The effect of inoculation with mycorrhiza: AM on growth, phenolics, tannins, phenolic composition and antioxidant activity in *Valeriana jatamansi* Jones

A.K. Jugran, A. Bahukhandi, P. Dhyani, I.D. Bhatt*, R.S. Rawal, S.K. Nandi, L.M.S. Palni

I.G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora - 263 643, Uttarakhand, India. *Corresponding author: idbhatt@gbpihed.nic.in

Abstract

The mycorrhizal inoculation often reported to increase plant growth and secondary metabolites production, however, little is known on the effect of arbuscular mycorrhizal fungi (AMF) on different growth parameters in *Valeriana jatamansi*, an important Himalayan medicinal herb. To evaluate mycorrhizal effect, *V. jatamansi* collected from natural population were planted and growth parameters, phytochemicals, antioxidant and phenylalanine ammonia lyase (PAL) activities were determined. Significantly ($p<0.05$) higher above ground fresh weight (AGFW), above ground dry weight (AGDW), below ground fresh weight (BGFW), below ground dry weight (BGDW) were recorded in plants after the 2nd year. The total phenolics were maximum in aerial [18.03 ± 0.05 (1st year) and 10.88 ± 0.52 mg GAE/g (2nd year)] and root portions [14.30 ± 0.04 (1st year) 7.92 ± 0.27 (2nd year)] of the treated plants as compared to corresponding portions of control plants in both the years. Similarly, the tannin content was higher in treated plants as compared to untreated controls. Variation in different phenolic compounds was also recorded and significantly higher contents of gallic acid, chlorogenic acid, Catechin, and Hydroxy benzoic acid during 1st year was observed. Antioxidant activity of aerial and root portion measured by three *in vitro* assays, showed significant variation ($p<0.05$) among plants of both the years. Significantly ($p<0.05$) higher PAL activity was recorded in treated over control plants in 2nd year. The results demonstrated that inoculation with AMF enhances both phenolics and tannins as well as stimulates antioxidant activity suggesting a positive influence of AMF inoculation on yield and for the production of nutraceuticals.

Keywords: Arbuscular mycorrhizal fungi (AMF), Phenolics, Tannins, antioxidant activity, *Valeriana jatamansi*

1. Introduction

Members of the family Valerianaceae are used both in the traditional as well as modern systems of medicine, the activity being attributed to various secondary metabolites, e.g., valepotriates, dihydrovaltrate, linalin-isovalerat, sesquiterpenoids, 6-methylapi-genin and hesperidin, and phenolics and flavonoids. One of the highly valued members of this family, *Valeriana jatamansi* Jones (English name - Indian valerian, Trade name - Tagar), has attracted much attention in recent years. It is a small herb, that grows widely in the temperate Himalaya between 1000-3000m above mean sea level (amsl); its roots/rhizomes have been used traditionally for the treatment of many ailments like snake bite, cold and cough; it is also a rich source of phenolics, flavonoids and antioxidants (Kalim *et al.*, 2010; Bhatt *et al.*, 2012; Jugran *et al.*, 2013). Large scale harvesting of this plant continues from the wild to meet the ever growing demand, thereby putting substantial pressure on the natural populations.

Although AMF association with the roots of *V. jatamansi* and some other alpine medicinal herbs has been reported to induce VAM (Vesicular arbuscular mycorrhizae) and hyphal infection, morphological and phytochemical parameters were not examined. The use of AMF to enhance the production of secondary metabolites has been reported (Nell *et al.*, 2010). Recently, it has been indicated that AMF treated *Valeriana officinalis* plants were successfully colonized and that such plants showed increased sesquiterpenic acid accumulation compared to untreated controls (Nell *et al.*, 2010). AMF also act as biological control agent and are often used in organic farming and play important role in improving the soil fertility and plant nutrition (Banuelos *et al.*, 2014; Caporale *et al.*, 2014; Channabasava *et al.*, 2015).

The biosynthesis of several secondary metabolites, such as carotenoids or lignin, may be mainly constitutive, the synthesis of numerous secondary metabolites like many phenolic compounds, can be induced and enhanced by abiotic and biotic stresses. It is well known that the accumulation of secondary metabolites may increase when plants are undergoing high or low temperatures, high light intensity, UV radiation, drought, mineral deficiencies, salinity or pathogen infection (Baslam *et al.*, 2013). A possible mechanism of increased phytochemical concentration in plants could be through improved nutrition. Amino acids like tyrosine and phenylalanine are important precursors to the production of various phenolics compound like rosmarinic acid and caffeic acid (Petersen and Simmonds, 2003). Therefore it could be possible that higher N assimilation in AM plants (Toussaint *et al.*, 2004) might contribute in the production of these amino acids, which subsequently stimulate higher production of the phenylalanine ammonia lyase, one of the main enzyme involved in the production of various phenolic compounds. Another possible mechanism could be the changes induced by AMF in the level of phytohormone such as cytokinin or gibberellin (Allen *et al.*, 1980). However, there possibilities are needed to be checked.

Phenolics are one of the major compounds which impart antioxidant activity in plants, and their biosynthesis involves the induction of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the first rate limiting enzyme in the phenyl propanoid metabolism (Rhodes, 1985). The accumulation of phenolics and other secondary metabolites in plant tissues has been known to be induced by abiotic stresses as well as by fungal infection (Zhi-Lin *et al.*, 2007). Therefore, there exists a potential for increased production of phytochemicals for developing commercial health

care products. As such, the influence of mycorrhizal inoculation on secondary metabolite production in Himalayan medicinal herbs has not been reported. Realizing this, the present investigation was undertaken using a highly valued medicinal herb, *V. jatamansi*, to assess the effect of mycorrhizal inoculation on (i) growth performance, (ii) secondary metabolites like total phenolics, tannins content, antioxidant activity, and (iii) establish relationship of phenylalanine ammonia lyase (PAL) with secondary metabolites in *V. jatamansi*.

