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In vitro propagation of *Blepharis sindica* ex T.Anders

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ABSTRACT: *Blepharis sindica*-T. Anders is a vulnerable species whose natural population is decreasing due to unscientific exploitation, scanty rainfall and habitat destruction. It has been notified as threatened species in the report of UNDP -CCF II Project and given A2cd Vulnerable category as per IUCN red list. So, this plant needs urgent efforts for conservation including both *in situ* and *ex situ*. In *ex situ* conservation the seeds were utilized first as an explant to develop complete seedling plants. Seeds were excised from fruit capsule after removing coat and inoculated in Basal modified MS medium supplemented with charcoal and ms medium supplemented with plant growth regulators, NAA, 2,4-D. Complete seedling plants and callus was achieved from seeds in basal modified MS and ms supplemented with 2,4-D, combination of 0.5mg/l NAA, 1.0mg/lBAP supplemented MS medium. Callus initiation and callus multiplication achieved from inoculation of cotyledons and subcultured callus parts. Callus initiation achieved by inoculation of cotyledons in combination of 0.5mg/l NAA, 1.0mg/l BAP, with only 1.0 mg/l 2,4-D supplemented MS medium. Multiplication of callus achieved from subculturing of callus parts in MS medium supplemented with 1.0mg/l&2.0 mg/l BAP. Enormous rooting and shooting along with leaf development showed on basal modified MS media. Better responses of rooting and shooting along with leaf development showed on Basal modified MS media supplemented with charcoal as compare to basal modified MS medium devoid of charcoal from seeds. Browning was also observed from cultures due to which callus appeared with brown colour after few days of subculture.

Key words: Tissue culture, *Blepharis sindica*, MS medium, Growth regulators.

INTRODUCTION

Blepharis sindica Stocks ex T. Anders is a herb plant which is known with local name of Billy Khojio, Bhangari, Unt-kantalo and belong to the Family: Acanthaceae. *Blepharis sindica* is a lignified annual growing on gravelly or sandy ground in the Thar Desert, northwestern India. Flowering begins in August and lasts for 3–4 months. This herb has desert adaptation including spinous teeth near the base and around sheetas (storage site of fruits) and leaves are narrow to prevent extra loss of water through evaporation.

This plant has medicinal values. If one or more organs of any plant which contain substances those can be used in cure purpose or it may be a precursor for synthesis of valuable drugs called a medicinal plant (Agarwal *et al.*, 2006). Methanolic and petroleum ether seeds extracts of *Blepharis sindica* are effective against bacterial strains *Streptomyces griceous*, *Basillus subtilis* and fungal strains *Trichoderma reesei* (Sharma & Roy 2018b). *Blepharis sinidica*'s roots are useful in urinary discharge and powdered plant useful in genital infection and burns. According to the World Health Organization, 80 percent of the world's total population, presently using herbal medicines for various aspects of primary health care.

Blepharis sindica is threatened species whose natural population is decreasing due to unscientific exploitation, scanty rainfall and habitat destruction. It has been declared as threatened species in the report of UNDP-CCF II Project and given A2cd Vulnerable category as per IUCN red list (Sharma & Roy 2018a,b). Because seeds of *Blepharis sindica* are dispersed by rain from the protected aerial seed banks adhere to the soil surface in rain flood water and germinated only after the excess water had reduced and these seeds germinated after a short dispersal. Due to absence of soil seed bank for this plant due to predatory and other reasons, availability of the new plants becomes rare. The plants which contains high medicinal values do not survive in all agro climatic conditions so the tissue culture methods like micropropagation and others are very useful to increase their availability. So, this plant need efforts for its conservation and in one of its mode *ex situ* conservation, plant tissue culture and seedling development tried in present research work. Plant growth regulators are chemicals which in trace amount is sufficient for growth of plants (Opik & Stephen 2005) and the development, differentiation of their cells and tissues. Auxins are a category of plant growth regulators and morphogens (phytohormone) which have an important role in coordination of many growth and behavioral processes throughout the plant life cycle (Mantell *et al.*, 1985; Suryvathana & Kumar 2010). The

pioneering work act as a source for concept of totipotency when regeneration of complete flowering in *Daucus carrota* took place by using its phloem cells as an explant (Chaturvedi *et al*., 2007). New approach was defined that both auxin and cytokine can regulate mechanism undergoing organogenesis that was evolved through balance between both of them (Miller, 1956; Skoog & Miller 1957). In these days, invitro propagation become a tool to assess the role of soil elements (N, P, K, Mg) in fertility of soil and in crop production to remove effect of change in environmental factors (Munthali *et al.,* 2022).

