

EX-SITU CONSERVATION OF *HAPLOPHYLLUM GILESII*: A CRITICALLY ENDANGERED AND NARROW ENDEMIC SPECIES OF THE KARAKORAM RANGE

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Abstract

The present study aimed to develop effective *In vitro* methods by means of callus and axillary buds for *ex-situ* conservation and rapid micro-propagation of a critically endangered, narrow endemic species *Haplophyllum gilesii* (Hemsl.) C.C. Townsend, present in Pakistan confined to Gilgit Baltistan region. Media used was MS basal with various combinations of growth regulators (kinetin, GA₃, BAP, IBA and NAA) for shoot multiplication, callus induction, regeneration and root induction. Supplementation of 2.5mgL⁻¹ BAP for 35 days of culture subsequently resulted in maximum number (6.8) of shoots. Healthy roots were induced effectively with addition of GA₃ (1.5mgL⁻¹) and (0.4mgL⁻¹) kinetin in half strength MS media. 2.5mgL⁻¹ NAA, 0.25mgL⁻¹ kinetin and 2.5mgL⁻¹ BAP exhibited effective initiation and proliferation of callus while best regeneration was obtained at the medium having 1mgL⁻¹ IBA. Acclimatized plantlets exhibited 100% survival rate for one week, after one month 60%, while survival rate after 5 months in pots was 45%. The protocol developed here can be used effectively for *in-situ* and *ex-situ* conservation as well as mass multiplication for production of *H. gilesii* plantlets and callus to assess its advantages without disturbing wild populations of this plant. There is still need to investigate secondary metabolic and molecular composition of this plant.

Key words: *Ex-situ* conservation, Endemic, Growth regulators, *Haplophyllum gilesii*.

Introduction

Endemic taxa occupy areas with specific ecological and physiographic factors in biogeographically confined region. Some plants are narrow endemics, which are more restricted and present as only few populations (Kruckeberg & Rabinowitz, 1985). Small and few populations, narrow geographical range, special niche demands complemented with anthropogenic activities making these endemic taxa more prone to the risk of extinction (Myers, 1988; Heywood & Watson, 1995).

The genus *Haplophyllum* A. Juss. having 70 species; with a majority restricted to narrow ranges often as small as a single mountain e.g., a narrow endemic *Haplophyllum telephioides* growing in few mountainous areas of central Anatolia; *Haplophyllum viridulum* present in Fars province of Iran (Townsend, 1986); *Haplophyllum gilesii* (Hemsl.) C. C. Townsend a narrow endemic is restricted to Gilgit Baltistan region, Pakistan (Alam & Ali, 2010).

Haplophyllum gilesii (Hemsl.) C.C. Townsend belongs to family Rutaceae a shrub branching vigorously with height reaching up to 3 feet. It grows in dry habitats with patchy populations confined to only three localities of Karakoram-Himalayan range of Pakistan i.e. Chupo Das, Juglot and along Karakoram highway at Astore (Alam, 2009; Alam & Ali, 2010). Fragmented populations of *H. gilesii* at these localities are threatened by various anthropogenic activities. Soil erosion is common problem because of dry habitat posing serious effects during rainy seasons. Construction and renovation of Karakoram highway coupled with careless utilization of the habitat by inhabitants has made the species Critically Endangered (CR) thus this taxon needs proper consideration and attention to minimize extinction threats (Alam & Ali, 2010).

Throughout the world, there are examples where strategies are developed, both *in-situ* and *ex-situ* conservation to protect and retrieve endangered taxa. *In-situ* conservation includes conservation of biodiversity in its natural habitat by establishment of protected areas, national parks and sanctuaries i.e., assigning land for maintenance and protection of habitat, associated natural resources and biodiversity. In *in-situ* conservation of whole ecosystem or community is the main focus of conservation. However, a species with evolutionary, ecological, economic or medicinal significance also deserve attention. *In-situ* conservation involves lot of human and financial inputs that are usually unavailable in most of the developing countries. Moreover, several factors such as access to remote areas, fewer human and economic resources, unawareness of local population and global climatic changes render *in-situ* conservation practices alone ineffective (Heywood & Dulloo, 2005).

On the other hand, *ex-situ* conservation involves establishment of gene banks, nurseries and botanical gardens where cultivation and protection of endangered taxa is carried out. However, development management and effectiveness of conservation in gene banks, botanical gardens and nurseries involves better understanding training and expertise of plant sciences (Blackmore *et al.*, 2011; Brutting *et al.*, 2013).

In-situ conservation alone had remained unsuccessful in case of many endangered species because of the failure of reproduction and propagation without effective assistance. For this *ex-situ* conservation practices are of prime importance for afterward reintroduction of an endangered species back in its habitat.

