Journal of Applied and Natural Science
10 (4): 1115 -1123 (2018)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Effect of mycorrhiza (*Glomus mosseae)* on morphological and biochemical properties of Ashwagandha (*Withania somnifera*) (L.) Dunal

Pratibha Parihar
Department of Biotechnology, Genetics and Bioinformatics, N.V. Patel College of Pure and Applied Sciences, Sardar Patel University, Vallabh Vidyanagar-388120 (Gujarat), India
Madhumati Bora*
Department of Biotechnology, Genetics and Bioinformatics, N.V. Patel College of Pure and Applied Sciences, Sardar Patel University, Vallabh Vidyanagar-388120 (Gujarat), India

*Corresponding author. E-mail: drmadhumatibora@gmail.com

Article Info
DOI:10.31018/jans.v10i4.1797
Received: June 13, 2018
Revised: September 4, 2018
Accepted: October 7, 2018

How to Cite
Parihar, P. and Bora, M. (2018). Effect of mycorrhiza (*Glomus mosseae)* on morphological and biochemical Properties of Ashwagandha (*Withania somnifera*) (L.) Dunal. *Journal of Applied and Natural Science*, 10(4): 1115 -1123

Abstract
Mycorrhizal inoculation in the plant causing increase in growth and production of phytochemicals is well reported, however little information is available related to the effect of mycorrhiza on morphological and biochemical properties of the medicinal plants like Ashwagandha. The present study is an attempt on diversity analysis in *Withania somnifera* with an aim to ascertain the nature and extent of genetic diversity present among different accessions in presence of mycorrhiza. The major biochemical constituents of Ashwaganda roots are with nolides which are well known for its medicinal properties. Mycorrhizal associations confer benefits like better nutrition acquisition, enhanced growth, defense enhancement and improved abiotic and biotic stress tolerance in plants. The present investigation was undertaken to assess genetic diversity among five different accessions of *W. somnifera* using morphological and biochemical markers and the effect of mycorrrhizal inoculation on these marker. The present study concluded that presence of mycorrhiza was effective on plant growth and phytochemical constituents more than non-treated plants. Amongst five selected germplasms IC 283662, JA 134, RAS 23, MPAS 6 and MWS 205 of *W. somnifera,* JA 134 showed best response in pretext of the selected morphological and biochemical features in presence of mycorrhiza.

Keywords: Arbuscular mycorrhiza, Flavonoid, Protein, Phenol, Sugar, Tannin

INTRODUCTION

Plant morphology has relevance to practically all disciplines of plant biology such as molecular genetics, physiology, ecology, evolutionary biology and systematics. *Withania somnifera* (L.) Dunal (Ashwagandha), is one of the important low input medicinal cash crops used in traditional system of Indian medicines. Lenin *et al*. (2010) showed that the inoculation of Arbuscular mycorrhiza (AM) fungi resulted in significant increase in germination percentage, shoot and root length, fresh and dry weight, number of leaves per plant and total leaf area, as compared to non-inoculated plants. Manila and Nelson (2014) identified that AM colonization led to significant increase in the phenolic content of tomato plant than control one. Allah *et al*. (2015) observed that Mycorrhiza triggers defense system of host plant by stimulating level of growth regulators. They found that mycorrhizal inoculation significantly improves the physiological and biochemical parameters as well as total contents of soluble sugar, phenol and amino acid like proline. RaziaShuab (2016) observed that plants inoculated with mycorrhiza showed significantly better morphological growth parameters regarding fresh and dry weight of plant and chlorophyll content as compared to non-mycorrhizal plants in *Crocus sativu*s.

Further, Vesicular-Arbuscular Mycorrhizal (VAM) fungi inoculation enables the increase in plant height, fresh shoot weight and fresh root weight in squash crop was reported by Al-Hmoud *et al*. (2017). They further claimed that double dose was the most effective treatment which increased the plant growth and root weight. Narwal *et al*. (2018) demonstrated the favorable effects of micorrhizal association in rice plants under aerobic conditions and showed positive correlation with the total chlorophyll content and nitrate reductase activity. The

present investigation aims to assess genetic diversity amongst five different germplasm of ashwgandha, *W. sominifera* on the basis of their phenotypic and biochemical characteristics and to study the mycorrhizal effect on these features.

MATERIALS AND METHODS

Collection of plant material and establishment of mycorrhiza: In the present investigation, seeds of different genotypes of *W. somnifera* (L.) Dunal were procured from "Botanical Garden, Department of Genetics and plant breeding, Chimanbhai Patel College of Agriculture, Saradar Krushinagar, Dantiwada Agricultural University, Saradarkrushinagar" which were formerly collected from different research stations in India. The genotypes of *W. somnifera* (L.) Dunal used in present investigation were IC 283662, JA 134, RAS 23, MPAS 6 and MWS 205.

