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In vitro Regeneration of a Vulnerable Medicinal Plant *Hibiscus lobatus* Kuntze. through Stem Callus Culture

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Abstract

Hibiscus lobatus Kuntze., an endemic and vulnerable medicinal plant of the Western Ghats, is on the verge of threatening status due to habitat destruction, prolonged dormancy, uprooting and over-exploitation of its stems and roots for their medicinal value. This investigation established a reliable *in vitro* micropropagation protocol by culturing stem explants. 2,4-D was the most effective growth regulator in inducing a callogenic response from the excised end of the stem explants at the concentration of 1 mg/l. Subculturing the stem callus to the media supplemented with 1.25 mg/l 2,4-D induced rhizogenesis. The interaction of BAP (3.0 mg/l.) and TDZ (0.5 mg/l.) induced multiple shoot differentiation from the stem calli. Subculturing the caulogenic nodules to lower concentrations of BAP promoted the development of root-intact plantlets in a single-phase culture. The stem callus regenerants were successfully hardened and 78% of the plants were survived in field condition.

Introduction

Hibiscus lobatus Kuntze. is a vulnerable annual herb belonging to the family Malvaceae. This species growing seasonally in the dry tropical biome of the Western Ghats of Karnataka, India. A thorough review of the literature has revealed a significant gap in the investigations regarding remarkable medicinal properties, phytochemical profiling pharmacological studies and lack of tissue culture studies on *H. lobatus*. In traditional medicine the whole plant of this species has been used to treat diabetes, debility, aphrodisiac, spermatorrhoea, inflammation, coughs, and wounds (Anil Kumar and Shivaraju 2016), but phytochemical screening and pharmacological properties of this species has not been investigated so far. Medicobotanical survey enlightened us that the traditional practitioner residing in the vicinity of the forests of Bhadra Wild Life Sanctuary near Sagara and Tirthahalli taluks, Karnataka State, India was using the whole plant extracts to cure sunburn, healing of burn wounds, gloving of the skin and as cosmetic properties. This research creates an opportunity to explore the medicinal

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importance and derive an *in vitro* protocol for micropropagation of this vulnerable species using stem explants.

Plant tissue culture is an important technique for producing many plants from a single sterile explant in a short period, regardless of seasonal changes (Chen 2016). Among various ornamental *Hibiscus* species, shoot explant culture was predominantly used for plant multiplication and regeneration (Davies et al. 2017, Debnath and Arigundam 2020). Previous researchers have successfully regenerated plantlets from stem explant culture of various *Hibiscus* species, including *Hibiscus rosa-sinensis* (Balla et al. 2009), *Hibiscus syriacus* (Yang et al. 2019, Sami et al. 2016), *Hibiscus cannabinus* (Srivatanakul et al. 2000, Ayadi et al. 2011) and *Hibiscus coddii* (Helena et al. 2021). In *H. lobatus*, the stem is hard, propagation occurs only through seeds, and the micropropagation protocol has not yet been standardized. This study presents the *in vitro* regeneration of plantlets from stem callus culture of *H. lobatus*.

Materials and Methods

The stem explants of *H. lobatus* were procured from robust specimens thriving within their native ecosystem and utilized for the purpose of indirect organogenesis. The explants were initially cleansed under a continuous flow of tap water utilizing a soft brush to eliminate dust and soil debris, followed by a thorough rinsing procedure lasting 5-10 min under running water. Thereafter, it underwent treatment with Bavistin (augmented with 2-3 drops of Tween-20) for a duration of 30 min, succeeded by three rinses with autoclaved water and subsequently exposed to 1% Tween-20 for 5 min prior to another series of three washes with autoclaved water. The explants were disinfested with 0.6% sodium hypochlorite treatment for 5 min, which was followed by exposure to 0.2% mercuric chloride for 1 min. Then treated with 0.1% gentamycin for 2 min, interspersed with three rinses using autoclaved water between each treatment. Within a laminar airflow chamber, the sterilization protocol was further extended with an additional rinse in sterile water for 2 min each. The explants were air-dried on sterile petri plates for 5 min under the laminar hood blower. Finally, the explants were trimmed and cut into 6 mm^2 pieces before being inoculated onto the culture MS medium.

