ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 16(2) 2017, 3–10

www.acta.media.pl ISSN 1644-0692

ORIGINAL PAPER

Accepted: 13.06.2016

ARBUSCULAR MYCORRHIZAL FUNGI PROMOTE ENHANCED GROWTH, TUBEROUS ROOTS YIELD AND ROOT SPECIFIC FLAVOUR 2-HYDROXY-4-METHOXYBENZALDEHYDE CONTENT OF *Decalepis hamiltonii* Wight & Arn.

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ABSTRACT

Potted seedling plants (SP) and micro-propagated potted plants (MP) of swallow root (*Decalepis hamiltonii*) were inoculated with mycorrhizal fungi (*Glomus mosseae*, *Glomus fasciculatum* and *Glomus monosporum*) to find out their influence on quality of tubers and their flavour content. Respective arbuscular mycorrhizal fungi (AMF) treatment in general supported better growth of SP and MP plants in terms of increased plant height, number of nodes, number of leaves, number of tubers, and fresh weight of tubers at harvesting stage. A maximum of 82.23% root specific flavour metabolite 2-hydroxy-4-methoxy benzaldehyde (2H4MB) improvement (4.5 mg g^{-100}) was found in tubers when MP plant of *D. hamiltonii* was given 50 g of *G. mosseae* treatment, followed by 71.43 and 20% improvement of 2H4MB for *G. fasciculatum* and *G. monosporum* respectively. The novelty of the present invention is that it provides for the first time an efficient method for improvement of growth and yield of flavour enhanced tubers of *D. hamiltonii* by using AMF. The symbiosis of mycorrhizal fungi and swallow root would be of benefit for qualitative and quantitative improvement of this endangered and endemic medicinally important climber.

Key words: *Glomus mosseae*, *Glomus fasciculatum*, micro-propagated, swallow root, symbiosis

INTRODUCTION

Swallow root (*Decalepis hamiltonii* Wight & Arn.) is a monogeneric climbing medicinal climber belong to Asclepidaceae family. It is reported to be highly endemic to forest areas of Western Ghats of India and also available in some parts of southern states of India [Wealth of India 1952]. This endangered plant's taxonomical position, morphological features, economic importance, biotechnological improvement and food technology intervention for value addition has been recently reviewed [Pradeep et al. 2016]. The fleshy tuberous roots are used as a culinary spice due to its high priced aromatic roots. The roots are markedly fleshy, cylindrical (1–6 cm diameter) and are characterized by a *sarasaparilla* like taste accompanied by a tingling sensation on the tongue [Wealth of India 1952]. The roots of *D. hamiltonii* are used as a flavouring principle [Wealth of India 1990], appetizer [Murthi nad Seshadri 1941], blood purifier [Jacob 1937], and preservative [Phadke et al. 1994]. Similarly the roots of this taxon as described by Nayar et al. [1978] are considered as "Sariva Bheda" in Ayurveda where it finds use as an alternative to roots of *Hemidesmus indicus* in the preparation of several herbal drugs like Amrutamalaka taila, Drakshadi

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churna, shatavari rasayana and yeshtimadhu taila. The roots contain 92% fleshy matter and 8% woody core. Of late the highly aromatic roots have been subjected to over exploitation by destructive harvesting that has endangered the survival of this plant. The potential antimicrobial, insecticidal, antifungal, antioxidant activity of root extracts were well established [Pradeep et al. 2016]. Efficient *in vitro* propagation methods for mass multiplication of *D. hamiltonii* followed by efficient *in vitro* rooting were optimized by researchers [Reddy et al. 2001, Giridhar et al. 2004, 2005].

Due to high cost of fertilizers and with a view to maintain the ecosystem of soil, addition of fertilizer has to be minimized which is done by adding biofertilizer in soil. Arbuscular Mycorrhizal Fungi (AMF) are well known for their beneficial and stimulating effects on plant growth, meeting nutritive deficiency of zinc, phosphorous and nitrogen as biofertilizers [Liu et al. 2007, Tanwarm et al. 2013] in soils of arid and semi-arid tropical countries, induced suppression of soil/root borne fungi and resistance of water stress etc. These AMF belong to the phylum Glomeromycota [Schüßler et al. 2001] and form symbiosis association with around 90% land plants in agricultural and natural ecosystems [Brundrett 2002].