Mycorrhizal spores (*Glomus mosseae)* were purchased from "The Energy and Resource Institute (TERI), New Delhi". Seeds of *W. somnifera* (L.) Dunal were surface sterilized with 10% ethanol for 1 minute, followed by a 10 minute treatment with 0.5% aqueous sodium hypochlorite solution (NaOCl) and finally three times rinsed with sterile distilled water. For initial germination, seeds were grown in petri dish on moist filter paper. The experiments were carried out under normal environmental conditions at temperature 25-32°C for ten days (Fig. 1). After two weeks seedlings grown were transferred in plastic pots containing sand which was sterilized at 121^{o}c for 2 hours and for nutritional support, Murashige and Skoog's basal medium (MS) without sucrose and agar Murashige and Skoog, 1962) was used for watering (Fig. 2). All the chemicals and reagents used in the present investigation were of high purity analytical grade, purchased from Hi Media Laboratories Pvt. Ltd.

All the five genotypes were grown in two sets i.e. control and mycorrhiza inoculated. In control set, Vesicular-Arbuscular Mycorrhizal was not inoculated while after 30 days of plants establishment, mycorrhizae spores were added to the second set of Ashwagandha accessions. The inoculum (0.5g fresh weight per pot containing approximately 100 spores) was placed in the pot at 2 cm depth closed to the roots, to facilitate fungal colonization on the plant root (Fig. 3).

Morphological characters evaluation: Data for nine quantitative traits was recorded for both the sets. The traits were plant height (cm), maximum leaf size (cm), fresh weight of leaf (gm), dry weight of leaf (gm), maximum root length (cm), fresh weight of root (gm), dry weight of root (gm), number of berries per plant and number of seeds per berry for each accession. Five plants were selected from each set on the basis of response in a genotype for recording the data. A two factor CRD individual analysis was performed to determine the significance differences between the mean of each parameter tested in the study. The mean value was used for statistical analysis. These statistical analyses were performed by using SPSS software (Version 15.0, SPSS Inc., USA). The significance was tested at 5% level.

Detection of arbuscular mycorrhizal fungi in roots of Ashwagandha: Roots were cleared by treating with 10 ml of 2M KOH solution at 60 °C for 1 hr then washed with water and treated with 3M HCl solution. Acidified root samples were stained with 0.01% trypan blue dye prepared in lactoglycerol (lactic acid and glycerol and water 1:1:1) for two hours. The roots were destained with lactoglycerol (without dye) overnight and finally were observed under compound microscope. Fungal structures like vesicles and arbuscules were observed under microscope as stained blue in the mycorrhizal inoculated root cells of Ashwagandha (Fig. 4).

Phytochemical Evaluation

Plant material: Fresh leaves collected during flowering stage (three months old plant) for all the five accessions of *W. somnifera* (L.) Dunal including both sets (control and mycorrhiza inoculated) were used for phytochemical analysis.

Sample preparation: Leaves were washed properly with tap water to remove soil and dirt and dried under shade at room temperature for two weeks and were ground to powder. The powder of the leaves was passed through a 0.5 mm metallic mesh to yield fine powder for the use of phytochemical estimation.

Quantitative profiling: The biochemical contents were estimated with calibrated curve method using UV-VIS spectrophotometer. The concentration of the samples were extrapolated from the calibration curve of absorbance and known concentrations of different standard. The details of the methods and reagents are as follows.

Sample preparation: 100 mg of dried and powered leaves of all the plant accessions were taken, and were extracted with ethanol (80%) on the boiling water bath for 30 minutes. The supernatant was preserved for analysis of sugar, protein, total phenol and tannins.

Quantification of total sugar: Anthrone method (Hodge, 1962).

0.2 ml of sample was taken with 1ml distil water and 4ml of freshly prepared Anthrone reagent was added to it. Warm for 8 min in the waterbath and cooled to room temperature. The absorbance of the green coloured complex was observed at 630nm.

Quantification of total protein: Lowry method (Lowry *et al*., 1951).

0.2 ml of sample was taken with 2 ml of alkaline copper sulphate reagent. After 10 min, 0.2 ml of

Folin Ciocalteau solution was added and incubated for 30 minutes. The absorbance of the colored complex was observed at 660nm.

Quantification of total phenol: Folin – Ciocalteau method (Ainsworth *et al*., 2007).

0.1ml extract was diluted to 2.5ml with distilled water in a test tube and mixed with 0.5ml of freshly prepared Folin – Ciocalteau reagent. The mixture was treated with 2ml Na_2CO_3 solution. It was boiled for 1 min and cooled to room temperature at running tap water. Absorbance was measured with the spectrophotometer at 650 nm.

Determination of total tannin: Folin Denis method (Makkar *et al*., 1993).

0.5g of powered plant material with 75ml water was boiled for 30 minutes in a conical flask and pelleted at 2000 rpm for 20 minutes. Supernatant was used for estimation. In one ml of sample, 5ml of Folin Denis reagent and 10 ml of sodium carbonate solution was added. It was heated for 1 minute and cooled to room temperature. Absorbance of the colored complex was measured at 700nm.

Quantification of total flavonoid: Boham and Kocipai- Abyazan (1974)

10 gms of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight. The flavonoid precipitated was recovered by filtration using weighed filter paper. The resulting weight difference gave the weight of flavonoid in the sample.

