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A review on progress and problems associated with *In vitro* propagation of *Commiphora wightii* (Arn.) Bhandari: an endangered medicinal plant species

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Abstract
The extinction comes near to the plant *Commiphora wightii* (Arn.) Bhandari, one the most pharmacologically and economically important slow-growing small tree belongs to a Genus *Commiphora* and the *Burseraceae* family. It is a flowering, dioecious plant, used in ayurvedic and Unani medicine in treating various ailments and disorders. It is commonly known as “Guggul” because of the presence of secondary metabolites “Guggulsterone” in oleo-gum resin. The species is now listed in the Red Data Book of IUCN because of the overexploitation of oleo-gum resin by the local population for economic benefits with no conservation efforts. This plant has much adequate attention on its conservation but it has not been focused yet. Conventional methods for the propagation of *Commiphora wightii* (Arn.) Bhandari are; the seeds, vegetative method through stem cuttings and air layering but they have many limitations too; seeds with slow-growing nature, poor seed set, poor seed germination due to hard endocarp, estimated 10% of seedling destroyed by insects and pests. Therefore, more advanced techniques involvement is important and beneficial to overcome these difficulties. Micropropagation is one of the most common and important biotechnological method which can develop a reliable and rapid protocol for its conservation. Despite many reports on the propagation of Guggul, tissue culture raised efficient plantlets for field performance has not been yet initiated. For commercial-scale efficient plantlet production, scientific studies are required to focus on the multiplication protocol and survival rates of plantlets in the greenhouse. An effective protocol for multiplication with the higher adaptive rate of the plant in field conditions can open the door for many future advances like breeding, embryogenic culture establishment, genetic transformation for resistance verities, higher production of oleo-gum resin, pharmaceutically active compound production and many more. Conservation efforts will require the involvement of local people and villagers. Scientific tapping practices are also required so that the plant is not killed after tapping.

Keywords: *commiphora wightii* (arn.) bhandari, endangered species, medicinal plant, micropropagation, oleo-gum resin

Introduction

Genus *Commiphora* contains approximately more than 200 species of balsamiferous trees and shrubs having the chromosome number 2n=26 [39]. The name *Commiphora* originates from the Greek words *Kommi* (meaning ‘Gum’) and *Phero* (meaning ‘to bear’) as the majority plants of the *Commiphora* genus which exude the fragrant ‘gum’ or ‘oleo-gum-resin’ from the damage made on the bark. The genus *Commiphora* belongs to the Burseraceae family which is also known as myrrh family and frankincense family which is a woody plant family [3, 50]. The genus comprises the plants which are slow-growing, drought-tolerant, and also adaptable to high temperature (45ºC). They prefer arid, semi-arid environments for natural rejuvenation where annual rainfall is 225-500mm [11].

The plants belong to *Commiphora* genus, are widely distributed or native to different countries of the world including tropical Asia [3, 48], India [10, 13, 40, 41, 49], Bangladesh and Pakistan [6, 40], South Africa, Tropical East Africa including Ethiopia, Kenya, Tanzania, Somalia, Bolivia, West Africa, Sri Lanka, and South America [22, 49, 50]. Approximately 190 species are African and are widely distributed in the desert area and dry habitat. The huge diversity of *Commiphora* plant species is found in the Somali-Masai region of Northeast Africa. The distribution further extends to Australia, Pacific Island [18], and Saudi Arabia [26].

