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SYNERGISTIC EFFECT OF AUXINS AND CYTOKININS ON PROPAGATION OF *ARTEMISIA AMYGDALINA* (ASTERACEAE), A CRITICALLY ENDANGERED PLANT OF KASHMIR

RAFIA RASOOL[1*], BASHIR A. GANAI[1], AZRA N. KAMILI[2], SEEMA AKBAR[3] AND AKBAR MASOOD[1]

[1]*Department of Biochemistry, University of Kashmir, Srinagar-190006, Jammu and Kashmir, India*
[2]*Plant Tissue Culture Laboratory, Centre of Research for Development, University of Kashmir, Srinagar-190006, Jammu and Kashmir, India*
[3]*Regional Research Institute of Unani Medicine, University of Kashmir Campus, Srinagar-190006, Jammu and Kashmir, India.*
[*]*Corresponding author e-mail: bbcganai@gmail.com; rasoolrafiya@gmail.com*

Abstract

Efficacy of different auxins and cytokinins was studied on critically endangered and endemic plant *Artemisia amygdalina* Decne. of Kashmir by exploiting the morphogenetic potential of shoot tip explants. Four phytohormonal combinations (NAA and BAP; Kn and 2, 4-D; BAP and 2, 4-D; NAA and Kn) were used to find the viable combination for its optimum proliferation and growth. Murashige and Skoog (MS) medium fortified with 2 µM of α- napthaleneacetic acid together with 1 µM of 6-benzyladenine produced up to 19 shoots per shoot tip after 6 weeks under optimal culture conditions. Regenerated shoots showed maximum rooting on MS basal media with an average of 10 roots per explant. Rooted plants were transferred to soil in green house where they exhibited normal growth. This is the first *In vitro* regeneration report of *A. amygdalina* which focuses on conservation of its unique germplasm.

Abbreviations: BAP: 6-benzyladenine, 2, 4-D: 2, 4, dichlorophenoxyaceticacid, Kn: Kinetin, MS: Murashige and Skoog, NAA: α- napthaleneaceticacid, PGR: Plant growth regulators.

Introduction

Artemisia amygdalina Decne. belonging to family asteraceae, was collected last in the Kashmir Himalaya, four decades ago. It is a critically endangered endemic species and has received no attention. The unique soil needs, habitats, and limited distribution make many world's rare plant species endemic, and meanwhile they are exploited by human activities. Endemic plant species are important to signify unique biodiversity and values to conservation biologists and ecologists. It helps in recognizing ways in which floras are different (Dar *et al*., 2006). This species was last collected from this region in 1971(Dar *et al*., 2006). Inspite of being an valuable ethno-medicinal angiosperm of our ecosystem, it is confined to specific belts of sub-alpine region in Kashmir which is isolated, less disturbed area and north west provinces of Pakistan (Dar *et al*., 2006 & www.eFloras.org). It is an erect 1.5 m tall perennial herb whose stem arise from base and leaves are simple, sessile and teeth curved. It thrives in sandy, relatively loose and moist soil habitats (Dar *et al*., 2006). *Artemisia* species have been reported to act as antihelmenthic, antiseptic, deobstruent, antimalarial, emmenagogue, febrifuge and tonic. Aqueous extract of leaves, inflorescence and seeds of some *artemisia* species are being used for gastric problems and to cure abdominal pain (Khan *et al*., 2011). This family largely contains flavonoids, acetylenic compounds and ascorbic acid (Sati *et al*., 2010) and most important sesquiterpene lactone artemesinin (Namdeo *et al*., 2006). Plants play a key role in maintaining our ecosystem and so their propagation and conservation, especially of critically endangered species should be a main concern. Two thirds of the world's plant species are in danger of extinction with pressure from the growing human population, habitat modification via deforestation, over-exploitation, spread of invasive alien species, pollution and the growing impacts of climate change (Murch *et al*., 2000, Anon., 2009). Other species of *Artemisia* (Mackay & Kitto, 1988; Mathe & Laszloffy, 1991; Liu *et al*., 2003; Sujatha & Kumari, 2007; Zia *et al*., 2007) have been studied in some details but nothing is known about this particular plant species. Therefore, a study was planned to obtain maximum regeneration potential of this plant using various phytohormonal regimes.