Distance Matrix and Cluster Analysis of Ashwagandha genotypes: The morphological and biochemical parameters were used to produce a dendrogram using cluster analysis subprogramme 'SimInt' of the software NTSYS-pC (Numerical Taxonomy and Multivariate Analysis System), which revealed the genetic linkage and proximity among all the genotypes investigated. The distance matrix calculated using software NTSYS-PC version 2.12.

RESULTS AND DISCUSSION

Statistical analyses were performed by using SPSS software (Version 15.0, SPSS Inc., USA).

An extreme degree of variability exist with respect to growth habit, morphological and biochemical characteristics of plants in different parts of the country.

Variation analysis: Study of variability in morphological characters based on nine quantitative parameters revealed maximum variability in the fresh weight and dry weight of root followed by fresh and dry weight of leaf and maximum leaf size and number of berries per plant. This pattern

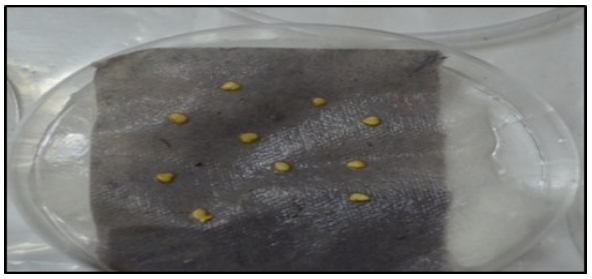

Fig.1. *Seed germination on moist filter paper.*

Fig. 2. *30 days old Ashwagandha plant in the plastic pot with sterilized sand*

Fig. 3. *50 days old Ashwagandha plants with and without mycorrhiza*

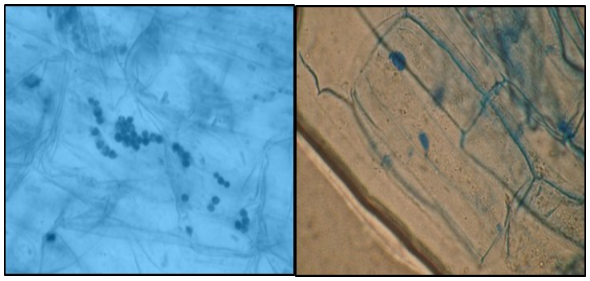

Fig. 4. *Trypan Blue stained roots of Ashwagandha under compound microscope*

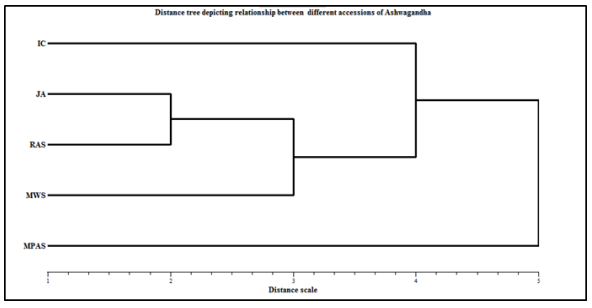

Fig. 5. *Phylogenetic relationship between different accessions based on their morphological and biochemical features in presence of mycorrhiza.*

Table 1. Morphological and phytochemical variation in quantitative characters of *Withania somnifera* (L.) Dunal (without Mycorrhiza).

	TRAIT	Mean	SD	SE	CV(%)
(i)	Plant height (cm)	57.8	6.242	3.12	10.8
(ii)	Maximum leaf length (cm)	7.2	1.72	0.860	23.9
(iii)	Fresh weight of leaf (gms)	0.728	0.229	0.114	31.5
(iv)	Dry weight of leaf (gms)	0.275	0.0854	0.042	30.9
(v)	Root length (cm)	11.2	1.93	0.96	17.3
(vi)	Fresh weight of root (gms)	2.05	0.732	0.366	35.6
(vii)	Dry weight of root (gms)	0.745	0.263	0.131	35.2
(viii)	Number of berries/plant	52	10.19	5.09	19.6
(ix)	Number of seeds/berry	20.8	2.31	1.15	11.13
(x)	Sugar (mg/gm dry weight of leaf)	33.5	4.52	2.26	13.4
(xi)	Protein (mg/gm dry weight of leaf)	71.72	10.88	5.44	15.1
(xii)	Phenol (mg/gm dry weight of leaf)	14.98	0.914	0.45	6.1
(xiii)	Tannin (mg/gm dry weight of leaf)	7.51	1.08	0.54	14.4
(xiv)	Flavonoid (mg/gm dry weight of leaf)	76.4	6.04	3.00	7.8

Table 2. Morphological and phytochemical variation in quantitative characters of *Withania somnifera* (L.) Dunal (with Mycorrhiza).