An important and effective biotechnological tool for both *in-situ* as well as *ex-situ* conservation is micro-propagation or plant tissue culture. This technique is superior to all *in-situ* and *ex-situ*

conservation techniques due to its robustness to produce a large number of diseased free plantlets in minimum time using only a minute amount of plant material. Direct or indirect organogenesis of endangered, endemic and rare plant species via tissue culture techniques has been used fruitfully to cope with the extinction problem without damaging wild populations (Bonness *et al.*, 1993; Carneiro *et al.*, 1999). These *In vitro* propagated plants are then cultivated in natural habitat for *in-situ* or in nurseries and botanical gardens for *ex-situ* conservation. Many endangered or endemic plants have been produced through tissue culture for conservation, few of them are *Vriesea reitzii* (Filho *et al.*, 2005), *Saussurea involucrata* (Guo *et al.*, 2006), *Ceropegia attenuata* (Chavan *et al.*, 2011), *Caralluma edulis* (Patel *et al.*, 2014) *Hypericum gaitii* (Swain *et al.*, 2015). Henceforth, plant tissue culture enhances the competence of both *ex-situ* and *in-situ* conservation approaches (Krogstrup *et al.*, 1992; Fay, 1992, 1994).

This study was aimed at conservation of narrow endemic and critically endangered plant (*Haplophyllum gilesii*) with objectives of (i) optimization of media for seeds germination and nodal culture and (ii) development of protocol for proliferation of callus and plantlets regeneration from callus.

Materials and Methods

Experiment was carried out at Plant Tissue Culture Laboratory, Department of Botany, Hazara University, Mansehra, Pakistan.

Plant material and sterilization: Plants were collected from Gilgit Baltistan region of Pakistan. Petiole, node and leaves were used as explant. Sterilization of explant was carried out by washing with tap water for 20 minutes followed by immersing the plant part for 30 minutes in 20% commercial bleach solution. Afterwards, bleach solution from explants was washed with sterile distilled water three times for five minutes each. This sterilized plant material was used for direct and indirect organogenesis.

Culture media: Murashige and Skoog (MS, 1962) basal medium solidified with 8gL⁻¹ agar- with growth regulators (NAA, IBA, BAP, Kn, GA₃) was used for multiplication of shoot, root induction, callus formation and regeneration of plantlets from callus (Table 1). pH of medium was adjusted at 5.8 and autoclaved at 15 psi at 121-122°C for 30 minutes. Cultures were incubated at 16/8 hour (light/dark) photoperiod at 27±2°C.

Media optimization: Media strength and concentration of growth regulators was optimized by setting up a pilot experiment as presented in Table 1.

Acclimatization

Acclimatization was carried out after 60 days of *In vitro* culture. Plantlets were kept in open culture tubes for 4 days. Roots were washed under running tap water to remove media. Plantlets were then moved to small containers in sterilized compost and soil mixture and kept in germ-free environment for one week before transferring to green house.

Table 1. Media utilized for shoot proliferation, callus induction, and regeneration of plantlets from callus (* represents half strength MS media).

Treatment	Concentrations of growth regulators	
1.	0.4 mgL ⁻¹ Kinetin, 1 mgL ⁻¹ GA ₃ *	Shoot proliferation
2.	0.4 mgL ⁻¹ Kinetin, 1 mgL ⁻¹ GA ₃	
3.	2.5 mgL ⁻¹ BAP	
4.	3 mgL ⁻¹ IBA	
5.	0.4 mgL ⁻¹ Kinetin, 1.5 mgL ⁻¹ GA ₃ *	Root induction
6.	0.5 mgL ⁻¹ GA ₃	
7.	0.4 mgL ⁻¹ kinetin, 1 mgL ⁻¹ GA ₃	
8.	0.25 mgL ⁻¹ 2, 4-D, 0.25 mgL ⁻¹ Kinetin	Callus production
9.	2.5 mgL ⁻¹ NAA, 0.25 mgL ⁻¹ kinetin, 2.5 mgL ⁻¹ BAP	
10.	30 gL ⁻¹ D-Sorbitol, 0.25 mgL ⁻¹ 2, 4-D, 2.5 mgL ⁻¹ BAP, 2.5 mgL ⁻¹ NAA	
11.	30 gL ⁻¹ D-Sorbitol, 1 mgL ⁻¹ NAA, 1 mgL ⁻¹ BAP	Regeneration
12.	1.5 mgL ⁻¹ Kinetin, 1 mgL ⁻¹ BAP, 0.5 mgL ⁻¹ GA ₃ , 0.25 mgL ⁻¹ NAA	
13.	1 mgL ⁻¹ IBA	

Statistical analysis

Software Statistix 8.1 was used to carry out statistical analysis (ANOVA and LSD) of data.

Results and Discussion

Many anthropogenic factors as well as natural phenomenon are responsible for global plant and animal extinction in the present time. Various conservation approaches (*in-situ* and *ex-situ*) are implemented to handle this problem of mass extinction. *In-situ* along with *ex-situ* conservation of plants are based on production of large number of plants. Through plant tissue culture technique small plant tissues for production of large number of disease free plants is possible, which is an important step toward the conservation of endangered taxa. Using plant tissue culture techniques seed dormancy and propagation difficulties are also affectively removed. For conservation and economic uses, many endemics as well as endangered plant species have been successfully propagated in the past. For *in-situ* conservation plants propagated through tissue culture are then cultivated in natural habitat while *ex-situ* is carried out through cultivation in botanical gardens (Blackmore *et al.*, 2011; Krogstrup *et al.*, 1992; Fay, 1992, 1994).