2. Materials and Methods

2.1. Screening of fungus in natural plants of *V. jatamansi*

Young growing plants (10 Nos.) were randomly collected from its natural population from Katarmal. Rhizomes/roots of each plant were washed in de-ionized water and cut into ~1cm segment and cleared in 10% KOH for two days at room temperature, rinsed several times with distilled water and stained with 5% ink- vinegar solution (Blank ink, Shaeffer) for 30 min at 80 °C. The rhizomes were subsequently destained with acidified water (using few drops of vinegar). Colonization was determined using magnified interaction method with minor modification (Cavagnaro *et al.*, 2001).

Rhizome/root pieces mounted on slides were examined at x100 magnification using light microscope. One hundred interactions between rhizome and crosshair were observed for presence or absence of AM structure (internal and external hyphae; arbuscules and/or vesicles). As no fungal growth was observed in the plants therefore the plants from this site was used for further experimentation.

2.2. Plant and fungal material

Young growing plants (120 Nos.) of *V. jatamansi* Jones were taken from their natural population in Katarmal forest, Almora, India (Lat. 29° 38' N, Long. 79° 36' E, altitude 1200m above mean sea level) and planted in the experimental plot at Surya Kunj - Nature Interpretation and Learning Center of G. B. Pant Institute of Himalayan Environment and Development, Kosi – Katarmal, Almora, India (Lat. 29° 46' N, Long. 79° 35' E; altitude 1150 m asl). The AMF preparation used in the present study was a consortia of three different isolates of *Glomus intradices* spp. (Tiwari and Adholeya, 2002) prepared and marketed by Majestic Agronomic Pvt. Ltd., Himachal Pradesh, India with brand name Mycorrhiza – AM. The Mycorrhiza-AM was a gift from Prof. Y. K. Bhoon (Sri Venkateswara College, New Delhi). The experimental plot was subdivided into 6 subplots (2X2 m); 3 subplots (size 12m² each; total plot size 24 m²) were used for growing control plants (i.e., without AMF inoculation) and the remaining 3 subplots were used for growing treated plants (20 plants/subplot). Plants growing in the control plots were treated with farmyard manure (4 kg/plot), while the plants growing in the treatment subplots were inoculated with AMF (400 g per dose) and the same amount of farmyard manure (4 Kg/plot). The treated plants of 1st year received two doses of inoculant, while plants in the 2nd year received 3 doses of inoculant. At the end of 1st year 60 plants (30 control and 30 treated ones) were harvested to record morphological parameters and for the determination of total phenolics, tannins and antioxidant activity. Remaining 60 plants were harvested at the end of 2nd year and all parameters, as in the first year, were recorded. The phenylalanine ammonia lyase (PAL) was, however, determined in the 2nd year plants only.

2.3. Morphological analyses

Various morphological parameters like leaf number (LN), leaf area (LA), plant height (PH), above and below ground fresh (AGFW and BGFW) and dry weight (AGDW and BGDW), rhizome length (RL) and width (RW), etc., were recorded for each individual. Leaf length and width of each plant was determined using 2 mature leaves per plant. AGFW and BGFW was recorded using electronic balance (Afcoset, India) and dry weight was determined after drying samples in an oven (40 °C till constant weight). RL and RW were measured using digital Vernier callipers (Mitutoya, Japan).

2.4. Extract preparation

Aerial and root portions of each plant were separately collected and washed first with running tap water and then with distilled water, dried (hot air oven, 40 °C) and ground into fine powder. Dried powder (1.0 g) was mixed with 25 ml aqueous methanol (80%, v/v); the suspension was stirred gently (12 h) and then sonicated (22 °C, 10 min; Model-ANIS 09001, Toshiba, New Delhi, India). The extract was centrifuged (10,000 rpm; 15 min, 22 °C) and the supernatants was collected, filtered and stored at 4 °C prior to use for analyses within 24 h.

2.5. Determination of total phenolic content

Total phenolic content in the methanolic extract of root and aerial portions was determined by Folin–Ciocalteu's colorimetric method (Singleton and Rossi, 1965). The extract (0.25 ml) was diluted with distilled water (2.25 ml) and then mixed with Folin–Ciocalteu's reagent (0.25 ml). After 5 min, this mixture was neutralized by adding 2.5 ml of sodium carbonate (7%, w/w) and allowed to stand in the dark (22±1°C,

90 min). The absorbance of resultant blue color was measured at 765nm using UV-VIS spectrophotometer (U-2001, Hitachi, Japan). The quantification of total phenolics was done on the basis of standard curve of gallic acid prepared in aqueous methanol (80%, v/v), and the results were expressed in mg gallic acid equivalent (GAE)/g dry weight.

2.6. Determination of total tannin content

This was estimated following the method of Nwinuka *et al.* (2005) with minor modifications. Methanolic extract (5 ml) was added to Folin–Dennis reagent (0.5 ml) and saturated with 1 ml sodium carbonate solution. The solution was diluted up to 10 ml with distilled water and allowed to stand (22±1°C, 20 min). The absorbance of resulting greenish blue color was measured at 700nm, and the quantification of total tannins was done on the basis of standard curve of tannic acid prepared in aqueous methanol (80%, v/v). Results were expressed in mg tannic acid equivalent (TAE)/g dry weight.

2.7. HPLC analysis of phenolic compounds

High Performance Liquid Chromatography (HPLC) analysis was carried out on a Merck Hitachi HPLC system equipped with L-7100 series pump, L-7400 series detector, 4.6 mm × 250 mm i.d., 5 µm, Purospher®, and C-8 column (Merck Pvt Ltd., Germany). The mobile phase water: methanol: acetic acid (80:20:1 and 70:30:1 ratio) with 0.8 ml/min flow rate and 20µl injection volume was used for isolation of phenolics and flavonoids. The compounds spectra were recorded at different wavelengths [(i.e., gallic acid, catechin and hydroxybenzoic acid (254 nm), and chlorogenic acid (370 nm)]. The concentration of phenolic acid and flavonoids were calculated with the help of a corresponding external standard and results expressed in mg/100 g dry weight.

