Agric Res (March 2013) 2(1):43–47
DOI 10.1007/s40003-012-0047-1

FULL-LENGTH RESEARCH ARTICLE

Arbuscular Mycorrhizal Fungi Induced Acclimatization and Growth Enhancement of *Glycyrrhiza glabra* L.: A Potential Medicinal Plant

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Received: 3 August 2012 / Accepted: 27 December 2012 / Published online: 19 January 2013

Abstract A bioinoculation experiment was performed to see the effect of inoculation of arbuscular mycorrhizal fungi (AMF) on post-transplanting performance and growth enhancement of micropropagated plantlets of *Glycyrrhiza glabra* L. The dominant AMF *Glomus mosseae* and *Acaulospora laevis* were isolated from the rhizosphere soil of *G. glabra* and mass produced in laboratory for further studies. In vitro raised plantlets were inoculated with single and in combined mycorrhizal treatments. Various morphological and biochemical parameters were measured after 120 days. All inoculated plantlets responded significantly different to all three mycorrhizal treatments. *G. mosseae* enhanced the survival rate of *G. glabra* plantlets to 100 %. Most growth related parameters (shoot length, leaf area, leaf number, fresh, and dry weight) were significantly affected by different fungal intervention. Treated plants typically showed more obvious modifications in their biochemical status also. The chlorophyll a, chlorophyll b, and protein content increased in AMF inoculated as compared to the control plants. The total soluble sugars and reducing sugars contents in the present study showed a minor decrease in mycorrhizal-treated plants. Overall results suggest that although, all mycorrhizal treatments showed good response, but the treatment with *G. mosseae* + *A. laevis* inoculation executed more pronounced response for enhancing the growth and development of this plant. These results emphasize the need for the incorporation of tissue culture protocols with mycorrhizal inoculation for ensuring better survival and improved growth of this valuable medicinal plant.

Keywords *Acaulospora laevis* · AM fungi · Bioinoculation · *Glomus mosseae* · *Glycyrrhiza glabra* · Micropropagation

Introduction

Glycyrrhiza glabra (family Leguminosae) is an important perennial cross-pollinated plant having diverse pharmacological effects including detoxication, antioxidant, antiulcer, anti-inflammatory, and antiviral properties [31]. The most important secondary metabolite isolated from roots and stolons of this plant is "Glycyrrhizin" which is used in the treatment of bronchial asthma, allergic, dermatitis, gastric ulcers, Adisson's disease, and eczema [21].

Because of its diverse medicinal properties, it is widely used in the confectionary, food, and pharmaceutical industries and there is an urgent need of new approaches of producing *G. glabra* to meet the increasing demands [36]. The conventional method for propagation is through rhizomes, stolons, or other cuttings which is a commercial valuable part of the plant. The regeneration rate is also very slow and low [9, 29].

Although studies on in vitro culture of *G. glabra* have been developed with different purposes, such as for rapid production of high quality, disease-free and uniform planting material [4, 7, 21, 29, 31]. During the acclimatization

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stage of micropropagation, the in vitro-raised plants are subjected to environmental stress due to poor root, shoot, and cuticular development [32]. The incorporation of mycorrhization at this stage represents a biologic solution for reducing the weaning stress by solving the problems of transient transplantation, thus shortening the acclimatization period [27].

Considering the beneficial effect of arbuscular mycorrhizal fungi (AMF) on medicinal plants, more attention should be paid toward the combining of appropriate mycorrhizal fungi with in vitro micropropagation techniques [28].

To our knowledge, there is no information available about the role of AMF on micropropagated plantlets of *G. glabra.* Our work aims to examine the effect of AMF inoculation on survival and growth enhancement of in vitro raised plantlets of *G. glabra* that can be used for sustainable utilization and mass cultivation in the future.

Materials and Methods

Plant Material and Micropropagation Procedures

Healthy nodal segments (1.0–1.5 cm) excised from the plants growing in Herbal Garden of Botany Department, Kurukshetra University, Haryana, India, served as source of explants for micropropagation. Complete plantlets of *G. glabra* consisting of well-developed roots and leaves were obtained by direct regeneration from nodal explants on Murashige and Skoog [22] medium supplemented with BAP (2.0 mg l^{-1}) following our modified protocol of Yadav and Singh [37].

Plant cultures were maintained in culture room, at a temperature of 25 ± 2 °C under photoperiod of 16 h having light intensity of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps with 60 % relative humidity.