	TRAIT	Mean	SD	SE	CV(%)
(i)	Plant height (cm)	61.2	5.30	2.65	8.6
(ii)	Maximum leaf length (cm)	8.6	2.05	1.02	23.9
(iii)	Fresh weight of leaf (gms)	0.817	0.273	0.136	31.4
(iv)	Dry weight of leaf (gms)	0.336	0.102	0.05	30.4
(v)	Root length (cm)	12.8	2.13	1.06	16.68
(vi)	Fresh weight of root (gms)	2.578	0.94	0.47	36.4
(vii)	Dry weight of root (gms)	1.160	0.417	0.208	35.9
(viii)	Number of berries/plant	56.8	10.9	5.48	19.3
(ix)	Number of seeds/berry	22.6	2.05	1.02	9.11
(x)	Sugar (mg/gm dry weight of leaf)	47.3	5.98	2.99	12.8
(xi)	Protein (mg/gm dry weight of leaf)	87.04	11.23	5.6	12.9
(xii)	Phenol (mg/gm dry weight of leaf)	24.592	4.3	2.15	17.4
(xiii)	Tannin (mg/gm dry weight of leaf)	10.9	1.51	0.75	13.9
(xiv)	Flavonoid (mg/gm dry weight of leaf)	86.5	8.7	4.3	10.1

Table 3. Correlation matrix of quantitative characters in control set.

Traits	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv
i) Plant height	1													
ii) Max. leaf size	0.059	1												
iii) Fresh wt. of leaf	0.269	0.85	1											
iv) Dry wt. of leaf	0.410	0.67	0.949	1										
v) Root length	0.598	-0.01	0.229	0.450	1									
vi) Fresh wt. of root	0.311	0.23	0.456	0.624	0.906	1								
vii) Dry wt. of root	0.3	-0.02	0.181	0.389	0.943	0.956	1							
viii) No. of berries/plant	0.452	0.362	0.302	0.354	0.809	0.776	0.769	1						
ix) No. of seeds/berry	0.813	0.361	0.313	0.341	0.632	0.435	0.410	0.813	1					
x) Sugar	-0.6	-0.1	0.05	0.141	0.188	0.482	0.481	-0.06	-0.551	1				
xi) Protein	-0.137*	-0.22	0.02	0.234	0.671	0.801	0.860	0.39*	-0.11	0.841	1			
xii) Phenol	-0.01	-0.37	-0.260	-0.05	0.748	0.730	0.883	0.56	0.127	0.592	0.892	1		
xiii) Tannin	-0.67	-0.317	-0.177	-0.06	0.05	0.281	0.351	-0.18	-0.69	0.868	0.776	0.562	1	
xiv) Flavonoid	-0.78	-0.189	-0.168	-0.14	-6.33	0.281	0.324	-0.09	-0.652	0.952	0.724	0.553	0.966	1

where, * correlation is significant at the 0.05 level (1-tailed) and ** correlation is significant at the 0.01 level (1-tailed)

of variation was found same for both controls set (Table 1) as well as for mycorrhiza treated set (Table 2). Morphological variability of characters showed the genotypic differences between the different accessions of the species studied. It can be used as an index to analyze the genotypic differences present within the species and such differences indicate the genetic diversity of the spe-

Table 4. Correlation matrix of quantitative characters in mycorrhiza inoculated set.

	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv
i) Plant height	1													
ii) Max. leaf size	0.611	1												
iii) Fresh wt. of leaf	0.509	0.905	1											
iv) Dry wt. of leaf	0.396	0.820	0.976	1										
v) Root length	0.863	0.44*	0.364	0.353	1									
vi) Fresh wt. of root	0.532	0.233	0.314	0.404	0.885	1								
vii) Dry wt. of root	0.580	0.336	0.443	0.560	0.858	0.975	1							
viii) No. of berries/plant	0.704	0.412	0.221	0.202	0.936	0.793	0.707	1						
ix) No. of seeds/berry	0.849	0.528	0.220	0.065	0.709	0.307	0.263	0.766	1					
x) Sugar	-0.292	-0.437	-0.196	-0.275	0.213	0.625	0.568	0.164*	-0.479	1				
xi) Protein	-0.181	-0.255	-0.07	0.117	0.362	0.735	0.664	0.355	-0.321	0.768	1			
xii) Phenol	-0.689	-0.3	-0.05	0.155	-0.277	0.183	0.171	-0.255	-0.801	0.783	0.75	1		
xiii) Tannin	-0.702	-0.490	-0.255	-0.363	0.64**	0.217	0.169	-0.215	-0.783	0.766	0.812	0.970	1	
xiv) Flavonoid	-0.426	-0.195	-0.09	0.096	0.130	0.504	0.413	0.238	-0.409	0.847	0.913	0.860	0.816	1

Where,* correlation is significant at the 0.05 level (1-tailed) and ** correlation is significant at the 0.01 level (1-tailed).

cies that has accumulated in the course of evolution of the species in different populations.

The selected five accessions were also subjected to estimation of phytochemical variability based on the five quantitative parameters (Table 2). Leaves were used as a sample for phytochemical analysis. The highest variability was found in protein followed by tannin and flavonoid in case of control set (without mycorrhiza), while in mycorrhiza treated set, variability was found to be highest in phenol and then in tannin and flavonoid. This indicates that association of mycorrhiza with the host plant gives a visible and remarkable increase in the production of secondary metabolites like phenol and tannin. Similar observations were also reported by Tejavathi *et al*. (2013) and Pusztahelyi *et al*. (2015). A significant increase in plant height, number of branches, number of leaves, fresh weight and dry weight of stem, and leaf and root over untreated plants is well documented by Ratti and Upadhyay (2012). Role of *G. intraradices* as bio-fertilizer in *W. somnifera* (L.) Dunal is established the report of Saikia *et al*. (2013).

