Research Journal of Agricultural Sciences
An International Journal

Res. Jr. of Agril. Sci. **12**(2): 629–636

P- ISSN: 0976-1675 | **E**- ISSN: 2249-4538

www.rjas.org

Research Paper

In vitro Shoot Regeneration of *Rhododendron anthopogon* D.Don from Seed Derived Callus and Acclimatization of *in vitro* Raised Plantlets using Mycorrhizal Inoculum

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Received: 28 Feb 2021 | Revised accepted: 06 Apr 2021 | Published online: 08 Apr 2021
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A B S T R A C T

Rhododendron anthopogon D. Don belongs to family Ericaceae, is an ornamental and medicinal plant which is commonly known as 'Talisfer' in Kashmir. *Rhododendron anthopogon* is globally distributed. It grows in the Himalayan range across Pakistan, India, Nepal and Bhutan between an altitudinal range of 3000-4800m asl. Within India, it has been found in Jammu & Kashmir, Himachal Pradesh, Uttar Pradesh, Sikkim and Arunachal Pradesh. During the present study, *in vitro* shoot regeneration has been achieved from callus obtained from seed explants. The seeds were inoculated on Woody Plant Medium supplemented with different concentrations of 2,4-D. Maximum callus production was observed on Woody Plant Medium supplemented with 2,4-D (1mg/l) within 35 days of culture with a percent response of 70%. Among different cytokinin -cytokinin combinations used, 2ip (3mg/l) + TDZ (4mg/l) gave best results with the production of maximum mean number (4.6±0.3) of shoots. The *in vitro* raised plantlets were first acclimatized using cocopeat & perlite having different ratios as well as garden soil under greenhouse conditions. In addition, mycorrhizal status of *Rhododendron anthopogon* was confirmed under compound microscope and later mycorrhizal inoculum was prepared. Lastly, micro-propagated plantlets inoculated with mycorrhizal inoculum were acclimatized under greenhouse conditions.

***Key words*:** Acclimatization, Micropropagation, Mycorrhizae, Inoculum, *Rhododendron*, Woody plant medium

Kashmir Himalaya is famous for its scenic beauty throughout the world. This Himalayan region is blessed by nature with huge biodiversity, enriched with the wealth of medicinal, ornamental as well as aromatic plants. Due to various activities such as; over exploitation, territory loss, over grazing, alien species invasion, etc. the degree of threat to these plants is increasing fast, due to which most of the plant species have become threatened. Besides this the threat of landslides, construction of roads and excessive tourist flow poses a great threat to the major portion of the flora of Kashmir Himalayas [1]. In entire Indian Himalayan region, the natural populations of one such genus *Rhododendron* are gradually diminishing because of many factors which include extensive natural and anthropogenic alterations of *Rhododendron* habitats, excessive dependence on some of the species for fuel wood purposes, road building activities, slash and burn agricultural practices, natural calamities like deficient or excessive rainfall, hot and prolonged summers, snowy winters and landslides [2].

The term '*Rhododendron*' has been derived from the Greek words "rhodo" and "dendron" meaning "rose–tree" [3] and was for the first time described by Carl Linnaeus in 1753. *Rhododendron* L. (*Ericaceae*) is represented by about 1025 species in the world [4] mostly concentrated in the temperate regions of Northern hemisphere especially in Sino Himalayas (Eastern Himalayas and Western China). There are about 80 species of *Rhododendron* growing in India which include 10 subspecies and 14 varieties [5] which are mostly distributed in different regions and altitudes in the Himalayas between 1500 – 5500 m asl.

During the present study, *Rhododendron anthopogon* D.Don was selected and *in vitro* strategies were utilized as a tool for its propagation. The *in vitro* raised plantlets were acclimatized under greenhouse conditions. Also, mycorrhizal status of *Rhododendron anthopogon* was examined under microscope and thereafter mycorrhizal inoculum was prepared. Lastly, micropropagated plantlets were acclimatized under greenhouse conditions using mycorrhizal inoculum.

Rhododendron anthopogon D.Don

Common Names: Hindi: Atarasu, Tazaktsum, Taalis-fer (Kashmiri).

