International Botanical Conference on 9-11 December, 2011, Dhaka, Bangladesh.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The author greatly grateful and indebted to Prof. N. C. Chatterjee, the Mycology and Plant Pathology laboratory, UGC Centre of Advanced Study, Dept. of Botany, The University of Burdwan for providing her the necessary laboratory facilities.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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