There is an evidence that AMF plays a major role by inducing changes in microbial populations in the mycorrhizosphere and modifications in the phytohormone balance in the roots of the host plants, such as cytokinins, gibberellins, ethylene, abscisic acid and jasmonates [López-Ráez et al. 2010, Martínez-Medina et al. 2011]. Among various microbial inoculants, AMF is one which stimulates plant growth in soils of low fertility providing phosphate to plants [Garg and Chandel 2010]. Moreover, secondary metabolites augmentation potential of AMF treated plants was demonstrated [Liu et al. 2007]. There are no prior reports on effective propagation of *D. hamiltonii* for commercial purpose. However recent findings on it's commercial importance for both food and medicinal applications prompted prospective growers for its large scale cultivation [Pradeep et al. 2016]. Moreover, there is a need for efficient hardening and successful field transfer of tissue cultured plants with their effective field growth, because micropropagated *D. hamiltonii* plants show better content of flavour in roots [Giridhar et al. 2005]. Accordingly the objective of this study was to develop a process for improvement of growth and yield of tubers of *D. hamiltonii* by using arbuscular mycorrhiza, in view of its wide range of applications and economic importance.

MATERIALS AND METHODS

Preparation of Mycorrhiza-based inoculants. Fresh seeds were collected from four year old *D. hamiltonii* Wight & Arn., plant grown in CSIR--Central Food Technological Research Institute, India and were sown in garden soil in greenhouse. Two month old seedlings with shoot length of approximately 12–15 cm were used for studying the effect of AMF. Three different strains of arbuscular mycorrhizal fungi viz. *Glomus mosseae*, *Glomus fasciculatum* and *Glomus monosporum* were used for the experiment. The starter inoculum of each arbuscular mycorrhizal strain was prepared by multiplying in sterile pots containing sterile soil by sowing the seeds of finger millet (*Eleusine coracana).* After 4 weeks the seedlings that emerged were taken out carefully and checked under microscope for percentage of arbuscular mycorrhizal infection of roots and counted the number of spores in roots per gram of soil. Subsequently, the prepared inoculum was used for different sets of treatments to *D. hamiltonii* plants. The treatments consisted of T1) *G. mosseae*, T2) *G. fasciculatum*, T3) *G. monosporum* and T4) uninoculated (controls). Pots were filled with mixture of soil: red earth: farm yard manure in the range of 2:1:1 (5700 cm^3 soil mixture pot^{-1}). Inoculation of arbuscular mycorrhizal fungi was done at the rate of 30, 50 and 70 g pot^{-1} (soil along with root pieces containing 15–16 spores per gram of soil) at the depth of 5 cm. Two months old *D. hamiltonii* seedlings were planted one per each pot. Similarly, to find out the influence of AMF on micro-propagated plants and hardened *in vitro* plants, two months old greenhouse plants were used. To establish micro-propagated plants earlier reported method was followed [Bais et al. 2000]. In brief; nodal explants excised from one year old garden grown plant were surface sterilized and inocu-

lated on Murashige and Skoog (MS) medium [Murashige and Skoog 1962] supplemented with plant growth regulators 2.0 mg l^{-1} of benzyladenine (BA) and 0.5 mg l^{-1} of α-naphthalene acetic acid (NAA) to get *in vitro* shoots in stage I and shoot elongation along with *in vitro* rooting on MS medium containing 2.0 mg l^{-1} of indole-3-butyric acid (IBA) in stage II [Bais et al. 2000], followed by their hardening in greenhouse for 2 months. These micro-propagated plants having 12–15 cm shoot length were also used to improve the growth and yield of flavour enhanced tubers. Respective AMF treatment was given as explained above. Only 50 g of each AMF inoculum independently per pot was used as the same has given better response for *D. hamiltonii* seedlings treatment.