Five species of *Commiphora* genus found in India including *Commiphora wightii* (Arn.) Bhandari which is previously known as *Commiphora mukul* Hook. ex Stock or *Commiphora roxburghii*, *Commiphora agollocha* Engl., *Commiphora stocksiana* Engl., *Commiphora berryi* (Arn). Engl and *Commiphora myrrha* [1, 3, 7]. *Commiphora agollocha* Engl. is found in the seasonally tropical area of Orissa (Baragarh) [43] whereas *Commiphora berryi* (Arn). Engl is grown as a hedge plant in Southern India Tiruchirapalli (Tamil Nadu) [31], Idukki (Kerala) as locally known as Mulkiluvai and also reported in Gwalior (Madhya Pradesh) [5, 18, 36]. *Commiphora stocksiana* Engl. is grown in Anand (Gujarat) while *Commiphora myrrha* is found in Jabalpur (Madhya Pradesh) and Anand (Gujarat). *Commiphora wightii* (Arn.) is native to India (Gujarat, Rajasthan, Madhya Pradesh, Karnataka, and Maharashtra), Pakistan (Sind and Baluchistan), and Bangladesh [1, 6, 13, 40, 44, 45]. *Commiphora wightii* (Arn.) Bhandari comparatively occurs in wider distribution other than four species. It is mainly found in arid rocky tracts, semi-arid, and sandy tracts in India[40]. In Gujarat, it is mainly found in the whole Kutch region comprising three main ranges: 1) Bhuj, 2) Nakatrana, 3) Anjar [1, 32], and also reported in some parts of Saurashtra regions [27].

In Rajasthan, it chiefly occurs in the western region, includes Sawai-Madhopur, Bundi, Kota, Jaisalmer, Barmer, Jalor, Siwana, Bhin Mal, Bisala, Jodhpur, Sirohi, Pali, Nagpur, Sikar, Churu, Bikaner, Alwar, Jhunjhunu, and Udaipur [1,43]. It is also found in some districts of Madhya Pradesh which includes Morena, Bhind, Shiv-puri, Sheopur, and Damoh.

In India, 'Guggul' or 'Oleo-gum-resin' is produced by the five species *Commiphora wightii* (Arn.) Bhandari, *Commiphora agollocha* Engl, *Commiphora stocksiana* Engl, *Commiphora berryi* (Arn). Engl and *Commiphora myrrha*. Out of these five species, *Commiphora wightii* (Arn.) Bhandari is a good quality producer of guggul [36]. It is often confusing to identify guggul from other resinous products obtained from the other species of *Commiphora* [1].

The plant name 'Guggul' originates from the Sanskrit Language. The generic name of the plant presented as a 'Guggul' means having an active steroidal ingredients 'Guggulesteron', found from the guggul gum and it was used to treat verities of disorders in the ancient history of Ayurvedic system of medicine. Locally, the plant is known as different names which include 'Guggul' in Hindi, 'Indian Bdellium' in English and 'Guggulu', 'Ahavabhishtha', 'Mahishaksha', 'Purah', 'Sarvasaha', 'Kumbholu' in Sanskrit while 'Gukkulu', 'Maishakshi', 'Kungiliyam' in Tamil, and 'Guggur' in Gujarati [18, 21, 23, 36].

The guggul plant species exist in three forms i.e. male, female, and polygamous and it is dimorphic. It is a small (3-3.5 meters), slow-growing tree or shrub and it takes 8-10 years to reach maturity. The flowers are small, subsessile, 2-3 together and reddish-pink and may be unisexual or bisexual. Leaves are trifoliate, alternate or fascicled and leaflets glabrous, rhomboid to ovate, terminal ones the largest. Branches are having sharp spines in end, crocked knotty, and aromatic. Fruits are single or 2-3 in a bunch, ovoid, 6-7 mm in diameter, and bright red when ripe. Seeds are bilobed, trilobed, and sometimes or rarely tetra lobed, ovoid, matured ones are yellowish white-black, black, and immatured reddish-brown. Physically, all seeds having no significant differences [11, 18, 25].

In India, Guggul plant species is considered as endangered or threatened and recorded in the Red Data Book of IUCN. It is listed as 'Data Deficient' plant in IUCN Red Data List ver. 3.1 (IUCN, 2015) because of a lack of knowledge about its conservation and also ruthless over-exploitation of oleo-gum-resin or guggul gum using unscientific tapping methods and it is also included in RET (Rare, Endangered, Threatened) by Government of India [28]. Only a few wild communities of this plant exist in the state of Gujarat and Rajasthan.

