Effect of Arbuscular Mycorrhizal Fungi on Growth and Nutrient Status of *Vigna unguiculata* (L.)

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Abstract Since last three decades, the increased use of chemical fertilizers in agriculture undoubtedly increased the food grain production and helped the country in achieving self-sufficiency in food grains. However, it also produced many harmful effects like water supply contaminations, shortage of quality of agricultural products and decrease in the amount of soil fertility. The study was performed to evaluate whether Arbuscular Mycorrhizal Fungi (AMF) improved Growth and Nutrient status in some important annual leguminous crops using Cow pea (*Vigna unguiculata* L.) as a study material. Arbuscular mycorrhizal fungi (AMF) are important soil organisms belonging to phylum Glomeromycota and form symbiotic association with the roots of 70-90 % plant species. A pot experiment was conducted at Botanical Garden, School of Studies in Botany Jiwaji University Gwalior during the year 2019 to study the effect of arbuscular mycorrhizal fungi viz *Glomus hoi* and *Acaulospora kentinensis* on seed germination, growth and biochemical contents of cow pea *Vigna unguiculata* L. The Plants were raised in triplicates through the pot culture. The seeds were surface sterilized by hypochlorite solution before sowing. Pots were placed at a sunny place after the seed sowing. And after the seed germination, plants were irrigated when required. After germination the inoculated plants along with their controls was sampled. The results showed that *Glomus hoi* and *Acaulospora kentinensis* significantly increased growth parameters like root and shoot length, total leaf area, fresh and dry weight of roots and shoots in *Vigna unguiculata* L. Inoculation of *Glomus hoi* and *Acaulospora kentinensis* significantly increases the biochemical constituents like

chlorophyll- a, chlorophyll-b, total chlorophyll, protein, carbohydrate, reducing sugar, non reducing sugar and total phenol. The present study pertains that AMF colonization improved positively the overall growth and development of cow Pea plant.

Keywords Cowpea, Arbuscular Mycorrhizal Fungi, Growth Parameters, Chlorophyll and Biochemical Constituents

1. Introduction

Cowpea (*Vigna unguiculata* L.) is an important crop commonly called as poor man's meat because of its sufficiently great source of vitamins, proteins and minerals for poor people who are not able to get such nutrients from animal source like fish and meat (Gondwe et al., 2019). The green pods and leaves are used as vegetable and the dried grain is used for the preparations of different foods. However, the production and productivity of cow pea is low because of lack of improved cultivators, limited input use, poor management practices and use of chemical fertilizers and pesticides (Kyei-Boahen et al., 2017). Hence for sustainable agriculture, all efforts should be streamlined to increase production and productivity of cowpea (Jain, 2018). In this context, now a day's bio fertilizers are gaining much importance in agriculture. Biofertilizers are not only organic material resulted from animal residues, plant residues and so on. They also include products results from microorganism's activities that act in relation to

