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INFLUENCE OF *GLOMUS* SPECIES ON ENHANCEMENT OF METABOLITES IN *IN VITRO* REGENERATED *WITHANIA SOMNIFERA* (L.) DUNAL.

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ABSTRACT

Withania somnifera (L.) commonly known as "Ashwagandha" is one of the promising herb with important multiple medicinal properties. The main active principle which is therapeutically known as Withanolides and steroidal lactones. The present study was aimed to investigate to effect of three arbuscular mycorrhizal (AM) fungi - *Glomus mosseae, G. fasciculatum* and *G. macrocarpum* to enhance the production of fresh and dry weight, primary and secondary metabolites as compared to uninoculted *Withania somnifera*. The micro propagated plants were derived from shoot tip explants cultured on MS medium supplemented with 2 mgl^{-1} of cytokinins and 0.5 mgl^{-1} of IBA were treated with three *Glomus* species to study the effects of AMF association on the production of metabolites. Quantitative analysis has revealed an increase in primary metabolites like proteins, carbohydrates, amino acids and secondary metabolite like Phenolics. The fresh and dry weight of leaves, stem and roots were significantly increased in AM treated plants compared to control plants, the highest in *G. mosseae* followed by *G. fasciculatum* and *G. macrocarpum*. While the contents of primary metabolites and Phenolics have concomitantly increased in micro propagated plants inoculated with *G. fasciculatum* followed by *G. mosseae* and *G. macrocarpum.* The present study AM fungi potentially represent an alternative way of promoting growth of this important medicinal plant and their chemical constituents.

INTRODUCTION

Withania somnifera a branching under shrub of family Solanaceae grows widely in South Africa, Egypt, Pakistan, Jordan and Afghanistan. It occurs in drier parts of subtropical India viz Uttar Pradesh, Madhya Pradesh, Punjab plains and North Western parts of India like Gujarat and Rajasthan. It is popularly Known as Ashwagandha is one of the major herbal components in Indian system of medicine. In Ayurveda the plant is used as potent aphrodisiac rejuvenative, life prolonging properties and used for the treatment of coughing, youthful vigor endurance and premature ageing. The plant extract used as an herbal tonic in Vedas in the form of Medharasayana (promoter of learning and memory) and considered as an Indian Ginseng in traditional system of medicines (Gupta and Rana, 2007). The roots and leaves exhibit pharmacological activities such as metabolic restoration and even used in anti ageing, nerve tonic and neurodegenerative disorders (Bhattacharya *et al.,* 2002), treatment of ulcers, dropsy, antimicrobial, anti-inflammatory, antitumor, analgesic and antioxidant (Mayura and Akanksha, 2010). It has received much attention in recent years due to presence of Withanolides (steroidal Lactones with Ergostone skeleton), Glycowithanolides, alkaloids which includes Withaferin - A, Withanolides, Sitoindosides and Withasomanine etc. found in roots. There are number of studies on the beneficial effects of AM fungal association on growth performances and metabolites of some medicinal plants (Gianinazzi and Gianinazzi-pearson, 1988; Raei and Weisany, 2013). Few studies have been reported by Ral and Lubraco (2003) and Halder and Ray (2006) on the root colonization and growth enhancement of *W. somnifera* treated with *Glomus* species. However, studies are limited on the influence of AM fungi on *in vitro* raised plantlets. Therefore the present study was aimed to investigate the effect of AM fungi (*Glomus mosseae, G. fasciculatum* and

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G. macrocarpum) association on enhancement of biomass and metabolites in micro propagated plants of *W. somnifera*.

MATERIALS AND METHODS

Plant source

The Seeds of *W. somnifera* were collected from Kolar, Karnataka in the month of April, 2010-11, grown on pots containing sterile soil mixture and maintained in green house conditions. Terminal and auxiliary shoot tips of 90 days old plants were used as explants for establishing *in vitro* cultures. The explants were surface sterilized and transferred to MS medium containing 3% sucrose (w/v) and 0.8% Agar and supplemented with cytokinins BA (0.5-4mgl^{-1}) and 0.5-1mgl^{-1} concentration of auxins (IBA). Cultures were maintained under standard conditions. Individual shoots derived from callus were transferred to proliferation MS medium containing 0.5-4mgl^{-1} of KN + 0.5-1mgl^{-1} of BA. *In vitro* regenerated shoots are separated and inoculated on MS medium containing 1mgl^{-1} of IBA for root induction. The Plantlets with well developed roots were washed thoroughly and transferred to green house conditions for hardening and acclimatization in nursery trays containing sterile coconut peat mass. After 45 days, plantlets were transferred to pots containing sterile farmyard manure, red soil and sand in the ratio of 2:1:1.