Thus, in *W. somnifera* Dunal, the role of AM fungi in growth and nutrient absorption is proven dynamic. Besides, AMF also gives a strong defense to plant by increasing the production of phenol required for secondary metabolite production.This study is further supported by the work of Ruiz-Lozano (2003), who reported that AMF enables not only the improvement in photosynthesis and water uptake, but also the production of secondary metabolites which provide resistance to plant for different stress.

Correlation coefficient analysis: Biological characters are controlled by genes which may be oligogenic in nature. Characters, which show continuous distribution, are polygenic in nature. Most of the plant characters related to growth, yield and productivity belong to this category. Being polygenic inherited trait, the yield of plant depends upon a number of other component traits. These characters showed different levels of interrelationships between them and such relationships can be identified by correlation analysis.

The extent of association and diversity between yield and its contributing characters may be exploited and quite helpful in breeding programs for making selections.

A critical observation of correlation coefficient (Table 3 and Table 4) between different traits revealed that plant height has positive and highly significantly correlation with root length, number of berries/plant and seed yield per berry. Maximum leaf size is correlated with fresh and dry weight of leaf whereas root length exhibited positive and more significant correlation with plant height

Table 5. Analysis of variance for morphological characters in Ashwagandha

Source of variation	d.f.	Plant height (cm)	Max. leaf length (cm)	Fresh wt. of leaf(gm/ gm fresh wt.)	Dry wt. of leaf (gm/gm dry wt.)	Root Length (cm)	Fresh wt. of leaf (gm/gm fresh wt.)	Dry wt. of leaf (gm/gm dry wt.)	No. of berries /plant	No. of seeds/ berry
Variety (V)	4	835.4*	160*	2.41*	0.5*	115*	20.81*	3.85*	1797*	88.1*
Treatment (T)	1	1132.8*	50*	0.59*	0.27*	800*	41.95*	26.68*	317.5*	343.2*
VT	4	486.1*	10	0.03*	0.01*	55*	2.08*	0.25*	37.0*	18.28
Error	40	84	58	0.04	0.0 1	84	0.73	0.48	102.8	126

Table 6. Analysis of variance for phytochemical characters in Ashwagandha.

Source of variation	Sugar (mg/gm dry wt.)	Protein (mg/gm dry wt.)	Phenol (mg/gm dry wt.)	Tannin (mg/gm dry wt.)	Flavonoid (mg/gm dry wt.)
Variety (V)	1319*	3280*	272.53*	2694*	56.54*
Treatment (T)	2379*	2437*	1149*	1271*	179.36*
VT	91.03*	1319*	208.98*	141.38*	0.71*
Error	90.24	85.45	86.18	80.18	37.25

d.f.- degree of freedom

and fresh and dry weight of root. Number of berries per plant had positive and significant association with plant height and weight of leaf.

In case of phytochemicals, both total sugar and protein showed highest correlation with flavonoid; whereas phenol revealed maximum correlation with tannin. However, majority of morphological characters showed negative correlation with the phytochemical traits except the fresh and dry weight of root and root length, which depicted significantly positive correlation with all the phytochemical traits *viz.* total sugar, protein, phenol, tannin and flavonoid. The significant value of correlation 0.01 levels (1- tailed) found between tannin and root length. On the other hand the significant value of correlation at 0.05 levels (1-tailed) found between number of berries per plant and total sugar and protein, maximum leaf size and root length and plant height and protein. The pattern of correlation is same for both control set as well as mycorrhiza inoculated set. This indicated that root length and root weight was found to be highly and positively associated with plant height, number of berries per plant, total sugar, phenol, protein, tannin and flavonoid. Thus it can be concluded that functionally related traits tend to be highly integrated morphologically and their phenotypic correlation confirmed the functional relationship.

A highly significant and positive association of plant height with root length, number of berries per plant and root weight was observed. Since they have originated in different habitats and have contributed significantly towards the adaptability and divergence of the species, such diversities can be considered as valuable sources of genes and genotypes for the selection of superior variety. Thus, variations identified can be considered as an important source of gene differences and can be exploited in propagation and breeding programs.

Analysis of variance (ANOVA): The data obtained for each character was analyzed by the usual standard statistical procedure ($P<0.05$). To provide comparisons, the sum of square was partitioned into varieties and treatments for different characters (Table 5, 6, 7 and 8). The results obtained indicate that the mean sum of squares in all the five varieties were found to be significant for all the characters, indicating considerable amount of variability among the control set (without mycorrhizal inoculation) and the set treated with mycorrhiza.

Significant result was found in all the characters except leaf size and number of seeds per berry. The significant mean sum of squares in control and treated set for all the traits indicated the presence of variability between the control and mycorrhizal inoculated set.

Plant height (cm): It was found that there is 16.46% increase in overall mean performance and when it was inoculated with mycorrhiza, all the five varieties showed significant increase in the plant height with 2.3% coefficient of variation.