nitrogen stabilization or phosphorus and other nutrient elements preparations that act in soil (Johnson *et al.*, 2015). One of the available ways to reach permanent agriculture is using some microorganisms that have important role in supplying food requirements like mycorrhizal fungi. Mycorrhizal fungi are used in conventional agriculture to improve crop production and productivity (Gianinazzi *et al.*, 2010). The tremendous advances in mycorrhizal research over the past years have led to a greater understanding of the multiple roles of mycorrhizal fungi in the agricultural ecosystem. Mycorrhizae are a symbiotic relationship between a fungus and roots of plants (Shuab *et al.*, 20016). On the basis of penetration there are two types of mycorrhiza viz Ecto mycorrhiza and Endo mycorrhiza. Ectomycorrhiza do not penetrate within the roots while Endo mycorrhiza penetrates cell wall and reaches into the cell membrane (Smith *et al.*, 2017). Arbuscular mycorrhizal fungus belongs to division Glomeromycota and forms symbiosis with eighty five percent of all plant families (Lone *et al.*, 2015). Arbuscular mycorrhizal fungi colonize roots and form two types of mycelium, intra radical mycelium (IRM) and extra radical mycelium (ERM). Arbuscules are the characteristic intra radical mycelium structures that are highly branched within host cells in order to mediate resource exchange between the symbionts. They are ephemeral structures and at the end of their life span, arbuscular branches collapse from the tip, fungal cytoplasm withdraws and the whole arbuscules shrinks into the fungal clumps. The exoskeleton of an arbuscule shrinks into the fungal clumps. The exoskeleton of an arbuscule contains structured chitin, which is a polymer of N-acetyl glucosamine; whereas collapsed arbuscule does not have chitin (Kobae *et al.*, 2015). Arbuscular mycorrhizal fungi benefit their host plant by improving the uptake of minerals, water and poorly mobile phosphorus in the soil. The fungus has shown to improve the tolerance of plants to drought stress (Van Der Heijden *et al.*, 2015). AMF are beneficial soil symbionts which establish mutualistic association with the roots of 80% of Plant species and the large majority of food crops including cereals, Legumes vegetables and fruits (Pepe *et al.*, 2018). Research suggests that arbuscular mycorrhizal fungi might foster upper range expansion of invasive tress over non-invaded higher elevations. The effect of these AMF communities on plant growth and nutrition shows that arbuscular mycorrhizal fungi can facilitate the growth of alien tress in non-invaded mountain ecosystems (Urcely, 2019). Inoculation of AMF optimizes the production of bioactive compounds in the plants which in turn increases the production of secondary metabolites. In those plants which show AMF association the flavonoid concentration increases as reported in *Libidubia ferrea* in the field conditions (Santos *et al.*, 2017). *Glomus hoi* is an arbuscular mycorrhizal fungi belongs to family Glomeraceae and division Glomeromycota. It forms symbiotic association with several plants and helps plants

in increasing growth and biochemical parameters. *Glomus hoi* is found in several agricultural regions of India like M.P, Himachal and Kashmir regions (S. Varga., 2009). *Acaulospora kentinensis* is a species of Arbuscular Mycorrhizal fungi belonging to the family Acaulosporaceae. It forms mycelium, vesicles and arbuscules with roots of several plant (Janos DP and Trappe JM., 1982). In the present study the above two species were used because they are native to Madhya Pradesh region as we have isolated these two fungi from rhizospheric regions of several agricultural sites of Gwalior region.

2. Materials and Methods

Seeds, Soil, Arbuscular Mycorrhizal Inoculation and Experimental Design

The seeds of *Vigna unguiculata* (L) were collected from Agriculture Collage Gwalior. Seeds were surface sterilized by dropping them in 4 % sodium hypochlorite for 10 minutes. Then seeds were washed 3-4 times by distilled water. Before sowing the seeds in pots, the germination rate was calculated. The germination percentage were calculated by the following equation

$$\text{Germination percentage} = \frac{\text{Seeds germinated}}{\text{Total seeds}} \times 100$$

Seeds were sown in pots in triplicates for each species along with control. Two types of Arbuscular Mycorrhizal Fungal Spores *Glomus hoi* and *Acaulospora kentinensis* were used separately and in combination. The two fungi were collected from different agricultural zones of Gwalior Region. The soil was collected from the rhizospheric regions of plant and isolation of fungi were done by the method of Gerdman and Nicholson (1963). Since these two Arbuscular Mycorrhizal fungi are non-culturable on any media, therefore we got the cultures of these two fungi from the energy and resource institute New Delhi under Material Transfer agreement.