Botanical description: This is most likely one of the smallest of *Rhododendrons*. It grows 2-3 ft in height. The flowers are white or yellow, tinged with pink which grows in small compact clusters of 4-6 and each flower is 2 cm across. The leaves are dark green and oval in shape which are strongly aromatic and densely scaly underneath. The seedlings

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being minute, having scanty endosperm which favour association with mycorrhiza to germinate as in the case of orchids (Plate 1).

A) Habitat **B)** Flowering Stage

Plate 1 *Rhododendron anthopogon*

Range of distribution

R. anthopogon is distributed in the Himalayan range across Pakistan, India, Nepal, Bhutan and SE Tibet at an altitudinal range of 3000-4800 m asl. In India, it has been reported from Jammu & Kashmir, Himachal Pradesh, Uttar Pradesh, Sikkim and Arunachal Pradesh.

Flowering and fruiting: March-May

Medicinal uses: In Nepal *R. anthopogon* is used in making an essential oil (Anthopogon oil) which is obtained by steam distillation of the aerial part of this shrub. This essential oil is used in gouty rheumatic conditions, as it is a stimulant and effects fibrous tissue, bones and nervous system.

MATERIALS AND METHODS

Alpine habitats of Kashmir Himalayan region at an altitude of above (1600-4000) m asl were surveyed for distribution of *Rhododendron anthopogon*. These habitats included Sinthan pass, kokernag (Anantnag); Afarwat (Gulmarg); Mahadev (Dhara, Sinagar). *Rhododendron anthopogon* was found growing in the Afarwat (Gulmarg) and in Sinthan pass region of Kashmir Himalaya. The alpine Kashmir Himalayan region where from collections made for further studies was Sinthan pass, Kokernag district Anantnag (Plate 2).

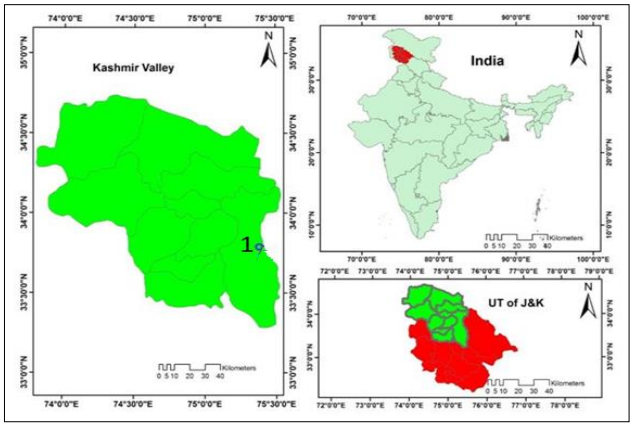


Plate 2 Map showing collection sites of (1) *Rhododendron anthopogon* D.Don from Sinthan pass, Kokernag (Anantnag) of Kashmir Himalaya

The collection was made in the form of seeds. Seeds were stored in poly bags at room temperature. The seeds of *Rhododendron anthopogon* are small in size and were kept first in falcon tubes with their mouth covered with muslin cloth. Seed washing with detergent solution "Labolene" 2% and with 2-3 drops of surfactant 'Tween-20' was carried out using 5ml syringe. The seeds were washed with tap water to remove the detergent and finally washed 2-3 times with double distilled water under laminar air flow hood and lastly surface sterilized by dipping in 2% aqueous solution of sodium hypochlorite for 10 minutes followed by washing 2-3 times with double distilled water. Woody Plant Medium [6] gelled with 1% agar and supplemented with different concentrations of auxins and cytokinins both individually and in combination were used. By adding few drops of 1N NaOH or 1N HCl, pH of the medium was adjusted to 5.8 and then autoclaved for 15-20 minutes at 121°C by maintaining 15lbs of pressure. The medium was finally allowed to cool and kept under aseptic conditions to check for any contamination until use. Auxins like IAA, IBA, NAA and 2,4-D and cytokinins like BAP,2ip, Kn and TDZ were used in concentration range of 0-5mg/l. Seeds were transferred into culture vials or conical flasks with the help of a sterilized brush. The culture vials or flasks were incubated under controlled conditions in the culture room. The culture racks are provided with cool fluorescent tube lights to provide 16 hours photoperiod of 3500 lux. The cultures were examined daily and the data was recorded on the various parameters. Statistical analysis of the data, collected on different parameters, was performed to determine the degree of authenticity of results in terms of mean and standard error using M.S excel 2010.