Sufficient amount of seeds of *Haplophyllum gilesii* are produced in wild but they fail to germinate possibly due to dormancy. Seedling survival rate is also very low resulting in decreased number of individuals. To the best of our knowledge, no work has been done for the propagation of *H. gilesii* and the present study is the first attempt of its kind to develop protocol for micropropagation of *H. gilesii*.

Shoot proliferation: Among tested cytokinins 2.5mgL⁻¹ BAP was dominant for shoot proliferation from explant. Buds proliferated after 6-7 days and maximum of 7 shoots with average 14 nodes per plant were produced in one month duration attaining a height of 0.874cm on average (Figs. 1-4, Table 2). However, stunted shoot growth was observed at concentrations higher than 2.5mgL⁻¹ of BAP resulting in clumpy appearance as reported by Sahoo & Chand (1998) in *Vitex negundo* L., Pattnaik & Chand

(1996) in *Ocimum americanum* and *O. sanctum* and Ahuja *et al.*, (1982) in *O. gratissimum* and *O. viride*. In few of the culture tubes, shoot tips dieback was detected but nodes under dead tip resumed normal growth. This dieback is attributed to the buildup of volatile compounds or ethylene as discovered in *Gymnema sylvestre*, *Holostemma ada-kodien* and *Rotula aquatica* (Komalavalli & Rao, 2000; Martin, 2002, 2003).

Table 2. LSD all-pairwise comparisons test for shoot length, No. of nodes and no. of shoots for shoot multiplication treatments.

Treatments	No. of shoots	No. of nodes	Shoot length
T1	2.8800 B	4.4400 C	0.2874 B
T2	2.8000 B	5.8800 BC	0.2824 B
T3	3.9600 A	8.0400 A	0.4740 A
T4	3.5600 A	5.7200 B	0.2190 B

Root induction and acclimatization: No root induction was observed on MS media deprived of growth regulators either half strength or full strength. Maximum of 9 roots per plant (Fig. 5) were produced at MS half strength medium with supplementation of 0.4mgL⁻¹ kinetin along with 1.5mgL⁻¹ GA₃ reaching to the maximum length of 2.34cm on average (Fig. 6). Media supplemented with NAA and IBA failed to induce roots in shoots. In previous studies root induction was reported by using half strength media without growth regulators or by with various concentrations of auxins either singly or in combination with low concentrations of cytokinins in *Verbascum thapsus*, *Santolina canescens*, *Rotula aquatica* and *Ruta graveolens* (Martin, 2003; Bohidar *et al.*, 2008). Contrary to these investigations *H. gilesii* showed unique response in case of rooting that might be due to idiosyncratic genotype of the explant.

Rooted shoots were acclimatized in green house and flowering was also observed in potted plants. Plantlets subjected to acclimatization showed 100% survival. However, although, 60% survived for one month and remaining 45% plants survived for five months (Fig. 7).

