Influence of *Glomus intraradices* on growth, biochemical changes and withanolides content of *Withania somnifera* L Dunal (Ashwagandha)

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Abstract

Withania somnifera L. Dunal (Ashwagandha) also known as Indian ginseng, of the family Solanaceae is an important ancient medicinal plant, used in the Indian traditional system of medicine. The medicinal importance of *W. somnifera* is attributed to the presence of steroidal lactones called withanolides which are present in the leaves and roots of the herb. The present set of pot experiment was designed to investigate the effect of *Glomus intraradices* inoculation on growth, biochemical parameters and withanolides content of *W. somnifera*. The results showed significant enhancement on plant growth, biochemical parameters and withanolide content in plant. Mycorrhizal treatment showed better vegetative growth in comparison to untreated plants. Significant increase in height, root length, and total dry weight (biomass) of AM inoculated plants as compared to non inoculated plants was observed. AM fungi considerably enhanced the levels of nitrogen, phosphorus and potassium as compared to that of non-mycorrhizal plants. A considerable increase in withaferin A and withanolide a content by mycorrhization was observed. Mycorrhizal inoculation significantly affected withanolide content of *W. somnifera*. The results suggested that *G. intraradices* is a beneficial biofertilizer for increasing yield of biologically important secondary metabolites in *W. somnifera*.

Keywords: Ashwagandha, *Glomus intraradices*, Withanolides, *Withania somnifera*, Arbuscular mycorrhizal fungi

Introduction

Withania somnifera (L.) Dunal (Family Solanaceae), commonly known as Ashwagandha, is one of the most valued medicinal plants with a number of pharmaceutical applications (Anonymous, 1962). The ethnopharmacological properties of this plant include adaptogenic, anti-sedative and anti-convulsion activities (Budhiraja *et al.*, 2000). The plant is recommended in many Ayurvedic recipes and has been employed in the treatment of neurological disorders, geriatric debilities, arthritis and stress and behaviour related problems (Gupta and Rana, 2007). The medicinal importance of *W. somnifera* is attributed to the presence of steroidal lactones called withanolides which are present in the leaves and roots of the herb (Ichikawa *et al.*, 2006; Misra *et al.*, 2008). Plant roots interact with a wide variety of microorganisms in the soil, among these arbuscular mycorrhizal fungi form an important component of

soil microflora. Arbuscular mycorrhizal fungi develop a symbiotic relationship with higher plants, and have been reported to enhance the plant growth by supplying phosphate, nitrogen and other nutrients from the soil and translocating them to the host plant (Bolan, 1991).

Beneficial effects of AM symbiosis on plant growth, nutrient uptake, and tolerance to environmental stresses have been extensively reported (Koide and Mosse, 2004). However, these fungi show a preferential colonization to hosts and thus the extent to which the host benefit depends of the fungal species involved in the symbiosis (Miller *et al.*, 1987).

The selection of efficient AM fungi is a key prerequisite for inoculation since there are different levels of compatibility between host plants and AM fungi (Smith and Read, 1997). The objective of the present study was to investigate the effect of *Glomus intraradices* on the growth and withanolide content of *Withania somnifera* under controlled environmental conditions.

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Material and Methods

Plant material and treatment of *Glomus intraradices*

Glomus intraradices was isolated from the rhizosphere soil of the plant and pure inoculum of the fungus was maintained on Rhodes grass roots in sterilized soil. A 90-days-old soil pot culture of *G. intraradices* maintained on Rhodes grass was used as inoculum. The infected root fragments and rhizospheric soil of the Rhodes grass containing 25 spores/g of soil was used as inoculum. Plants were harvested after 90 days. Dry weight of shoot, root and leaf was recorded after drying in an oven at 40°C for 7 days. Fresh weight of root, shoot, leaves and height of the plants was recorded at the time of harvesting.

Plant growth conditions

Surface sterilized seeds of *Withania somnifera* L. Dunal obtained from College of Horticulture, Mandsaur (M.P) were sown in earthen tray containing sterilized soil. The tray was inoculated with 10 g of soil-based AM inoculant (*Glomus intraradices*) containing approximately 250 chlamydospores of *G. intraradices* at the time of sowing. Seeds sown in sterilized soil without AM fungi served as controls. Twenty seeds were sown in each tray and were grown for 30 days in glasshouse with day/night regimes of 12 h at 30°C $\pm$ 2°C and 45–60% relative humidity. Germinated seedlings were transplanted in earthen pots, one plant in a pot (6" size) filled with autoclaved soil (pH 8.0, EC 0.062 ms/cm, available P 0.12%, organic carbon 0.79%, nitrogen 0.06%, potassium 0.87%). There were five replicates for each treatment. Plants were watered when necessary. Roots of the seedlings were periodically checked for AM colonization by standard method of Phillips and Hayman (1970).