Acclimatization

The *in vitro* raised plantlets were gently pulled out of the culture vials, with a blunt forcep under laminar air flow hood. The basal portion of the plantlets was thoroughly washed with double distilled water, in order to remove the adhering medium and then transferred to plastic pots containing garden soil. The pots also contained cocopeat and perlite in 1:1, 1:2 and 2:1 ratios. Plantlets were maintained in high humidity conditions (90 – 100%). Survival percent frequencies of *in vitro* regenerated plantlets was also recorded.

Studies on Mycorrhizal status

Roots of *Rhododendron anthopogon* were collected randomly from the rhizosphere of the plants from their natural habitat. There are dimorphic roots which include both frame work as well as hair roots. Fresh or preserved dimorphic root from the study site were washed thoroughly with distilled water and then separated into frame work and hair roots. Staining was performed using an acidic glycerol solution (500 ml glycerol, 450ml H_2O, 50 ml of 1% HCI) containing 0.05% trypan blue. Roots were put in this solution and heated upto 90°C on a water bath for 15-30 minutes. Transverse section of frame work root samples were taken and observed under compound microscope, for the presence of mycorrhizal growth. Hair root samples were also cut into approximately 1cm long pieces and mounted on glass slides in polyvinyl alcohol lacto-glycerol mixture (PVLG) for the preparation of semi-permanent mounts [7]. The slides were examined under compound Microscope. Observations were recorded and the following scorings were made: no fungal material, vesicles, endophytes only. Percent of mycorrhizae colonization was estimated using the following formula:

$$\% \text{ Root colonization} = \frac{\text{No. of positive segments}}{\text{No. of segments observed}} \times 100$$

Preparation of mycorrhizal inoculum

Crude mixed consortium of rhizosphere soil of *Rhododendron anthopogon* plants collected from alpine region was multiplied using *Zea mays* as the trap plant. Maize seeds were surface sterilized by immersing in 70% ethanol for 1 minute followed by immersing in 6% NaOCl for 5 minutes and finally rinsing with sterile distilled water for 7-10 times. These surface sterilized maize seeds were sown in earthen pots containing sterilized soil, sand and mixed consortium of rhizosphere soils (1:1:1) and allowed to germinate for 10 days. The germinated seedlings were transplanted to pots containing mixed consortium of rhizosphere soil of *Rhododendron anthopogon* and regularly watered. These plants were allowed to grow for 90 days and thereafter harvested. Ten days before the harvest, the trap plants (maize seedlings) were cut down at the base of their stem and watering was stopped. This killed the plant and triggered the production of reproductive spores of mycorrhizal fungi. Thereafter, the roots of the trap plants were pulled out, chopped roughly into 1-2cm pieces. For preparing the mycorrhizal inoculum, chopped roots were mixed back into the rhizosphere soil present in pots in which maize seedlings were allowed to grow earlier.

Acclimatization of micro-propagated plantlets using mycorrhizal inoculum

In an attempt to study the role of mycorrhizal fungi for the acclimatization of micro propagated plantlets, the *in vitro* raised plantlets were thoroughly washed in order to remove the adhering medium and were transferred to pots containing inoculum and autoclaved sterile soil in 2:1 ratio. Pots were kept in the greenhouse conditions. The plantlets were allowed to grow and no pesticide or fertilizer was added during the course of the experiment. Percentage survival as well as vegetative growth response were monitored regularly.

