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Microbial Associates of *Hippophae rhamnoides* (Seabuckthorn)

Shiv Kumar and Anand Sagar
Department of Biosciences, Himachal Pradesh University, Summer Hill, Shimla-171005, India

Abstract: Seabuckthorn (*Hippophae rhamnoides*), a multipurpose shrub and native of higher Himalayas is also known as cold desert gold due to its high potential as a bio resource for wetland reclamation, soil erosion, food medicinal and cosmetic industries. Studies conducted on the microbial associates of this plant revealed the presence of 26 fungal species in its rhizosphere. Three fungal entophytes (i.e., *Aspergillus niger*, *Mortierella minutissima* and a sterile mycelium) and four species of VAM spores (i.e., *Glomus albidum*, *Glomus fasciculatum*, *Glomus macrocarpum* and *Gigaspora margariata*) has also been isolated from different plant parts (Root, stem, leaves and bark) and soil samples, respectively.

Key words: Seabuckthorn, rhizosphere, VAM, Endophytes

INTRODUCTION

Seabuckthorn (*Hippophae rhamnoides*, Family Elaeagnaceae) is a multipurpose deciduous, dioecious, thorny and nitrogen fixing shrub-tree growing widely on high altitude regions of Himachal Pradesh, Jammu and Kashmir, Sikkim and Uttranchal. It is tolerant of extremes of temperature (−43 to +45°C), resistant to drought conditions and well adapted to the salinity and alkalinity. Because of its benefits to the mountain communities, it is called wonder plant or cold desert gold (Kumar, 2003; Jodha *et al*., 1992). Seabuckthorn is supposed to be a store house of nutrients and many items like jams, soft drinks, sauces, pickles, jelly and biscuits are made from it. Its fruits are good source of vitamins. A part from this it is used as fire wood, as fencing plant on river side for soil conservation purpose (Naithani, 2004).

Research on medicinal properties and other aspects of Seabuckthorn has received much attention in recent past ((Gurevick, 1956; Gurmeet, 1997; Dorjey, 1998; Eccleston *et al*., 2002; Kumar, 2003; Singh, 2006; Yang and Kallio, 2006), but there is little information available on the rhizosphere, VAM and endophytic fungi of this plant. This communication records observations on such microbial association of Seabuckthorn so as to suggest strategies (by inoculating with suitable mycorrhizal fungi) for growing it on degraded and abandoned land for sustainable development of the traditional mountain societies.

MATERIALS AND METHODS

Site of sample collection: Various samples were collected from District Lahaul-Spiti, of Himachal Pradesh (December, 2004-July, 2005), lying between North latitude 33°44' and 33° 42' and east latitude 76°56' and 78°41'. This District is referred to as 'cold desert' and composed of rugged and different mountain terrains, which is a part of great Himalayan region.

Morphoanatomy of root: Root samples were collected from three different plants from different localities and were stored in F:A:A: (Formaldehyde: Acetic acid: Alcohol) in the ratio 5:5:9. For morphological study, root color, root diameter, presence or absence of nodule, size of nodule and any change in morphology due to an ectomycorrhiza were taken in to account. To reveal anatomical detail, thin sections of these roots were double stained and observed (Marx and Davey, 1969).

Isolation and identification of rhizosphere fungi: Rhizosphere soil samples were collected from three plants in sterile polythene bags. The isolation of rhizosphere fungi from these was done by dilution plate method of Wakesman (1927) and Warcup (1950). This method includes shaking of 1 g soil in 100 mL of distilled water followed by further dilutions under asceptic conditions. Various dilutions were cultured on Potato Dextrose Agar medium and Czapek's Dox medium (Raper and Thom, 1949). Subculturing was done and the fungi were identified following Barnett and Hunter (1972), Gilman (1967) and Nagmani *et al*. (2005).

VAM spores isolations: Isolation of VAM spores was done by Wet sieving and Decanting Technique (Gerdemann and Nicolson, 1963). A pinch of soil was shaked in distilled water and was filtered through sieves 300, 200 and 100 μm followed by filtration through

Corresponding Author: Shiv Kumar, Department of Bio Sciences, Himachal Pradesh University, Summer Hill, Shimla-171005, India Tel: 091 177-2830946

Whattman No. 1 filter paper. This filter paper was observed directly under binocular microscope. Each spore was picked with micro needle and identified following (Mortan, 1988; Schenck and Perez, 1988; Trappe, 1982; Walker, 1983, 1986).