Materials and Methods

Fresh plants of *A. amygdalina* were collected from very higher reaches of Gurez Nallah in north Kashmir during September 2009. The plant specimens were confirmed by comparison with reference standards at Centre of Plant Taxonomy (COPT), Department of Botany, University of Kashmir. Voucher specimen numbers of the deposited plants were 1013 (KASH) and 1014 (KASH). Actively growing shoot tips 1-1.5 cm in length were collected from COPT garden. The explants were immersed in solution containing labolene and a few drops of Tween-20 (0.1% w/v) (Himedia, Mumbai, India) followed by washing under tap water. Explants were surface-sterilized with 0.05% (w/v) mercuric chloride for 10 min, followed by three rinses with sterile distilled water. 25 ml of molten media were dispensed into culture vials (100 ml) plugged with non absorbent cotton plugs sterilized at 121°C for 15 min. Media was supplemented with 30 g l^{-1} sucrose (Himedia) as carbon source, 0.8% (w/v) agar (Himedia) for gelling. pH was adjusted to 5.5-5.8 by 0.1M NaOH or HCl before autoclaving at 1.06 kg cm^{-2} (121°C) for 20 min. Explants were inoculated on basal MS media (Murashige & Skoog, 1962) (Himedia) as well as on media fortified with series of combination of various plant growth regulators 6- benzyladenine (BAP), α- napthaleneaceticacid (NAA), Kinetin (Kn) and 2, 4,

dichlorophenoxy acetic acid (2, 4-D) (2µM to 15µM) (Sigma-Aldrich, St Louis, USA). Cultures were maintained at 25 ± 3°C temperature, 55-65% relative humidity and exposed to 16 h photoperiod provided by cool fluorescent tubes (37.5 µmol m^{-2} s^{-1}). Shoots obtained were subjected to various phytohormonal treatments. Cultures were subcultured after four-week interval on to fresh medium which consists of same media composition for six-weeks. Regenerated shoots were simultaneously inoculated on MS (full strength), ½ MS (half strength) media for rooting. Vials were gradually opened for acclimatization for a week. Plants with healthy roots were transferred to small pots containing perlite, sand and garden soil in 1:1:4 ratios. They were irrigated with distilled water during first few days and later on with tap water. Pots were transferred to misting chamber of green house under high humidity for their further growth and development. Experiments were set up in randomized block design (RBD) and each treatment had 15 replicates. Data was scored at the end of the six weeks culture period. Sub culturing was carried out at an interval of 6 weeks. The parameters evaluated were: number of shoots per explants, shoot length, type of shooting, morphology of callus and rooting. The data were analyzed by ANOVA (analysis of variance) and means ± standard deviation were compared through Tukey's test.

Results

Effect on basal media: Shoot tips of *A. amygdalina* when inoculated on media devoid of any plant growth regulator (PGR), two elongated shoots emerged from the base of explant (Fig. 1a).

Effect of auxins and cytokinins: Induction of series of PGR's in single or in combination exhibited varied effect on the organogenesis of *A. amygdalina*. Exogenous individual supply of cytokinins; BAP or Kn and auxins; NAA or 2, 4-D (2 µM to 15 µM) respectively displayed no apparent effect on shoot bud initiation (Tables 1 & 2). The combined response of auxins and cytokinins increased frequency of multiplication and resulted in callus formation typically on all phytohormonal treatments (Tables 1- 4). Among four combinations (NAA + BAP; Kn + 2, 4-D; BAP + 2, 4-D and NAA + Kn) of auxins and cytokinins tested, frequency of shoot induction response was optimal in BAP and NAA. Greater number (19.67 ± 7.0) of *de novo* multiple shoots were recorded from shoot tip explants in MS medium containing NAA (2 µM) + BAP (1 µM) (Fig. 1b) followed by 10.67 ± 1.15 in MS supplemented with NAA (10 µM) + BAP (10 µM) (Fig. 1c & Table 1). The other three combinations of auxins and cytokinins gave significantly lesser number of shoots (Tables 2-4).

