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An *in Vitro* Approach for the Conservation of *Meizotropis pellita*: An Endangered and Endemic Plant

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ABSTRACT

An efficient protocol for high frequency *in vitro* regeneration of *Meizotropis pellita* an endangered and endemic plant was developed. *In vitro* germination of the seeds of Patwa was achieved successfully after incubation for 10 - 15 days and plantlets up to a height of 5 - 7 cm with profuse rooting were observed after incubation for 7 weeks in hormone free MS medium. This *in vitro* germination totally reduced contamination that used to occur in the explant taken from the field. Callus induction and proliferation were observed in leaf explants after 15 - 20 days of incubation in MS medium containing 2 - 4, D (9.06 µM) alone or in combination with 2 - 4, D (9.06 µM) + 2-iP (7.38 µM). Shoot regeneration was achieved from 2 months old callus explant in MS medium supplemented with BA (17.6 µM) + GA3 (1.0 µM). Shoot multiplication was also achieved from cotyledonary node of *M. pellita* in MS medium supplemented with Kinetin + GA3 (4.6 µM + 1.0 µM) or BA (13.2, 17.6 µM) + GA3 (1.0 µM) after 30 - 45 days of incubation. IBA (4.9 µM) was more effective in root regeneration from micro shoots. The plantlets after acclimatization over a period of 1 month were further hardened in a polyhouse for two months. On the basis of available literature this is the first and significant study regarding the comparative effect of different PGRs on *in-vitro* propagation of *Meizotropis pellita* by using different explants and their subsequent effect on rooting. This significant study could be useful for large scale production of successfully hardened plants and conservation of this shrub.

Keywords: Patwa; Patwadanger; *Meizotropis pellita*

1. Introduction

Patwa (*Meizotropis pellita Wall. Ex Hook*, *F & Grev*.) as called in local dialect, blooms just 12 km. from Nainital in a place known as Patwadanger, Uttarakhand. Patwadanger village derives its name from this plant. It was discovered in 1925 when a British Botanical expert Osmoston reported its existence after a visit to the village. The expert's records say that Patwa existed in the Kali-Kumaun *and Dhoti district of Nepal as well. However*, *the species couldn*'*t be traced in those regions.* Patwa is an angiosperm belonging to the family Fabaceae (Papilionaceae). This plant is a shrub with stout, woody perennial rootstock from which several erect shoots up to 6 feet high and 0.75 inch diameter are annually produced. Stems are ribbed with large pith. Leaves are 18 - 30 inches long. Flowers are 0.5 - 1 inch long in fascicles of usually 3 - 5, arranged in erect terminal and axillary simple raceme. Corolla has bright red wings, keel changing to orange towards the base inside. The plant will reappear in a year from the root stock in April/May. This species occurs more gregariously on flat hill tops as well as on the valley slopes near dry rides and in open chir forest at around 5000 feet in May-June [1]. There are three major sites in Patwadanger where it is blooming in groups of some plants and at present an estimated 150 - 300 plants of *M. pellita* are left which too are in the verge of extinction. In nature it is under critical selection because of increased deforestation, habitat fragmentation, forest fires, human interference, ignorance of the people and changing climatic conditions. This species has very small population and grows in very specialized and sensitive habitats; therefore any further change and ecological disturbance are bound to cause their total extinction from this region [1].

Tissue culture is used for conservation of biological diversity of endangered species that have extremely

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small populations, for species with reproductive problems and for recovery and reintroduction [2]. Seeds of *M. pellita* are very large and recalcitrant in nature. Mostly, in nature it propagates by its root stock. So, *in vitro* regeneration of this plant was a typical task. *M. pellita*, an endemic Himalayan species needs to be protected from extinction so that the plant is given maximum opportunity to cover barren slopes to safeguard our environment. However till date there is no report of *in vitro* micropropagation of *Meizotropis pellita*. In view of above facts, the present work was aimed to develop an efficient protocol for *in vitro* micropropagation of *Meizotropis pellita* and its establishment *ex situ and in situ.*