1.1 Medicinal importance of *C. wightii*

Atharva Veda (2000 B.C) has been mentioned that Guggul is one of the old Ayurvedic drugs. As per Sushrut Samhita, guggul is, when taken orally, healing of obesity, liver dysfunction, internal tumors, malignant sores and ulcers, urinary related complaints, fistula-in-ano, intestinal worms, leucoderma, sinus, edema, and unexpected paralytic seizures. It is additionally viewed as a cardiac tonic [29, 30]. Guggul was introduced to the medication world in 1966 by G. V. Satyavati. Her investigations of guggul on rabbits were directly inspired by Ayurvedic content, in which guggul was prescribed for the treatment of atherosclerosis, and in 1986, guggul was recommended as Hypolipidemic drug in India [2, 19]. In the middle of the 1990s, guggul was presented as a recipe for hypercholesterolemia and other cardiovascular diseases in the western clinical world. In the 1990s, novel medications from guggul were created to treat and manage different cardiovascular issues, for example, hypolipidemic, and so on [24].

Oleo-gum-resin of Guggul helps in decreasing elevated cholesterol since it brings down harmful low-density lipoproteins while elevating the valuable high-density lipoproteins. It helps to prevent blood platelet aggregation and separates previously formed blood clumps. In this manner, it aids to prevent cardiac illness and stroke. It is utilized in the treatment of muscular rheumatism. Guggul is useful as a weight reduction specialist, stimulates the action of white platelets in the body, development of the immune system, dispose of expelling dead tissues, wastes, toxins from the body, in indolent ulcers as a gargle in pyorrhoea alveolaris, chronic tonsillitis, pharyngitis, relieve coughing, lung congestion, and other respiratory issues.

Various most important ayurvedic remedies of guggul are Chandraprabha Vati, Yogaraja guggul, Vyoshadi guggul, Trayodasanga guggul, Kaisora guggul, Gokshuradi guggul, Lakshadi guggul, Punarnava guggul, Sadanga guggul, Vatari rasa, Amrita guggul, Kanchanara guggul, Triphala guggul, Shimhanada guggul, etc [33].

2. Progress on *In vitro* propagation of *C. wightii*

Several techniques are being developed for the conservation of a Red Data book enlisted endangered plant species *Commiphora wightii* (Arn.) Bhandari. From those techniques, Plant tissue culture is one of the most promising techniques which are used for plant multiplication and also proved beneficial in the conservation of several endangered species. Micropropagation is a technique to produce many progeny plants within a short period by multiplying stock plant material in an artificially controlled environment. Most significant events of micropropagation are listed below:

1. Organogenesis (Direct regeneration)
2. Callus Induction (Indirect regeneration)
 I. Organogenesis
 II. Embryogenesis

2.1 Major steps involved in Micropropagation are mentioned below

0. Preparation stage (Selection of mother plants and various explants)
1. Initiation stage
2. Multiplication Stage
3. Rooting Stage
4. Acclimatization

2.1.1 Selection of elite mother plants

No systematic shreds of evidence are available for selection and screening of elite mother plant but careful consideration should be given to the selection of elite mother plants before commencing micropropagation. It should be healthy, free from disease, and true to type and also should be certified for desirable characters by certifying agency. Most probably good attentions for mother plants are given to the controlled environment growing stock plants for mass production of the plant. These precautions provide more responsive explants and produce a contamination-free culture. The selection of elite mother plants is the most important factor for the successful establishment of any plant driven mass production protocol for micropropagation. These all are the general precautions which should be taken for the selection of mother plant for any plants.

Specifically, for *C. wightii*, Brave and Mehta [3] selected or screened 48 trees according to the variation in the ketonic and guggulsterone fractions from the three different localities. Mishra and Kumar [20] reported they have screened elite plants of *C. wightii* based on different locations along with oleo-gum-resin content.