The callogenic media was augmented with various concentrations 2,4-D (0.5-1.5 mg/l). The caulogenic media consists of combinations of BAP (0.5-3 mg/l) and TDZ (0.1-0.5 mg/l). Following a 30-day incubation period, the callogenic cultures were subsequently transferred to a caulogenic medium fortified with 3.0 mg/l BAP and 0.5 mg/l TDZ. The process of shoot organogenesis was assessed through the quantification of shoots per unit of callus mass, and the resultant data were subjected to statistical analysis (mean ± SD) utilizing GraphPad Prism version 8.4.3 software. The cultures were maintained under fluorescent illumination at a temperature of 24 ± 2°C and a relative humidity of 65%. The cultures were subjected to a photoperiod comprising 16 hrs of illumination (25 $\mu mol \cdot s^{-1} \cdot m^{-2}$) succeeded by 8 hrs of darkness and systematic daily observations of the cultures were conducted.

Under aseptic circumstances, the fully regenerated plantlets that were produced from stem calli were carefully removed from the culture bottles and cleaned with distilled water that had been sterilised to get rid of remaining media. The regenerants were transferred to garden soil consists of soil, sand and organic fertiliser in the ratio of 1:2:1 and transparent polythene bags were used to cover the pots to preserve humidity. During primary hardening the plants were first housed in a culture room for a period of two weeks to help them adjust and prevent environmental shock. The polythene bags were taken off and the plants were relocated to field conditions.

Results and Discussion

The primary experimental results demonstrated that among different growth regulators tested, 2,4-D was the effective growth regulator in stimulating the callogenic potency of the stem explants at concentrations ranging from (0.75 -1.5 mg/l). Callus initiation was first observed from the excised cut end of the stem explant in the form of whitish, nodular mass (Fig. 1A). Callusing was gradually spread over the excised end and the dorsal surface of the stem explant (Fig. 1B) and finally, callus was transformed in to whitish hard nodular mass (Fig. 1C). The results indicated that the optimal callogenic response was noticed at the concentration of 1.25 mg/l of 2,4-D and the percentage of callus formation was also more at this concentration (Table 1). The stem calli was mass multiplied at the concentration was subcultured to caulogenic media to induce shoot differentiation from the calli.

Table 1. Effects of auxins and cytokinins on callogenesis and plantlet regeneration from the stem callus culture of *Hibiscus lobatus.*

Treatments			% of response	Mean no shoots/explant	% of rooting
2,4-D (mg/ml)	BAP (mg/ml)	TDZ (mg/ml)			
0.5	-	-	6 ± 0.6	-	-
0.75	-	-	32 ± 1.2	-	-
1.0	-	-	84 ± 4.4	-	32 ± 2.0
1.25	-	-	70 ± 4.8	-	76 ± 5.6
1.5	-	-	58 ± 3.8	-	65 ± 5.2
-	1.0	0.25	36 ± 1.2	0.2 ± 0.04	-
-	1.5	0.50	70 ± 5.4	0.8 ± 0.03	-
-	2.0	0.50	74 ± 4.8	1.2 ± 0.1	-
-	2.5	0.50	88 ± 6.2	2.5 ± 0.4	-
-	3.0	0.75	75 ± 5.2	1.7 ± 0.2	-
0.75	2.5	0.50	81 ± 4.8	2.4 ± 0.2	63 ± 5.4
1.0	2.5	0.50	85 ± 5.6	2.6 ± 0.5	78 ± 4.4
1.25	2.5	0.50	65 ± 3.3	1.5 ± 0.2	47 ± 2.8
1.5	2.5	0.50	41 ± 2.2	0.6 ± 0.08	35 ± 1.6

In tissue culture of *Hibiscus* species, Sami et al. (2016) also noticed the high frequency of calllogenic potency from stem explants of *H. syriacus* induced by 2.4-D. However, at increasing the concentration of 2,4-D callogenic potency of the stem explants was declined.