For the current study 40 pots were used. Ten pots were filled with soil inoculated with *Glomus hoi*, Ten with *Acaulospora kentinensis*, ten with combination of *Glomus hoi* and *Acaulospora kentinensis* and ten pots were filled with sterilized soil without inoculation of spores. Finally, four sets of Pots were made viz Species A, Species B, Species A+B and Control

[Where

A= Pots filled with *Glomus hoi*

B = Pots filled with *Acaulospora kentinensis*

C = Pots filled with the combination of *Glomus hoi* and *Acaulospora kentinensis*

And

D= Pots filled with no spores]

All the pots were filled with sterilized soil which autoclaved twice at 15 lbs pressure at 120°C temperature for 45 minutes. The pot experiment was conducted at Botanical Garden of School of studies in Botany, Jiwaji University Gwalior. The pots were placed in sunny area. After germination the pots were irrigated when needed. After germination the inoculated plants along with their controls was sampled at 30 days for growth and other biochemical constituents.

Table 1. Some physical properties of soil which was used in pot culture

Colour	Reddish brown
Texture	clay loamy
pH	7.12
Electric conductivity(dsm-1)	0.24
Organic carbon (percent)	0.39
Moisture content (percent)	6.34
Water holding capacity (percent)	2.32
Density(mg. m-3)	1.21

Table 2. Macro and Micronutrient analysis of soil used for the experiment

Nitrogen (kg. ha-1)	160
Phosphorous (kg. ha-1)	14.60
Potassium (kg. ha-1)	230.42
Iron (ppm)	5.40
Zinc (ppm)	1.23
Manganese (ppm)	6.20
Copper (ppm)	1.66

Plant Growth Parameters and AMF Colonization

Plant Height (cm), Plant Width at Base (cm), Total number of leaves per plant, Largest Leaf length Base to tip (cm) and Root length Bulb base to tip. (cm) were recorded. Roots was collected and placed in 4% isopropanol solution for analysis of AMF colonization. The roots were stained with 0.05% trypan blue stain using the method suggested by Phillips and Hayman (1970).

Estimation root colonization was carried by using Biermann and Linderman (1981) method. Percent root colonization was calculated using following equation

$$\text{Root colonization (\%)} = \frac{\text{Number of arbuscular mycorrhizal positive segments}}{\text{Total number of segments studied}} \times 100$$

AMF from rhizosphere

For the separation of AMF spore from the rhizospheric region of the soil the method of wet sieving and decanting proposed by Gerdemann and Nicolson (1963) were used. 100 gram rhizosphere soil was suspended in 1000 ml of tap

water. The mixture was stirred for half an hour and the heavier soil and sand particles were allowed to settle down to the bottom. The soil water mixture was decanted through sieves arranged in descending order (240µm, 120µm, 60µm, 30µm,). Top sieve captured roots and debris. Debris from the remaining sieves was collected separately in beakers. Spores were collected in a petriplate of 10 cm diameter and were examined under stereo-microscope. The manual provided by Schenck and Perez (1990) were used for the identification of different species of AMF.

Chlorophyll and Other Biochemical parameters

For Chlorophyll a, Chlorophyll b and total chlorophyll Method of Arnon (1949) and Witham et al. (1971) was used. Following equation was used to determine the chlorophyll present in the extract as mg chlorophyll per gram green tissue using the following equations for each fraction;

$$\text{For chlorophyll a} \\ \text{mg chlorophyll a per gm tissue} = \frac{12.7 (A_{663}) - 2.69 (A_{645})}{V}$$

$$\times 1000 \times W$$

$$\text{For chlorophyll b} \\ \text{mg chlorophyll b per gm tissue} = \frac{12.7 (A_{645}) - 2.69 (A_{663})}{V}$$

$$\times 1000 \times W$$

$$\text{Total chlorophyll} \\ \text{mg total chlorophyll} = \frac{20.2 (A_{645}) + 8.02 (A_{663}) \times V}{V}$$

$$\times 1000 \times W$$

Where

A= absorbance at specific wavelength

V= final volume of chlorophyll extract in 80% acetone

W= fresh weight of tissue extracted

Biochemical parameters

Protein estimation by Lowry's et al., (1951) method.