2. Materials and Methods

2.1. Establishment of Aseptic Seedlings

The fresh, mature and dried seeds of *Meizotropis pellita* were collected from plants growing at forest of Patwadanger. The seeds are covered with leathery seed coat, for such species the scarification of seed coat and pretreatment by immersion in water at 90˚C soaked for 24 h is essential. After removal of seed coat, thorough washing of the seeds was done with running tap water for 20 min followed by immersion in fungicide (bavistin) solution for 30 min. again washing and then treated with 0.1% Tween-20 (detergent) for 5 min. The seeds were then washed with distilled water repeatedly and soaked for 24 hrs. The seeds were chemically sterilized with 70% (v/v) ethanol in laminar air flow for 1 min. and seeds were again sterilized with freshly prepared 0.1% $HgCl_2$ (w/v) for 4 min followed by repeated washing with double distilled water. The sterilized seeds were germinated under aseptic conditions in hormone free MS medium (3) and incubated in dark. After the immergence of whole plant the plant was maintained in culture room conditions.

2.2. Culture Medium and Conditions

The nutrient medium consisted of MS Salts, vitamins supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar was used in all experiments. The pH of the medium was adjusted to 5.8 by 1N NaOH and 1N HCl. The culture vials containing the media were autoclaved at 121˚C at 106 $kg\cdot cm^{-1}$ for 20 min. All the cultures (except seeds) were maintained at 25˚C ± 2˚C and 60˚C ± 5˚C relative humidity in the culture room conditions under 16 h photoperiod with photosynthetic photon flux density of 40 μ $mol\cdot m^{-2}\cdot s^{-1}$ fluorescent lamps.

2.3. Callus Induction

The nodes, juvenile leaves and stem sections of the plant germinated aseptically from seeds were used as explant. Explants were inoculated for callus induction in MS medium supplemented with growth regulators auxin (2, 4-D) either alone or in combination with cytokinins (2-iP) at different concentrations. No prior treatments to explants were given because the seeds were germinated *in vitro*.

2.4. Shoot Regeneration

The callus/cotyledonary nodes from *in vitro* germinated seeds were used as explant and transferred to shoot regeneration medium. Treatments were avoided because the seeds were germinated aseptically. MS medium supplemented with different growth regulators like BA and Kn either alone or in combination with GA3 were used in different combinations with other growth regulators as well, as they have been reported to be potent shoot inducers. The best combinations in MS medium were used for shoot multiplication.

2.5. Root Induction

The shoots (3.0 - 7.0 cm in length) were transferred to MS medium supplemented with IBA (2.45, 4.9, 9.8 μM) and NAA (2.69, 5.38, 10.76 μM) for root induction.

2.6. Acclimatization of Plantlets to Soil

Shoots with well-developed roots were taken out from the flasks and gently washed under running tap water to remove traces of medium. The plantlets after recording of data of rooting response were then transferred to small pots containing soil (collected from natural habitat of plant), sand and farmyard manure (3:1:1 ratio). Potted plants were placed inside culture room under 16-h photoperiod (60 $\mu mol\cdot m^{-2}\cdot s^{-1}$) at 25˚C ± 2˚C and 60% relative humidity. Plants were watered on alternate days and gradually acclimatized over a period of 1 month. The plants were then transferred to pots containing garden soil and kept for further hardening in a polyhouse for two months. Well hardened plants were then transferred to garden for further growth.

2.7. Statistical Analysis

The experiments were repeated thrice. Data were analyzed statistically by ANOVA using Microsoft Office Excel-2007, with the level of significance set at 5%. The results are expressed as the means ± standard error of three experiments.