RESULTS AND DISCUSSION

Callus production from seed explants

Seeds of *Rhododendron anthopogon* were inoculated on basal Woody Plant Medium (WPM) and on WPM supplemented with various auxins and cytokinins (1mg/l to 5mg/l) in different combinations and concentrations. Out of various combinations and concentrations of growth regulators used, callus production was observed on Woody Plant Medium supplemented with 2,4-D (1.0,1.5 and 2mg/l) within 35,48 and 50 days respectively with a percent response of 70%, 60% and 40% respectively as the results are depicted in (Plate 3A-C, Table 1). The callus formed was friable and light brown in colour.

Table 1 *Rhododendron anthopogon*: Effect of different hormone concentrations on callus production

Medium used	Callus formation	Callus characteristics	Formation of shoots	Number of days taken	% culture response
WPM + 2,4-D (1mg/l)	+	Friable and light brown in colour	-	35	70
WPM + 2,4-D (1.5mg/l)	+	Friable and light brown in colour	-	48	60
WPM + 2,4-D (2mg/l)	+	Friable and light brown in colour	-	50	40
WPM + 2,4-D (2.5mg/l)	-	-	-	-	-

10 replicates per treatment

A) Callus production on WPM +2, 4-D (1.0 mg/l) **B)** Callus production on WPM +2, 4-D (1.5 mg/l) **C)** Callus production on WPM +2, 4-D (2mg/l)

Plate 3 *Rhododendron anthopogon*: *In vitro* studies

Shoot regeneration

Callus obtained from seeds was sub-cultured on WPM supplemented with different cytokinins in different concentrations (1mg/l to5mg/l) and combinations. Among different cytokinin-cytokinin combinations used, 2ip (3mg/l) + TDZ (4mg/l) gave best results with the production of maximum mean number (4.6 ± 0.3) of shoots with maximum mean height (1.8 ± 0.13cm) in a mean time period of 9.66 days (Table 2). The percent culture response was also maximum in this case (80%). A drop in the growth response was observed when the medium was fortified with 2ip (3mg/l) + TDZ (5 mg/l) leading to decrease in the mean shoot number and height to (2.3 ± 0.3) and (1.3 ± 0.06cm) respectively (Plate 4, Fig 1). However, shoot regeneration did not occurred when seed callus was sub-cultured on WPM supplemented with different auxins and cytokinins either individually or in combinations.

Table 2 *R. anthopogon*: Effect of cytokinin-cytokinin combinations on shoot regeneration from seed derived callus

Medium used	Mean number of shoots ± SE	Mean height of shoots (cm) ± SE	Mean number of days taken	% culture response
WPM basal	-	-	-	-
WPM + 2ip (3mg/l) + TDZ (1mg/l)	-	-	-	-
WPM + 2ip (3mg/l) + TDZ (2mg/l)	-	-	-	-
WPM + 2ip (3mg/l) + TDZ (3mg/l)	2.6±0.3	1.5±0.12	11.33	70
WPM + 2ip (3mg/l) + TDZ (4mg/l)	4.6±0.3	1.8±0.13	9.66	80
WPM + 2ip (3mg/l) + TDZ (5mg/l)	2.3±0.3	1.3±0.06	14	60

Rooting

Shoots regenerated from seed callus were transferred to both full strength Woody Plant Medium (WPM) as well as half Strength WPM containing various auxins at different concentrations and combinations so as to induce root development in the shoots. Root regeneration occurred on full strength Woody Plant Medium (WPM) containing Indole acetic acid (IAA) only.

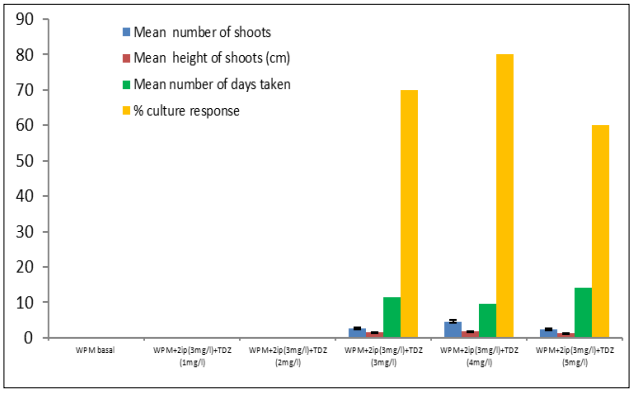
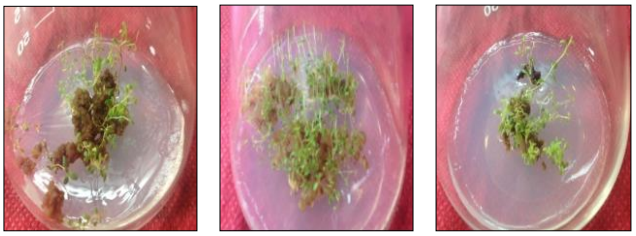