Subculturing: When *In vitro* raised callus and micro cuttings were placed onto different culture media, no differentiation was observed in callus but multiple shoot (12.1 ± 1.6) formation together with intense callus formation at base of explant was observed again on MS medium augmented with NAA (2 µM) + BAP (1 µM) (Table 5). Likewise microcuttings when cultured on MS and MS half strength resulted in formation of direct extensive rooting as well as multiple shooting (Figs. 1d & e). The average numbers of adventitious and axillary shoots obtained were 5.16 ± 2.2, length of longest shoot being 26 ± 3 cm.

Rooting and acclimatization: The average number of roots obtained were 10 ± 0.1, length of longest root being 21 ± 1.7 cm. Plants with well developed roots were let open as such for a week and then transferred to pots containing perlite, sand and garden soil (1: 1:4). Plant after transferring to green house looked healthy and acclimatized normally (Fig. 1f). The establishment percentage of plants was 95%.

Discussion

The main crux of the present study was to bridge the gap between other explored species of *Artemisia* and this lesser known, scarce plant of Kashmir valley and Pakistan. A system was framed to evaluate the response of various phytohormones on the *In vitro* propagation strategy of *A. amygdalina* so that its further loss from our biodiversity can be prevented. Further it could be characterized biochemically and that might reveal its novel use to us. In the present study shoot tip was used as an explant because it possesses several axillary buds, has more survival chances and grows rapidly (George & Sherrington, 2004). It is evident from (Tables 1& 2) that MS medium individually supplied with either cytokinins or auxins does not induce any effect on explant. Possible reason might be that auxins together with cytokinins treatments are considered to be the best shoot proliferators. Levels of endogenous auxin and cytokinin in the explant were probably high because lesser concentrations of exogenous auxins and cytokinins significantly enhanced shoot proliferation together with callusing and introduction of higher concentrations decreased multiplication. Moreover BAP and NAA favored multiple shoot regeneration via callus redifferentiation. Such findings are in accordance with Mackay & Kitto, 1988; Nin *et al*., 1996 who also reported NAA and BAP good for multiple shooting and callus formation in *A. dracunculus* and *A. absinthium* respectively. Contrary to this, only cytokinins (BAP and Kn) were considered optimal for shoot proliferation in *A. vulgaris* by Sujatha and Kumari, 2007. In *A. pallens*, callus formation was reported on MS containing BAP and 2, 4-D (Benjamin *et al*., 1990) which is in line with our results (Table 3). In contrast to this, callusing was observed on 2, 4-D (4.5 µM) alone (Paniego & Giuliette, 1996). Subculturing was also carried and frequency of multiplication was maintained except in callus (Table 5). Callus did not show any signs of regeneration even when subcultured on new or fresh medium. Possibly regeneration potential was not expressed because of altogether different threshold level of phytohormones in callus. Heavy rooting and elongation was observed on MS basal medium (Figs. 1d & e), which is again in accordance with Benjamin *et al*., 1990 in *A. pallens* and in contradiction to Liu *et al*., 2003 where induction of indole butyric acid (IBA) induced rooting in *A. judaica*.