3. Observations and Results

3.1. *In Vitro* Germination of Seeds

In vitro germination of seeds of Patwa was obtained successfully after incubation for 15 - 20 days and plantlets

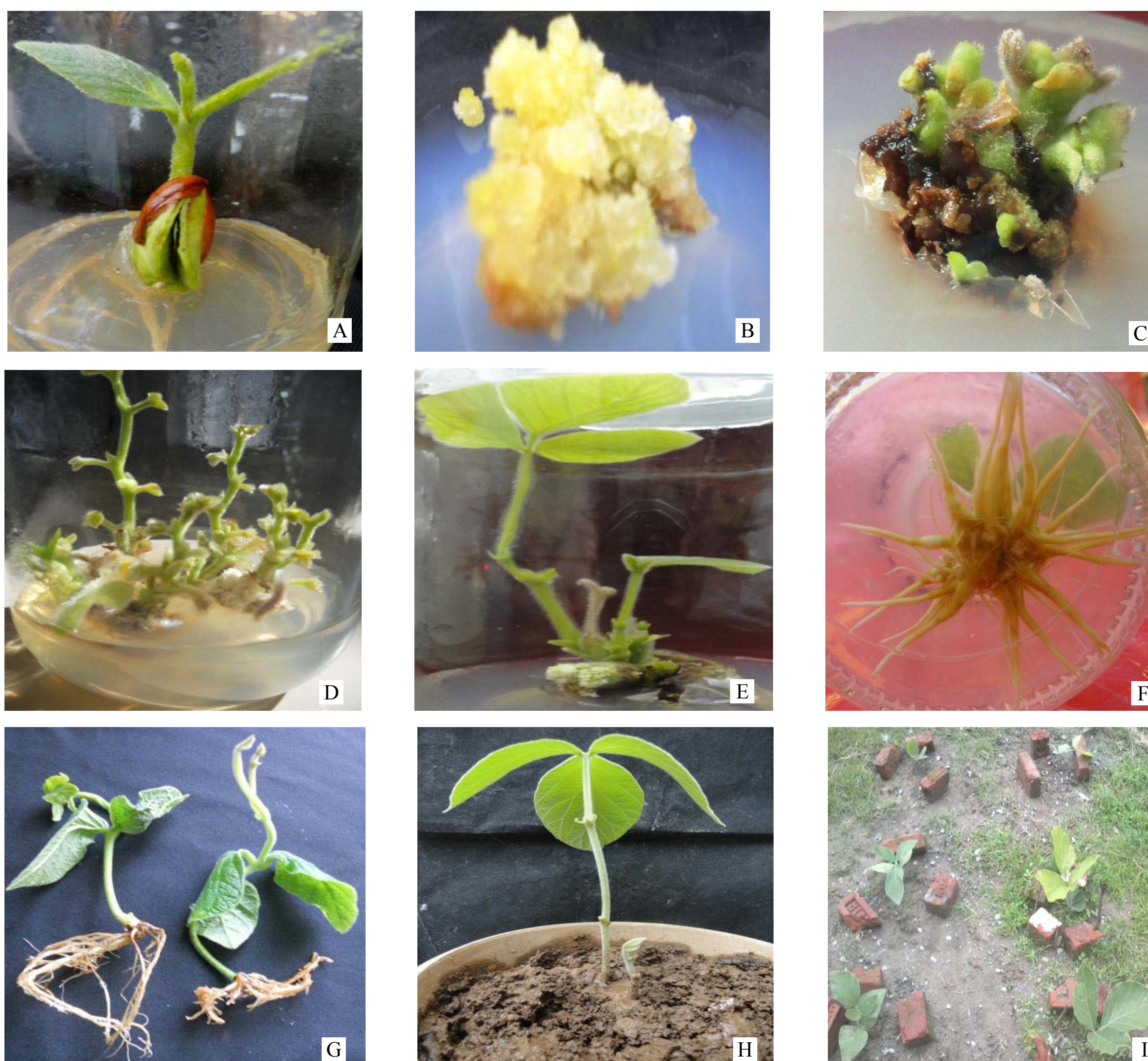
up to height of 5 - 7 cm. with profuse rooting were observed after incubation for 7 weeks in hormone free MS medium [**Photoplate 1(A)**]. This *in vitro* germination totally reduced contamination that used to occur in the explant taken from the field.