Isolation of endophytes: To isolate endophytes, various plant parts (i.e., roots, leaves, bark, stem) collected from different plants were screened out following Hot water treatment and Three step methods. Small pieces of tissue were treated with 25, 50 and 75% methanol for 5, 3 and 2 min, respectively. Then these were inoculated on half strength PDA medium. Pure cultures of each endophyte were subcultured at regular intervals.

RESULTS

Morphological studies on the roots of *Hippophae rhamnoides* Linn: *Hippophae rhamnoides* Linn. is a non-leguminous nitrogen fixing plant. It has stout, deep feeding and dichotomously branched roots. The roots are brown in colour. Average diameter of roots ranges from 4 mm-1.5 cm. Roots are nodulated. Nodules actually represent modified lateral root. Each nodule is highly branched and varies from 4.0-9.0 mm in size. Nodule harbours an actinorhizal microorganism called *Frankia*, which help in nitrogen fixation.

Anatomical features of roots of seabuckthorn: In transverse section, the roots of *Hippophae rhamnoides* Linn. showed outer most torned bark layer which is deep brown in colour. Next to it somewhat squarish yellow coloured cells of cork are seen. In these layers a network of branching filaments were seen. Next to cork layers, are present cortical cells, which are loosely arranged with large intercellular spaces in between.

The intercellular spaces of cortex reveal the presence of branching filaments. Some of filaments penetrate the cortical cells and form vesicles within the cells. These vesicles are formed by an Actinomycete; *Frankia* and these were present in large number in the cortical cells. The presence of *Frankia* in the root nodules of Seabuckthorn confirms the Actinorhizal type of association between both the partners. Inter stellar infection is absent (Fig. 1a and b).

Qualitative assessment of mycoflora from rhizosphere of *Hippophae rhamnoides* Linn. during Winter season (December, 2004): Fungi were isolated from rhizosphere of *Hippophae rhamnoides* Linn. using dilution plate technique. During winter season 22 species of fungi were isolated. These fungal isolates fall into 13 genera. One isolate is represented by sterile mycelia.

The different fungal genera isolated from soil samples collected in winter season are *Absidia*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Cunninghamella*, *Epicoccum*, *Fusarium*, *Macrophomina*, *Mucor*, *Penicillium*, *Rhizoctonia*, *Rhizopus* and *Trichoderma*.

The genus *Penicillium* is represented by four species (i.e., *P. funiculosum*, *P. nigricans*, *P. chrysogenum* and *Penicillium* sp.). The genera *Aspergillus* and *Fusarium* are represented by three species each (i.e., *A. flavus*, *A. niger*, *A. versicolor*, *F. moniliforme*, *F. oxysporum* and *Fusarium* sp.). The genus *Cladosporium* is represented by two species (i.e., *C. herbarum* and *C. sphaerospermum*). Other nine genera (i.e., *Absidia*, *Alternaria*, *Cunninghamella*, *Epicoccum*, *Macrophomina*, *Mucor*, *Rhizoctonia*, *Rhizopus* and *Trichoderma*) are represented by one species each (i.e., *Absidia ramosa*, *Alternaria alternata*, *Cunninghamella echinulata*, *Epicoccum nigrum*, *Macrophomina phaseoli*, *Mucor hiemalis*, *Rhizoctonia solani*, *Rhizopus negricans* and *Trichoderma viride*).

The fungi isolated from rhizosphere soil samples of *Hippophae rhamnoides* Linn. belong to the fungal subdivisions Zygomycotina, Ascomycotina, Deuteromycotina and Basidiomycotina. Out of these four genera belong to Zygomycotina (*Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*), two belong to Ascomycotina (*Aspergillus* and *Penicillium*), seven to Deuteromycotina (*Alternaria*, *Epicoccum*, *Fusarium*, *Rhizoctonia*, *Cladosporium*, *Macrophomina* and *Trichoderma*). Basidiomycotina is represented by a single sterile mycelium. The maximum representatives are of Deuteromycotina (7 genera), followed by Zygomycotina (4 genera) and Ascomycotina (2 genera) which in return are followed by Basidiomycotina (Table 1). This study shows that during winter season, maximum number of species is that of *Penicillium* followed by *Aspergillus* and *Fusarium* while subdivision Deuteromycotina is represented most.