The Cultures of *Glomus* species procured from Department of Microbiology, U.A.S., G.K.V.K Campus, Bangalore. The cultures were multiplied and maintained in pots containing sterilized soil and sand (1:1) on Rhodes grass (*Chloris gayana*). The inoculum of three *Glomus* species was placed 2 cm below the soil for pots with inoculums potential of 0.18×10^4 g^{-1} infective propagules of *G. mosseae* and 0.19×10^4 g^{-1} infective propagules of *G. fasciculatum* and *G. macrocarpum*, i.e., 10g of inoculum per pot. The experiment was laid out in replicated randomized block design with three replications of seedlings from nursery trays were transplanted to each of the fifteen pots are as follows,

T_1- Normal plants without fungi
T_2- Micro propagated plants without fungi
T_3- Micro propagated plants inoculated with *G. mosseae*
T_4- Micro propagated plants inoculated with *G. fasciculatum*
T_5- Micro propagated plants inoculated with *G. macrocarpum*

Fresh and dry weight

The root, stem and leaves of **c**ontrol and micro propagated *Glomus* treated Plants were harvested after 48 weeks and biomass was recorded in terms fresh and dry weight.

Assessment of AM fungal association in roots

The percent infection of both normal and micro propagated plants inoculated with *Glomus* species was estimated according to Philips and Hayman (1970). The cleaned roots cut into 1 cm long and fixed in FAA (5:5:90) for 24 hours. The root pigments were depigmented by autoclaving with 10% KOH at 15Psi for 10 mins. The alkalinity was neutralized with HCl and stained with 0.03% Tryphan blue in lacto phenol. The stained samples were observed under the microscope for AM mycelium, vesicles and arbuscles. The percent infection was calculated by the following formula.

$$\text{Percent of root colonization} = \frac{\text{No. of root bits shows colonization}}{\text{Total no. of root bits observed}} \times 100$$

Spore count

The AM fungal spores were extracted using wet sieving and decanting method (Gerdemann and Nicholson, 1963). 500mg of soil from each treatment was suspended in water and stirred thoroughly; soil suspension was passed through sieves of 1000- 45µm size, kept one below the other. Spores from the bottom sieve were collected and observed under a microscope.

Estimation of primary metabolites

Root, Stem and leaves were selected for quantitative estimation of primary metabolites viz. Total Carbohydrates and Reducing sugars by Anthrone and DNS methods (Mahadevan and Sridhar, 1986). Total proteins (Lowry *et al*., 1951) and chlorophyll content by Arnon method (1949) were analyzed. The secondary metabolites like total Phenolics and Free amino acids were analyzed by Nitsch and Nitsch (1962) and Moore and Stein (1948).

Data analysis

The data obtained was subjected to statistical analysis by two way ANOVA, significant 'F' ratios between groups means were further subjected to Duncan's multiple range tests using SPSS version 21. Probability values <0.05 were considered as significant.

RESULTS AND DISCUSSION

In vitro regeneration and plantlets development

The compact callus derived from shoot tip explants were cultured on MS media supplemented with 0.5 mgl^{-1} of IBA and 2 mgl^{-1} of Cytokinins (Fig. 1). Numbers of shoots were increased when KN was combined with IBA. Percentage of shoot induction was higher in KN (2 mgl^{-1}) + IBA (0.5 mg l^{-1}) compared to 2 mgl^{-1} of BA + 0.5 mgl^{-1} of IBA (Fig. 2).