2.8. Estimation of antioxidant activity

Total antioxidant activity was measured by improved ABTS (ethyl benzothiazoline, 6-sulphonic) method (Cai *et al.*, 2004). ABTS (7.0 μ M) and potassium persulphate (2.45 μ M) were added in an amber colored bottle for the production of ABTS cation (ABTS^{•+}) and kept in the dark for (16 h, 22 $\pm$ 1°C). ABTS^{•+} solution was diluted with ethanol (80%, v/v) till an absorbance of 0.700 $\pm$ 0.05 at 734 nm was obtained. For sample analysis 3.90 ml of diluted ABTS^{•+} solution was added to 0.10 ml of methanolic extract. The resulting solution was mixed thoroughly and allowed to stand for 6 min in the dark at 22 $\pm$ 1 °C. Absorbance was recorded at 734 nm using UV-VIS spectrophotometer. Samples were diluted with aqueous methanol (80%, v/v) to obtain 20–80% reduction in absorbance at 734nm with respect to blank. A standard curve using various concentrations of ascorbic acid in aqueous methanol (80%, v/v) was prepared for the quantification of antioxidant potential. Results were expressed in millimole (mM) ascorbic acid equivalent (AAE)/100 g dry weight.

Study followed traditional DPPH (2, 2- diphenyl-1-picrylhydrazyl) cations assay (Brand-William *et al.*, 1995) with minor modifications. Equal volume of 400 mM DPPH, 0.2M MES buffer (pH 6.0 adjusted with NaOH) and ethanol (20%, v/v) was added to the mixture. DPPH cation solution (2.7 ml) was mixed with sample extract (0.9 ml) and kept in the dark at room temperature (22 $\pm$ 1 °C) for 20 min. Absorbance was recorded at 520nm using UV-VIS spectrophotometer. Results were expressed in mM AAE/100 g dry weight.

Ferric reducing antioxidant power (FRAP) assay was performed using the method of Faria *et al.* (2005) with minor modifications. FRAP reagent was prepared by adding 300 mM acetate buffer (100 ml) (i.e., 3.1 g sodium acetate and 16 ml glacial acetic acid/l);

10 mM 2, 4, 6-tri-2-pyridyl- 1, 3, 5-triazin (TPTZ) in 40 mM HCL (10 ml); 20 mM ferric chloride (10 ml). The mixture was pre - warmed at 37 °C in water bath (Labtech, Korea) and 3.0 ml of the mixture was added to 0.1 ml methanolic extract and kept at 37 °C for 8 min. Absorbance was then recorded at 593 nm. A blank sample was prepared using ascorbic acid and the results were expressed in mM AAE/100 g dry weight.

2.9. Phenylalanine ammonia lyase (PAL) estimation

PAL activity was estimated using the method of Zucker *et al.* (1968) with following modifications. Freshly harvested aerial and root portions (500 mg) were homogenized in 5 ml of ice cold 0.1M sodium borate buffer (pH 8.8) containing 1.4mM of 2-mercaptoethanol. The extract was filtered through Whatman filter paper (No. 42) and the filtrate was centrifuged (15,000 rpm, 4 °C, 15 min). The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm.

Samples containing 0.3 ml of enzyme extract were incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 40 °C. The amount of *trans*-cinnamic acid formed was calculated as micromoles of *trans*-cinnamic acid using a standard curve developed for *trans*-cinnamic acid, and expressed as Ug⁻¹ fw (μ moles *trans*-cinnamic acid min⁻¹ mg fw⁻¹). One unit of PAL was defined as the formation of 1 μ M cinnamic acid per hour.

2.10. Statistical analysis

All determinations of antioxidant activity by ABTS, DPPH, FRAP assays and the measurements of total phenols and tannins were conducted using 4 replicates.

The value for each sample was calculated as the mean $\pm$ SE. Statistical analyses [paired sample t test and Pearson correlation coefficients (r)] were carried out using Microsoft Excel 2007 and SPSS version 7.5.

3. Results

3.1. Morphological variations

Variations in the morphological parameters of control and treated plants have been shown (Table 1). Significant ($p < 0.05$) variations were observed in respect of RW ($t = 11.59$, $p < 0.01$) and RL ($t = 9.9$, $p < 0.01$) of the 1st year treated and control plants. The 1st year control plants showed higher values for all morphological

parameter as compared to treated ones except for RL. Similar results were obtained in the 2nd year plants for all morphological parameters except for LN and PH that showed higher values in treated ones. However, except for RL ($t = 3.53$; $p < 0.01$), BGFW ($t = 2.57$; $p < 0.01$), and BGDW ($t = 2.97$; $p < 0.01$) no significant differences were recorded in morphological parameters between control and treated plants of 2nd year. The comparison between 1st and 2nd year control plants revealed significant difference in AGFW ($t = 3.90$; $p < 0.00$), BGFW ($t = 4.47$; $p < 0.01$), LL ($t = 2.42$; $p < 0.01$), LW ($t = 3.54$; $p < 0.01$), LA ($t = 3.33$; $p < 0.01$) (Table 1). Whereas the comparison between 1st and 2nd year treated plants exhibited significant difference in all morphological parameters, except LN, LL and LW.

Table 1. Comparative analysis of morphological parameters of control and AMF treated plants of *V. jatamansi*.