2.1.2 Selection of various explants from the plant

The explant is a piece of tissue obtained from the vigorous mother plant to be cultured in a controlled environment by providing necessary nutritional values.

The selection of different types of explants from the elite mother plant is an art itself because a limited number of selected explants are taken from the healthy mother plant which is having thousands of different types of explants. The quality of explants determines the response and establishment of contamination-free culture.

Fresh explants consisting of Nodal segments, inter-nodal segments, leaves, shoot tips, auxiliary buds, and embryos (fruit) of *C. wightii* are used for the tissue culture applications. (Table 1) Brave and Mehta [3] reported seasonal effects on nodal explants collection. Explant collection during April-October determines the most suitable time to initiate culture. Sprouting was reduced when explants collected between July-August in comparison with April-June whereas poor growth of explants was observed during September-October. Soni.V [40] also reported that April is favorable for culture initiation when the plant is growing with the full flowering stage. Shweta.N [35] mentioned that the sampling during different seasons from different regions was affected on the growth of *in vitro* plants. She has also mentioned that pre-monsoon seemed more favorable than post-monsoon for the development of callus from the nodal explants and also for the production of secondary metabolites.

Kumar and Kant [13] developed a technique for the selection of nodal explants from the mature elite tree. They reported that the young juvenile branches of the plant were divided into three-part; 1. Immature, 2. Semimature, and 3. Mature from these categories semimature young new branches (having 6-7 nodes) selected and these nodes were excised after removing the initial 5-6 cm terminal portion after that each one used as an explant.

Kant *et al.* [10] used the water submergence selection process for seeds (mature fruits) selection. They mentioned that the fruits either remained submerged or floated and were accordingly classified as sinkers and floaters. Mostly floaters have empty locules with small, inappropriately developed or no seed so the floaters were rejected while sinkers are further classified into three classes according to their weight. High weight seeds were isolated from the fruits and used for further investigation.

Table 1: Various explants of *C.wightii* used for micropropagation.

Explants	Reference
Micropropagation	
Apical and Axillary Meristems	Bhardwaj and Alia [4]
Nodes, shoot Apexes, and leaves	Verma *et al.* [47]
Nodes, shoot Apexes, and leaves	Jakhar *et al.* [8]
Nodes and Shoot Apexes	Shekhawat *et al.* [34]
Nodes and Leaves	Kumavat *et al.* [17]
Nodes	Joshi and Mathur [9]
Nodes	Shweta.N [35]
Nodes	Kumar and Kant [13]
Nodes, shoot tips, and axillary buds	Tejovathi *et al.* [42]
Nodes	Soni.V [40]
Nodes, inter-nodes, and leaves	Singh *et al.* [38]
Fruits (Seeds), Nodes and Micro shoots	Kant *et al.* [10]
Nodes, Leaves	Mishra and Kumar [20]
Nodes	Yusuf *et al.* [51]
Nodes	Brave and Mehta [3]
Somatic Embryogenesis	
Fruits	Mishra and Kumar [20]
Immature fruits	Kumar *et al.* [14]
Immature fruits	Kumar *et al.* [15]
Immature fruits	Kumar *et al.* [16]
Immature Zygotic embryos	Singh *et al.* [37]

2.1.3 Different surface sterilization chemicals are used to avoid contaminations

Usage of different chemical solutions for surface sterilization process is an important step to remove contaminants from the explants. A large amount of microflora is known in woody and mature plants as growing in the open field and collected explants from these plants are difficult to sterilise [35]. To find a crucial sterilization process is very difficult in the establishment of any plant tissue culture protocol. Generally, $Hgcl_2$, NaOcl, and sometimes fungicide is also used to sterilize plant material.

(Table 2.)