After 75 days, when adequate rooted shoots were obtained, the in vitro raised plantlets (14 cm) after washing with fresh water to remove agar particles were transferred from the culture tubes to plastic cups (15 × 8 cm) holding 500 g of sterilized soil and sand (3:1) mixture. The cups were covered with transparent polythene bags (to maintain humidity) with holes (to provide aeration) and kept in culture room under the same light and temperature conditions provided during micropropagation.

Choice of AMF

Two dominant AMF, *Glomus mosseae* and *Acaulospora laevis*, were isolated from the rhizosphere soil of *G. glabra* by wet sieving and decanting technique given by Gerdemann and Nicolson [8].

Multiplication of AMF Cultures

The starter inoculum or pure culture of each selected dominant AM fungus was raised by "Funnel Technique" of Menge and Timmer [20].

AMF Inoculation Procedure

The acclimatized plantlets were transplanted into experimental earthen pots (30 × 30 cm) having sterilized sand:soil mixture (300:1,500 g). To each pot, 10 % inoculum consisting of AMF spores and fine roots of maize having mycelium/arbuscules/vesicles alone and in combination were added. The inoculum was put very close to rhizosphere of transplanted plantlets in the experiments and was transferred in glasshouse having constant temperature (28–30 °C) and relative humidity (65–75 %) for further studies. Light was provided by cool white fluorescent lamps (8,000 lux) under a 16-h photoperiod. The polyhouse also received sunlight. Plants were watered regularly and 50 ml per pot Hoagland nutrient solution (without KH_2PO_4) was given every 7 days.

The experiments were carried out in alone and combined inoculations. In the control, no inoculum was added.

Different growth and biochemical parameters were studied after 120 days of inoculation (DOI).

Growth and Morpho-Physiologic Parameters

Among various plant growth parameters, plant height (cm), number of leaves per plant, leaf area (cm^2), fresh shoot weight (g), fresh root weight (g), dry shoot weight (g), and dry root weight (g) were observed. Plant dry weight was determined after drying the tissue in an oven at 80 °C for 48–72 h. Leaf area was measured using a leaf area meter (Leaf Area Meter 211; Systronics Ltd, Ahmadabad, India).

Biochemical Assays

Different biochemical parameters, viz., chlorophyll, total sugars, reducing sugars, and proteins, were estimated from the leaf tissues. The content of chlorophyll (*a*, *b*, and total) was measured according to Arnon [3], reducing and total sugars according to Hart and Fisher [10], and proteins by the method of Bradford [6].

Statistical Analyses

Each experiment consisted of 5 replicates per treatment and was repeated thrice. Data were analyzed for significance by one-way analysis of variance and the differences contrasted

Table 1 Effect of AM fungi on the survival rate of micropropagated *G. glabra* after 120 days post-inoculation

Treatments		Survival rate (%)
T1	Control	66.66 ± 48.79^{b}
T2	A	80.0 ± 41.40^{ab}
T3	G	100 ± 0.00^{a}
T4	G + A	93.33 ± 25.81^{a}

Values represent mean ± standard error, $n = 15$

Means were compared by the least significant difference (LSD) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$

G Glomus mosseae, *A Acaulospora laevis*

by a Duncan's multiple range test (DMRT) at $p \leq 0.05$. All statistical analyses were performed by means of the Statistical Package for Social Sciences (SPSS, version 11.5).

Results and Discussion

In vitro methods of propagation provide an alternate and effective means for rapid multiplication of species by the continuous production to meet the demand for commercial exploitation [15–17]. The beneficial effect of mycorrhizal inoculation has also been reported earlier in other micropropagated medicinal plant [11, 32, 33, 38, 39].

The survival rate and development of micropropagated plantlets of *G. glabra* have been improved by introducing effective mycorrhizal symbiosis with the plant. Table 1 shows the survival rate of *G. glabra* plants improved significantly when inoculated with different treatments of AMF at the beginning of the acclimatization stage. Inoculation with *G. mosseae* contributed to 100 % of plantlet survival as opposed to 66.6 % of survival in control (Table 1).