Morphological parameters	1 st year plants		t value	2 nd year plants		t value	t value between 1 st and 2 nd year control plants	t value between 1 st and 2 nd year treated plants
	Control	Treated		Control	Treated			
LN	20.63 $\pm$ 1.7	18.23 $\pm$ 1.19	ns	20.23 $\pm$ 1.51	22.57 $\pm$ 2.44	ns	ns	ns
LL (cm)	3.25 $\pm$ 0.14	3.43 $\pm$ 0.10	ns	3.70 $\pm$ 0.14	3.91 $\pm$ 0.34	ns	2.42*	ns
LW (cm)	3.07 $\pm$ 0.14	3.44 $\pm$ 0.10	ns	3.76 $\pm$ 0.19	3.83 $\pm$ 0.26	ns	3.54**	ns
LA (cm ²)	9.98 $\pm$ 0.02	11.82 $\pm$ 0.01	ns	13.92 $\pm$ 0.03	14.98 $\pm$ 0.09	ns	3.33**	2.48*
RW (cm)	10.53 $\pm$ 0.4	3.52 $\pm$ 0.33	11.59**	5.50 $\pm$ 0.39	4.83 $\pm$ 0.30	ns	ns	2.93**
RL (cm)	4.87 $\pm$ 0.33	11.00 $\pm$.51	9.92**	4.82 $\pm$ 0.30	4.97 $\pm$ 0.29	3.53**	ns	19.88**
AGFW (g)	6.20 $\pm$ 0.52	5.72 $\pm$ 0.54	ns	12.99 $\pm$ 1.56	12.33 $\pm$ 1.38	ns	3.90**	4.54**
AGDW (g)	0.76 $\pm$ 0.06	0.74 $\pm$ 0.08	ns	1.52 $\pm$ 0.15	1.65 $\pm$ 0.13	ns	ns	7.70**
BGFW (g)	8.47 $\pm$ 0.81	7.00 $\pm$ 0.79	ns	16.04 $\pm$ 1.43	12.18 $\pm$ 1.57	2.57**	4.47**	2.77**
BGDW (g)	1.60 $\pm$ 0.15	1.45 $\pm$ 0.16	ns	3.56 $\pm$ 0.45	2.93 $\pm$ 0.37	2.97**	ns	9.06**
PH(cm)	6.57 $\pm$ 0.45	5.76 $\pm$ 0.29	ns	7.31 $\pm$ 0.42	7.98 $\pm$ 0.66	ns	ns	2.99**

Level of significance: * $p < 0.05$; ** $p < 0.01$

LN, Leaf Number; LL, leaf length; LW, leaf width; LA, leaf area; RW, rhizome width; RL, rhizome length; AGFW, above ground fresh weight; AGDW, above ground dry weight; BGFW, below ground fresh weight; BGDW, below ground dry weight; PH, plant height

Figure 1. Variation in phytochemicals and antioxidant activity in control and treated plants of *V. jatamansi* in 2 successive years; A- total phenolics and tannin content; B- antioxidant activity by ABTS, DPPH and FRAP assays [AP, aerial portion; RP, root portion; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2, 2'-diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power activity].

t value for the 1st year - Aerial portion- ABTS ($t = -0.15^{ns}$); DPPH ($t = -16.95^*$); FRAP ($t = -9.21^*$); Root portion -ABTS ($t = 6.75^*$); DPPH ($t = -10.35^*$); FRAP ($t = 4.87^*$); 2nd year- Aerial portion- ABTS ($t = 3.17^*$); DPPH ($t = 7.31^*$); FRAP ($t = 1.60^{ns}$); Root portion- ABTS ($t = 1.41^{ns}$); DPPH ($t = 6.23^*$); FRAP ($t = 0.87^{ns}$)

3.2. Variation in the total phenolic and tannin contents

Higher total phenolic content was recorded in control and treated plants of 1st year as compared to values recorded for 2nd year plants (Figure 1A). Total phenolic content of 1st year plants was higher in aerial (18.03 ± 0.05 1st year and 10.88 ± 0.52 mg GAE/g 2nd year) and root portions (14.30 ± 0.04 1st year and 7.92 ± 0.27 mg GAE/g 2nd year) of treated plants as compared to controls (aerial 1st year- 16.61 ± 0.04 ; 2nd year: 6.63 ± 1.20 , and roots 1st year - 11.94 ± 0.03 ; 2nd year - 6.66 ± 0.48 mg GAE/g); however, the quantity was higher in the aerial portion. Similarly, the tannin content was recorded to be higher in both aerial [10.26 ± 0.40 (1st year) and

12.99 ± 0.26 mg TAE/g (2nd year)] and root portions [9.21 ± 0.27 (1st year) and 15.18 ± 0.41 mg TAE/g (2nd year)] of treated plants as compared to control (Figure 1A). Comparison between 1st and 2nd year plants (control and treated) revealed significantly higher tannin content in plants of 2nd year (Figure 1A). However, phenolics in aerial and root portions of treated and control plants of 2nd year did not reveal significant difference ($t = 1.503$, $p < 0.17$). Similarly, tannin content in root portion of treated and control plants of 2nd year did not show significant ($t = 0.96$, $p < 0.36$) difference. However, significant ($t = 3.35$, $p < 0.01$) difference was found in respect of tannin content of aerial portions of 2nd year treated and control plants.

Table 2. Phenolic composition identified in control and treated plants of *V. jatamansi*

	Plant portion	Control	Treated	t	p
1st Year					
Gallic acid					
	Aerial	10.76 $\pm$ 1.47	67.67 $\pm$ 0.87	-25.030	0.002**
	Roots	22.66 $\pm$ 5.46	14.99 $\pm$ 2.56	-2.563	0.124
2nd Year					
	Aerial	13.90 $\pm$ 0.85	13.98 $\pm$ 0.85	0.98	0.430
	Root	21.06 $\pm$ 1.32	23.91 $\pm$ 0.56	-1.72	0.228
Chlorogenic acid					
1st Year					
	Aerial	311.95 $\pm$ 3.45	404.19 $\pm$ 72.09	-1.988	0.185
	Roots	41.15 $\pm$ 13.42	51.89 $\pm$ 0.16	-2.753	0.110
2nd Year					
	Aerial	264.37 $\pm$ 5.68	264.37 $\pm$ 5.68	0.700	0.556
	Root	111.58 $\pm$ 10.83	92.80 $\pm$ 15.33	-0.802	0.502
Catechin					
1st Year					
	Aerial	6.76 $\pm$ 0.58	16.29 $\pm$ 1.96	-9.699	0.010**
	Roots	12.90 $\pm$ 0.89	19.47 $\pm$ 1.19	-13.160	0.006**
2nd Year					
	Aerial	9.25 $\pm$ 0.40	10.25 $\pm$ 0.40	0.667	0.573
	Roots	13.84 $\pm$ 0.59	16.06 $\pm$ 0.11	3.202	0.085
Hydroxy benzoic acid					
1st Year					
	Aerial	313.80 $\pm$ 1.69	358.71 $\pm$ 109.14	-0.992	0.426
	Roots	45.95 $\pm$ 6.07	51.89 $\pm$ 0.16	-1.021	0.415
2nd Year					
	Aerial	360.53 $\pm$ 29.80	381.53 $\pm$ 0.41	-0.479	0.042*
	Roots	88.60 $\pm$ 14.92	111.80 $\pm$ 10.60	-0.675	0.569