Analysis of phosphatase activity, macro and micronutrients, protein, chlorophyll content, crude cytokinin content and withanolides

Macro and micronutrients were analyzed from stem, root and leaf parts of the plant by the standard method of Jackson (1973). Assay of acid and alkaline phosphatase activity was done by the modified method of Bergmeyer (1974). Protein content was estimated by the standard method of Lowry (1951). Chlorophyll content was estimated by the age old method of Arnon (1949). Crude

cytokinin was estimated by the method of Thiagarajan and Ahmad (1994). Withanolides were extracted from leaf and root of mycorrhizal and non-mycorrhizal plants by the method of Khajuria *et al.*, (2004) and their qualitative analysis was done by reverse phase analytical high performance liquid chromatography (HPLC).

Percentage root colonization and number of spores/100 g soil

The root samples were stained with trypan blue in lactoglycerol by standard method (Phillips and Hayman, 1970). The percentage of infected root length was evaluated by the gridline intersect method of Giovannetti and Mosse (1980). Spores were isolated from the soil samples by wet sieving and decanting method (Gerdemann and Nicolson, 1963) and quantified by eelworm counting slide.

Results and Discussion

Effect of AM fungi on plant growth

Inoculation of *G. intraradices* significantly affected plant growth, nutrient content, acid and alkaline phosphatases, protein and withanolides content of *W. somnifera* (Table 1-2, Fig. 1). AM fungi inoculation significantly enhanced shoot length, root length and dry weight of plant as compared to that of non-mycorrhizal control (Table 1). Percent increase in shoot length and root length of AM inoculated plants over control was 24.94%, and 30%, respectively. Total dry weight/plant of AM fungi inoculated plants was higher than control. Percent increase in total dry weight of mycorrhizal plant was 106.03%. Earlier workers have reported significant increase in growth parameters due to mycorrhizal inoculation in medicinal plants (Earanna *et al.*, 2002; Boby and Bagyaraj, 2003). Similar type of result was obtained by Haldar and Ray (2006) in *W. somnifera* inoculated with *Glomus fasciculatum*. Percent root colonization and number of spores in plants inoculated with *Glomus intraradices* was 63.84 and 188.14/100 g rhizosphere soil, respectively (Table 1). There was no AM fungi colonisation in control plants.

Effect of AM fungi on biochemical changes

There was a increase in chlorophyll a, chlorophyll b, total chlorophyll, protein, acid phosphatase, alkaline phosphatase and kinetin content

Table 1: Effect of *G. intraradices* inoculation on growth of *Withania somnifera*

Treatments	Height (cm)	Root length (cm)	Dry wt/plant (g)	Percent Root Colonization	No. of spore/100 g soil
Control	34.48±2.90	6.0±0.38	2.32±0.33	--	--
Mycorrhizal	43.08±3.65	7.8±0.49	4.78±0.32	63.84±3.28	188.14±4.09
P<0.05	0.0033	0.0002	2.17E-06	8.59E-11	8.9E-14

± = Standard Deviation

Table 2: Effect of *G. intraradices* inoculation on biochemical changes of *Withania somnifera*

Treatments	Protein content leaf (µg/mg fresh wt)	Protein content root (µg/mg fresh wt)	Alkaline phosphatase activity root (µm p-nitrophenol /g fresh wt/min)	Acid phosphatase activity root (µm p-nitrophenol /g fresh wt/min)	Chl a (mg/g fresh wt)	Chl b (mg/g fresh wt)	Total chl (mg/g fresh wt)
Control	0.23±0.02	0.15±0.004	1.13±0.08	26.47±2.03	0.757±0.04	0.442±0.024	1.212±0.02
Mycorrhizal	0.46±0.01	0.26±0.007	2.32±0.09	61.09±0.73	0.810±0.04	0.612±0.029	2.012±0.18
P<0.05	1.06E-08	8.81E-10	1.94E-08	4.32E-12	0.081	7.1E-06	9.13E-06

Figure 1. Withaferin A and Withanolide A content in mycorrhizal and non-mycorrhizal roots of *Withania somnifera*

in AM fungi treated plants compared with non-mycorrhizal plants (Table 2). Inoculation of AM fungi increased 66.01% of total chlorophyll content as compared to that of control. Chlorophyll a was 7.0% higher in AM inoculated plants than that of control. The present results are in agreement with earlier workers who reported increased chlorophyll content in mycorrhizal plants (Morte *et al.*, 2000). AM fungi colonization significantly increased production of acid and alkaline phosphatases (expressed as $\mu\text{m p-nitrophenol g}^{-1}$ fresh wt min^{-1} of root tissue) as compared to that of non-mycorrhizal plants. Acid phosphatase activity was dominated over alkaline phosphatase in both the mycorrhizal and non-mycorrhizal plants. Acid phosphatase activity was 130.79% higher in AM inoculated plants than that of control (Table 2). The present results are in agreement with several authors who also observed increased activity of acid phosphatase and alkaline phosphatase in mycorrhizal roots as compared to nonmycorrhizal roots. Fries *et al.*, (1998) observed that acid phosphatase and alkaline phosphatase activities in maize roots were closely correlated to levels of AMF colonisation of roots. Selvaraj (1998) found that due to inoculation of AM fungi *G. fasciculatum* acid phosphatase activity increased in leaves and roots of *Prosopis juliflora*. Higher uptake of phosphorus in mycorrhizal *Withania somnifera* plants may be due to the increased levels of phosphatase enzymes which ultimately resulted in enhanced growth response of plant. Mycorrhizal fungi have been reported to improve P availability by solubilising inorganic