Biometric observations

Isolation and estimation of flavour compound. After three months of growth biometric observations like per cent root infection, plant height, number of nodes, number of leaves, number of tubers, size of tubers, fresh weight of tubers and the flavour content of tubers along with chlorophyll content of leaves was recorded. The tubers were separated from plant and washed in water to remove the adhering soil particles. Then the washed tubers were mechanically dissected into small pieces of 0.5–1.0 cm diameter and subjected to steam distillation for 5 hours. The steam condense was extracted with dichloromethane (50 ml × 4). The combined extracts were passed through a funnel containing anhydrous sodium sulphate to remove the water content, concentrated in a flash evaporator and dissolved in 1 ml ethanol and stored in closed vials. Quantification of the flavour compound was determined by gas chromatographic analysis (GC) using flame ionization detection (FID).

Initially 2-hydroxy-4-methoxybenzaldehyde (2H4MB) content was qualitatively evaluated by spotting the root extracts on TLC plate along with standard (Fluka Chemicals, Switzerland) and run in a solvent system comprising Hexane: Benzene (1:1). Rf value of spot coinciding with that of standard (2H4MB) (0.47) was eluted in solvent and UV spectrum was measured on a Perkin-Elmer UV-Vis recording spectrophotometer UV-160. Maximum absorption was obtained at 278 nm. Quantitative detection was done by gas chromatographic analysis (GC) using flame ionization detection (FID). The constituent was identified by matching the mass spectra with GC-MS library user generated mass spectral libraries, and also confirmed by comparison with GC retention time of standard sample.

The concentrated volatiles were separated by GC, flame ionization detector (FID) with capillary column and GC-MS analysis using a Shimadzu, GC-14B coupled with QP 5000 MS system under the following conditions SPB-1 column (Supelco, USA, 30 m × 0.32 mm, 0.25 μM film thickness); oven temperature programme, 60°C for 2 min, rising at 2°C/min to 250°C, held for 5 min; injection port temperature 225°C; detector temperature, 250°C; carrier gas helium, flow rate 1 ml min^{-1}. The amount of solution injected was 1 μl for analysis.

Statistical analysis. The experiment was repeated twice with five replicates for each treatment and three replicates of roots for flavour metabolite 2H4MB analysis. The mean ±S.E. values were calculated by two-way ANOVA using Duncan multiple comparison. Also, ANOVA for seedling plants and micropropagated plants for growth characteristics and flavour content of tubers in response to 50 g per pot AMF treatment was calculated ($p < 0.05$).

RESULTS AND DISCUSSION

The data obtained in this study, revealed that, among the three arbuscular mycorrhizal fungi used, *D. hamiltonii* plants grown in soil containing *G. mosseae* outperformed those grown in presence of either *G. fasciculatum* or *G. monosporum* and the response is again inoculum quantity dependent. When 30 g pot^{-1} AMF inoculum was used for treatment, among these fungi used *G. mosseae* found to be the most efficient in colonizing the roots, and improved the biometric characters like (tab. 1) plant height (52.2 ±1.85 cm), number of nodes (9.0 ±0.75), number of leaves (17.2 ±1.02), number of tubers (6.5 ±1.0), fresh weight of tubers (13.5 ±0.38 g) compared to *G. fasciculatum* and *G. monosporum* and controls. *G. mosseae* treated plant leaves showed high content of chlorophyll (tab. 1).