Qualitative assessment of mycoflora from rhizosphere soil samples of *Hippophae rhamnoides* Linn. during Rainy season (July, 2005): The samples of soil collected during July, 2005 (rainy season), from Lahaul Spiti district of Himachal Pradesh revealed the presence of

Table 1: Distribution of fungal genera isolated from the rhizosphere of *Hippophae rhamnoides* Linn. during winter season into different subdivisions of fungi

Sr. No.	Sub-division	Genus
1	Zygomycotina	*Absidia, Cunninghamella, Mucor, Rhizopus*
2	Ascomycotina	*Aspergillus, Penicillium*
3	Basidiomycotina	*Sterile mycelia*
4	Deuteromycotina	*Alternaria, Cladosporium, Epicoccum, Fusarium, Macrophomina, Rhizoctonia, Trichoderma*

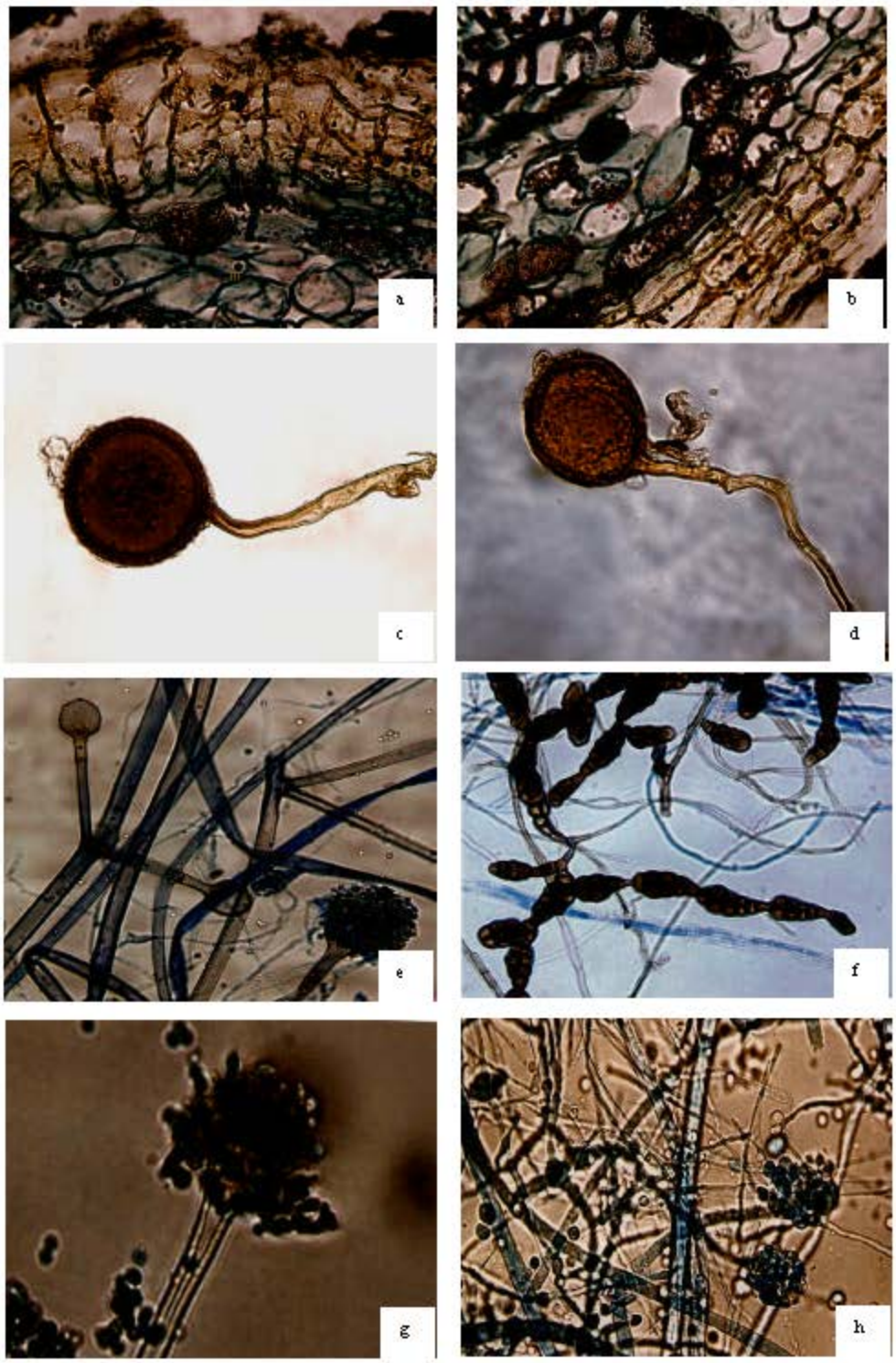

Fig. 1: TS Root of *Hippophae rhamnoides* Linn. Showing Branching Filaments and Vesicles of *Frankia* (a and b), *Glomus macrocarpum* (c), *Glomus fasciculatum* (d), *Absidia ramosa* (e), *cunninghamella echinulata* (f), *Aspergillus niger* (g), *Mortierella minutissima* (h)

Table 2: Comparison of occurrence of different fungal species during winter and rainy season

Sr. No.	Name of fungi isolated	Winter season	Rainy season
1	*Absidia ramosa*	+	+
2	*Alternaria alternata*	+	+
3	*Aspergillus flavus*	+	+
4	*Aspergillus niger*	+	+
5	*Aspergillus versicolor*	+	+
6	*Cephalosporium acrimonium*	-	+
7	*Cladosporium herbarum*	+	+
8	*Cladosporium sphaerospermum*	+	+
9	*Cunninghamella echinulata*	+	+
10	*Drechslera tetramera*	-	+