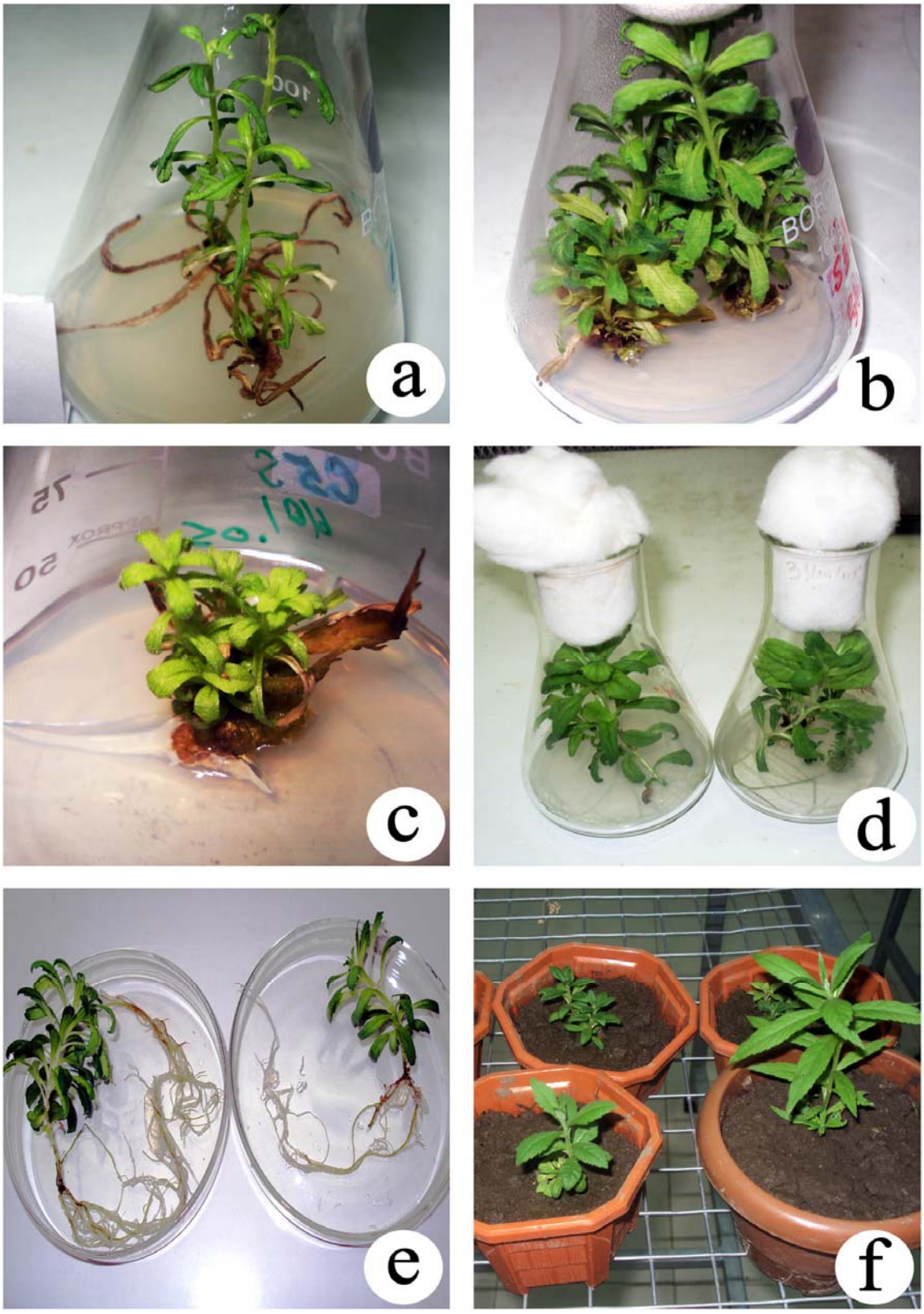

Fig. 1. *In vitro* regeneration of *A.amygdalina* Decne.
a. Elongated shoots on medium devoid of PGR's, b. Multiple shoots on MS + NAA (2 µM) + BAP (1 µM), c. Multiple shoots on MS + NAA (10 µM) + BAP (10 µM), d. Subculturing and subsequent multiplication on MS basal medium, e. Plants showing development of roots on MS basal medium, f. Acclimatized healthy elongated micropropagated plants established in green house

Table 1. Effect of NAA and BAP on shoot proliferation and growth of *A. amygdalina* using shoot tip explant.