However, the best response was evident for 50 g AMF inoculum treatment per pot, where in, *G. mosseae* exhibited maximum improvement in the plant height (72.2 ±1.78 cm), number of nodes (13.4 ±0.89), number of leaves (26.2 ±1.78), number of tubers (10.6 ±1.51) and fresh weight of tubers (16 ±0.72 g) compared to *G. fasciculatum* and *G. monosporum*. Even chlorophyll content of leaf (24.18 ±1.26 mg g^{-1}) was high for *G. mosseae* treated plant (tab. 1). In case of 70 g pot^{-1} AMF inoculum treatment, the response was less to 50 g pot^{-1} treatment though it is significantly better to 30 g pot^{-1} treatment. At 70 g pot^{-1} treatment, *G. mosseae* improved the plant height (70.5 ±1.65 cm), number of nodes (12.9 ±0.97), number of leaves (24 ±1.0), number of tubers (10.0 ±0.50), fresh weight of tubers (15.2 ±0.28 g) compared to *G. fasciculatum* and *G. monosporum* and controls (tab. 1).

Table 1. Effect of AMF inoculation on *in vivo* growth and yield of *Decalepis hamiltonii*

Plant source	Concentration	Shoot length (cm)	Number of nodes	Number of leaves	Total chlorophyll (mg g^{-1} FW)	Number of tubers	Range of tuber diameter (cm)	Range of tuber length (cm)	Fresh weight of tubers (g)	Flavour content (mg g^{-100})
Control		14.2 ±1.46g	5.16 ±0.40f	10.4 ±0.89f	13.88 ±1.60d	4.2 ±0.45e	0.9 ±0.16c	2.9 ±0.16e	9.59 ±0.85h	0.6 ±0.16d
G. mosseae	30	52.2 ±1.85c	9.0 ±0.75d	17.2 ±1.02d	19.4 ±1.65b	6.5 ±1.00bc	1.4 ±0.22b	4.8 ±1.2cd	13.5 ±0.38c	2 ±0.22b
	50	72.2 ±2.86a	13.4 ±0.89a	26.2 ±1.78a	24.18 ±1.26a	10.6 ±1.51a	2.2 ±0.36a	8.06 ±0.13a	16.0 ±0.72a	3 ±0.25a
	70	70.5 ±1.65b	12.9 ±0.97b	24 ±1.00b	23.7 ±1.40a	7.6 ±0.50b	1.93 ±0.22a	7.8 ±0.34a	15.2 ±0.28b	3 ±0.34a
G. fasciculatum	30	20 ±2.14f	5.0 ±0.78f	10 ±1.0f	17.2 ±1.42c	4.2 ±0.66e	1.03 ±0.16b	4.23 ±0.23d	10.3 ±0.78g	1 ±0.18c
	50	30 ±3.16d	10.4 ±1.01c	20.2 ±1.88c	24.14 ±1.32a	6.1 ±0.84bcd	1.16 ±0.23b	5.5 ±0.36bc	13.3 ±0.80d	2 ±0.26b
	70	28.5 ±0.65d	9.0 ±0.50d	18 ±0.50d	23.8 ±0.92a	5.90 ±0.70cd	1.16 ±0.18b	5.53 ±0.33bc	12.8 ±0.68e	2 ±0.11b
G. monosporum	30	14.95 ±0.98g	4.0 ±0.82g	8.2 ±1.75g	17.4 ±0.88c	4.5 ±0.98de	1.03 ±0.13b	5.2 ±0.2c	11.6 ±0.35f	0.7 ±0.14d
	50	25.95 ±1.5e	8.4 ±5.16e	16.8 ±2.22e	19.19 ±0.98b	6 .0 ±0.70cd	1.1 ±0.26b	6.23 ±0.26b	10.4 ±0.63g	0.9 ±0.18c
	70	26.3 ±0.95e	8.5 ±0.65e	16.0 ±0.50e	19.3 ±0.78b	6.0 ±0.50cd	1.2 ±0.17b	6.16 ±0.21b	11.5 ±0.85f	1.5 ±0.23c

Data recorded after 3 months of growth of two months old seedlings given with AMF treatment. Results are an average of 5 samples (mean ±SE). Flavour values are represented as mean ±SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.05$, and values with same superscript were found not significantly different from each other. Amount of AMF inoculum used 30, 50, 70 g pot^{-1}

Table 2. Effect of AMF inoculation on the growth and yield of *in vitro* micropropagated plants of *D. hamiltonii*