Fig 1 *R. anthopogon*: Effect of 2ip and TDZ combinations on shoot regeneration from seed derived callus



A) 2ip (3mg/l) + TDZ (3mg/l) **B)** 2ip (3mg/l) + TDZ (4mg/l) **C)** 2ip (3mg/l) + TDZ (5mg/l)

Plate 4 *R. anthopogon*: Shoot regeneration from callus on WPM containing

Effect of IAA on rooting of regenerated shoots

In order to find out the optimum concentration of IAA for inducing rooting, regenerated shoots were transferred to WPM containing IAA in concentration range of 1mg/l to 5mg/l. Initially there was no root regeneration on WPM + IAA (1mg/l) and WPM + IAA (2mg/l). Shoots transferred to WPM + IAA (3mg/l) developed a mean number of (4.0 ± 0.5) roots with a mean length of (1.2 ± 0.1cm) in 100% cultures within a mean number of (10.6) days. Increase in the concentration of IAA to 4mg/l resulted in decrease in the mean root number to 3.0 ± 0.5. (Table 3) depicts that further increase in concentration of IAA decreased the overall culture response (Plate 5, Fig 2).

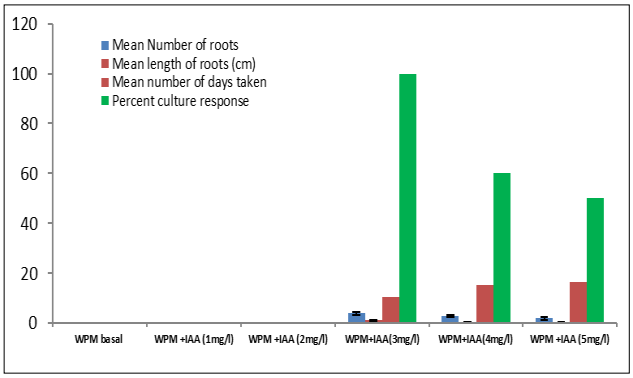


Fig 2 *R. anthopogon*: Effect of IAA on rooting of regenerated shoot

Table 3 *R. anthopogon*: Effect of IAA on rooting of regenerated shoots on Woody Plant Medium

Medium used	Mean Number of roots ± S.E	Mean length of roots (cm) ± S.E	Mean number of days taken	Percent culture response
WPM basal	-	-	-	-
WPM + IAA (1mg/l)	-	-	-	-
WPM + IAA (2mg/l)	-	-	-	-
WPM + IAA (3mg/l)	4.0±0.5	1.2±0.1	10.6	100
WPM + IAA (4mg/l)	3.0±0.5	0.5±0.03	15.3	60
WPM + IAA (5mg/l)	2±0.5	0.45±0.03	16.6	50

10 replicates per treatment

A) IAA (3mg/l) **B)** IAA (4mg/l) **C)** IAA (5mg/l)

Plate 5 *R. anthopogon*: Root regeneration from shoots on WPM containing

Acclimatization

For acclimatization 30 days old plantlets with well-developed shoots and roots were taken out of the culture vials, the medium adhering to the basal end portion of plantlets was washed off with double distilled water. After washing the rooted plantlets were transferred to small pots containing garden soil and also in pots containing coco peat and perlite in the ratio of 1:1, 1:2 and 2:1. The pots were then maintained in green house under controlled conditions of temperature (22 ± 4°C) and relative humidity (60%). The plantlets survived in pots containing garden soil and in cocopeat and perlite (1:1 ratio) with a frequency of 15 % and 10% respectively for 14 days and 8 days respectively (Plate 6, Table 4).