3.2. Effect of PGRs Oncallus Induction

Callus induction was successfully observed in the leaf/shoot/root explants taken from *invitro* germinated seedlings of Patwa. The callus was subcultured at regular intervals of 30 days into same medium and pH of medium was maintained at 5.8. The lower hormone concentration in media didn't have any effect on callus induction but as the concentration increases the induction and growth was better but further increase in PGR concentration the growth ceases. Best callus induction and proliferation was observed after 15 - 20 days of incubation in MS medium containing 2 - 4, D (9.06 μM) alone or in combination with 2, 4- D (9.06 μM) + 2-iP (7.38 μM) [**Photoplate 1(B)**]. The leaf explant was most effective in callus induction as compared to stem and roots.

3.3. Effect of Explants and PGRs on *in Vitro* Shoot Regeneration

The result showed that callus showed increase in size and growth when transferred to MS medium containing only cytokinins. Shoot induction was observed when callus was transferred to MS medium containing BA in combination with GA3. Best Shooting response was achieved from 2 months old callus after 35 days of incubation in MS medium supplemented with BA (13.2 μM) + GA3 (1.0 μM) [**Photoplates 1(C)** and **(D)**].



Photoplate 1. (A) *In vitro* seeds germination of *M. pellita*; (B) Induction of callus; (C) Shoot regeneration through callus; (D) Shoot multiplication; (E) Shoot multiplication though cotyledonary node; (F) Root induction after 45 days; (G) Well rooted *in vitro* regenerated plant; (H) 6 month old well acclimatized plant inside poly house; (I) Field transfer of *in vitro* raised plants.

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Shoot multiplication was also achieved from cotyledonary node of *M. pellita* in MS medium supplemented with Kinetin + GA3 (4.6 μM + 1.0 μM) or BA (13.2, 17.6 μM) + GA3 (1.0 μM) after 30 - 45 days of incubation **[Photoplate 1(E)]**. While no shoot induction was observed when Kn and BA alone were used in MS medium. The explants were subcultured at regular intervals of 18 - 25 days into same medium and pH of media was maintained at 5.8. The result of mean value showed that among all the parameters studied number of shoot (2.66) was maximum at BA (17.6 μM) + GA3 (1.0 μM) while shoot length (3.10 m), number of leaves (3.82) and longest shoot (3.46 cm) was maximum at BA (13.2 μM) + GA3 (1.0 μM) concentration respectively The results of one way analysis of variance (ANOVA) showed that F-factor and P-value for most of the parameters were significant at 0.05% [**Table 1**; **Figure 1**].

3.4. Effect Auxins on *in Vitro* Root Induction.

The results showed that IBA was more effective in root regeneration among all the auxins used [**Photoplate 1(F)**]. The mean value of *in vitro* rooting response for all the parameters at different PGR's concentration showed that

Table 1. Effect of various PGRs on *in vitro* shoot regeneration of *M. pellita*.

SN	PGRs	Avg. shoot no./explant	Shoot length	Avg. no. of leaves	longest Shoot
1.	Control	00	00	00	00
2.	BA + GA3(2.2 + 1.0μM)	0.91 ± 0.24	0.82 ± 0.23	1.83 ± 0.55	1.03 ± 0.33
3.	BA + GA3 (4.44 + 1.0μM)	0.90 ± 0.21	1.70 ± 0.40	1.83 ± 0.28	1.95 ± 0.35
4.	BA + GA3 (8.88 + 1.0μM)	1.26 ± 0.38	2.45 ± 0.43	2.25 ± 0.53	3.08 ± 0.46
5.	BA + GA3 (13.2 + 1.0μM)	2.55 ± 0.46	3.10 ± 0.98	3.91 ± 0.16	4.28 ± 1.19
6.	BA + GA3 (17.6 + 1.0μM)	2.66 ± 1.33	2.53 ± 1.23	3.82 ± 1.39	3.46 ± 1.44
7.	BA + GA3 (22.0 + 1.0μM)	1.85 ± 0.15	1.85 ± 0.40	3.25 ± 0.44	2.47 ± 0.18
8.	Kn + GA3 (2.3 + 1.0μM)	1.75 ± 0.38	1.64 ± 0.40	2.40 ± 0.33	2.00 ± 0.34
9.	Kn + GA3 (4.6 + 1.0μM)	2.50 ± 0.41	3.20 ± 0.74	4.25 ± 0.63	4.53 ± 0.69
10.	Kn + GA3 (9.2 + 1.0μM)	1.68 ± 0.58	2.55 ± 0.39	3.08 ± 0.77	2.58 ± 0.32
11.	Kn + GA3 (13.8 + 1.0μM)	1.60 ± 0.21	1.40 ± 0.41	2.20 ± 0.41	1.90 ± 0.61
12.	Kn + GA3 (18.4 + 1.0μM)	1.25 ± 0.37	0.88 ± 0.17	1.75 ± 0.55	1.56 ± 0.29
13.	Kn + GA3 (23.0 + 1.0μM)	1.25 ± 0.58	1.00 ± 0.48	1.20 ± 0.54	1.40 ± 0.57
	p value < 0.05	**0.244**^{ns}	**0.071***	**0.019***	**0.011**^{ns}