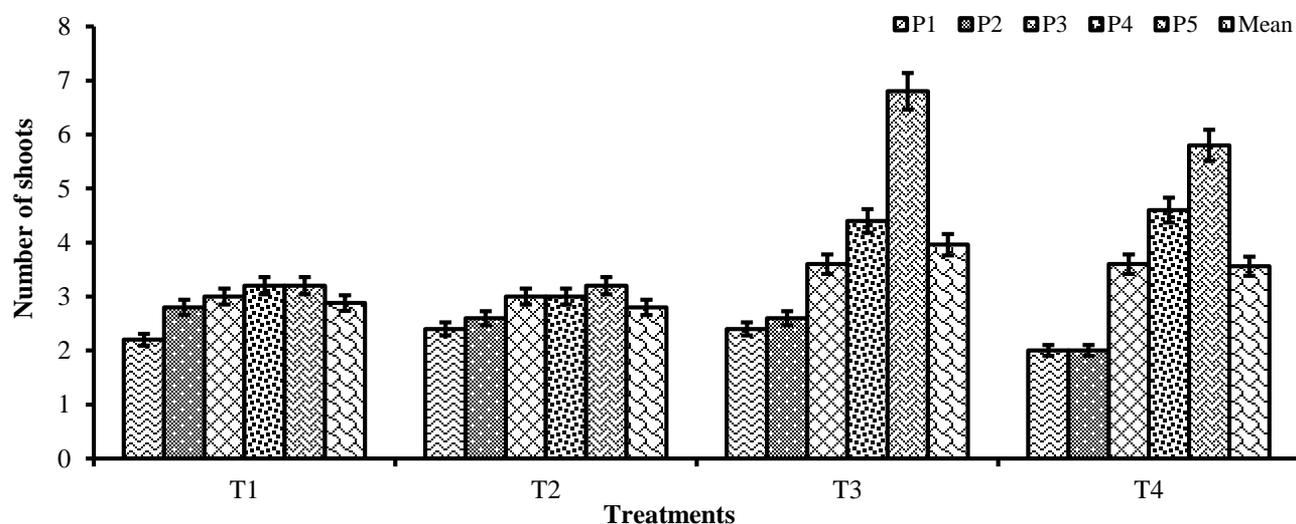


Fig. 1. Effect of different media treatments *i.e.* T1 (MS half strength supplemented with 1mgL⁻¹ GA₃ and 0.4mgL⁻¹kinetin), T2 (MS full strength with kinetin 0.4mgL⁻¹ and GA₃ 1mgL⁻¹), T3 (BAP 2.5mgL⁻¹) and T4 (IBA 3mgL⁻¹) on number of shoots plant⁻¹. The data represent mean values of 5 replicates ± SE.

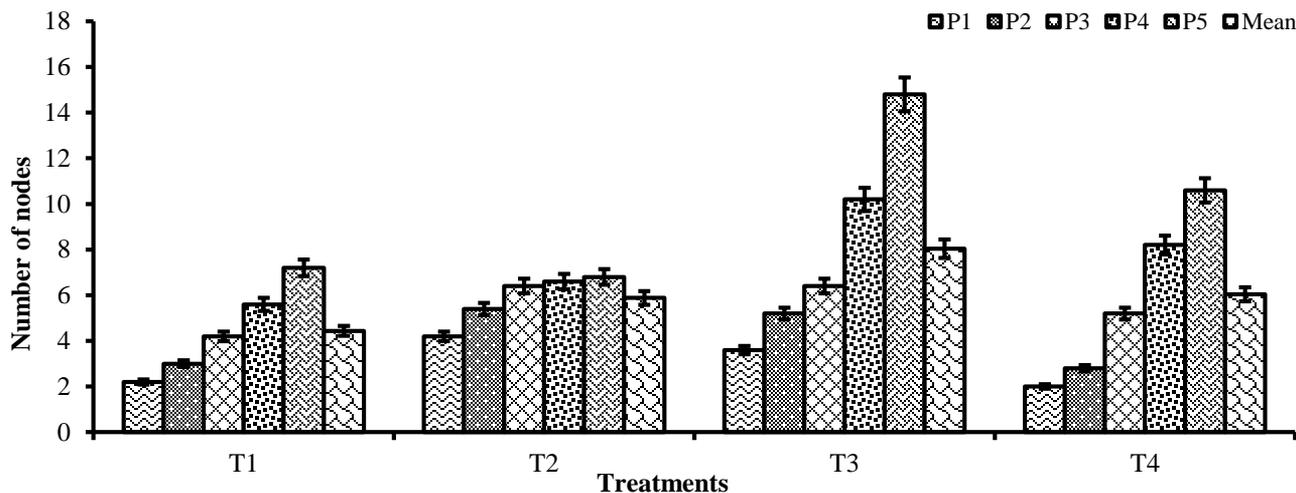


Fig. 2. Effect of different media treatments *i.e.* T1 (MS half strength with 0.4mgL⁻¹ kinetin and 1mgL⁻¹ GA₃), T2 (MS full strength with kinetin 0.4mgL⁻¹ and GA₃ 1mgL⁻¹), T3 (BAP 2.5mgL⁻¹) and T4 (IBA 3mgL⁻¹) on number of nodes plant⁻¹. The data represent mean values of 5 replicates ± SE.