Table 4 *R. anthopogon*: Morphological features of *in vitro* raised plantlets in pots containing different substrates

Substratum	8 days	14 days	20 days	26 days
Garden soil	All the plantlets (40) survived and were healthy. Leaves having full turgor pressure.	6 plantlets survived out of 40. Leaves having full turgor pressure.	Leaves turned yellow. Leaves lost turgor pressure.	None of the plantlets survived.
Cocopeat and perlite (1:1)	4 plantlets survived out of 40. Leaves having full turgor pressure.	Leaves turned yellow. Leaves lost turgor pressure.	None of the plantlets survived.	-

A) In vitro raised Plantlets

B-C) Plantlets transferred to pots containing garden soil

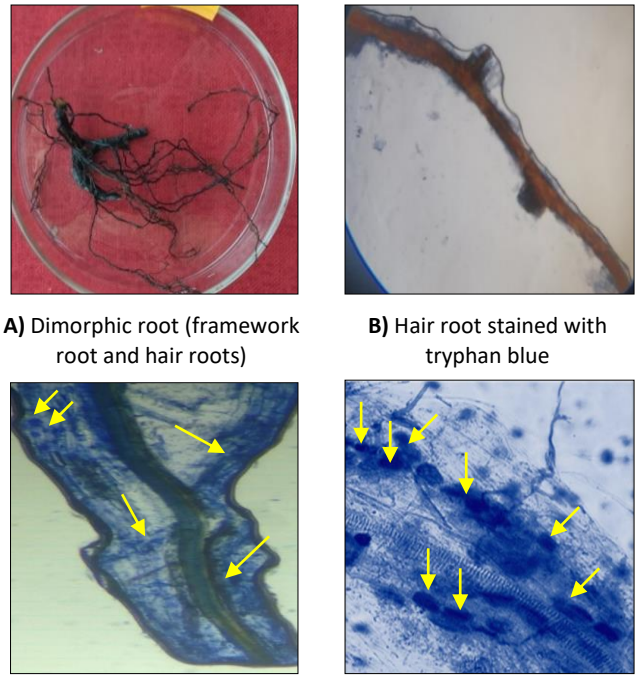
D) Plantlets in pots containing cocopeat and perlite 1:1

Plate 6 *R. anthopogon:* Acclimatization of *in vitro* raised plantlets

Table 5 *R. anthopogon*: Mycorrhizal status

Status of mycorrhizal association	Form of colonization	% colonization	
		Frame work	Hair root
+	Endophytes	40	80

+: Present



A) Dimorphic root (framework root and hair roots)

B) Hair root stained with tryphan blue

C-D) Well colonized hair root cells showing fungal endophytes

E) Transverse section of framework root

F) Cortical cells of frame work roots stained with tryphan blue showing colonized cells

Plate 7 *R. anthopogon*: Mycorrhizal status

Mycorrhizal status: R. anthopogon

Root samples of *R. anthopogon* were collected from natural habitat (Sinthan pass) of Kashmir Himalayan region during growing season (June). These roots are dimorphic i.e., frame work roots and hair roots (Plate 7A). Hair roots were stained with tryphan blue (Plate 7B). For mycorrhizal studies, 1cm long hair root segments were stained with trypan blue [9] before observation were made under compound microscope which showed presence of endophytic fungi (Plate 7C-D). To determine frame work root mycorrhizal fungi, transverse sections of roots were cut by freehand sectioning and photomicrographs were taken with a compound microscope (Plate 7E-F). The dimorphic roots exhibited the presence of mycorrhizal fungi in growing season (June) but % colonization varied in both frame work roots and hair root cells. In the present investigation, the framework roots exhibited the presence of 40% colonization and hair roots exhibited high percentage (80%) of fungal colonization (Table 5).