11	*Epicoccum nigrum*	+	+
12	*Fusarium moniliforme*	+	+
13	*Fusarium oxysporium*	+	+
14	*Fusarium* sp.	+	+
15	*Macrophomina phaseoli*	+	+
16	*Mucor hiemalis*	+	+
17	*Penicillium chrysogenum*	+	+
18	*Penicillium funiculosum*	+	+
19	*Penicillium nigricans*	+	+
20	*Penicillium* sp.	+	+
21	*Rhizoctonia solani*	+	+
22	*Rhizoctonia* sp.	-	+
23	*Rhizopus negricans*	+	+
24	*Stachybotrys atra*	-	+
25	*Trichoderma viride*	+	+
26	*Sterile mycelium*	+	+

+: Indicating presence of the fungus in a particular season, -: Indicating absence of the fungus in a particular season

26 species of fungi. These fall into 16 genera. The fungal genera isolated from the rhizosphere soil samples of Seabuckthorn collected during rainy season are *Absidia*, *Alternaria*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Drechslera*, *Epicoccum*, *Fusarium*, *Macrophomina*, *Mucor*, *Penicillium*, *Rhizoctonia*, *Rhizopus*, *Stachybotrys* and *Trichoderma*.

In the case of rainy season *Penicillium* is also represented by four species (i.e., *Penicillium funiculosum*, *P. negricans*, *P. chrysogenum* and *Penicillium* sp.). The genera *Aspergillus* and *Fusarium* are represented by three species each (i.e., *A. flavus*, *A. niger*, *A. versicolor*, *F. moniliforme*, *F. oxysporum* and *Fusarium* sp.). The genera *Rhizoctonia* and *Cladosporium* are represented by two species each (i.e., *R. solani*, *Rhizoctonia* sp., *C. herbarum* and *C. sphaerospermum*).