Treatments	No. of shoots per explant*	Shoot length (cm)*	Type of shoots	Nature of callus	Fresh weight of unorganized callus (g)*
Control	2 ± 1^{c}	4.75 ± 1^{ab}	Adv	–	–
BAP 2 µM	–	–	–	–	–
BAP 4 µM	–	–	–	–	–
BAP 10 µM	–	–	–	–	–
BAP 15 µM	–	–	–	–	–
NAA 2 µM	–	–	–	–	–
NAA 4 µM	–	–	–	–	–
NAA 10 µM	–	–	–	–	–
NAA 15 µM	–	–	–	–	–
NAA 1 µM + BAP 2 µM	9 ± 3^{c}	3 ± 0.5^{bf}	Adv	Green hard nodular	–
NAA 2 µM + BAP 2 µM	4 ± 2.64^{b}	2.5 ± 0.5^{c}	Adv	Brownish green hard nodular	–
NAA 2 µM + BAP 1 µM	19.67 ± 7.0^{a}	5.33 ± 1.15^{a}	Adv/ax	Creamy hard nodular	–
NAA 4 µM + BAP 4 µM	4.16 ± 0.28^{g}	1.66 ± 0.15^{g}	Adv	Light green hard nodular	–
NAA 5 µM + BAP 10 µM	5.33 ± 2.5^{e}	1.5 ± 0.5^{h}	Adv/ax	Fast green nodular	–
NAA 7.5 µM + BAP 10 µM	6.33 ± 2.08^{d}	2.5 ± 0.5^{d}	Adv/ax	Creamy hard nodular	–
NAA 10 µM + BAP 7.5 µM	5 ± 1^{f}	1.4 ± 0.3^{f}	Adv	Cream colored nodular hard	–
NAA 10 µM + BAP 10 µM	10.67 ± 1.15^{b}	2.16 ± 0.28^{e}	Adv	Pale hard nodular	–
NAA 5 µM + BAP 15 µM	–	–	–	Green hard nodular	0.67 ± 0.03^{b}
NAA 10 µM + BAP 15 µM	–	–	–	Creamy non nodular	5.183 ± 0.46^{a}

* Statistical analysis was done using one way ANOVA. Means ±SD followed by same letters are not significantly different ($p<0.05$) using Tukey's test. Adv (adventitious); Ax (axillary)

Table 2. Influence of Kn and 2, 4- D on *In vitro* response of *A. amygdalina* using shoot tip explant.

Treatments	No. of shoots per explant*	Shoot length (cm)*	Type of shoots	Nature of callus	Fresh weight of unorganized callus (g)*
Control	2 ± 1^{a}	4.75 ± 1^{b}	Adv	–	–
Kn 2 µM	1.66 ± 1.1^{a}	1.1 ± 0.1^{a}	Adv	–	–
Kn 7 µM	–	–	–	–	–
Kn 10 µM	1.66 ± 0.57^{b}	1.16 ± 0.15^{a}	Adv	–	–
Kn 15 µM	–	–	–	–	–
2, 4-D 2 µM	–	–	–	–	–
2,4-D 7 µM	–	–	–	–	–
2,4-D 10 µM	–	–	–	–	–
2,4-D 15 µM	–	–	–	–	–
2,4-D 5 µM + Kn 10 µM	–	–	–	Brown nodular	0.15 ± 0.005^{a}
2,4-D 7 µM + Kn 10 µM	–	–	–	Brown hard nodular	0.46 ± 0.2^{a}
2,4-D 10 µM + Kn 10 µM	–	–	–	–	–
2,4-D 10 µM + Kn 15 µM	–	–	–	Light brown hard nodular	0.35 ± 0.05^{b}

* Statistical analysis was done using one way ANOVA. Means ± SD followed by same letters are not significantly different ($p<0.05$) using Tukey's test. Adv (adventitious); Ax (axillary)

Table 3. Effect of BAP and 2, 4- D on *In vitro* culture of *A. amygdalina* using shoot tip explant.

Treatments	No. of shoots per explant*	Shoot length (cm)*	Type of shoots	Nature of callus	Fresh weight of unorganized callus (g)*
Control	2 ± 1^{c}	4.75 ± 1^{a}	Ax	–	–
2,4-D 1 µM + BAP 2 µM	2.43 ± 0.4^{a}	1.5 ± 0.5^{b}	Ax	Cream hard nodular	–
2,4-D 2 µM + BAP 2 µM	2.26 ± 0.2^{b}	1.53 ± 0.5^{c}	Ax	Cream hard nodular	–
2,4-D 2 µM + BAP 1 µM	2 ± 0.5^{a}	3 ± 0.15^{b}	–	Cream hard nodular	–
2,4-D 4 µM + BAP 4 µM	–	–	–	Cream hard nodular	2.82 ± 0.02^{a}
2,4-D 7 µM + BAP 10 µM	–	–	–	Cream hard nodular	1.2 ± 0.9^{b}
2,4-D 10 µM + BAP 10 µM	–	–	–	Cream hard nodular	0.31 ± 0.02^{b}
	–	–	–	Cream soft nodular	0.416 ± 0.1^{d}
	–	–	–	Cream hard nodular	0.483 ± 0.17^{c}