A) Germinated maize seeds in pots containing sterilised soil, sand and mixed consortium of rhizosphere soil (1:1:1 ratio)

B) Maize plants in earthen pots containing mixed consortium of rhizosphere soil

Plate 8 *R. anthopogon*: Preparation of mycorrhizal inoculum

Plate 9 *R. anthopogon*: Roots of the trap plants

Preparation of mycorrhizal inoculum

After ensuring the presence of mycorrhizal fungi in roots of *Rhododendron anthopogon*, the mixed consortium of rhizosphere soil was collected. Maize plants were used as trap plants for multiplying crude mixed consortium of rhizosphere soils. After following standard protocol for surface sterilization of maize seeds, these were sown in earthen pots containing sterilized soil, sand and mixed consortium of rhizosphere soil of *R. anthopogon* in ratio of 1:1:1 (Plate 8A). The seeds were allowed to germinate for 10 days. The germinated seedlings were transplanted to pots containing mixed consortium of rhizosphere soil of *R. anthopogon* (Plate 8B). After following the standard protocol for harvesting of trap plants, inoculum was prepared (Plate 9).

Acclimatization of micro-propagated plantlets using the mycorrhizal inoculum

In vitro raised plantlets of *Rhododendron anthopogon* were gently pulled out of the culture vials with a blunt forcep under laminar air flow hood. The basal portion of the plantlets was thoroughly washed off with double distilled water in order to remove the adhering medium and were then transferred to pots containing inoculum and autoclaved sterile soil in 2:1 ratio. Pots were kept in the greenhouse conditions. The plantlets were allowed to grow and no pesticide or fertilizer was added during the course of the experiment. From the present study it was depicted that the survival period of plants of *R. anthopogon* was increased upto 30 days with percent frequency of 30% (Plate 10, Table 6).

Table 6 *R. anthopogon*: Morphological features of *in vitro* raised plantlets after treatment of mycorrhizal inoculum

Treatment	10 days	20 days	30 days	40 days	50 days
Mixed mycorrhizal inoculum	All the plantlets (40) survived and were healthy. Leaves having full turgor pressure.	All the plantlets survived and were healthy. Leaves having full turgor pressure.	12 plantlets survived out of 40. Leaves having decreased turgor pressure.	Leaves start turning yellow. Leaves lost turgor pressure	None of the plantlets survived.

A) In vitro raised plantlets in pots containing inoculum and autoclaved soil (2:1 ratio) after 30 days.

Plate 10 *In vitro* raised plantlets in pots containing inoculum and autoclaved soil (2:1 ratio) after 30 days

A protocol for seed culture in which indirect shoots regeneration was achieved with an intervening callus phase was standardized. During our study, callus production was observed on Woody Plant Medium supplemented with different concentrations of 2,4-D in which 70% culture response was shown on 2,4-D at concentration of 1 mg/l within 35 days of culture. Our findings are similar to [9] in *Rhododendron ponticum* where they obtained heavy callus formation when seeds were cultured on Anderson *Rhododendron* medium supplemented with different hormones.

The effect of two cytokinins in combination was assessed on shoot regeneration from seed callus. The study depicted that out of all cytokinin – cytokinin combinations used, 2ip (3mg/l) + TDZ (4mg/l) gave best results with maximum mean number of (4.6 ± 0.3) shoots regenerating with maximum mean height (1.8 ± 0.13cm) in a time period of 9.66 days.

The findings of [10] on tissue culture of *Rhododendron indicum* in which Anderson medium supplemented with 10 mg/l 2ip + 0.2 mg/l TDZ was the best treatment for increasing shoot number are in accordance with our findings. Our results are also in accordance with those of [11] as they observed shoot multiplication on Schenk-Hildebrandt (SH) medium supplemented with 9.84 µM 2iP and 1.00 µM TDZ on leaf cultures of *Rhododendron tomentosum*. However, studies made by [12] in *Rhododendron catawbiense* and [14] in *R. maddeni* revealed that 2ip and IAA combinations results in active proliferation of shoots.