Extraction of protein from Sample: Extraction is usually

carried out with buffers used for the enzyme assay. Weigh 500mg of the sample and grind well with a pestle and mortar in 5-10mL of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of Protein:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0ml of the working standard into a series of test tubes.

2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. 3. Make up the volume to 1.0 ml in all the test tubes. A tube with 1.0ml of water serves as the blank. 4. Add 5.0 ml of reagent C to each tube including the blank. Mix well and allowed to standing for 10mins. 5. Then add 0.5 ml of reagent D, Mix well and incubate at room temperature in the dark for 30min, blue colour is developed. Take the reading at 660nm. Draw a standard graph and calculate the amount of protein in the sample.

Estimation of sugar content (Total, reducing sugar and Non reducing sugar)-

The total and reducing sugar contents were analyzed in host plant shoot and root parts by the Nelson-Somogy method (Nelson, 1944).

Procedure:

(i) Total sugars content

To 1ml alcoholic aliquot, 1ml 1N H₂SO₄ was added and heated at 490C in water bath for 30 minutes for hydrolysis of the mixture. 1-2 drop of methyl red indicator was added. 1N NaOH was added dropwise for the neutralization (Colour was to yellow from pink). 1ml Nelson Somogy's reagent was added to it and the tube was kept in boiling

water bath for 20 minutes. After cooling of the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water and optical density was noted at 540nm. Blank was prepared in the same manner. A standard curve was prepared using glucose in µg per ml.

(ii) Reducing sugars contents

To 1ml alcoholic aliquot, Nelson Somogy's reagent was added and kept in boiling water bath for 20 min. After cooling of the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water and optical density was noted at 540nm. Blank was prepared in the same manner.

(iii) **Non-reducing sugar**= Total sugar – Reducing sugar. The result was expressed as mg/ g-1 plant material.

3. Results

Root Colonization

The germination percentage of seeds was 100 percent. The roots of the cowpea plants in the control pots did not show any of the AMF structures. Pots inoculated with *Glomus hoi* shows minimum root colonization. Plant root percentage colonization of AMF was higher in the pots inoculated with the combination *Glomus hoi* and *Acaulospora kentinensis* (as shown in the following Figure). Mycorrhizal Colonization is confirmed in all pots inoculated with AMF. This finding is based on the presence of AMF in various morpho-presentations like hyphae, arbuscules or vesicles, in the infected roots.

Growth parameters

Plants height of all treatments was measured at the flowering/fruiting stage (45 days after sowing). To evaluate shoot and root parameters, all plants were harvested at the flowering/fruiting stage. Plants without AMF inoculation don't show any significant increase in plant width, number of branches per plant, leaf length and root length whereas plant inoculated with different mycorrhizal species shows significant increase in several growth and developmental parameters. Pots inoculated with *Acaulospora kentinensis* shows maximum increase in plant height while as pots inoculated with the combinations of *Glomus hoi* and *Acaulospora kentinensis* showed increase in plant width and total number of leaves. The largest root length was found in plants inoculated with Species *Glomus hoi* and largest leaf area was also found in plants inoculated with the combinations of species *Glomus hoi* and *Acaulospora kentinensis* (Table 3)

Table 3. Various Growth and developmental parameters of cow pea plant as affected by the presence of AMF

Specie	Plant height (cm) (Average± Standard error)	Plant width (cm) (Average± Standard error)	Total number of leaves (Average± Standard error)	Largest leaf area (cm) (Average± Standard error)	Root length(cm) (Average± Standard error)
<i>Glomus hoi</i>	29.5±0.01	1.3±0.03	2.0±0.02	6.5±0.03	13±0.02
<i>Acaulospora kentinensis</i>	30.4±0.02	1.3±0.01	2.0±0.03	7.1±0.01	11.4±0.02
<i>Glomus hoi</i> and <i>Acaulospora kentinensis</i>	30±0.03	1.2±0.02	2.4±0.02	6.810.7±0.02	12.2±0.01
control	25.5±0.01	1±0.04	1.2±0.01	6.8±0.03	10.7±0.03