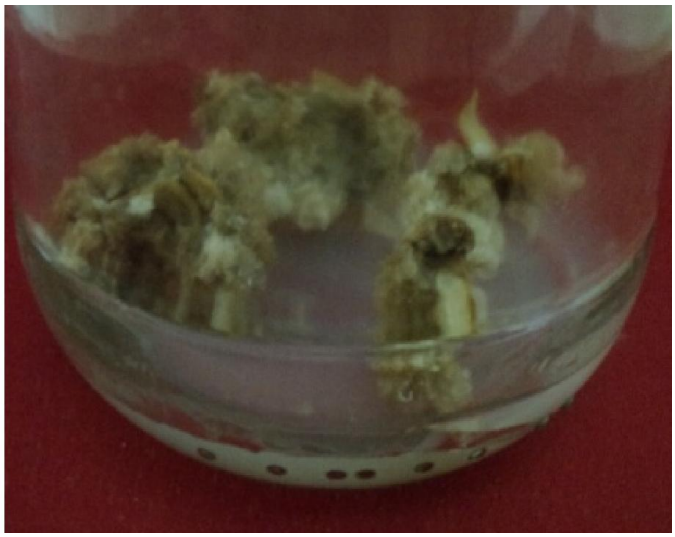

Fig.1. MS+ IBA (0.5 mg l^{-1}) + BA (2 mgl^{-1})

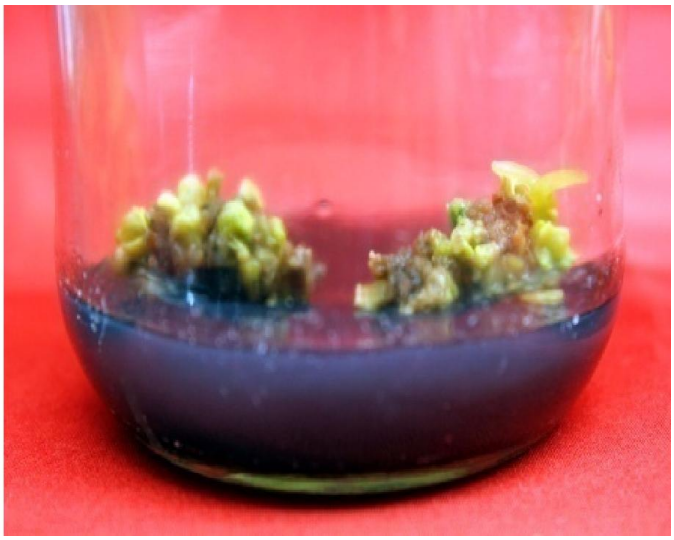

Fig. 2. MS + KN (2 mgl^{-1}) + IBA (0.5 mg l^{-1})

Fig. 3. MS+ KN (2 mg l^{-1}) + BA (0.5 mgl^{-1})

Fig. 4. MS + IBA (1 mg l^{-1})

Fig. 5 & 6. Hardened plants are maintained in green house

Multiple shoots were obtained maximum when supplemented with 2 mgl^{-1} of KN + 0.5 mgl^{-1} of BA (Fig. 3). Similar results were reported by Vadawale *et al.* (2004) from nodal explants, maximum shoots from apical buds (Sivanesan, 2007) of *W. somnifera*. Maximum root formation was induced when multiple shoots were cultured on MS medium containing 1mgl^{-1} of IBA (Fig. 4). Sivanesan and Murugesan (2008) attributed the maximum root induction at lower concentration of IBA in *W. somnifera.* The results are concurrent with studies of (Tulin and Biswajit, 2012) where maximum roots were induced with IBA (1mgl^{-1}) within 30 - 40 days. The well rooted plantlets were transferred to pots and maintained in the green house condition as represented in Fig. 5 & 6.

Assessment of plant growth and root colonization

Table 1 represents the fresh and dry weight of root, stem and leaves of normal, micropropagated and micropropagated plants inoculated with three *Glomus* species. The biomass was significantly more in the plants inoculated with *Glomus* species compared to the control plants. However, *G. mosseae* treated plants showed a higher biomass with respect other species. This may be due to higher photosynthetic rates of mycorrhizal plants and also by uptake of N, P and K. Similar

Table 1. Influence of *Glomus* species on Fresh and Dry weight of plants at 120th day