First successful shoot development *in vitro* in *Blepharis* species was initiated with the stem of *Blepharis repens* on MS medium that was supplemented with varying concentration of different growth hormones namely IBA, BAP, GA3 respectively (Suryvathana & Kumar 2010) and recently, it was achieved with somaclonal variation studies in other plant of acanthaceae family in *Phlogacanthus thyrsiformis* Nees (Baro & Das 2022).

MATERIALS AND METHOD

First plant materials, Nodal segment and seeds of *Blepharis sindica* was collected from Ramsisar village in Churu from actively growing field. These plant materials were used as explant to raise callus and *in vitro* plantlets. Culturing and sub culturing process were carried out by using MS medium with different growth factors in different concentrations.

The explants were washed thoroughly with tap water and kept under running tap water for 3-5 minutes after that the explants were given preliminary washing of detergent to remove adhering contaminations on the surface of explants which was followed by through washings under running tap water to remove the detergent totally. After that explants were surface sterilized with 0.1% (w/v) $HgCl_2$ (2 min). Finally, the explants (root, internodes, seeds, cotyledons) were washed thoroughly for three times with double distilled water. The surface sterilization procedure was carried out under aseptic condition in laminar flow (Murashige & Skoog 1962). For all experiments MS (Murashige & Skoog 1962) medium was used. The phytohormones were added to the nutrient medium not before autoclaving they are added after as per the need of the experiments. The medium was sterilized in an autoclave at 15 psi for 15 min. 16 hrs photoperiod provided with white fluorescent light and observed at regular intervals (Suryavathana & Kumar 2010). After which callus parts and cotyledons were isolated and transferred on nutrient medium (Roy & Kumar 1990). Culture were raised on modified MS medium to study the influence of various plant growth factors (Roy & Kumar 1985, 1990).

RESULTS AND DISCUSSION

Tissue culture of this herb was carried out first by utilizing root, node, stem, leaves but they had not shown any further response then seeds and cotyledons were utilized as explants. This was the first time report of this plant that seeds, seedling explants(cotyledons) shown responses in the form of development of callus. According to table Enormous rooting (huge clump of roots) and shooting along with leaf development showed on basal modified MS media from seeds. Better responses of rooting and shooting along with leaf development showed on Basal modified MS media supplemented with charcoal as compare to basal modified MS medium devoid of charcoal. Callus initiation and callus multiplication achieved from inoculation of cotyledons and subcultured callus parts. Callus initiation achieved by inoculation of cotyledons in combination of 0.5mg/lNAA,1.0mg/lBAP, with only 1.0 mg/l 2,4-D supplemented MS medium. Multiplication of callus achieved from subculturing of callus parts in MS medium supplemented with 1.0mg/l&2.0 mg/l BAP. Enormous rooting and shooting along with leaf development showed on basal modified MS media. Better responses of rooting and shooting along with leaf development showed on Basal modified MS media supplemented with charcoal as compare to basal modified MS medium devoid of charcoal from seeds. Browning was also observed from cultures due to which callus appeared with brown colour after few days of subculture.

Table 1: Effect of various nutrient media manipulations on development of callus and seedlings in *Blepharis sindica*.

Sr. No.	Combination	Concentration	Callus development	Special comments
1.	Mod.MS	Basal	-	Enormous rooting, much length along with leaves.
2.	Mod. MS	Basal+Charcoal	-	Complete rooting along with enormous leaves in the whorl of four from cotyledons.
1	Mod. MS(comb-1)	0.5mg/lBAP+1mg/l NAA	C^{+}	Callus developed from cotyledons, seeds
2	Mod.MS(comb-2)	1.0mg/l BAP+0.5mg/l NAA	C^{++}	Small amount of callus with cotyledons and multiplied callus after subculturing
3	Mod. MS	2.0mg/l BAP	C^{+++}	Huge callus with subculturing
5.	Mod. MS	2.0mg/l 2,4-D	C^{++}	Limited Callus achieved with subculturing
6.	Mod. MS	1.0mg/l 2,4-D	C^{+}	Low amount of Callus