Other eleven genera i.e., *Absidia*, *Alternaria*, *Cephalosporium*, *Cunninghamella*, *Drechslera*, *Epicoccum*, *Macrophomina*, *Mucor*, *Rhizopus*, *Stachybotrys* and *Trichoderma* are represented by single species each (i.e., *A. ramosa*, *A. alternata*, *C. acrimonium*, *C. echinulata*, *D. tetramera*, *E. nigrum*, *M. phaseoli*, *M. hiemalis*, *R. negricans*, *S. atra* and *Trichoderma viride*). These fungi belong to subdivision Zygomycotina, Ascomycotina, Deuteromycotina and Basidiomycotina. Out of these, four genera belong to Zygomycotina (*Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*), three to Ascomycotina (*Aspergillus*, *Drechslera* and *Penicillium*), nine to Deuteromycotina (*Alternaria*, *Cephalosporium*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Macrophomina*, *Stachybotrys* and *Trichoderma*). Basidiomycotina is represented by three white sterile mycelia. During rainy season, the number of fungal species isolated from rhizosphere is more than that isolated during winter. The new genera reported during rainy season are *Cephalosporium*, *Stachybotrys* and *Drechslera*. One more species of *Rhizoctonia* is reported (Table 2).

Table 3: Distribution of fungal genera isolated from the rhizosphere of *Hippophae rhamnoides* Linn. during Rainy season into different subdivisions of fungi

Sr. No.	Sub-division	Genus
1	Zygomycotina	*Absidia, Cunninghamella, Mucor, Rhizopus*
2	Ascomycotina	*Aspergillus, Drechslera, Penicillium*
3	Basidiomycotina	*Sterile mycelia*
4	Deuteromycotina	*Alternaria, Cephalosporium, Cladosporium, Epicoccum, Fusarium, Macrophomina, Rhizoctonia, Stachybotrys, Trichoderma*

The maximum representative fungal genera belongs to Deuteromycotina (nine genera), followed by Zygomycotina (four genera) and Ascomycotina (three genera). Basidiomycotina is also represented by three sterile mycelia (Table 3).

Isolation and identification of VAM fungal spores from rhizosphere of *Hippophae rhamnoides* Linn.: The soil samples from rhizosphere of *Hippophae rhamnoides* Linn. were analyzed for the presence of various VAM fungi. The fungal spores were isolated by wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963) and were identified at Department of Botany, University of Delhi, New Delhi by Prof. K.G. Mukerji.

Spores of four species of VAM fungi were isolated from samples taken during December 2004 (winter season) and July, 2005 (Rainy season). These species belong to two genera namely, *Glomus* and *Gigaspora*.

Out of four species isolated, three belonged to *Glomus* and one to *Gigaspora*. Thus the genus *Glomus* was more predominant among these genera. The four species isolated are: *Glomus albidum*, *Glomus fasciculatum*, *Glomus macrocarpum* and *Gigaspora margarita*.

Relative frequency of occurrence of VAM fungal species: In total, 17 spores of VAM fungi were isolated. Out of these, 13 spores (76.47%) were of *Glomus fasciculatum*, 2 spores (11.76%) were of *Glomus albidum* and only 1 spore (5.88%) was each of *Glomus macrocarpum* and *Gigaspora margarita*, respectively.

Table 4: Relative percentage frequency of occurrence of VAM fungi

Sr. No.	Fungal sp.	No. of spores of individual species isolated	Total No. of VAM spores isolated	Occurrence frequency (%)
1	*Gigaspora margarita*	1	17	5.88
2	*Glomus albidum*	2	17	11.76
3	*Glomus fasciculatum*	13	17	76.47
4	*Glomus macrocarpum*	1	17	5.88

This shows that most abundant genus of VAM fungi in the rhizosphere of *Hippophae rhamnoides* Linn. is *Glomus* among two genera isolated i.e., *Glomus* and *Gigaspora* during this study. Among the three species of *Glomus* (i.e., *G. albidum*, *G. fasciculatum* and *G. macrocarpum*), *G. fasciculatum* is most abundant followed by *G. albidum* and *G. macrocarpum* (Table 4).