Treatments	Fresh weight (g)			Dry weight (g)		
	Root	Stem	Leaves	Root	Stem	Leaves
T1	6.1±4.16^{a}	9.23±0.24^{a}	13.5±0.40^{a}	1.6±0.61^{a}	1.72±0.13^{a}	1.33±0.06^{a}
T2	6.68±1.18^{b}	11.53±0.18^{b}	15.31±0.32^{b}	1.44±0.19^{a}	1.84±0.313^{a}	1.24±0.25^{a}
T3	8.57±0.14^{e}	15.82±0.23^{e}	33.3±0.32^{e}	1.86±0.40^{c}	3.07±0.17^{c}	2.83±0.27^{c}
T4	6.95±0.26^{d}	16.69±0.19^{d}	21.54±0.55^{d}	1.51±0.37^{b}	2.87±0.25^{b}	1.92±0.23^{b}
T5	6.04±0.30^{c}	13.91±0.39^{c}	15.82±0.94^{c}	1.59±0.212^{b}	2.82±0.25^{b}	1.7±0.44^{b}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

Table 2. Percentage of root colonization and spore count in 500mg of soil

Treatments	No of vesicles/root	No of arbuscles/root	No of spores
T1	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
T2	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
T3	7.33±0.57^{b}	7.33±1.52^{b}	232.66±4.04^{b}
T4	7±2^{b}	7.66±0.57^{b}	284.66±8.62^{c}
T5	6.33±2.51^{b}	6.33±2.08^{b}	293.66±6.42^{c}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

Table 3. Estimation of primary metabolites in *W. somnifera* treated with *Glomus* species

Treatments	Carbohydrates			Reducing sugars		
	Root	Stem	Leaves	Root	Stem	Leaves
T1	4.87±6.34^{a}	4.916±7.09^{b}	5.43±2.08^{a}	3.04±6.92^{a}	3.66±6.11^{a}	3.22±0.10^{a}
T2	6.405±6.57^{b}	4.34±8.505^{a}	6.25±0.13^{b}	3.133±6.11^{a}	3.61±2.30^{a}	3.58±0.10^{b}
T3	7.97±1.36^{cd}	6.43±2.00^{c}	7.86±3.00^{d}	4.4±4.00^{c}	4.92±0.17^{b}	5.49±0.20^{e}
T4	8.655±0.16^{d}	6.58±2.51^{d}	8.56±0.49^{e}	3.64±6.92^{b}	5.33±0.46^{c}	4.96±0.10^{d}
T5	6.95±1.38^{bc}	6.43±2.51^{c}	7.19±6.08^{c}	4.8±0.10^{d}	4.57±4.61^{b}	4.58±6.11^{c}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

Table 4. Estimation of primary metabolites in *W. somnifera* treated with *Glomus* species

Treatments	Proteins			Free amino acids		
	Root	Stem	Leaves	Root	Stem	Leaves
T1	3.94±6.11^{a}	5.21±0.10^{b}	4.77±8.32^{a}	368±8.00^{a}	518.66±10.06^{a}	484±8.00^{ab}
T2	4.18±8.32^{b}	4.98±6.11^{a}	4.98±0.12^{b}	356±8.00^{a}	535.06±67.70^{a}	396±34.17^{a}
T3	6.21±0.12^{d}	5.86±6.11^{c}	7.36±6.92^{d}	390.66±18.03^{b}	609.33±12.22^{b}	548±99.51^{b}
T4	6.86±4.61^{e}	5.92±4.00^{c}	6.65±0.11^{c}	438.66±8.32^{c}	650.66±6.11^{b}	585.33±102.31^{b}
T5	5.42±0.14^{c}	4.92±8.00^{a}	6.48±0.14^{c}	405.33±12.22^{b}	598.66±6.11^{b}	584±21.16^{b}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

Table 5. Estimation of Chlorophyll contents in *W. somnifera* treated with *Glomus* species (mg/g)