Plant source	Shoot length (cm)	Number of nodes	Number of leaves	Total chlorophyll (mg g^{-1}FW)	Number of tubers	Range of tuber diameter (cm)	Range of tuber length (cm)	Fresh weight of tubers (g)	Flavour content (mg g^{-100})
Control	15.5 ±0.96d	6.0 ±0.85d	12 ±0.95d	14.2 ±0.86d	4.5 ±0.50c	0.9 ±0.17c	0.9 ±0.17 c	9.85 ±0.95d	0.8 ±0.19c
G. mosseae	80.5 ±1.50a	15.0 ±0.50a	30.0 ±0.65a	24.8 ±1.85a	11.5 ±0.85a	2.8 ±0.18a	9.4 ±0.18a	18.65 ±0.85a	4.5 ±0.21a
G. fasciculatum	36.7 ±0.58b	11.0 ±0.45b	22.0 ±0.85b	24.1 ±0.52b	6.5 ±0.98b	1.6 ±0.18b	6.5 ±0.21b	14.5 ±0.35b	2.8 ±0.15b
G. monosporum	33.3 ±0.56c	9.2 ±0.85c	18.0 ±0.38c	21.6 ±0.58c	6.2 ±0.55b	1.4 ±0.16b	6.5 ±0.23b	12.6 ±0.46c	1 ±0.09c

Data recorded after 3 months of growth of two months old seedlings given with AMF treatment. Results are an average of 5 samples (mean ±SE). Flavour values are represented as Mean ± SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.05$, and values with same superscript were found not significantly different from each other. Amount of AMF inoculum used 50 g pot^{-1}

It is quite interesting to note that *D. hamiltonii* micro-propagated plants grown in presence of 50 g pot^{-1} AMF treatments exhibited superior growth characteristics, tuber yield and also flavour content for all the three different AMF used, wherein, *G. mosseae* showed better response by improving the plant height (80.5 ±1.50 cm), number of nodes (15.0 ±0.50), number of leaves (30 ±0.65), number of tubers (11.5 ±0.85), fresh weight of tubers (18.65 ±0.85 g) and leaf chlorophyll content (24.8 ±1.85 mg g^{-1}) compared to *G. fasciculatum*, *G. monosporum* and control respectively (tab. 2).

2H4MB content in tubers. The GC(FID) profiles indicated that there was an improvement in the flavour content (2-hydroxy-4-methoxy benzaldehyde) in tubers of both treated and control plants (tab. 1). There was 25% improvement in 2H4MB content in tubers of *D. hamiltonii* micro-propagated plants grown in greenhouse (0.8 ±0.19 mg g^{-100}) compared to seedling control plants (0.6 ±0.16 mg g^{-100}). A significant enhancement in 2H4MB content of tubers was observed with all the three AMF viz., *G. mosseae*, *G. fasciculatum* and *G. monosporum* respectively at all the three different inoculum concentrations. In the case of biometric parameters, the trend was mostly same for 2H4MB content of tubers for all the treatments. A maximum of 82.23% 2H4MB (4.5 ±0.21 mg g^{-100}) improvement was found in tubers when micro-propagated *D. hamiltonii* plant was grown in pot containing 50 g of *G. mosseae*, followed by 71.43 and 20% improvement of 2H4MB for *G. fasciculatum* and *G. monosporum* respectively. Similarly, in case of seedling based plants, there was 80, 70 and 44.44% rise in 2H4MB content of *D. hamiltonii* upon growing in presence of 50 g pot^{-1} inoculum of *G. mosseae*, *G. fasciculatum* and *G. monosporum* respectively (tab. 3).