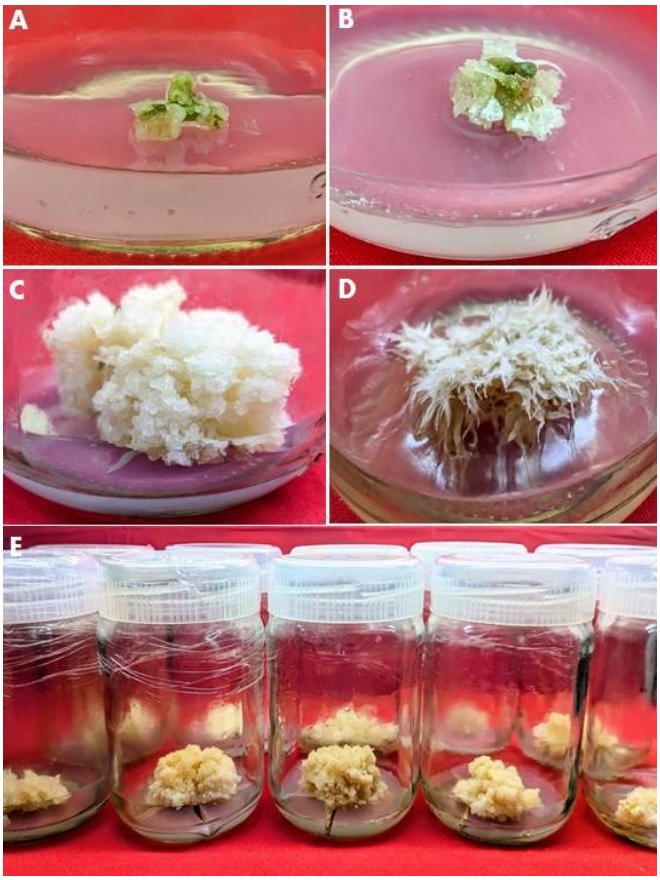

Fig 1. Morphogenesis of callus from stem explants of *H. lobatus:* (A) Callogenesis from the excised end of the stem explant on MS +1.25 mg/l of 2,4-D, (B) Extensive growth of the callus, (C) Luxuriant mass of whitish callus on MS + 1.25 mg/l of 2,4-D, (D) Rhizogenesis from the stem callus on MS 0.5mg/l 2,4-D, (E) Mass multiplication of stem callus on MS +1 mg/l of 2,4-D.

Subculturing of the stem calli onto the media fortified with a lower concentration of 0/l 2,4-D (0.5 mg/l) resulted in the organogenesis of tuft of root initials from the stem calli (Fig. 1D). In the culture of stem calli of *Caesalpinia bonduc* (Kumar et al. 2012) rhizogenesis from the stem calli was also reported. After four weeks of incubation tuft of whitish roots were differentiated from the stem calli (Fig. 1E). However, upon incubation on the same media, there was no sign of caulogenesis from the calli, but luxuriant proliferation of compact mass of calli was noticed at the concentration of with 1 mg/l of 2,4-D (Fig. 1E).

The application of plant tissue culture has become an important biotechnological tool which offers additional advantages for mass production of calli, genetic improvement of the species and isolation of therapeutic compounds from the calli grown in *in vitro* condition. The reports are very scare on the phytochemical profiling of *H. lobatus*. Chowdhury et al. (2017) reported the usage of *H. lobatus* leaves mucilage as pharmaceutical additives and cytoprotective and antidiabetic properties. The research conducted by Li et al. (2020) indicates that the phytochemical assessment of various *Hibiscus* species has identified a diverse spectrum of bioactive constituents, encompassing flavonoids, anthocyanins, terpenoids, steroids, polysaccharides, alkaloids, amino acids, lipids, sesquiterpenes, quinones and naphthalene derivatives. These constituents are associated with an array of pharmacological properties, including antibacterial, anti-inflammatory, antihypertensive, antifertility, hypoglycemic, antifungal and antioxidant activities. Mass-propagated stem calli of *H. lobatus* may function as a significant pharmacological resource for the pharmaceutical sector, thereby contributing to the alleviation of the excessive harvesting of this at-risk species.

The objective of this study is to establish an efficient and effective protocol for the micropropagation of *H. lobatus*. Currently, there are no reports available detailing methods for the *in vitro* propagation of this species. Callus-mediated plantlet regeneration appears to be a promising approach and could contribute to the conservation and improvement of endangered and vulnerable species. The callus cultures obtained from stem explants were transferred to fresh MS medium supplemented with 1.0 mg/l to 5.0 mg/l of each BAP and TDZ respectively showed caulogenic responses. The interaction of higher concentration of BAP (2.5-3 mg/l) with lower concentration of TDZ (0.5 mg/l) favoured shoot bud differentiation from the calli. Within a week of incubation, the primary white mass of callus became hard nodular creamy mass. After four weeks of incubation on the same media photosynthetic loci were differentiated from the callus (Fig. 2A), later the entire callus became photosynthetic with greenish lobelike structures (Fig. 2B). However, simultaneously differentiation of root initials was also noticed from the shoots and from the base of callus mass.