It is obvious that the integration of AMF was found to have a significant effect on the growth and development of micropropagated *G. glabra* plantlets. However, this improvement was considerably different with regard to the type of fungal strain used. In the present investigation, it was found that mycorrhizal inoculation increased the height of the plant in comparison to the control. The most effective results were observed in the dual combination of *A. laevis* + *G. mosseae* after 120 days of inoculation. Similar trend was observed in the case of leaves number per plant (Table 2). Significant increment in root length was observed with *G. mosseae* followed by dual combination of *G. mosseae* + *A. laevis*. The maximum leaf area was found in with the *A. laevis* treatment followed by dual combination of *G. mosseae* + *A. laevis*. A significant

Table 2 Effect of AMF on the various growth parameters of micropropagated *G. glabra* after 120 days of inoculation

Treatments	Shoot length (cm)	Root length (cm)	Leaves (no./plant)	Leaf area (cm^2/plant)	Fresh weight (g/plant)		Dry weight (g/plant)	
					Shoot	Root	Shoot	Root
T1 Control	31.7 ± 0.41^{d}	15.6 ± 0.31^{d}	16.2 ± 0.84^{d}	11.8 ± 1.73^{d}	0.95 ± 0.04^{d}	4.42 ± 0.33^{c}	0.12 ± 0.00^{d}	1.84 ± 0.03^{d}
T2 A	36.9 ± 0.34^{c}	20.7 ± 0.41^{c}	19.2 ± 0.84^{c}	30.4 ± 1.34^{a}	1.66 ± 0.05^{c}	7.01 ± 0.26^{a}	0.40 ± 0.01^{c}	3.15 ± 0.05^{b}
T3 G	41.8 ± 0.35^{b}	23.3 ± 0.30^{a}	22.8 ± 1.30^{b}	24.6 ± 1.63^{c}	2.84 ± 0.05^{b}	7.33 ± 0.45^{a}	0.59 ± 0.01^{b}	3.35 ± 0.06^{a}
T4 G + A	45.9 ± 0.30^{a}	22.2 ± 0.35^{b}	25.6 ± 1.14^{a}	26.9 ± 0.33^{b}	3.15 ± 0.05^{a}	6.40 ± 0.42^{b}	0.64 ± 0.01^{a}	2.72 ± 0.08^{c}

Values represent mean ± standard error, $n = 15$

Means were compared by using the least significant difference (LSD) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$

G Glomus mosseae, *A Acaulospora laevis*

Table 3 Effect of AMF on the various biochemical parameters (mg/100 mg fresh wt.) of micropropagated *G. glabra* after 120 days post-inoculation

Treatments		Chlorophyll "*a*"	Chlorophyll "*b*"	Total sugars	Reducing sugars	Proteins
T1	Control	1.14 ± 0.04^{c}	0.29 ± 0.01^{d}	0.16 ± 0.02^{a}	0.06 ± 0.00^{a}	0.08 ± 0.00^{c}
T2	A	1.22 ± 0.06^{c}	0.36 ± 0.00^{c}	0.08 ± 0.00^{d}	0.03 ± 0.00^{d}	0.08 ± 0.00^{c}
T3	G	2.08 ± 0.03^{a}	0.64 ± 0.03^{a}	0.15 ± 0.02^{b}	0.05 ± 0.00^{b}	0.11 ± 0.00^{a}
T4	G + A	1.69 ± 0.10^{b}	0.47 ± 0.06^{b}	0.10 ± 0.01^{c}	0.04 ± 0.00^{c}	0.09 ± 0.00^{b}

Values represent mean ± standard error, $n = 15$

Means were compared by using the least significant difference (LSD) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$

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increment in leaf area registered with inoculated plant may be due to increased phosphorus uptake resulting in enhancement of biosynthesis processes, determining a faster growth and development, which leads to a greater leaf area [30].

Biomass of all the inoculated plants of *G. glabra* increased significantly in terms of fresh and dry shoot weight after 120 days of inoculation. A significant increment in shoot biomass was recorded in dual combination of *G. mosseae* + *A. laevis* followed by *G. mosseae* (Table 2). AMF play a dominant role in increasing phosphorus solubilization and uptake of P, N, Ca, S, K, Mg, Mn, and Cl by plants [35]. It may be due to the reason that AMF hyphae growing through soil pore spaces can affect phosphate absorption beyond the depleted zone. The fungal hyphae transport phosphate over large distance into the root cortical cells [13, 24]. They also produce growth-promoting substances such as indole acetic acid-, cytokinins-, and gibberellin-like substances [12, 14, 25, 26].

The results of the present study clearly revealed the significant variations among different biochemical parameters treated with different AM fungus (Table 3). It can be stated that the leaf chlorophyll content recorded in the mycorrhizal plants was typically higher than non-treated control. Micropropagated plantlets inoculated with *G. mosseae* yielded the highest amount of total chlorophyll. In this study, increased chlorophyll "a" and chlorophyll "b" in plants is similar to the findings of Bavaresco and Fogher [5] on the effect of *G. mosseae*. Mycorrhizal-treated plants show a greater increase in the rate of photosynthesis than their controls which may be due to increase in the content of total chlorophyll [18]. Increased chlorophyll content after AMF inoculation may be due to the higher concentrations of Mg, Fe, and Cu in foliar tissues thereby influencing chlorophyll synthesis [19, 33].