*significant at 0.05 levels; ^{ns}: not significant. Data are in an average of 24 explant (3/flask) after 45 days of subculturing in shooting media.

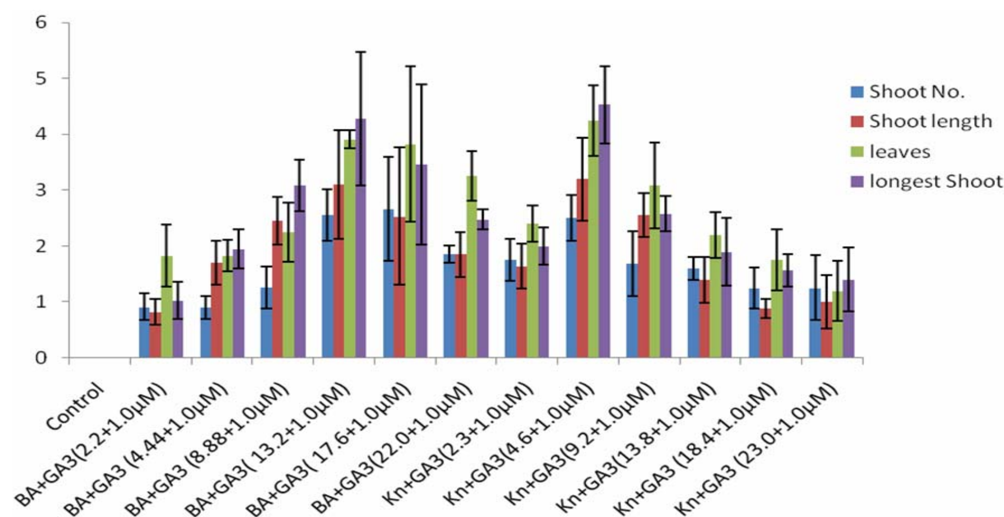

Figure 1. Effect of PGRs on *in vitro* plantlet enhancement: Effect of different hormone combination on induction of adventitious shoots. Bars correspond to means and lines correspond to standard errors of the mean at 5% level of probability.

average number of root (8.25), average root length (3.65 cm) and longest root (5.47) was maximum at IBA (4.9 µM) concentration. The survival rate were recorded after 30 days of acclimatization and was maximum 75% at IBA (4.9 µM) concentration respectively [**Photoplate 1(H)**]. About 55% - 65% survival was achieved when these *in vitro* raised plants were exposed to environment [**Photoplate 1(I)**]. The results of one way analysis of variance (ANOVA) showed that the p-value and f- factor for most of the parameters under *in vitro* rooting response of *M. pellita* were significant at 0.05% level [**Table 2**; **Figure 2**]. Half strength MS medium supplemented with IBA (4.9 µM) was more effective than full strength MS medium.