Maximum leaf length (cm): According to the results of ANOVA, genotypes inoculated with mycorrhiza showed non-significant increase in leaf size as compared to control plants. High variability was observed in leaf size for selected genotypes of *Withania somnifera* (L.) Dunal with range from 4.5 - 9.5 cm. Overall result of mean showed an increase of 28.5% in leaf size after mycorrhiza inoculation and 15.5% increase in coefficient of variation. The genotypes belong to different geographical region with high variation in climatic conditions. This also proves that leaf size is one of the important characteristic of Ashwagandha plant affected by geographical variations.

Fresh weight of leaf (gm): On comparison of

Table 7. Variation in the trait after the inoculation of mycorrhiza.

	Plant height (cm)	Max. leaf length (cm)	Fresh weight of leaf (gm/gm fresh wt.)	Dry weight of leaf (gm/gm dry wt.)	Root length (cm)	Fresh weight of root (gm/gm fresh wt.)	Dry weight of root (gm/gm dry wt.)	No. of berries/ plant	No. of seeds / berry	Sugar (mg/gm dry wt.)	Protein (mg/gm dry wt.)	Phenol (mg/gm dry wt.)	Tannin (mg/gm dry wt.)	Flavo-noid (mg/gm dry wt.)
V1	70	9.5	1.11	0.49	17	3.78	1.86	65	26	38.5	81.8	17.7	75	8.3
V2	61	9	1.02	0.46	18	3.8	1.68	65.5	24	45	89.2	22	92	10.2
V3	63	4.5	0.5	0.21	15.5	2.95	1.53	54.6	23	44.3	77.4	20.7	85	10.1
V4	60	8.5	0.99	0.44	13.5	2.13	1.08	50	21.9	31.2	64.5	16.6	71.3	7.8
V5	58	8.5	1.04	0.45	16	2.7	1.3	57.5	23.5	43.2	80.4	22	84.3	10.3
SEM	0.46	0.38	0.01	0.01	0.45	0.04	0.03	0.5	0.56	0.47	0.46	0.46	0.44	0.3
CD	1.31	1	0.03	0.02	1.3	0.12	0.1	1.44	1.6	1.35	1.32	1.32	1.28	0.8
T1	57.7	7	0.82	0.33	12	2.16	0.76	56	21.1	33.5	71.7	15	76.4	7.5
T2	67.2	9	1.04	0.48	20	3.69	2.22	61	26.3	47.3	85.6	24.5	86.5	11.2
SEM	0.29	0.24	0.01	0.01	0.3	0.27	0.02	0.05	0.35	0.3	0.3	0.3	0.28	0.2
CD	0.82	0.68	0.02	0.01	0.82	0.07	0.06	0.14	1.01	0.85	0.83	0.83	0.8	0.55
VxT	Signifi-cant	Non Significant	Significant	Significant	Significant	Significant	Significant	Signifi-cant	Non Significant	Significant	Significant	Signifi-cant	Signifi-cant	Significant
CV%	2.3	15.5	3.54	4.13	9.06	7.38	7.35	2.74	7.48	3.72	1.86	7.42	1.74	9..28

Table 8. Variation in the traits of all the varieties in control and mycorrhiza inoculated set.

	Plant height (cm)		Max. leaf length (cm)		Fresh weight of leaf (gm/ gm fresh wt.)		Dry weight of leaf (gm/gm dry wt.)		Root length (cm)		Fresh weight of root (gm/gm fresh wt.)		Dry weight of root (gm/gm dry wt.)		No. of berries/ plant		No. of seeds / berry		Sugar (mg/gm dry wt.)		Protein (mg/gm dry wt.)		Phenol (mg/gm dry wt.)		Tannin (mg/gm dry wt.)		Flavo-noid (mg/gm dry wt.)	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T 2	T1	T2	T1	T2	T1	T2	T1	T2
V1	69	71	8	11	1	1.2	0.4	0.6	14	20	3	4.6	1.0	2.7	62	68	24	28	31	46	75	89	15	20	70	80	7	10
V2	52	70	8	10	1	1.1	0.4	0.5	12	24	2.8	4.8	0.9	2.4	63	68	21	27	38	51	82	97	16	28	84	99	8	12
V3	57	69	4	5	0.4	0.6	0.1	0.2	12	19	2	4	0.8	2	51	58	20	26	35	54	80	75	16	25	80	90	8	12
V4	58	61	8	9	1	1	0.4	0.5	10	17	1.5	2.7	0.3	1.8	49	51	20	23	26	36	51	78	14	19	68	74	6	10
V5	52	65	7	10	0.9	1.1	0.4	0.5	12	20	1.5	4	0.6	2	55	60	20	27	37	50	71	90	14	30	80	88	8	12
SEM	0.6		0.53		0.015		0.01		0.64		0.06		0.04		0.71		0.8		0.67		0.65		0.65		0.63		0.43	
CD	1.8		NS		0.04		0.03		1.85		0.17		0.14		2.04		NS		1.92		1.86		1.87		1.81		1.62	

overall mean value of mycorrhiza inoculated variety with non-inoculated one, there was an increase in 26.83% in fresh weight of leaves. The variation was found to be significant with the coefficient of variation 3.54%.