Level of significance: * $p < 0.05$; ** $p < 0.01$

3.3. Phenolic composition

Significantly ($p < 0.05$) higher gallic acid (67.67 ± 0.87 mg/100 g dw) content was detected in first year treated plants as compared to control plants in aerial portion while in case of root portion it was lower (14.99 ± 2.56 mg/100 g dw; Table 2). However in case of 2nd year plants it was non-significantly higher in aerial (13.98 ± 0.85) and root portion (23.91 ± 0.56 mg/100 g dw) of treated plants. In case of chlorogenic acid higher value was recorded in aerial and root portion of treated plants of first year. In case of 2nd year plants aerial portion exhibits higher value in control plants but root portion of treated showed non-significant higher value. Catechin content was significantly ($p < 0.01$) higher (16.29 ± 1.96 mg/100 g dw) in aerial and root portion (19.47 ± 1.19 mg/100 g dw) of first year treated plants. While in case of 2nd year plants no significant variations was recorded in control and treated plants. Although Hydroxy benzoic acid content was higher in aerial and root portion of 1st and 2nd year treated plants but the variations were non-significant.

3.4. Antioxidant activity

Antioxidant activity in aerial and root portions of *V. jatamansi*, measured by three *in vitro* assays, showed a significant difference ($p < 0.05$) among plants of both the years. Results revealed that treated plants exhibited higher antioxidant activity over control plants (Figure 1B). Significant increase ($p < 0.05$) was found in antioxidant activity measured by ABTS and FRAP assays. Comparison between 1st year control and treated plants revealed difference (control aerial vs treated aerial and control roots vs treated roots) by all assays except ABTS in aerial portion. In case of 2nd year plants, significant differences were recorded by DPPH assay in aerial ($t = 7.31$, $p < 0.01$) and root portions ($t = 6.23$, $p < 0.01$) of treated and control plants.

Both the treated and control plants also showed significant difference in ABTS activity in aerial portion ($t = 3.17$, $p < 0.01$). Higher antioxidant activity (ABTS- 11.83 ± 0.02 , DPPH- 11.07 ± 0.10 , FRAP- 8.85 ± 0.01 mM AAE/100g dw) was, however, recorded in aerial portion of treated over control plants of the 1st year. Similarly, antioxidant activity measured by DPPH (1.06 ± 0.90 mM AAE/100g dw) and FRAP (2.02 ± 0.25 mM AAE/100g dw) assays was higher in aerial portion of treated plants of 2nd year, but lower (2.43 ± 0.28 mM AAE/100g dw) in ABTS assay. Invariably, higher antioxidant activity was recorded in the root portions of treated as compared to control plants.

3.5. PAL activity

Lower PAL activity was recorded in aerial (11.06 ± 1.78 U g^{-1} fw) and root portions (6.47 ± 0.43 U g^{-1} fw) of control plants, while in treated plants, roots exhibited higher (17.12 ± 6.24 U g^{-1} fw) PAL activity as compared to aerial portion (11.84 ± 1.68 U g^{-1} fw; Figure 2). The variations of PAL activity between control and treated plants across plant parts was not significant (aerial: $t = 0.54$, $p < 0.59$; roots: $t = 1.85$, $p < 0.09$).

3.6. Relationship among morphological and phytochemical attributes

Various relationships have been depicted in case of 1st year control plants for different morphological and phytochemical parameters (Table 3). AGFW showed positive relationship with AGDW ($r = 0.85$) and PH ($r = 0.70$). There were positive relationships with AGDW and PH ($r = 0.60$; $p < 0.05$) and between BGFW and BGDW ($r = 0.97$; $p < 0.05$). TP (aerial portion) showed positive relationship with DPPH (aerial portion; $r = 0.88$). FRAP (aerial portion) showed positive relationship with TP (aerial portion; $r = 0.44$). DPPH (aerial portion) showed positive relationship

with FRAP ($r = 0.62$) and ABTS ($r = 0.54$) aerial portion. TP (aerial portion) showed positive relationship with DPPH aerial portion ($r = 0.88$). However, in control plants of 2nd year only DPPH (aerial portion) exhibited significant positive ($p < 0.05$) relationship with DPPH (root portion; $r = 0.92$).

In treated plants, significant relationships were found among different morphological parameters of 1st year treated plants (Table 4); AGFW showed significant positive relationship with AGDW ($r = 0.66$), BGFW ($r = 0.82$), BGDW ($r = 0.78$), and PH ($r = 0.62$). AGDW exhibited positive relationship with BGFW ($r = 0.60$) and BGDW ($r = 0.60$). BGFW also showed

positive relationship with BGDW ($r = 0.90$), and PH ($r = 0.52$). Among different phytochemicals analyzed in the aerial and root portions of treated plants, FRAP (aerial portion) showed significant ($p < 0.05$) positive relationship with TP (aerial portion; $r = 0.66$). Similarly, ABTS (root portion) showed positive relationship with TP (aerial portion; $r = 0.53$). TP (root portion) showed positive relationship with FRAP (root portion; $r = 0.72$); DPPH (root portion) showed positive relationship with FRAP (root portion; $r = 0.80$). However, in treated plants of 2nd year only TP (aerial portion) showed significant positive ($p < 0.05$) relationship with TP (root portion; $r = 0.83$).