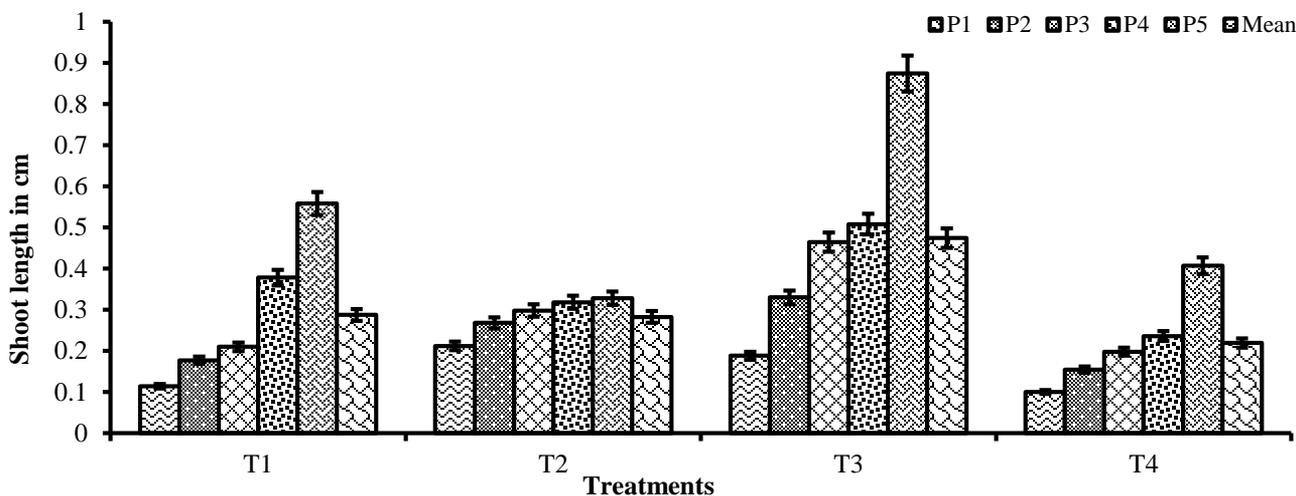


Fig. 3. Effect of different media treatments *i.e.* T1 (MS half strength with 1mgL⁻¹ GA₃ and 0.4mgL⁻¹ kinetin), T2 (MS full strength with 1mgL⁻¹ GA₃ and 0.4mgL⁻¹ kinetin), T3 (BAP 2.5mgL⁻¹) and T4 (IBA 3mgL⁻¹) on shoot length of plantlets. The data represent mean values of 5 replicates ± SE.

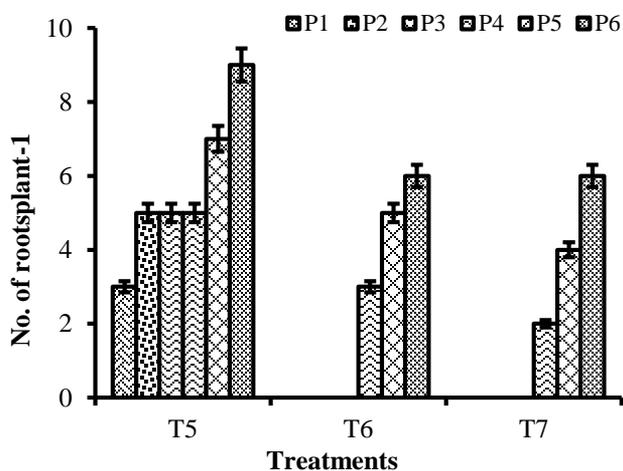


Fig. 5. Effect of different treatments *i.e.* T5 (1.5mgL⁻¹ GA₃ and kinetin 0.4mgL⁻¹), T6 (0.5mgL⁻¹ GA₃) and T7 (GA₃ 1mgL⁻¹ and kinetin 0.4mgL⁻¹) on number of roots plant⁻¹. The data represent mean values of 3 replicates ± SE.

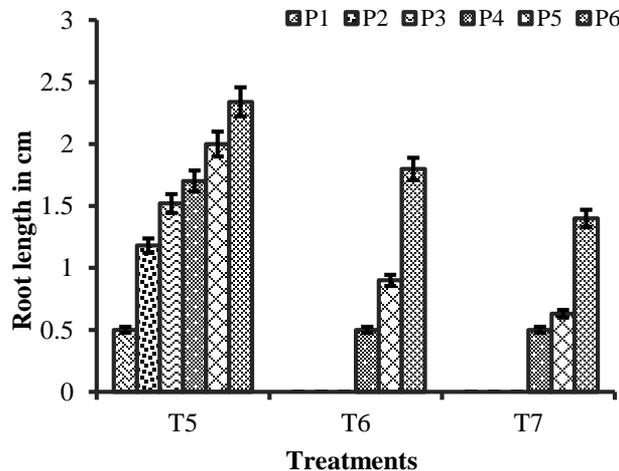


Fig. 6. Effect of different treatments *i.e.* T5 (GA₃ 1.5mgL⁻¹ and kinetin 0.4mgL⁻¹), T6 (0.5mgL⁻¹ GA₃) and T7 (GA₃ 1mgL⁻¹ and kinetin 0.4mgL⁻¹) on root length of plantlets in six passages duration. The data represent mean values of 3 replicates ± SE.