4. Discussion

Requirement of contamination free explant is essential for plant tissue culture, because throughout the study, the surface sterilized seeds and seedlings derived plant parts were used as explants [4]. Seed dormancy is a temporary failure or block of a viable seed to complete germination [5]. The seed coat is so rigid in case of *M. pellita* that it inhibits the rate of seed germination therefore its removal is necessary. The seeds of *M. pellita* after removal of seedcoat were soaked in water for 24 hrs. to break the dormacy of seeds and facilitate its germination . Chemicals that accumulate in the seed-coat during development and remain in the seed can act as germination inhibitors. Some of the substances associated with inhibition are various phenols, coumarin and abscisic acid, which can be leached out by soaking in water [6]. Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins). The specific concentration of plant regulators needed to induce callus, varies

Table 2. Results showing Mean (±SE) various parameters studied under the effect of different auxins on rooting response of *in vitro* multiplied shoots of *M. pellita*.

SN	PGRs	Avg. no. of root	Avg. root length	Longest root	Survival %
1	IBA (2.45 µM)	2.48 ± 0.21	2.53 ± 0.93	3.48 ± 1.3	58.3 ± 16.1
2	IBA (4.9 µM)	8.25 ± 2.3	3.65 ± 0.98	5.47 ± 1.25	75 ± 16.0
3	IBA (9.8 µM)	2.63 ± 1.23	1.4 ± 0.52	1.97 ± 0.75	58.5 ± 21.0
4	NAA (2.69 µM)	2.24 ± 1.11	1.75 ± 0.72	2.38 ± 0.96	50 ± 21.6
5	NAA (5.38 µM)	2.0 ± 1.38	1.32 ± 0.45	1.65 ± 0.61	50 ± 21.6
6	NAA (10.76 µM)	1.4 ± 0.8	1.27 ± 0.5	1.6 ± 0.7	50 ± 21.6
	LSD (p < 0.05)	**0.020***	**0.181^{ns}**	**0.073^{ns}**	**0.938^{ns}**

*significant at 0.05 levels; ^{ns}: not significant. Data are in an average of 15 microshoots (1shoot /flask) after 45 days of subculturing in rooting media (MS + Auxins); while % survival data were recorded after 30 days of acclimatization .

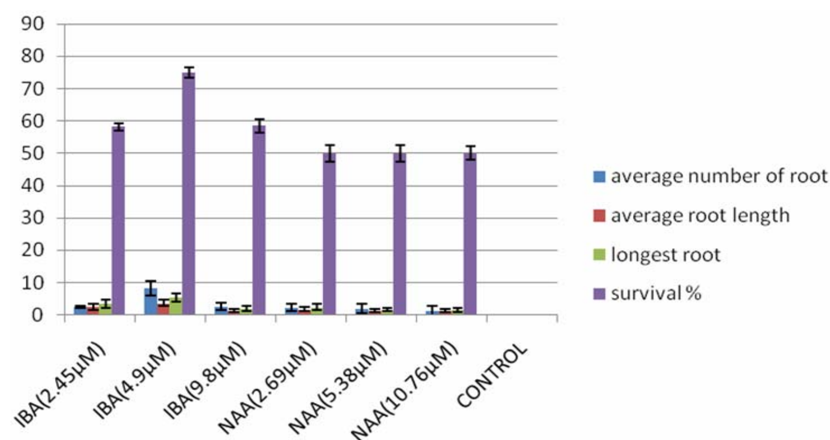

Figure 2. Results showing mean values for different parameters studied during the effect of different auxins on rooting response of *in vitro* multiplied shoots of *M. pellita.* Bars correspond to means and lines correspond to standard errors of the mean at 5% level of probability.

from species to species and even depends on the source of explant [7]. It has been demonstrated in many cases that 2,4-D is usually the choice of auxin for callus induction and subculture of grasses [8]. Lately more and more experimental results indicate that the addition of a low concentration of cytokinin in callus culture medium often enhances callus regeneration [9]. The results of present study in *Meizotropis pellita* showed that MS medium supplemented with 2,4-D (9.06 μM) and combination of auxin with low concentration of cytokinins 2,4-D (9.06 μM) and 2-iP (7.38 μM) is quite suitable for callus induction. Leaf explants readily develop callus in comparison to stem and roots. When the concentration of 2,4-D was increased from 4.53 to 9.06 μM, the degree of callus formation was also increased. However, when the concentration of 2,4-D was further increased to 9.06 to 13.6 μM, there was a decrement in the degree of callus formed. This decrease in callus formation could be due to the herbicidal property of 2,4-D. In addition, high auxin concentrations promote the biosynthesis of ethylene by increasing the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase [10]. Ethylene is able to stimulate senescence of leaf, inhibit leaf abscission and shoot growth. 2-iP is a cytokinin and is generally incurporated in tissue culture medium for cell division and differentiation of adventitious shoots from callus and organs [11].