Chlorophyll Contents

Chl a content in plants inoculated with species *Acaulospora kentinensis* was more as compared to plants inoculated with species *Glomus hoi* and in combinations of *Glomus hoi* and *Acaulospora kentinensis*. The plants which were inoculated with the *Glomus hoi* and combinations of *Glomus hoi* and *Acaulospora kentinensis* showed increase in chlorophyll b. Total chlorophyll was high in the plants which were inoculated with *Acaulospora kentinensis*. Plants without AMF inoculations show minimum concentrations of Chl a, Chl b and total chlorophyll (Table 4).

Table 4. Leaf chlorophyll contents in the plant with and without the presence of AMF at various growth stages (mg/g fresh weight)

Species	Chlorophyll a (Average ± Standard error)	Chlorophyll b (Average ± Standard error)	total chlorophyll (Average ± Standard error)
<i>Glomus hoi</i>	0.06±0.00	0.12±0.02	0.19±0.00
<i>Acaulospora kentinensis</i>	0.07±0.00	0.10±0.00	0.21±0.00
<i>Glomus hoi</i> and <i>Acaulospora kentinensis</i>	0.47±0.1	0.12±0.00	0.19±0.00
control	0.06±0.00	0.09±0.00	0.16±0.00

Fresh and dry weight

The fresh and dry weight of cow pea plants shows increase with the inoculation of different AMF species. Plants inoculated with species *Acaulospora kentinensis* shows significant increase in fresh and dry weight of root and shoot. (Table 5).

Table 5. Fresh and dry matter content with and without AMF in the plant (gram/plant)

Species	Root fresh (Average± Standard error)	Shoot fresh (Average± Standard error)	Total fresh (Average± Standard error)	Root Dry (Average± Standard error)	Shoot Dry (Average± Standard error)	Total Dry (Average± Standard error)
<i>Glomus hoi</i>	0.12±0.22	1.34±0.04	1.46±0.12	0.088±0.01	0.357±0.03	0.445±0.68
<i>Acaulospora kentinensis</i>	0.49±0.22	2.25±0.03	2.74±0.13	0.089±0.02	0.543±0.01	0.632±0.59
<i>Glomus hoi</i> and <i>Acaulospora kentinensis</i>	0.26±0.23	2.91±0.04	3.17±0.14	0.092±0.03	0.651±0.12	0.743±0.58
control	0.13±0.20	1.20±0.01	1.33±0.12	0.086±0.02	0.338±0.03	0.424±0.53

Table 6. Biochemical parameters (mg/ gram fresh weight)

Species	Reducing sugar (Average± Standard error)	Non Reducing Sugar (Average± Standard error)	Total Carbohydrate (Average± Standard error)	Total Protein (Average± Standard error)	Total Phenol (Average± Standard error)
<i>Glomus hoi</i>	4.69 ±0.24	174.29 ±5.24	178.96 ±7.68	4.23 ±0.32	0.070±0.03
<i>Acaulospora kentinensis</i>	5.19 ±0.14	183.29 ±4.31	198.96 ±6.58	5.33 ±0.23	0.083±0.02
<i>Glomus hoi</i> and <i>Acaulospora kentinensis</i>	5.13 ±0.14	184.29 ±4.21	199.96 ±6.48	5.43 ±0.33	0.093±0.03
control	10.19 ±0.32	109.60 ±4.62	119.79 ±5.14	3.39 ±0.12	0.630±0.03