As compared to other biochemical traits, reducing sugar and total sugar contents showed a decrease was observed in different AMF inoculation than control (Table 3). It may be because the AMF always serves as a strong sink for sugars and utilize 10–20 % of net photosynthate in exchange for the transfer of nutrients to the host to lead a symbiotic life [1, 23].

Leaf protein showed a minor increase in *G. mosseae* inoculated plants followed by *G. mosseae* + *A. laevis* (Table 3). This is in accordance with Vazquez et al. [34] according to which the total soluble protein content was significantly higher in mycorrhizal than non-mycorrhizal plants Mycorrhizal formation is known to increase the expression of low molecular weight proteins as reported by Arines et al. [2] in *Pisum sativum.*

This technology may also be applied to improve the post-transplanting performance of many other multipurpose valuable plants for the sustainable cultivation.

Acknowledgments The authors are grateful to University Grants Commission, New Delhi, for financial assistance and to Kurukshetra University, Kurukshetra, for providing laboratory facilities to carry out the work.

References

1. Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge
2. Arines J, Quintela M, Vilarino A, Palma JM (1994) Protein patterns and superoxide dismutase activity in nonmycorrhizal and arbuscular mycorrhizal *Pisum sativum* L. plants. Plant Soil 166:37–45
3. Arnon DI (1949) Copper enzymes in isolated chloroplasts polyphenol-oxidase in *Beta vulgari*. Plant Physiol 24:1–15
4. Arya S, Rathi N, Arya ID (2009) Micropropagation protocol for *Glycyrrhiza glabra* L. Phytomorphology 59:71–76
5. Bavaresco L, Fogher C (1996) Lime-induced chlorosis of grapevine as affected by rootstock and root infection with arbuscular mycorrhiza and *Pseudomonas fluorescens*. Vitis 35:119–123
6. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye-binding. Ann Biochem 72:248–254
7. Fu C, Lei C, Gan L, Li M, Yang Y, Yu L (2010) Optimization of embryogenic-callus induction and embryogenesis of *Glycyrrhiza glabra*. Afr J Biotechnol 9:5823–5829
8. Gerdemann JW, Nicolson YH (1963) Spores of Mycorrhiza *Endogone* species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc 46:235–244
9. Gupta V, Kak A, Singh BB (1997) Studies of germination and seedling vigour in licorice (*Glycirrhiza glabra*). J Med Aromat Plant Sci 2:412–413
10. Hart FL, Fisher HJ (1971) Modern food analysis. Springer, New York
11. Joshee N, Mentreddy SR, Yadav AK (2007) Mycorrhizal fungi and growth and development of micropropagated *Scutellaria integrifolia* plants. Ind Crop Prod 25:169–177
12. Karthikeyan B, Jaleel CA, Changxing Z, Joe MM, Srimannarayan J, Deiveekasundaram M (2008) The effect of AM fungi and phosphorus level on the biomass yield and ajmalicine production in *Catharanthus roseus*. EurAsian J Biosci 2:26–33
13. Kaushih S, Kumar A, Aggarwal A (2011) Influence of hosts and substrates on mass multiplication of *Glomus mosseae*. Afr J Agric Res 6:2971–2977
14. Kaushih S, Kumar A, Aggarwal A, Parkash V (2011b) Influence of inoculation with the endomycorrhizal fungi and *Trichoderma viride* on morphological and physiological growth parameters of *Rauwolfia serpentina* Benth. Ex. Kurtz. Ind J Microbiol. doi: 10.1007/s12088-011-0215-1
15. Kumar S, Rai MK, Singh N, Mangal M (2010) Alginate-encapsulation of shoot tips of jojoba [*Simmondsia chinensis* (Link) Schneider] for germplasm exchange and distribution. Physiol Mol Biol Plants 16:379–382
16. Kumar S, Mangal M, Dhawan AK, Singh N (2011) Assessment of genetic fidelity of micropropagated plants of *Simmondsia chinensis* (Link) Schneider using RAPD and ISSR markers. Acta Physiol Plant 33:2541–2545
17. Kumar S, Mangal M, Dhawan AK, Singh N (2012) Biotechnological advances in jojoba [*Simmondsia chinensis* (Link) Schneider]: recent developments and prospects for further research. Plant Biotechnol Rep 6:97–106
18. Manoharan PT, Pandi M, Shanmugaiah V, Gomathinayagam S, Balasubramanian N (2008) Effect of vesicular arbuscular mycorrhizal fungus on the physiological and biochemical