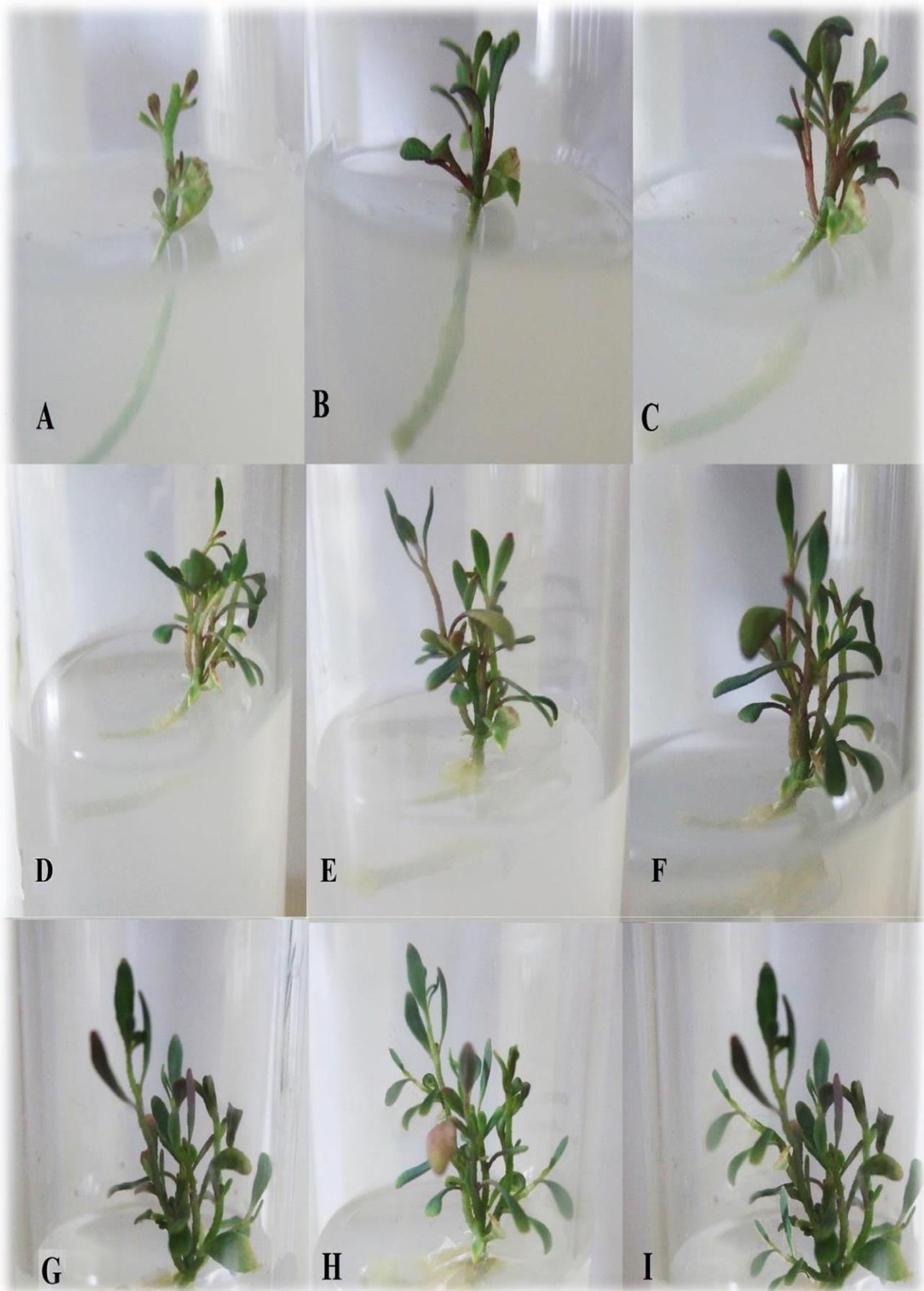


Fig. 4. Different stages of shoot multiplication. (A. Bud proliferation, B-I shoot growth during 8 passages).



Fig.7. Acclimatization of plantlets. A. after one week, B. two weeks, C. four weeks, D two months and E. five months.

Callus induction: Callus initiation was noted after average 7-10 days of inoculation of explant on media regardless of source of explant. This is the indication that all parts of plant exhibit similar degeneration response towards various growth regulators. Efficient growth of callus was recorded at media containing 0.25mgL^{-1} kinetin, 2.5mgL^{-1} BAP and 2.5mgL^{-1} NAA (Figs. 8, 9). NAA was more efficient in callus formation than 2, 4-D as reported in *Citrus reticulata* by Ill *et al.*, (1995). Cut ends of shoots explants also produced callus when cultured on media containing cytokinins. Similar observation was recorded by Saini & Jaiwal (2000) and Martin (2002) which can be the result of auxins deposition at injured ends of explant when combined with cytokinins of media results in callus production (Marks & Simpson, 1994).

Shoot regeneration: In our analysis, 1mgL^{-1} IBA exhibited best shoot regeneration response with 65% regeneration and more than 50 shoots were recorded in each culture flask after 2 months of culture (Fig. 10). BAP and NAA also initiated shoots buds but no further growth or elongation was observed in those shoots. Our results disagreed with previous studies that reported regeneration of shoots from citrus callus in the presence of kinetin and BAP (Singh *et al.*, 2011).

On the basis of results of current study, it may be concluded that supplementation of media with 2.5mgL^{-1} BAP for shoot multiplication is effective. While commencement and proliferation of callus supplementation of media with kinetin (0.25mgL^{-1}), (NAA) 2.5mgL^{-1} and BAP (2.5mgL^{-1}) is suitable. Similarly, MS half strength media supplemented with 1mgL^{-1} IBA in media is most appropriate for shoots

regeneration from callus. However, 2 mgL^{-1} GA₃ with MS half strength media was best for introduction of healthy rooting system amongst tested media.

Compelling evidences suggests that global climate changes are inevitable, and this is to alter species physiology, phenology, biotic interactions and distribution at large (Urban, 2015). Further, as the temperature rises it is likely that plant species will start altitudinal migration and many of the plant species that are evolutionarily related but distinctly placed could make fertile interspecific hybrids (Gómez *et al.*, 2015). No doubt, on one hand such creative hybrids could be useful for widening the genetic base of plant species, but for narrow endemic species, it could couple the threats of its extinction due to genetic assimilation and outbreeding depression (Hails & Morley, 2005; Soltis & Soltis, 2009). It is likely that *H. gilesii* may follow the same fate. Thus the current work is of utmost importance and it is imperative to develop efficient and non-conventional means of reproducing such narrow endemic species; so that such plant species may be reintroduced into the natural habitats that they once dominated.