* Statistical analysis was done using one way ANOVA. Means ±SD followed by same letters are not significantly different ($p<0.05$) using Tukey's test. Adv (adventitious); Ax (axillary)

Table 4. Response of NAA and Kn on *In vitro* response of *A. amygdalina* using shoot tip explant.

Treatments	No. of shoots per explant*	Shoot length (cm)*	Type of shoots	Nature of callus	Fresh weight of unorganized callus (g)*
Control	2 ± 1^{b}	4.75 ± 1^{b}	–	–	–
NAA 1 µM + Kn 2 µM	–	4.83 ± 1.04^{a}	–	Cream hard nodular	2.7 ± 0.25^{b}
NAA 2 µM + Kn 2 µM	–	3.16 ± 0.7^{ac}	–	Brown or light cream hard nodular	2.8 ± 0.2^{a}
NAA 2 µM + Kn 1 µM	–	1.5 ± 0.5^{c}	–	Brown nodular	0.6 ± 0.2^{d}
NAA 4 µM + Kn 4 µM	–	–	–	Green nodular	2.6 ± 0.26^{a}
NAA 7 µM + Kn 10 µM	4.4 ± 0.4^{a}	2.46 ± 0.45^{d}	–	Brown hard nodular	1.96 ± 0.05^{c}
NAA 10 µM + Kn 10 µM	–	–	–	–	–
NAA 10 µM + Kn 15 µM	–	–	–	Pale nodular	0.9 ± 0.1^{d}
NAA 5 µM + Kn 15 µM	–	–	–	–	–

* Statistical analysis was done using one way ANOVA. Means ±SD followed by same letters are not significantly different ($p<0.05$) using Tukey's test. Adv (adventitious); Ax (axillary)

Table 5. Sub culturing of *In vitro* raised plants of *A. amygdalina*.

Treatments	No. of shoots per explant*	Shoot length (cm)*	Type of shoots	No. of roots per explant	Length of longest root (cm)	Nature of callus	Fresh weight of unorganized callus (g)*
Basal	5.16 ± 2.2^{c}	26 ± 3^{a}	Adv/Ax	10 ± 0.1	21 ± 1.7	–	–
NAA 1 µM + BAP 2 µM	7 ± 1^{b}	3 ± 0.5^{c}	Adv	–	–	Brownish green hard nodular	7.78 ± 1.4^{b}
NAA 2 µM + BAP 2 µM	1.6 ± 0.5^{d}	3.5 ± 0.5^{b}	Adv	–	–	Brownish green hard nodular	8.04 ± 1.13^{c}
NAA 2 µM + BAP 1 µM	12.1 ± 1.6^{a}	3 ± 0.5^{d}	Adv/Ax	–	–	Creamy hard nodular	9.86 ± 0.57^{b}
NAA 7.5 µM + BAP 10 µM	–	–	–	–	–	Creamy hard nodular	3.05 ± 0.05^{d}
NAA 10 µM + BAP 7.5 µM	1.66 ± 1.15^{c}	1.5 ± 0.5^{b}	–	–	–	Light green nodular hard	12.65 ± 0.02^{a}
NAA 10 µM + BAP 10 µM	2.66 ± 1.5^{b}	1.5 ± 0.5^{e}	–	–	–	Cream colored nodular	6.15 ± 0.05^{c}

* Statistical analysis was done using one way ANOVA. Means ± SD followed by same letters are not significantly different ($p<0.05$) using Tukey's test. Data was recorded after 6 weeks. Adv (adventitious); Ax (axillary)

Conclusion

In conclusion this *In vitro* protocol would provide an effective way for the conservation and building up populations of this critically endangered plant species. This species can be a good source of bioactive principles as is evident from the therapeutic importance and medicinal properties of its related species.

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