Dry weight of leaf (gm): The mean value was studied and compared. Mycorrhizal presence showed an increase in dry weight of leaf by 35.35%. This increase was found significant with 4.13% coefficient of variation.

Root length (cm): The significant increase in root length (66.65%) was observed after inoculation with mycorrhiza. The coefficient of variation (CV%) was 9.06 which indicates root length to be significant factors affected by the presence of mycorrhiza.

Fresh weight of root (gm): The calculated mean value was compared between control and mycorrhiza inoculated variety. It showed an increase by 66.2% in fresh weight of root after mycorrhiza treatment. This variation is found to be significant with CV% value as 7.38.

Dry weight of root (gm): In all the five selected varieties, the comparative analysis of mean value depicted that dry weight was also influenced by mycorrhiza. The calculated increase in percentage from the mean value was 64.66%. The coefficient of variation was found to be significant with the value 7.35%.

Number of berries per plant: It was found that there was 8.9 % increase overall mean performance in number of berries when it was inoculated with mycorrhiza. All the five varieties showed significant increase in berries number with 2.74% coefficient of variation.

Number of seeds per berry: Statistically, all genotypes of plant when inoculated with AM showed non-significant increase in number of seeds per berry as compared to control plants. The high variability was found in number of seeds/berry ranging between 20-26. Overall result of mean showed an increase of 24.6% in number of seeds after mycorrhiza inoculation and 7.48% increase in coefficient of variation.

Total sugar (mg/gm dry weight of leaf): All the selected Ashwagandha varieties were analyzed and compared for total sugar content. It was found that inoculation of AM in Ashwagandha significantly increased the total sugar content. The estimated increase in percentage from the mean value was 41.19 %. The coefficient of variation was found to be significant with the value 3.72%.

Total protein (mg/gm dry weight of leaf): An increase of 19.38 % overall mean performance in the total protein estimated was observed when inoculated with mycorrhiza. All the five varieties showed significant increase in total protein with 1.86% coefficient of variation.

Total phenol (mg/gm dry weight of leaf): Significant increase in phenol concentration after inoculation with mycorrhiza was found to be 63.3%. The coefficient of variation was 7.42% which showed that phenol is one of the significant factor affected by the presence of mycorrhiza.

Total tannins (mg/gm dry weight of leaf): The increase in tannin concentration was found to be 13.21 after mycorrhizal treatment. In all the five varieties, tannin showed significant increase in coefficient of variation (1.74%).

Total flavonoid (mg/gm dry weight of leaf): The sum of squares mean value of both control and mycorrhiza inoculated varieties was compared. It showed an increase of 49.3% in flavonoid content after mycorrhiza treatment. This variation was found to be significant with CV% value as 7.38.

In the above results, high significant growth diversity was found in all the studied traits between mycorrhizal inoculated and control plants. Phenol and flavonoid contents showed maximum variation, followed by root length, dry and fresh weight of root. The following result indicated that mycorrhiza play a significant role in secondary metabolite production. It also suggested that the production secondary metabolite in response to the inoculation with AMF may vary in different germplasms of same species. Dos Santos *et al*. (2017) identified that the mycorrhizal association result in activation of the expression of genes encoding for enzymes Phenylalanine-ammonia-lyase (PAL) and Chalcone synthase (Chs) linked with the production of flavonoids. Presence of mycorrhizal association with *C. leucanthemum* detected with significant increase in total phenol and flavonoids content (Noori *et al*. 2012). Zhang *et al*. (2013) reported colonization with mycorrhiza enhance phenolic compounds synthesis due to alteration of gene expression of PAL and CHS enzymes.

Al-Karaki and Clark (1999) indicated that shoot and root dry matter were higher for mycorrhizal infected wheat plants than non-infected plants which were ascribed to an already established phenomenon of higher phosphorus (P) uptake by AMF infected roots for the plants. The present study suggests that AMF colonization improved positively the overall growth and development of Ashwagandha. It also emphasizes the fact that AMF is responsible for an increase in different morphological and biochemical trait parameters and also the tolerance power of plant to fight against stress conditions through an enhanced production of secondary metabolites.

Cluster analysis using phyolegentics tree of Ashwagandha genotypes: The data obtained from morphological and biochemical parameters for all the five accessions *viz.* IC 283662, JA 134, RAS 23, MPAS 6 and MWS 205 were further used to construct distance matrix with fourteen different characters using software NTSYS-PC version 2.12. All the five

genotypes of Ashwagandha were grouped in major and minor clusters based on the distance coefficient. JA 134 and RAS 23 showed highest similarity with lowest distance coefficient value 2.0. JA 134 and RAS 23 together showed high similarly with MWS 205 at a distance coefficient of 3.0. Finally IC 283662 followed by MPAS 06 showed less similarity with high distance coefficient 4.0 and 5.0 respectively. Thus, among all the five genotypes, JA 134 and RAS 23 showed the maximum similarity for all the 14 morphological and biochemical characters in the presence of mycorrhiza as revealed by phylogenetic tree (Fig. 5).