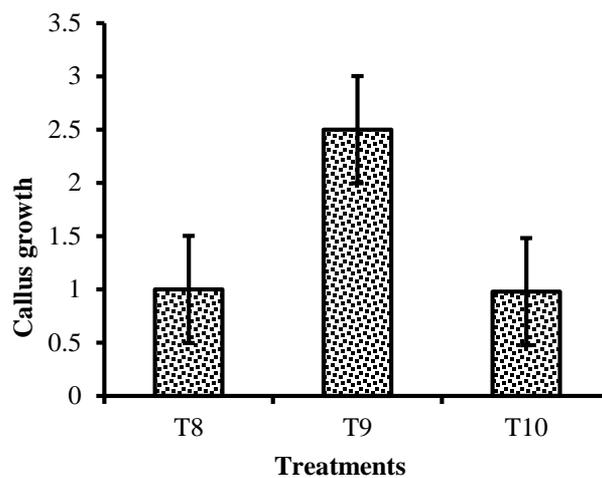


Fig. 8. Mean growth of callus at different treatments *i.e.* T8 (0.25 mgL^{-1} 2, 4-D and 0.25 mgL^{-1} kinetin), T9 (2.5 mgL^{-1} BAP, 0.25 mgL^{-1} kinetin and 2.5 mgL^{-1} NAA) and T10 (2.5 mgL^{-1} BAP, 0.25 mgL^{-1} 2, 4-D, 2.5 mgL^{-1} NAA and 30 gL^{-1} D-Sorbitol) after 14 days of culture. The data represent mean values of 3 replicates \pm SE.