phosphorus which is mediated through release of organic acids and phosphatases (George *et al.*, 1995). Earlier workers have suggested that alkaline phosphatase in mycorrhizal root played an important role in poly P degradation and thus the release of inorganic phosphate (Pi) from arbuscules to root cells (Ezawa *et al.*, 2001). Phosphorus translocated from external hyphae which is normally present in the form of polyphosphate can be hydrolysed by alkaline phosphatase. Leaf and root protein level expressed as $\mu\text{g protein mg}^{-1}$ fresh weight of mycorrhizal inoculated plants showed a greater increase than the non-mycorrhizal plants (Table 2). Protein content of mycorrhizal plants was nearly two-fold than that of non-mycorrhizal plants. Mycorrhization increased 103.10% and 80.14% protein content in leaf and root over non-mycorrhizal plants (Table 2). The beneficial effect of mycorrhizal fungi on total protein content has been reported on plants of *Catharanthus roseus* inoculated with *G. mosseae* (Ratti *et al.*, 2010). Accumulation of protein in the mycorrhizal roots has been reported in many plants (Gianinazzi-Pearson and Gianinazzi, 1989).

Effect of AM fungi on nutrient content

The effect of AM fungi in the total nitrogen, phosphorus and potassium content is presented in Table 3. Concentration of nitrogen, phosphorus and potassium in root and leaves of mycorrhizal plants was significantly higher than that of non-mycorrhizal ones (Table 3).

Table 3: Effect of *G. intraradices* inoculation on nutrient content of *Withania somnifera*

Treatments	Concentration of nutrients (%)								
	Nitrogen			Phosphorus			Potassium		
	S	R	L	S	R	L	S	R	L
Control	1.17± 0.02	1.43± 0.02	1.40± 0.04	0.11± 0.02	0.14± 0.03	0.18± 0.03	2.10± 0.05	1.53± 0.04	3.37± 0.06
Mycorrhizal	1.97± 0.10	2.31± 0.07	2.25± 0.09	0.28± 0.06	0.31± 0.03	0.37± 0.07	3.52± 0.04	3.21± 0.03	5.55± 0.07
P<0.05	1.6E- 07	5.55E- 09	3.85E- 08	0.000 242	1.39E -05	0.000 427	2.64E- 11	7.14E- 13	1.65E -11

S = Stem; R = Root; L = Leaf

Mycorrhizal infestation increased 68.38%, 60.71% and 61.54% nitrogen in stem, leaf and root as compared to non-mycorrhizal plants. Phosphorus content in stem, leaf and roots of mycorrhizal plants was 154.55%, 105.56% and 121.43% higher than non-mycorrhizal ones. The results of the present study coincide with the reported findings of Jackobsen *et al.*, (1992), who have reported that the fungal hyphae growing beyond the rhizospheric soil increase the absorptive surface area of the root, which result in a greater efficiency of nutrient absorption, especially slowly diffusing mineral ions like phosphorus (Kothari *et al.*, 1991). Transport of P into host plants and its release to root cells is an important function of AM fungi (Ryan *et al.*, 2007). Increased P absorption is usually attributed to increased surface area and increased soil exploration by the root AM fungal association. Mycorrhizal fungi significantly increased potassium content as compared to that of non-mycorrhizal plants. Percent increase in potassium content of mycorrhizal plants was 67.62% in stem, 64.69% in leaf and 109.80% in roots as compared to that of non-mycorrhizal plants.

Effect of AM fungi on withanolide content

Mycorrhizal inoculation significantly affected withanolide content of *W. somnifera* (Fig. 1). The concentration of withaferin-A was 3.53 mg and 5.67 mg g⁻¹ dry weight root in non-mycorrhizal and mycorrhizal plants, respectively. Similarly the withanolide A content was 2.67 mg in non-mycorrhizal and 4.26 mg g⁻¹ dry wt. in mycorrhizal plants (Fig 1). Percent increase in Withaferin-A was 60.62 and Withanolide-A was 59.55 as compared to that of non-mycorrhizal plants. The potential of AM fungi has been reported to enhance isoflavone content in red clover (Khaosaad *et al.*, 2008). Results of the present study indicate that *G. intraradices* inoculation significantly enhanced plant growth, changed biochemical parameters and increased biologically active secondary metabolites in *W. somnifera*.

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