changes of five different tree seedlings grown under nursery conditions. Afr J Biotechnol 7:3431–3436
19. Mathur N, Vyas A (1995) Influence of VA mycorrhizae on net photosynthesis and transpiration of *Ziziphus mauritiana*. J Plant Physiol 147:328–330
20. Menge JA, Timmer LW (1982) Procedures for inoculation of plants with vesicular–arbuscular mycorrhizae in the laboratory, greenhouse, and field. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St. Paul, pp 59–68
21. Mousa N, Siaguru P, Wiryowidagdo S, Wagih ME (2006) Rapid clonal propagation of licorice *(Glycyrrhiza Glabra)* by in vitro Shoot culture. Sugar Technol 8:292–298
22. Murashige T, Skoog F (1692) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
23. Muthukumar T, Udaiyan K (2000) The role of seed reserves in arbuscular mycorrhizal formation and growth of *Leucaena leucocephala* (Lam.) de Wit. and *Zea mays* L. Mycorrhiza 9: 323–330
24. Parkash V, Aggarwal A (2009) Diversity of endomycorrhizal fungi and their synergistic effect on the growth of *Acacia catechu* Willd. J For Sci 55:461–468
25. Parkash V, Aggarwal A, Sharma V (2011) Rhizospheric effect of vesicular arbuscular mycorrhizal inoculation on biomass production of *Ruta graveolens* L.: a potential medicinal and aromatic herb. J Plant Nutr 34:1386–1396
26. Parkash V, Sharma S, Aggarwal A (2011) Symbiotic and synergistic efficacy of endomycorrhizae with *Dendrocalamus strictus* L. Plant Soil Environ 57:447–452
27. Rai MK (2001) Current advances in mycorrhization in micropropagation. In Vitro Cell Dev Biol Plant 37:158–167
28. Rathore S, Yadav K, Kumar S, Singh N (2012) Biotechnological approaches to induce salt resistant in plants. Ann Biol 28:141–150
29. Sawaengsak W, Saisavoey T, Chuntaratin P, Karnchanatat A (2011) Micropropagation of the medicinal herb *Glycyrrhiza glabra* L., through shoot tip explant culture and glycyrrhizin detection. Int Res J Plant Sci 2:129–136
30. Schemidt B, Domonkos M, Sumalan R, Biro B (2010) Suppression of arbuscular mycorrhiza's development by high concentration of phosphorus at *Tagetes patula* L. Res J Agric Sci 42:156–162
31. Shams-Ardakani M, Mohagheghzadeh A, Ghannadi A, Barati A (2007) Formation of glycyrrhizin by in vitro cultures of *Glycyrrhiza glabra*. Chem Nat Compd 43:353–354
32. Sharma D, Kapoor R, Bhatnagar AK (2008) Arbuscular mycorrhizal (AM) technology for the conservation of *Curculigo orchioides* Gaertn.: an endangered medicinal herb. World J Microbiol Biotechnol 24:395–400
33. Singh NV, Singh SK, Singh AK, Meshram DT, Suroshe SS, Mishra DC (2012) Arbuscular mycorrhizal fungi (AMF) induced hardening of micropropagated pomegranate (*Punica granatum* L.) plantlets. Sci Hortic 136:122–127
34. Vazquez M, Cesar S, Azcon R, Barea JM (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272
35. Vestberg M, Estaun V (1994) Micropropagated plants, an opportunity to positively manage mycorrhizal activities. In: Gianinazzi S, Schüepp H (eds) Impact of AMF on sustainable agriculture and natural ecosystems. Birkhauser Verlag, Basel, pp 217–225
36. Wang ZY, Athar M, Bickers DR (2000) Licorice in foods and herbal drugs: chemistry, pharmacology, toxicology and uses. In: Mezza G, Oomah BD (eds) Herbs, botanicals & teas. Technomic Publishing Co. Inc, Lancaster, pp 321–353
37. Yadav K, Singh N (2012) Factors influencing in vitro plant regeneration of liquorice (*Glycyrrhiza glabra* L.). Iranian. J Biotechnol 10(3):161–167
38. Yadav K, Singh N, Aggarwal A (2011) Influence of arbuscular mycorrhizal (AM) fungi on survival and development of micropropagated *Acorus calamus* L. during acclimatization. J Agric Technol 7:775–781
39. Yadav K, Singh N, Aggarwal A (2012) Arbuscular mycorrhizal (AM) technology for the growth enhancement of micropropagated *Spilanthes acmella* Murr. Plant Protect Sci 48:31–36