Conclusion

Morphological and biochemical variability of characters of Ashwagandha, *W. somnifera* shows the genotypic differences between the different accessions of the species studied. It can be used as an index to analyze the genotypic differences present within the species and such differences indicates the genetic diversity of the species that has accumulated in the course of evolution of the species in different populations. Since these genotypes have originated in different habitats they have contributed significantly towards the adaptability and divergence of the species. It can be considered as valuable sources of genes and genotypes for the selection of superior accessions leading to their use in conservation, propagation and economical exploitation. Such variation can also be exploited both for commercial and plant breeding purposes.

REFERENCES

1. Ainsworth, E.A. and Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nat. Protocols* 2: 875 – 877.
2. Allah, M., Safwat., El-Bassiouny, H., Bakry, B.A. andSadak, M.A. (2015). Effect of arbuscular mycorrhiza and glutamic acid on growth, yield, some chemical composition and nutritional quality of wheat plant grown in newly reclaimed sandy soil. *RJPBCS,* 6 (3):1038-1054.
3. Al-Karaki, G.N. and Clark, R.B. (1999). Mycorrhizal influence on protein and lipid of durum wheat grown at different soil phosphorus levels. *Mycorrhiza*, 9:97-101
4. Al-Hmoud G, Al-Momany A (2017). Effect of Four Mycorrhizal Products on Squash Plant Growth and its Effect on Physiological Plant Elements. *Adv Crop Sci Tech* 5: 260.
5. Boham, B. A., Kocipai-Abyazan, R. (1974). Flavonoids and condensed tannins from leaves of Hawaiian Vaccinium vaticulatum and V. calycinium. *PacificScience* 48: 458-463.
6. Dos Santos, E. L., Alves da Silva, F., and Barbosa da Silva, F. S. (2017). Arbuscular Mycorrhizal Fungi Increase the Phenolic Compounds Concentration in the Bark of the Stem of *Libidibia Ferrea* in Field Conditions. *The Open Microbiology Journal*, 11:283–291
7. Narwal, E. Annapurna K, Choudhary J and Sangwan S (2018). Effect of Arbuscular mycorrhizal Fungal Colonization on Nutrient Uptake in Rice Aerobic Conditions. *Int.J.Curr.Microbiol.App.Sci.* 7(04):1072-1093
8. Hodge, J.E. andHofreiter, B.T. (1962). Methods in Carbohydrate Chemistry, (Eds. Whistler, R.L. and Be Miller, J.N.), Academic Press, New York.
9. Lenin, M., Selvakumar, G., Thamizhiniyan, P. andRajendiran, R. (2010) Growth and biochemical changes of vegetable seedlings induced by arbuscular mycorrhizal fungus. *J. Exp. Sci.*, 1(4):27-31.
10. Lowry, O.H., Rosbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin Phenol reagent. *J. Biol. Chem., 193:* 265.
11. Makkar, H.P.S., Blummel, M., Borowy, N.K. and Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J. Sci. Food Agric.,*61:161-165
12. Manila, S. and Nelson, R. (2014). Biochemical changes induced in tomato as a result of arbuscular mycorrhizal fungal colonization and tomato wilt pathogen infection. *Asian J. of Plant Sci. and Res.*, 4 (1):62-68.
13. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant,* 15(3): 473-497.
14. Noori A.S., Maivan H.Z., Alaie E (2012).Changes in total phenol and flavonoid contents in Chrysanthemum leucanthemum under crude oil contamination. *Adv. Environ. Biol*., 6(12): 3057-3064.
15. Pusztahelyi, T., Holb, I. J. and Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Front. Plant Sci,.* 6:573. doi: 10.3389/ fpls.2015.00573
16. Ratti, N. and Upadhyay, A. (2012). Effect of *Glomus intraradices* on growth and biochemical constituents of *W. somnifera* (Ashwagandha). *Mycorrhiza News* 24(1)
17. RaziaShuab (2016). Arbuscular Mycorrhizal Fungal Symbiosis with saffron (Crocus sativus L.) Plant. *JNBR* 5(1): 59 – 67.
18. Ruiz-Lozano, J.M. Mycorrhiza (2003) 13: 309. https:// doi.org/10.1007/s00572-003-0237-6.
19. Saikia, S.K., Tiwari,S. and Pandey R. (2013). Rhizospheric biological weapons for growth enhancement andMeloidogyne incognita management in *Withania somnifera* cv. Poshita. *Biological Control,* 65 (2013) 225–234.
20. Tejavathi, D.H. and Jayashree, D.R. (2013). Phytochemical screening of selected medicinal herbs inoculated with arbuscular mycorrhizal fungi. *IJBPAS*, 2 (11): 2090-2106.
21. Zhang, R.Q., H. Zhub, H.Q. Zhao and Q. Yao (2013). Arbuscular mycorrhizal fungal inoculation increases phenolic synthesis in clover roots via hydrogen peroxide, salicylic acid and nitric oxide signaling pathways. *J Plant Physiol.* 1;170(1):74-9.