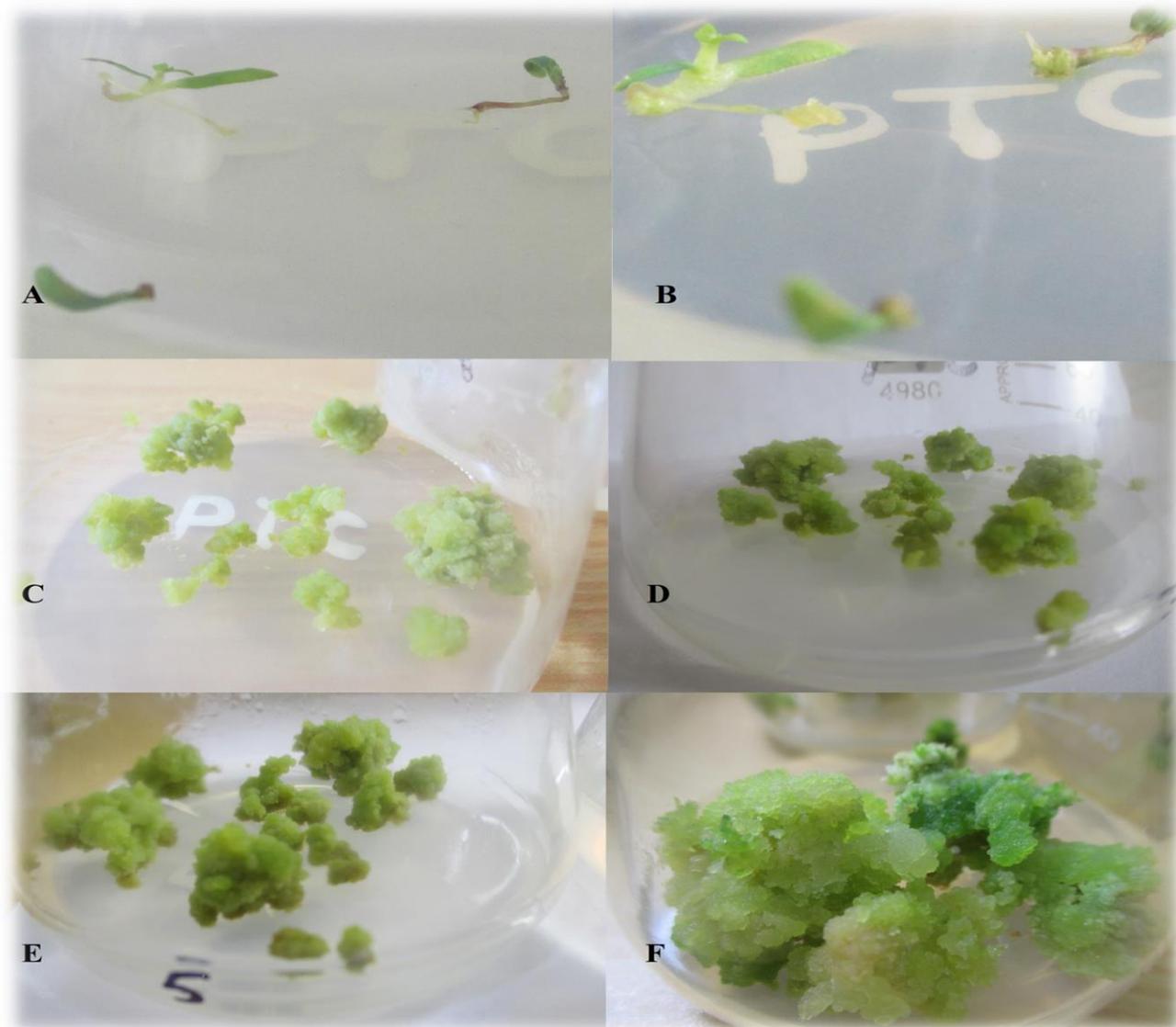


Fig. 9. Stages of initiation and growth of callus. A. freshly inoculated explants, B. callus initiation, C-F. proliferation of callus.

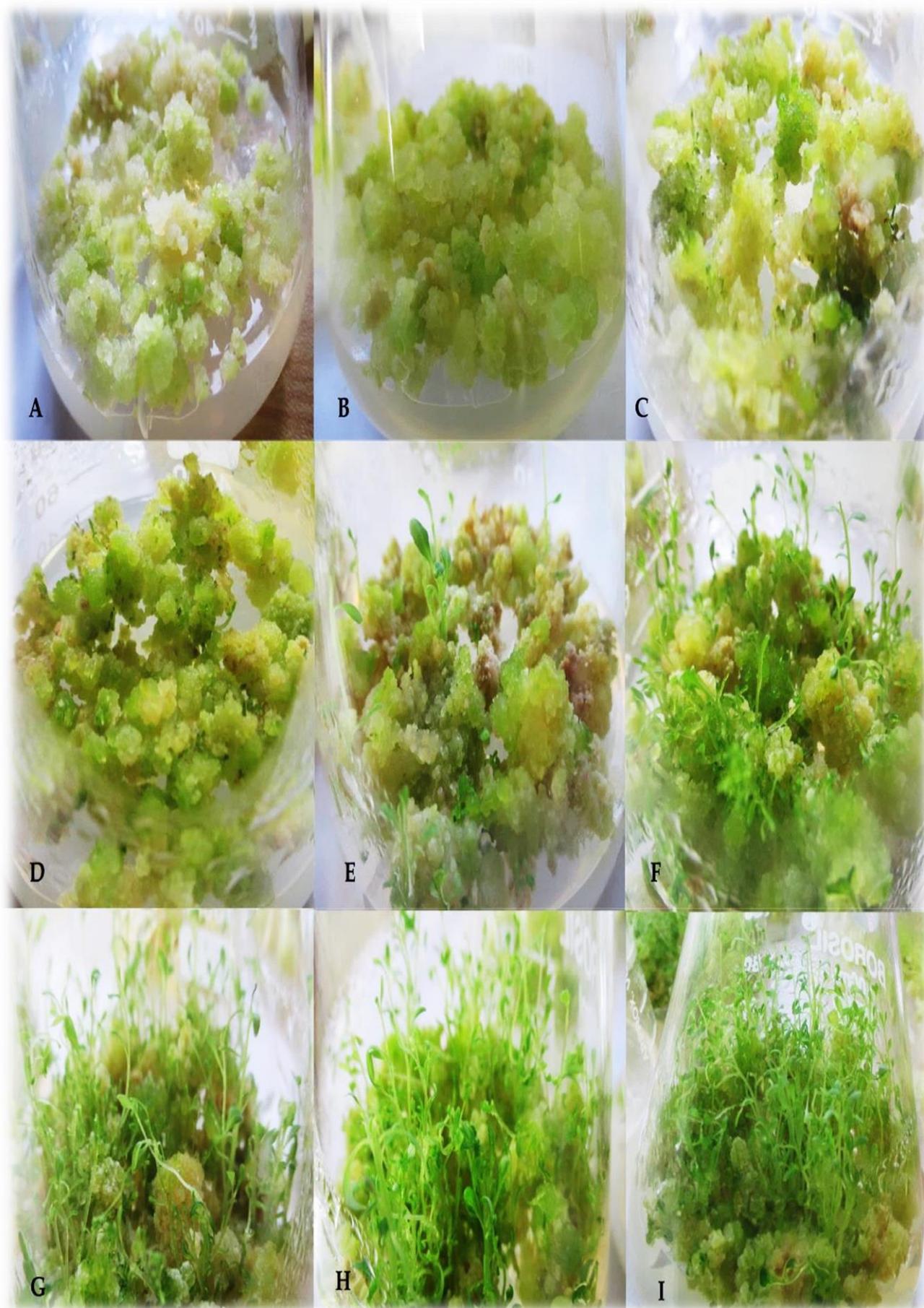


Fig. 10. Regeneration of plantlets from callus, A and B inoculated callus, C and D initiation of shoots, E-I growth and multiplication of shoots.

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