Biochemical parameters

Inoculation of AMF spores increases the biochemical parameters of cow pea plant. Non reducing sugars, total sugars, total protein and total phenol were high in plants inoculated with the combinations of species *Glomus hoi* and *Acaulospora kentinensis*. While reducing sugars was high in the plants inoculated with *Acaulospora kentinensis* plants without the inoculation of AMF species shows minimum concentration of reducing sugars, non reducing sugars, total sugars, total protein and total phenol (Table 6)

production of secondary metabolites. In those plants which show AMF association the flavonoid concentration increases as reported in *Libidubia ferrea* in the field conditions (Santos *et al.*, 2017). Arbuscular mycorrhiza fungi (AMF) colonize roots of host plants and promote plant growth due to improved uptake of nutrients like P, Mn, K, Cu, and Fe in Chickpea (Farzaneh *et al.*, 2011). Arbuscular mycorrhizal fungi are a good candidate for promoting plant growth and essential oil composition and improving Phosphorous uptake in low fertility soils (Tarraf *et al.*, 2017).

4. Discussion

Modern cowpea cultivars are still responsive to mycorrhizal inoculation suggesting that modern breeding programs are not deleterious AMF symbiosis (Oruru *et al.*, 2018). AMF are beneficial soil symbionts which establish mutualistic association with the roots of 80% of Plant species and the large majority of food crops including cereals, Legumes vegetables and fruits (Pepeet *et al.*, 2018). Arbuscular mycorrhizal fungi increase the growth of some plants like *Spilanthes calva*, *Withania somnifera*, *Cartanospermum austral*, *Cymbopogon martini*, *Phyllanthus amarus*, *Gloriosa superba* and *Pueraria tuberosa* (Shenbagam, 2008). Arbuscular mycorrhizal fungi contribute to the primary and secondary metabolism of plants and increases active ingredients of medicinal and aromatic plants (Tarraf *et al.*, 2017). Arbuscular mycorrhizal fungi contribute to the primary and secondary metabolism of plants and increases active ingredients of medicinal and aromatic plants (Tarraf *et al.*, 2017). Arbuscular mycorrhizal fungi enhance the production of secondary metabolites in plants like alkaloids, terpenoids and phenolics, which are very much useful to humans. The role of Arbuscular mycorrhizal fungi in the amplification of secondary metabolite content has attained enormous recognition for sustainable cultivation of medicinally important plants. Therefore, AMF plays a vital role in the better accumulation of bioactive compounds in host plants (Pandey *et al.*, 2018). Inoculation of AMF optimizes the production of bioactive compounds in the plants which in turn increases the

5. Conclusions

Cowpea is an important legume grown primarily in semi-arid area. Its production is generally inhibited by various abiotic and biotic stresses. The use of beneficial microorganisms (e.g., plant growth promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF)) can enhance agricultural production, as these microorganisms can improve soil fertility and plant tolerance to environmental stresses, thus enhancing crop yield in an eco-friendly manner. The application of beneficial soil microorganisms can be a viable approach for sustainable cowpea production in precision agriculture scenarios (Ma *et al.*, 2019) Cow pea has a good mycotropic status and it has arbuscules and vesicles are found in its roots. It shows the symbiotic association with *Glomus hoi* and *Acaulospora kentinensis*. The partners in this association are members of fungi (Basidiomycetes, Ascomycetes, and Zygomycetes) and most of the vascular plants. Fitter and Moyersoen (1996) provided a succinct definition -"a sustainable nonpathogenic biotrophic interaction between a fungus and root". In the mycorrhizal literature, the term symbiosis is used to describe a highly interdependent mutualistic relationship where the host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon atoms. The present study pertains that AMF colonization improved positively the overall growth and development of cow Pea plant. Chlorophyll, Reducing sugars, Non Reducing sugars, Total sugars, Total protein and Total phenol content too was found higher in AMF inoculated than control.

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Author Contribution

SAT and SP designed the study. SAT performed the practical work, analyzed the data and wrote the manuscript. SP supervised the study.

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Compliance with Ethical Standards Conflict of Interest

The authors declare that they have no conflict of interest

Ethical Approval

The study does not involve any research on Humans or Animals.

Informed Consent

Informed consent was obtained from all individual participants included in the study

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