The response for overall plant growth of *D. hamiltonii* along with significant increase in root specific flavour compound 2H4MB, however the response is varied with the AMF inoculum quantity. In general, AMF through symbiosis association with roots of plants are known for their potential role in improvement of plant growth, and also has profound influence on a number of important ecosystem processes, including plant productivity, plant diversity and soil

Table 3. ANOVA analysis for growth characteristics and flavour content (2-Hydroxy-4-Methoxy Benzaldehyde) of tubers in response to 50 g pot^{-1} AMF treatment for seedling plants and micro-propagated plants of *D. hamiltonii*

Source	d.f.	F-values								
		shoot length (cm)	number of nodes	number of leaves	total chlorophyll (mg g^{-1} FW)	number of tubers	range of tuber diameter (cm)	range of tuber length (cm)	fresh weight of tubers (g)	flavour content (mg g^{-100})
Seedling (A)	02	2.23**	414.62**	786.82**	162.89**	13.7**	10.25**	24.46**	131.74**	39.5**
Micropropagated (B)	02	2.42**	385.3**	447.52**	160.76**	394.74**	48.15**	152.38**	404.11**	419.11**
Interaction										
A × B	05	4.87**	300.4**	313.56**	84.64**	129.51 ns	21.36ns	101.9**	296.96**	129.57**

ns – non-significant
d.f. – degrees of freedom
FW – fresh weight
** F – values and significance at p = 0.05

structure [van der Heijden et al. 1998]. The better performance of plants under AMF treatment in tuberous crop such as yam and potato was reported [Tchabi et al. 2010, Hijri 2016]. In the present study all the three AMF viz., *G. mosseae*, *G. fasciculatum* and *G. monosporum* were found useful in improving the overall performance of *D. hamiltonii* plants growth. This was in concurrence with similar studies in other plants wherein, AMF such as *G. mosseae* improved chick pea plant vegetative growth [Sohrabi et al. 2015] and papaya plant growth, fruit quality [Vázquez-Hernández et al. 2011].

In general, plants respond to the respective microbes of the rhizosphere in different ways and detection of pathogen-derived elicitors triggers plant signalling cascades that lead to various responses [Glazebrook 2005]. In this regard, plant responses to fungal-derived signals at early and mature symbiosis stages with AMF were reviewed [Garg and Chandel 2010]. AMF benefit plants by aiding phosphorus uptake from soil [Paszkowski 2006]. In addition AMF can take up and transfer significant amounts of inorganic nitrogen (NH^{+4} or NO^{-3}) to their host plants [Paszkowski 2006]. All these factors contribute to the improvement in growth of plants. Secondary metabolites augmentation potential of AMF treated plants of licorice and yam was demonstrated [Liu et al. 2007]. Plants performance under stressful conditions and role of AMF was reported [Smith et al. 2010]. It is a well established fact that, elicitor stress induce or augment plant secondary metabolites such as phenylpropanoid pathway intermediates [Prasad et al. 2006], apocarotenoids [Parimalan et al. 2011], and isoflavonoids [Saini et al. 2013] as shown in various plants. Sailo and Bhagyaraj [2005] reported the influence of AMF on secondary metabolites such as forskolin production *Coleus forskohlii*. Similarly, Selvaraj and Sumitra [2011] demonstrated the efficiency of *Glomus* sp. on growth and metabolites production in *Glycerhhiza glabra*. In the present study, up to 82.23% improvement in root specific vanillin flavour metabolite 2H4MB production was recorded in AMF treated plants. Functional diversity in AMF with reference to the role of gene expression, phosphorus nutrition and symbiotic efficiency plays a key role for such responses in plants [Fedderman et al. 2010].

CONCLUSIONS

1. From this study it can be concluded that the principal effect of arbuscular mycorrhizal fungi on *D. hamiltonii* plants was on yield and plant growth along with significant increase in flavour metabolite.

2. *G. mosseae* treatment exhibited higher response than *G. fasciculatum* and *G. monosporum* for better growth characteristics of *D. hamiltonii*.

3. The symbiosis of mycorrhizal fungi and swallow root would be of benefit to crop growth, tuber yield and flavour metabolite improvement.

ACKNOWLEDGMENT

The authors are thankful to Council of Scientific and Industrial Research, Government of India, New Delhi for the research grant (12 FYP-Network Program-BSC0106).

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