Figure 2. Variation in Phenylalanine ammonia lyase (PAL) activity in control and treated plants of *V. jatamansi* after 2 years of cultivation.

t value for the 1st year - Aerial portion- Total phenolics - TP ($t = -1.84^{ns}$); Tannins ($t = 3.35^*$); Root portion - TP ($t = -4.51^*$); Tannins (0.97^{ns}); 2nd year - Aerial portion - TP ($t = 1.50^{ns}$); Tannins ($t = 3.35^*$); Root portion - TP ($t = -2.021^{ns}$); Tannins (0.96^{ns})

Table 3. Relationship (Pearson Correlation) among morphological and phytochemical parameters of 1st year control plants of *V. jatamansi*.

S. No.	LN	AGFW	AGDW	BGFW	BGDW	PH	RL	LA	TPA	DPPHA	FRAPA	ABTSA	TPR
LN	1.00												
AGFW	0.49**	1.00											
AGDW	0.46*	0.85**	1.00										
BGFW	-0.09	0.42*	0.41*	1.00									
BGDW	-0.10	0.40*	0.39*	0.97**	1.00								
PH	0.31	0.70**	0.60**	0.39*	0.42*	1.00							
RL	-0.10	0.03	-0.00	0.55**	0.63**	0.17	1.00						
LA	0.07	0.61**	0.54**	0.51**	0.47**	0.62**	0.21	1.00					
TPA	-0.16	-0.37*	-0.30	-0.13	-0.08	-0.21	0.33	-0.19	1.00				
DPPHA	-0.11	-0.36*	-0.31	-0.20	-0.19	-0.22	0.25	-0.17	0.88**	1.00			
FRAPA	-0.04	-0.12	-0.22	-0.25	-0.24	-0.14	-0.03	0.09	0.44**	0.62**	1.00		
ABTSA	0.02	-0.17	-0.18	0.17	0.13	0.09	0.39*	0.08	0.35	0.54**	0.33	1.00	
TP R	-0.04	-0.04	0.10	0.24	0.21	0.13	0.18	0.06	-0.10	-0.08	-0.21	-0.04	1.00
DPPHR	0.10	-0.09	-0.01	-0.27	-0.22	-0.14	0.03	-	0.00	-0.01	-0.25	-0.01	0.07
FRAPR	0.30	0.01	-0.08	-0.17	-0.14	0.07	0.09	-0.16	0.29	0.18	-0.12	0.09	-
ABTSR	-0.23	-0.13	-0.03	0.12	0.10	0.10	0.07	0.06	-0.01	0.03	0.10	-0.07	0.23

Level of significance: * $p < 0.05$; ** $p < 0.01$. LN, Leaf Number; AGFW, above ground fresh weight; AGDW, above ground dry weight; BGFW, below ground fresh weight; BGDW, below ground dry weight; PH, plant height; LA, leaf area; RL, rhizome length; TPA, total phenolics in aerial portion; DPPHA, DPPH activity in aerial portion; FRAPA, FRAP activity in aerial portion; ABTSA, ABTS activity in aerial portion; TPR, Total phenolics in root portion; DPPHR, DPPH activity in root portion; FRAPR, FRAP activity in root portion; ABTSR, ABTS activity in root portion.

Table 4. Relationship (Pearson Correlation) among morphological and phytochemical parameters of 1st year

S. No	LN	AGFW	AGDW	BGFW	BGDW	PH	RL	LA	TPA	DPPHA	FRAPA	ABTSA	TPR	DPPHR	FRAPR	BTSR
LN	1.00															
AGFW	0.63**	1.00														
AGDW	0.41*	0.66**	1.00													
BGFW	0.42*	0.82**	0.60**	1.00												
BGDW	0.35	0.78**	0.60**	0.90**	1.00											
PH	0.25	0.62**	0.44*	0.52**	0.43*	1.00										
RL	0.10	0.08	0.06	0.36	0.29	0.06	1.00									
LA	0.51**	0.65**	0.45*	0.57**	0.58**	0.53**	0.35	1.00								
TPA	0.01	0.04	-0.05	0.10	0.13	0.07	0.35	0.04	1.00							
DPPHA	-0.03	0.03	-0.18	0.07	0.08	-0.14	0.01	0.02	0.22	1.00						
FRAPA	-0.05	0.02	-0.02	0.04	0.16	0.03	-0.01	-	0.66**	-0.05	1.00					
ABTSA	-0.27	-0.26	-0.04	-0.24	-0.19	-0.08	-0.03	-	-0.10	0.13	-0.05	1.00				
TPR	-0.27	-0.12	0.18	0.02	0.11	0.08	0.37*	0.14	0.37*	0.09	0.15	-0.12	1.00			
DPPHR	-0.31	-0.18	0.06	-0.04	-0.08	0.01	0.28	0.01	0.27	0.02	0.01	-0.09	0.80**	1.00		
FRAPR	-0.38	-0.34	-0.04	-0.06	-0.06	-0.07	0.44*	0.01	0.25	0.05	0.02	-0.00	0.72**	0.80**	1.00	
ABTSR	-0.18	-0.05	-0.23	0.21	0.13	-0.02	0.41*	0.02	0.53**	0.58**	0.19	0.09	0.14	0.05	0.18	1.00

Level of significance: * $p < 0.05$; ** $p < 0.01$. LN, Leaf Number; AGFW, above ground fresh weight; AGDW, above ground dry weight; BGFW, below ground fresh weight; BGDW, below ground dry weight; PH, plant height; LA, leaf area; RL, rhizome length; TPA, total phenolics in aerial portion; DPPHA, DPPH activity in aerial portion; FRAPA, FRAP activity in aerial portion; ABTSA, ABTS activity in aerial portion; TPR, Total phenolics in root portion; DPPHR, DPPH activity in root portion; FRAPR, FRAP activity in root portion; ABTSR, ABTS activity in root portion.