Direct shoot regeneration is preferred since it reduces the possibility of somaclonal variation common in plants regenerated from cultured cells or tissues [12]. Direct-Shoot induction was achieved from cotyledonary node explants of *M. pellita* in MS medium supplemented with Kn + GA3), BA + GA_3 while no shoot induction was observed when Kn and BA alone were used in MS medium. Plant regeneration from cotyledonary node explant was observed in mungbean [13] and peanut [14]. Growth regulators, particularly cytokinins have been reported to be important in inducing organogenesis from cotyledons. BA as a cytokinin proved to be effective *in vitro* with many woody species. Also, it is the most frequently used compound in enhancing the production of proliferated shoots [15]. It was reported that an excess of synthetic cytokinin like BA is the most effective for the *in vitro* micropropagation of Acacia mangium [16]. The pH of the culture medium is an important factor for promoting *in vitro* shoots in *M. pellita*. A change of medium pH may have various effects that may influence performance and development of explants [17]. In *M. pellita*, a better performance in all parameters on shoot development was found at pH 5.8. pH plays important role in enhancing the activities of growth regulators and enzymes that affect the function of cells as well as whole plants. The explants of *M. pellita* for *in vitro* shoot regeneration were regularly subcultured after 3 weeks and transferred to the same medium. The reduction of the duration of the subculture from 4 - 6 weeks to 3 weeks was very effective in the deterioration of the yellowing of the explants. Frequent subculture of explants at constant intervals (25 - 30 days) makes significant improvement in enhancing the number of multiple shoots [18]. Repeated sub-culturing caused activation and conditioning of meristems. Transferring the cultures to fresh medium after three weeks was found to be essential to prevent culture deterioration and sustained shoot growth in *M. pellita*. Growth regeneration decreased with further increase in cytokinin concentration. It is concluded that BA is very important for the multiplication of shoots but if added with high concentrations it causes yellowing and necrosis of the explants.

IBA was more effective in root regeneration. Half strength MS medium supplemented with IBA was more effective than full strength MS medium. The success of IBA in promoting efficient root induction has also been reported earlier in *C. ternatea* [19], Psoraleacorylifolia [20], Withaniasomnifera (Dunal) L., [21] Morusindica [22], Murrayakoenigii [23] and Sterculiaurens [24].

5. Conclusion

Uttarakhand India is a hot spot of biodiversity, and every native organism needs to be conserved especially since these organisms are crucial for ecological balance of forest. Besides, these are a source of crucial stress resistant genes which give them ability to survive the harsh climatic condition like chilling, drought, high altitude etc. to name a few prevailing in most of the Himalayan region. The high production costs of nursery plants and the time required for restored plants to complete their life cycle are commonly considered to barriers to successful propagation. In case of *M. pellita*, the problem is further compounded by low viability and poor germination of seeds. Moreover, in natural conditions it propagates by its root stock *in situ*. Thus, this protocol offers potential *in vitro* system that can be used for improvement, conservation and efficient mass multiplication of clones of *M. pellita* an endangered and endemic shrub. Further, *in vitro* mass propagation reduces cost and completion of life cycle can be attained within reasonable time, reducing risk of extinction to the endangered wild population. In the present case an estimated 150 - 300 plants are left which, too are in the verge of total extinction and the study will provide an alternative for their conservation.

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Abbreviations

2,4-D—2,4-dichlorophenoxyacetic acid;
2-iP—6-(y,y,dimethylallylamino)purine;
IBA—Indole-3-butyric acid;
Kn—Kinetin;
BA—6-benzyladenine;
NAA—Naphthalene acetic acid;
MS—Murashige and Skoogs (1962).

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