The frequency of shoot bud differentiation was more at the concentration of 3.0 mg/l BAP and 0.5 mg/l TDZ. The effect of interaction of BAP and TDZ on caulogenic potency of the stem callus is shown in the Table 1. As the concentration of TDZ increased the organization of photosynthetic loci were also decreased. On the contrary, BAP alone was in effective in the differentiation of shoot buds. Even after three subcultures onto the same media only the growth of photosynthetic mass of calli was noticed.

Numerous investigations have highlighted that 6-Benzylaminopurine (BAP) represents the most efficacious cytokinin, recognized for its capacity to stimulate cellular division, promote cellular elongation, and facilitate the genesis of shoots from callus or directly from explants (Bhalla et al. 2009, Bekircan et al. 2018, Faisal et al. 2020). The interplay between an optimal concentration of externally administered cytokinin and intrinsic growth regulators engenders organogenic responses from the cultured tissues.

Fig. 2. Interaction of BAP and TDZ on Multiple shoot differentiation from the stem callus of *Hibicus lobatus.* (A) Organogenesis of shoot buds (B) Multiple Shoot differentiation. (C) Growth of shoot with Palmate lobed leaves (D) Root intact regenerant (E) Hardened and soil acclimatized plantlets.

Research focused on the micropropagation of *Hibiscus sabdariffa*, *H. moscheutos* and *H. rosa-sinensis* has demonstrated that the induction of shoots is contingent upon the concentration of BAP, the specific plant species, and the nature of the explant employed (Kumar et al. 2016, Faisal et al. 2020, Lobodina et al. 2020). The photosynthetic caulogenic mass of calli was aseptically excised in to small pieces and subculture on to the media supplemented with 2.0 mg/l BAP and 0.5 mg/l TDZ showed the photosynthetic buds in to shoots (Table 1). After four weeks of incubation, the primary leaf primordial were developed in to palmately lobed leaves and root intact plantlets were developed (Fig. 2E). In the present study organization shoot and root initials noticed in single stage culture. The percentage of survival of regenerated plantlets was found to 78% in the field condition. This reproducible protocol is useful for ex situ conservation and genetic improvement of threatened and vulnerable plant species. Similar investigations on the derivation of micropropagation protocol of threatened and vulnerable medicinal plants

have been reported on *Entada pursaetha* (Vidya et al. 2005), *Caesalpenea bunduc* (Kumar et al. 2012), *Hydrastis canadensis* (Bedir et al. 2003), *Paulownia kowakamii* (Taha et al. 2008) *Silene fabaria (*Sarropoulou and Maloupa 2019), *Hibiscus coddii* (Helena et al. 2021).

H. lobatus is a vulnerable and endemic medicinal plant species of the Western Ghats, known to be a rich source of therapeutic compounds and holding significant potential as an anti-aging agent due to its antioxidant and skin-protective properties. Conventional methods of improving and selecting medicinal plants have limited potential to meet the growing demands of the industry and reforestation programs. Micropropagation, offers an efficient approach for large-scale production in a short time using minimal maternal material. Consequently, tissue culture techniques are commonly employed for the propagation of rare plant species, especially when the number and regenerative capacity of the mother plant are limited.

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References

Anil Kumar K and **Shivaraju H** (2016). A study on traditional knowledge and medicinal applications of the endemic herbal species in the Western Ghats of Shimoga Region, Karnataka, India. (IJRCE) **2**: 1-13.

Ayadi R, Hamrouni L, Hanana M, Bouzid S, Trifi M and **Khouja M L** (2011) *In vitro* propagation and regeneration of an industrial plant kenaf (*Hibiscus cannabinus* L.*).* Indust. Crops and Products **33**(2): 474-480. https://doi.org/10.1016/j. indcrop.2010.10.025.

Bedir E, Lata H, Schaneberg B, Khan IA and **Moraes RM** (2003) Micropropagation of *Hydrastis canadensis*: Goldenseal a North American endangered species. Planta medica. **69**(1): 86-88. https://doi.org/ 10.1055/s-2003-37039

Bekircan T, YaşarA, Yildirim S, Sökmen M and **Sökmen A** (2018) Effect of cytokinins on *in vitro* multiplication, volatiles composition and rosmarinic acid content of *Thymus leucotrichus* Hal. shoots. 3 Biotech. **8(**180): 1-9. https://doi.org/10.1007/s13205-018-1206-2

Bhalla S, Abdullah JO, Sreeramanan S and **Karuthan C** (2009) Shoots induction from *Hibiscus rosa-sinensis* nodal explant using N6-benzylaminopurine (BAP*).* Res. J. Agric. Biol. Sci. **5(**4): 403-410.