*C^{+++} -High amount of callus, C^{++} -Moderate callus, C^{+} -low amount of callus

Mod.Basal MS

Mod. Basal MS +charcoal

Roots in Mod.Basal+charcoal

Combination-2

Subculturing response(comb-2)

Subculturing response (2.0mg/l BAP)

Fig. 1. Different responses from explants (seeds) in modified basal MS medium and in charcoal added medium and Cotyledons in combination-2 (1.0mg/l BAP+0.5mg/l NAA) and callus multiplication from sub-cultured callus parts in mod. MS medium supplemented with combination-2 (1.0mg/l BAP+0.5mg/l NAA) and in 2.0mg/l BAP.

DISCUSSION

Because previous tissue culture work is reported by Suryvathana and Kumar (2010) in *Blepharis repens* which are related species of *Blepharis* in which *in vitro* shoot development was initiated. In the stem of *Blepharis repens* on MS medium supplemented with varying concentration of different growth hormones namely IBA, BAP, GA3 respectively. The results show induction of shoot with the growth regulators BAP/IBA and GA3. MS+4 mg/l BAP+1.0 mg/l IBA+0.5 mg/l GA3, MS+4 mg/l BAP+0.5 mg/l IBA+0.5 mg/l GA3 & MS+3.5 mg/l BAP+1.0 mg/l IBA+0.5 mg/l GA3 were different media combination which was responsible for shoot induction in *Blepharis repens* (Suryvathana & Kumar 2010). Pattar *et al.* (2011) reported *in vitro* shoot regeneration in *Blepharis molluginifolia*, Pers. (Acanthaceae) by using nodal explants. Same nutrient media tried in *Blepharis sindica* but stem segment did not show fruitful response with these concentrations so seeds, seedlings (cotyledons) were used as explants. Because *Blepharis sindica* have fruit with hard coat. Seeds always remain surrounded by this coat due to which it is hard to take initial developmental steps (caulogenesis, rhizogenesis) in culture media .So for early tissue culture responses, it is necessary to make a cut in fruit with scalpel blades prior to transfer in culture media to uncover it by removing its fruit coat for nourishment of seeds . After which when seeds were cultured, they show (Table 1) high amount of callus development with MS+2mg/l BAP, combination-2 [MS+1mg/lBAP+0.5mg/l NAA] and MS+1mg/l 2,4-D show moderate amount of callus development. But both combinations 2 (subcultured with cotyledon), 2 (direct with seed) shows callus development along with well developed shoot.

Combinations of BAP and NAA have been used to induce shoot formation in numerous species (Moore, 1986; Tripepi, 1997; Huang *et al.*, 2000; Pattar *et al.*, 2011). In *Blepharis sindica* (Table 1) BAP is seems to be differ with MS with 1.0 mg/l BAP + 0.5 mg/l NAA. But in Blepharis repens BAP along with GA3 and IBA promoting shoot regeneration. In *Blepharis sindica* 2,4-D seems to be beneficial for promoting callus in low concentration MS+1mg/l 2,4-D (same reported by Panigrahi *et al.* (2017) in *Adhatoda vasica* Nees). NAA and IAA are promoted callusing when utilized in high concentration (Noor Camellia *et al.*, 2009). Most important thing happened that enormous amount of rooting take place on basal MS Media in *Blepharis sindica* (also same reported by Mii *et al*. (1988) in *Actinidia chinensis*) along with well developed shoots with leaves in whorl of four.

CONCLUSIONS

Through this research work it is concluded that addition 2,4-D or NAA and BAP in nutrient medium(modified ms) are responsible for callus initiation from seed and cotyledonary part of seedling plant. Due to hard coat around seeds in *Blepharis sindica*, it is not easy to initiate response from seed, so a cut by scalpel is required in coat to remove it and seed isolation. Basal MS with little change is sufficient to initiate and develop complete seedling plants with roots, epicotyl, hyhpocotyl and leaves in whorl of four which is necessary for its conservation due to its vulnerable status and typical habitat. Callus multiplication can be achieved with low concentration of BAP in ms medium from subculturing of callus parts and cotyledonary parts.

FUTURE SCOPE

Because very less research work is attempted so far for this medicinal plant but it requires more research efforts specially in the area of conservation and genomics due to its special habitat, desert adaptations and continuously decline in population.

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