Observations on the endophytic fungal isolates of *Hippophae rhamnoides* Linn: Tissues taken from the various parts of the plant of *Hippophae rhamnoides* Linn. (i.e., leaf, bark, stem and root) were analyzed for the presence of fungal endophytes. In total three fungal endophytes were isolated which fall into two genera (i.e., *Aspergillus* and *Mortierella*) and one is represented by a non sporulating mycelium. Each of these (*Aspergillus* and *Mortierella*) is further represented by a single species i.e., *A. niger* and *M. minutissima*. The isolated endophytes belong to Zygomycotina (*M. minutissima*), Ascomycotina (*A. niger*) and Basidiomycotina (non sporulating mycelium) sub-divisions of fungi.

DISCUSSION

Morphology of roots of Seabuckthorn revealed simple and nodulated roots with no sign of ectomycorhiza, where the root tip becomes swollen and shows dichotomous branching. Harley and Smith (1983) have also concluded that while ectomycorrhizal fungi change the root morphology, VAM fungi do not alter root's shape and size. The nodules on the surface of roots of Seabuckthorn are due to *Frankia-actinorhizal* association, which is a non-leguminous N_2 fixing plant (Singh *et al*., 2003). The presence of vesicles and inter and intracellular branching filaments in the root cortical cells suggest actinorhizal type of association between Seabuckthorn and *Frankia*. Similar findings have been reported by earlier workers (Katiyar *et al*., 1989, Vijaylakshmi and Rao, 1988; Gardner *et al*., 1984; Gauthier *et al*., 1983) in most of the angiospermic plants. Regarding rhizosphere fungi of Seabuckthorn, it may be mentioned that it is the first report of its kind.

Slight fluctuation in the number of fungal species was observed during winter and rainy seasons. These rhizosphere organisms experience fluctuations due to change in pH, environmental extremes and root exudates (Molina and Amaranthus, 1990). Many interactions occur between plant root and rhizosphere fungi and the dominance of few of the micro-organisms in the rhizosphere and disappearance of other was also reported by Linderman (1988). The rhizosphere fungi are not merely passive prisoners of the abiotic environment rather they play an important role in the creation and maintenance of favourable growth conditions (Perry *et al*., 1987). Rhizosphere fungi have been isolated with the aim of their exploitation for the overall establishment and welfare of the plant. Because the organisms present in rhizosphere influence plant growth positively by enhancing nutrient uptake, storage, pathogen suppression, development of soil structure and protection against environmental extremes (Linderman, 1986).

VAM fungi help to increase the soil root interphase area and hence increase the nutrient uptake. These fungi are also known to increase the Phosphate and moisture uptake by the plant. The plants which are infected with mycorrhizal fungi exhibit significant increase in the yield as compared to non mycorrhizal plants (Gerdemann, 1968). The four types of endogenous spores obtained from the rhizosphere of Seabuckthorn indicate adaptability of these fungi to varied soil and environmental conditions. Sagar *et al*. (1993) and Kaur *et al*. (1997) while conducting studies on *Celtis australis* and *Grewia optiva*, respectively reported the presence of *Glomus*, *Sclerocystis*, *Acaulospora* and *Gigaspora* from the rhizosphere of these plants.

Endophytes are be being studied extensively since some of them may a source of novel biomolecules of pharmaceutical importance (Dreyfuss and Chapela, 1994). Present investigations have also revealed the presence of three endophytes (i.e., *Aspergillus niger*, *Mortierella minutissima* and one sterile mycelium) which were found closely associated with the tissue (roots, leaves, stem and bark) of *Hippophae rhamnoides*. Clelland *et al*. (1983) have reported the presence of *Glomus fasciculatum* as an endophytic fungus of *Hippophae rhamnoides*. The same fungus has also been isolated from the rhizosphere of Seabuckthorn during present study.

These microbial associates (rhizosphere fungi, VAM spores and endophytes) of Seabuckthorn can be exploited for its mass multiplication with the ultimate aim of transplanting the resultant tailored seedlings in degraded and treeless land of cold desert areas. Stone *et al*. (2000) have also regarded VAM endophytes as sources of novel

metabolites for therapeutics, as potential biocontrol agents and helping the establishment of seedlings at new sites by providing disease resistance and enhancing growth.

Present study stress the need to undertake indepth scientific researches on the role of microbial associates and their exploitation so as to suggest strategies for growing Seabuckthorn on degraded and abandoned land which will not only help them to improve their ecological environment but also help in the sustainable development of the traditional mountain societies.

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