Root regeneration was observed on WPM supplemented with IAA. The results showed that WPM + IAA (3mg/l) developed 4.0 ± 0.5 number of roots with a length of 1.2 ± 0.1cm in 100% cultures. The findings of [14] Fei *et al.* (2003) in *Rhododendron hybridun*, [15] in *R. maddenii*, [16] in *R. dalhousiae*, [17] in *Rhododendron dalhousiae* var. rhabdotum, *R. elliottii* and *R. johnstoneanum* in which they found that rooting was obtained on medium supplemented with IBA. The findings of [18] in *Rhododendron catawbiense*, [19] in *Rhododendron fortune* suggested that NAA was best (auxin) hormone for regeneration of roots from shoots. However, [20] found that best hormone for rooting in R*hododendron micranthum* was IAA (0.5 mg/l) with rooting rate up to 92.5. After following the standard protocol for acclimatization of plantlets, 15% plantlets survived in pots containing garden soil and 10% plantlets in cocopeat and perlite having 1:1 ratio.

During the present study, mycorrhizal inoculum was prepared by following standard protocol for its preparation. The *in vitro* raised plantlets were then transferred to pots containing inoculum and autoclaved sterile soil in 2:1 ratio. The plantlets were allowed to grow and no pesticide or fertilizer was added during the course of the experiment. *Rhododendron anthopogon* were assessed for morphological features. It was depicted that the survival period of plantlets of *R. anthopogon* was increased upto 30 days with 30% survival frequency.

The findings of [21] are in accordance to our study. They studied effects of mycorrhizae on seed germination and seedling transplant survival rate of *Rhododendron annae*. They found that seed germination of inoculation treatments was earlier than non-inoculation treatment and germination potential, germination rate, seedling and their transplant survival rate were increased by inoculation. From this study it was revealed that mycorrhizae played a significant role in seed germination and post survival of transplant of *Rhododendron annae*.

Our study is supported by [22] who studied ability of the Rf32 strain (*Cryptosporiopsis*, Helotiales) to form ericoid mycorrhiza symbioses with *Rhododendron* species. The study showed that *Cryptosporiopsis* sp. Rf32-inoculated *Rhododendron* seedlings grew vigorously and also hyphal complex was found in cortical cells of root associations. Our findings are also in accordance to [23] who studied impact of inoculation with mycorrhizal fungi *in vitro* on growth and resistant enzymes of *Rhododendron moulmainense*. The findings revealed that ericoid mycorrhizal fungi could form mycorrhizal symbionts with *Rhododendron moulmainense* and that different fungi had different impact on growth of host plants.

CONCLUSION

Rhododendron anthopogon D.Don is ornamental as well as medicinally important plants growing in the alpine region of Kashmir Himalaya at an altitudinal range of 3700 m -3837m asl. Woody Plant Medium (low salt medium) proved effective for developing *in vitro* propagation protocols in *Rhododendron anthopogon*. Seed explants in *R. anthopogon* produced callus on WPM supplemented with 2,4-D (1.0,1.5 and 2mg/l) with maximum callus production on 2,4-D (1mg/l) within 35 days with a percent response of 70%. Shoot regeneration from callus of seed explants occurred on WPM supplemented with cytokinin –cytokinin combinations; 2ip (3mg/l) + TDZ (4mg/l) was the optimum concentration for maximum shoot regeneration. The present study, revealed that the callus obtained from seed explants has a differentiation potential in *Rhododendron anthopogon.* Root regeneration occurred on full strength WPM containing IAA; the best concentration for root regeneration is 3mg/l, with maximum mean number (4.0 ± 0.5) of roots having maximum mean root length (1.2 ± 0.1cm) with 100% culture response. *In vitro* raised plantlets survived for 14 days in pots containing garden soil and in pots containing peat and perlite in ratio of 1:1 for 8 days with 15% and 10% frequency rate respectively. The plants have dimorphic roots (frame work and root hairs) which exhibited mycorrhizal fungal association but percent colonization varies in both frame work and hair root cells during growth season. The present study revealed that fungal association plays important role for survival of plantlets under greenhouse conditions by using mycorrhizal inoculum. The survival period of *in vitro* raised plantlets increases from 14 days to 30 days.

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