Chen C (2016) Cost analysis of plant micropropagation of *Phalaenopsis*. Plant Cell Tiss. Org. Cult. **126:** 167-175. https://doi.org/10.1007/s11240- 016-0987-4.

Chowdhury M, Sengupta A, Datta L and **Chatterjee** S (2017) Role of mucilage as pharmaceutical additives and cytoprotective agent. J. Innovat. Pharma. Biolo. Sci. **4**: 46-52.

Davies FT, Geneve R L, Wilson S B, Hartmann H T, Kester DE and **Hartmann (**2017) Plant propagation**:** principles and practices. 9. ed. New York: Pearson Education, 1024 p.

Debnath SC and **Arigundam U** (2020) *In vitro* propagation strategies of medicinally important berry crop, Lingon berry (*Vaccinium vitis-idaea* L.). Agronomy **10**(5): 744. https://doi.org/ 10.3390/ agronomy10050744.

Faisal M, Alatar AA, Abdel-Salam EM and **Qahtan AA (**2020) Effects of 4-CPPU on *in vitro* multiplication and sustainable generation of *Hibiscus rosa-sinensis* L. 'White Butterfly'. *Saudi* J. Biolo. Sci. **27(**1): 412-416.

Du Plessis HJ, Nikolova RV, Egan BA and **Kleynhans R** (2021) Preliminary study on *in vitro* shoot culture of *Hibiscus coddii*sub sp. *barnardii*, an indigenous South African flowering plant. https://doi.org/10.1590/2447-536X.v27i3.2353.

Kumar SR, Krishna V, Venkatesh PK and **Gnanesh UA** (2012) Indirect organogenesis from stem derived callus of *Caesalpinia bonduc* (l.) roxb–a medicinal plant of western ghats.

Kumar SS, Manoj P and **Giridhar P** (2016) Micropropagation for mass multiplication and enriched production of ascorbic acid in tissue culture foliage of roselle (*Hibiscus sabdariffa* L.). In Vitro Cellular & Developmental Biology. **52**: 427-436. https:// doi. org/ 10.1007/s11627-016-9785-2.

Li J, Lu YR, Lin IF, Kang W, Chen HB, Lu HF and **Wang HMD** (2020) Reversing UVB-induced photoaging with *Hibiscus sabdariffa* calyx aqueous extract. J. Sci. Food Agri. **100**(2): 672-681.

Lobodina E, Suprun I, AL-Nakib E, Avakimyan A and **Tyschenko E (**2020) Micropropagation of hardy *Hibiscus* (*Hibiscus moscheutos* L.). BIO Web of Conferences Bioengineering **25**: 1-6. https:// doi.org/10.1051/bioconf/20202505008

Saikia M, Shrivastava K and **Singh SS (**2013) Effect of culture media and growth hormones on callus induction in *Aquilaria malaccensis* Lam., a medicinally and commercially important tree species of North East India. Asian. J. Biolo. Sci. **6**: 96-105.

Sami AM, Hashish KHI, Sawsan SS and **Lobna ST (**2016) *In vitro* propagation protocol of *Hibiscus syriacus*L. plants. Int. J. Pharm. Tech. Res. **9**(11): 178-186.

Sarropoulou V and **Maloupa E** (2019) Micropropagation and *ex situ* conservation of *Silenefabaria*(L.) Sm. in Sibth. & Sm. subsp. Domokina Greuter (Caryophyllaceae); an important endemic plant in Greece with medicinal and ornamental value**.**

Srivatanakul M, Park SH, Sanders JR, Salas MG and **Smith RH** (2000) Multiple shoot regeneration of kenaf (*Hibiscus cannabinus*L.) from a shoot apex culture system. Plant Cell Reports **19**: 165-1170. https://doi.org/10.1007/s002990000256.

Lobna TS, Ibrahim SMM and **Farahat MMA** (2008) Micropropagation Protocol of *Paulownia kowakamii* through *in vitro* culture technique*.* Australian J. Basic Appl. Sci. **2**(3): 594-600.

Vidya SM, Krishna V, Manjunatha BK and **Shankarmurthy K** (2005) Micropropagation of *Entada pursaetha* DC-An endangered medicinal plant of Western Ghats.

Yang JE, Ngo HT, Hwang E, Seo SA, Park SW and **Yi TH** (2019) Dietary enzyme-treated *Hibiscus syriacus* L. protects skin against chronic UVB-induced photoaging via enhancement of skin hydration and collagen synthesis. Archives of biochemist. Biophys. **662**: 190-200.

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