Treatments	Chlorophyll-a		Chlorophyll-b		Total chlorophyll	
	Stem	Leaves	Stem	Leaves	Stem	Leaves
T1	1±0.00^{a}	1.21±0.00^{b}	6.66±5.77^{ab}	2.07±1.00^{b}	8.36±5.77^{ab}	1.35±1.75^{b}
T2	2.33±0.77^{b}	0.952±0.44^{a}	6±0.00^{a}	1.58±0.81^{a}	8.3±1.00^{a}	1.27±1.20^{a}
T3	3±0.00^{bc}	1.24±1.00^{c}	7.33±5.77^{bc}	2.19±0.107^{c}	8.26±2.08^{a}	1.41±1.75^{c}
T4	2.33±0.71^{b}	1.51±1.05^{d}	7.66±5.77^{c}	2.66±1.85^{d}	8.63±1.52^{b}	1.61±1.52^{d}
T5	3.33±.72^{c}	0.96±0.00^{a}	7±0.00^{bc}	2.14±1.95^{bc}	8.13±2.51^{a}	1.32±1.50^{b}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

Table 6. Estimation of total Phenolics in *W. somnifera* treated with *Glomus* species

Treatments	Phenolics		
	Root	**Stem**	**Leaves**
T1	11.42±0.11^{b}	7.06±6.11^{b}	16.85±3.05^{c}
T2	10.42±0.34^{a}	7.28±5.29^{c}	15.7±3.05^{b}
T3	11.74±0.34^{bc}	8.36±1.52^{d}	18.68±5.03^{d}
T4	12.18±0.21^{c}	8.31±1.52^{d}	19.49±3.05^{e}
T5	11.24±0.63^{b}	6.9±5.29^{a}	15.26±4.00^{a}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level.

observations were reported by (Halder and Ray, 2006) wherein the biomass production was increased when the plants were treated with *Glomus* species compared to normal plants. The percentage of root colonization of vesicles, arbuscles and spores were represented in Table 2. There is in significant changes observed between *Glomus* treated plants in percentage of vesicles and arbuscles in colonized roots. The number of spores was significantly more in colonized roots of *G. macrocarpum* fallowed by *G. fasciculatum* treated plants. The root colonization percentage was higher in *Glomus* treated plants which may be due to the effect of rhizosphere soil that favored the growth of AM fungi in roots. The increased root colonization and spore formation may be due to improved mineral nutrition and also the colonization of fungal hyphae in roots to produce Arbuscles, and vesicles. The results are comparable with the studies of Hosamani *et al.* (2011) where root colonization and spore number in *W. somnifera* are increased in *Glomus* species treated plants.

Primary metabolites

The carbohydrates are significantly higher in *G. fasciculatum* treated plants and reducing sugars showed significant variations in different parts of *Glomus* treated plants (Table 3). The protein content was significantly increased in root and stem of *G. fasciculatum* treated plants. While in the leaves the protein content was higher in *G. mosseae* treated plants (Table 4). However, the free amino acids do not exhibit significant changes between the treated plants (Table 4). The increase in amino acids and proteins may be due to reverse translocation of the carbon compound to the host. The increase in the level of amino acids is correlated to the percent root colonization of AM fungi as reported by Fattah and Mohamedin (2000). The amount of chlorophyll-a, chlorophyll-b and total chlorophyll were represented in Table 5. The *G. fasciculatum* treated plants showed a higher response in increasing the chlorophyll content in the stem and leaves. The results obtained were similar to the studies of Tejavathi *et al.* (2011) where the chlorophyll content was increased in *Glomus* treated plants of *Andrographis paniculata.*

Phenolics content

The total Phenolics content was also increased in *G. fasciculatum* treated plants compared to other treated and control plants as shown in the Table 6. Phenolics are to be of major importance in pathogenic interactions between plant and fungi. Increased phenolic concentration in plant tissues may be pathogenic attack is one of the important mechanisms by which pathogenic activity may limit or decreased Morandi, (1996). Devi and Reddy (2002) also observed the increase in Phenolics acids in groundnut plants inoculated with *Glomus* species.

Conclusion

In the present study the AM fungi treated micro propagated plants significantly increases in the biomass, primary metabolites and Phenolics in *W. somnifera*. The *G. fasciculatum* appeared to be more effective than other species of *Glomus* in enhancing the primary metabolites in micropropagated treated plants, which are precursors for the production of important secondary metabolites produced in the plant. The study also provides a scope for *in vitro* mass multiplication of the plants and treated with AM fungi which can be useful in accumulation of secondary metabolites in order to fulfill the global demands and have a high prospective in pharmaceuticals.

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