4. Discussion

The higher levels of phenolics, tannins, and the anti-oxidant and PAL activity in *V. jatamansi*, following AMF treatment, correspond well with the report of increased production of phytochemicals due to infection of AMF (Toussaint *et al.*, 2008). The study on the association of AMF with a cultivar of lettuce (Batavia Rubia Munguia) revealed that accumulation of phenolics in leaves increased significantly under optimal irrigation (Baslam and Goicoechea, 2012). In another species of *Valeriana* (*V. officinalis*) enhanced secondary metabolite (sesquiterpenic acids) production has been observed after mycorrhizal inoculation along with a decrease in plant biomass; the nutritional improvement in such treated plants, in general, resulted in positive growth response (Nell *et al.*, 2010). The root portion of the plant seems to be an important factor for the production of active ingredients and products of pharmaceutical importance. Several reports on medicinal and aromatic plants confirm increased phosphorus content and biomass in AMF treated plants (Khaosaad *et al.*, 2006). The observed reduction in biomass related parameters of the 1st year plants, treated with AMF, agreed with earlier reports and suggested that the AMF association negatively influences or independent of biomass production (Hausmann and Hawkes, 2010). This, however, does not follow the general observation of increased biomass production following treatments (Smith and Read, 2008). Further, clear trends of increased value of morphological parameters were observed following 2nd year growth, except for BGDW. This is supported by reports on other medicinal plants (Khaosaad *et al.*, 2006).

A large number of studies have shown that growth responses to AMF colonization depend both on the host plant and fungal species, as well as on the experimental conditions (Huang *et al.*, 2013). Evidences indicate that AMF induce changes in the primary me-

tabolism, including photosynthesis, water uptake and drought tolerance (Ruiz-Lozano, 2003). Changes in secondary metabolism such as phytohormone dynamics, structural modifications and activation of defense mechanism were also reported in *Bouteloua gracilis* and *Ocimum basilicum* (Allen *et al.*, 1982).

Increased phenolics, tannins, phenolic composition and antioxidant activity in AMF treated 1st year plants can be explained in terms of defense response to fungal infection. Further, the higher antioxidant activity of the samples from treated plants appears to be on account of higher content of phenolics and tannin which are known to exhibit positive relationship with antioxidant activity (Bhatt *et al.*, 2012). As such, antioxidant properties of phenolic compounds play a vital role in the defense mechanism of biological systems (Macheix and Fleuriet, 1998). In the present study also total phenolics exhibited significant positive correlation with antioxidant activity in treated as well as control plants. Earlier reports also indicated that the antioxidant potential of medicinal plants may be related to the concentration of phenolic compounds which include phenolic acid, flavonoids, anthocyanins and tannins (Bhatt *et al.*, 2012; Jugran *et al.*, 2013). These compounds are of great value in preventing the onset and/or progression of many human diseases (Muanda *et al.*, 2009). Antioxidative enzymes in leaves of pepper indicated that AMF (*Glomus deserticola*) can alleviate the deleterious effect of *Verticillium dahliae* on the growth and yield of plant during pathogen attacks (Garmendia *et al.*, 2004).

Moreover, the high phytochemical content in AMF treated plants could be associated with induction of PAL activity following exposure of plants with AMF for the first time, and thereby resulting in higher production of secondary metabolites. However, no further increase in activity after 1st year would indicate possible adaptation of plants after the first exposure. The observed increase in PAL activity levels in fresh aerial and root parts might have resulted in increased phenolic

content and antioxidant activity in treated plants. Study on the mycorrhizal treated plants of tomato has also shown increased growth, PAL activity, phenol concentration and foliar trichome density as compared to control plants (Kapoor, 2008).

5. Conclusions

Present study provides evidence that AMF inoculation can be a simple and useful method for obtaining higher content of phenolics tannins and phenolic composition, and consequently increased antioxidant activity in *V. jatamansi*. As the species is facing ever increasing demand for quality herbal products, the situation necessitates standardization of methods for obtaining adequate raw material for pharmaceutical purposes and to minimize pressure on natural populations by way of promoting cultivation. The production of higher yield and quality in medicinal plants, through conventional methods, often requires external inputs such as fertilizers and pesticides. In this context, use of mycorrhizal inoculation, a natural alternative to chemical fertilizers, is likely to boost the production of active ingredients. In this study clear cut growth and enhancement were recorded in morphological and phytochemical attributes, which indicate that AMF can be utilized for higher biomass production as well as for the higher production of antioxidants and phenolics. The use of AMF could thus be recommended as a part of the package of practice for growers.

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References

- Allen, M.F., Moore, T.S., Christensen, M. 1980. Phytohormone changes in *Bouteloua gracilis* infected by vesicular–arbuscular mycorrhizae. I: Cytokinin increases in the host plant. *Can. J. Bot.* 58, 371-374.
- Allen, M.F., Moore, T.S., Christensen, M. 1982. Phytohormone changes in *Bouteloua gracilis* infected by vesicular–arbuscular mycorrhizae. II: Altered levels of gibberellin- like substances and abscisic acid in the host plant. *Can. J. Bot.* 60, 468-471.
- Banuelos J., Alarcón A., Larsen J., Cruz-Sánchez S., Trejo D. 2014. Interactions between arbuscular mycorrhizal fungi and *Meloidogyne incognita* in the ornamental plant *Impatiens Balsamina*. *J. Soil Sci. Plant Nutri.* 14, 63-74.
- Baslam, M., Garmendia, I., Goicoechea, N. 2013. Enhanced Accumulation of Vitamins, Nutraceuticals and Minerals in Lettuces Associated with Arbuscular Mycorrhizal Fungi (AMF): A Question of Interest for Both Vegetables and Humans. *Agriculture* 3, 188-209.
- Baslam, M., Goicoechea, N. 2012. Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. *Mycorrhiza*. 22, 347-359.
- Bhatt, I.D., Dauthal, P., Rawat, S., Gaira, K.S., Jugran, A., Rawal, R.S., Dhar, U. 2012. Characterization of essential oil composition, phenolic content, and antioxidant properties in wild and planted individuals of *Valeriana jatamansi* Jones. *Sci. Horticult.* 136, 61-68.

- Brand-William, W., Cuveiler, M.E., Berset, C. 1995. Use of free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol. 28, 25-30.
- Cai, Y., Luo, Q., Sun, M., Corke, H. 2004. Antioxidant activity and phenolic compounds in 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 75, 2157-2184.
- Caporale A.G., Sarkar D., Datta R., Punamiya P., Violante A. 2014. Effect of arbuscular mycorrhizal fungi (*Glomus* spp.) on growth and arsenic uptake of vetiver grass (*Chrysopogon zizanioides* L.) from contaminated soil and water systems. J. Soil Sci. Plant Nutri. 14, 955-972.
- Cavagnaro, T.R., Smith, F.A., Lormer, M.F., Haskard, K.A., Ayling, S.M., Smith, S.E. 2001. Quantative development of *Paris*-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum*. New Phytol. 149, 105-113.
- Channabasava, A., Lakshman, H.C., Jorquera, M.A. 2015. Effect of fungicides on association of arbuscular mycorrhiza fungus *rhizophagus fasciculatus* and growth of proso millet (*panicum miliaceum*). J. Soil Sci. Plant Nutr. 15, 35-45.
- Faria, A., Oliveira, J., Neves, P., Gamero, P. Santos-Buelga, C., de Freitas, V., Mateus, N., 2005. Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. J. Agric. Food Chem. 53, 6896-6902.
- Garmendia, I., Goicoechea, N., Aguirreolea, J. 2004. Antioxidant metabolism in asymptomatic leaves of Verticillium-infected Pepper associated with an arbuscular mycorrhizal fungus. J. Phytopathol. 152, 593-599.
- Hausmann, N.T., Hawkes, C.V. 2010. Order of plant host establishment alters the composition of arbuscular mycorrhizal communities. Ecology. 91, 2333-2343.
- Huang, J.C., Lai, W.A., Singh, S., Hameed, A., Young, C.C. 2013. Response of mycorrhizal hybrid tomato cultivars under saline stress. J. Soil Sci. Plant Nutr. 13, 469-484.
- Jugran, A., Rawat, S., Dauthal, P., Mondal, S., Bhatt, I.D., Rawal, R.S. 2013. Association of ISSR markers with some biochemical traits of *Valeriana jatamansi* .Jones. Ind. Crop Prod. 44, 671-676.
- Kalim, M.D., Bhattacharyya, D., Banerjee, A., Chattopadhyay, S. 2010. Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine. BMC Compl. Alt. Med. 10,77.
- Kapoor, R. 2008. Induced resistance in mycorrhizal tomato is correlated to concentration of Jasmonic Acid. J. Biol. Sci. 8, 49-56.
- Khaosaad, T., Vierheilig, H., Nell, M., Zitterl-Eglseer, K., Novak, J. 2006. Arbuscular mycorrhiza alter the concentration of essential oils in oregano (*Origanum* sp., Lamiaceae). Mycorrhiza. 16, 443-446.
- Macheix, J, Fleuriet. 1998. Flavonoids in health and diseases. Marcel Dekker Inc. New York, pp 839-868.
- Muanda. F., Kone, D., Dicko, A., Soulimani, R., Younos, C. 2009. Phytochemical composition and antioxidant capacity of three Malian medicinal plant parts. eCAM; doi: 10.1093/ecam/nep109.
- Nell, M., Wawrosch, C., Steinkellner, S., Vierheilig, H., Kopp, B., Losse, A., Franz, C., Novak, J., Zittere – Eyeseer, K. 2010. Root colonization by symbiotic arbuscular mycorrhizal fungi increases sesquiterpenic acid concentration in *Valeriana officinalis* L. Planta Med.76, 393-398.
- Nwinuka, N.M., Ibeh, G.O., Ekeke, G.I. 2005. Proximate composition and levels of some toxicants in four commonly consumed species. J. Applied Sci. Environ. Management 9,150-155.

- Petersen, M., Simmonds, M.S.J. 2003. Rosmarinic acid. *Phytochemistry*. 62, 121-125.
- Rhodes, M. 1985. The physiological significance of plant phenolic compounds. *Ann. Proceed. Phytochem. Soc. Europe*. 25, 99-118.
- Ruiz-Lozano, J.M. 2003. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. *Mycorrhiza*. 3, 309-317.
- Schadt, C.W., Mullen, R.B., Schmidt, S.K. 2001. Isolation and phylogenetic identification of a dark-septate fungus associated with the alpine plant *Ranunculus adoneus*. *New Phytol.* 150, 747-755.
- Singleton, V.L., Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American J. Enol. Vitic.* 16, 144-158.
- Smith, S.E., Read, D.J. 2008. *Mycorrhizal symbiosis*, 3rd edition. London, UK: Academic Press.
- Tiwari, P., Adholeya, A. 2002. *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. *FEMS Microbiol. Lett.* 206, 39-43.
- Toussaint, J.P., Kraml, M., Nell, M., Smith, S.E., Smith, F.A., Steinkellner, S., Schmiderer, C., Vierterheilig, H., Novak, J. 2008. Effect of *Glomus mosseae* concentrations of rosmarinic and caffeic acids and essential oil compounds in basil inoculated with *Fusarium oxysporum* f. sp. basilici. *Plant Pathol.* pp. 1109-1116.
- Toussaint, J.P., Starnaud, M., Charest, C. 2004. Nitrogen transfer assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri DNA roots of *Dacus carota* L. in an in vitro compartmented system. *Can. J. Microbiol.* 50, 251-260.
- Zhi-Lin, Y., Chuan-Chao, D., Lian-Quing, C. 2007. Regulation and accumulation of secondary metabolites in plant-fungus symbiotic system. *African J. Biotechnol.* 6, 1266-1271.
- Zucker, M. 1968. Sequential induction of phenylalanine ammonia lyase and a lyase inactivation system in potato tuber disks. *Plant Physiol.* 43, 365-374.