Table 2: Various chemicals are used to sterilize various plant materials (explants)

Explants	Chemical agents	Reference
Apical and Axillary Meristems	Fungicide (Bavistin) (60 min), liquid soap solution, 70% Ethanol (30 sec), 0.1% $Hgcl_2$ (5 min)	Bhardwaj and Alia [4]
Nodes, shoot Apexes, and leaves	Rankleen (Liquid detergent) (20 min), Fungicide (Bavistin) (5-10 min), 90% Ethanol (30 sec), 0.1% $Hgcl_2$ (2-5 min)	Verma *et al.* [47]
Nodes, shoot Apexes, and leaves	Rankleen (Liquid detergent) (10 min), Fungicide (Bavistin) (5-10 min), 0.1% $Hgcl_2$ (2-5 min)	Jakhar *et al.* [8]
Nodes and Shoot Apexes	Rankleen (Liquid detergent) (10 min), 0.1% $Hgcl_2$ (3-4 min)	Shekhawat *et al.* [34]

Nodes and Leaves	Rankleen (Liquid detergent) (10 min), 0.1% $Hgcl_2$ (1-2 min)	Kumavat *et al.* [17]
Nodes	Tween 20 (10 min), Fungicide (Bavistin) (5-10 min), 70% Ethanol (30 sec), 0.1% $Hgcl_2$ (3-6 min)	Joshi and Mathur [9]
Nodes	Tween 20, 0.1% $Hgcl_2$ (1-2 min)	Shweta.N [35]
Nodes	Tween 80 (10 min), Fungicide (Bavistin 200mg + Streptomycin 50mg in 100ml RO water) (10 min), 2.5% NaOCl (7-10 min)	Kumar and Kant [13]
Nodes	0.1% $Hgcl_2$ (6 min)	Soni.V [40]
Nodes, inter-nodes, and leaves	Liquid detergent, 0.1% $Hgcl_2$ (3-5 min)	Singh *et al.* [38]
Fruits (Seeds), Nodes and Micro shoots	Tween 80 (10 min), Fungicide (Bavistin 200mg + Streptomycin 50mg in 100ml RO water) (10 min), NaOCl (providing 5% available chlorine) (5 min)	Kant *et al.* [10]
Nodes, Leaves and embryos	Tween 20, 0.1% $Hgcl_2$ (5 min), Mature seeds treated with 0.1 N HCL	Mishra and Kumar [20]
Seeds, Nodes	Seeds: 70% Ethanol containing nystatin and streptomycin (10ppm) (1min), 0.1% $Hgcl_2$ (20 min), 0.1% $Hgcl_2$ (10 min) once more after 48h, Nodes: Mild detergent solution, 70% ethanol containing nystatin and streptomycin (10ppm) (5 sec), 0.1% $Hgcl_2$ (2-5 min)	Brave and Mehta [3]

2.1.4 Detailed information about various explants, its suitable medium with different combinations of plant growth-promoting regulators and other additives which are added to support the culture

2.1.4.1. Nodes culture

Selection techniques of mother plants and their juvenile parts (explants) are mentioned earlier.

Various chemicals are used to sterilize the explants for reducing the chances of contamination in culture medium and distilled or autoclaved distilled water washes (2-3 times) are given to the explants after applying each chemical treatment.

In *C. wightii* different combinations of auxins and cytokinins with additives in media were tried for the establishment of the Nodal explants in controlled environmental conditions.

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2.1.4.1.1 Organogenesis (Direct regeneration)

Initiation of Nodal culture and Multiplication of Shoots: Incorporation of 18.6 µM (4 mg/l) Kinetin (Kn) and 17.8 µM (4 mg/l) benzyladenine (BA) in the MS media supplemented with additives i.e. 100 mg/l glutamine, 10 mg/l thiamine HCL, and 0.3% activated charcoal (MS-1) was found better medium and achieved highest frequency (75%) of shoot formation. Seedling material nodal explants induced a higher number of shoots (4.1 shoots) per explants and shoot length (1.7 cm) as compared to the adult material nodal explants (2.8 shoots/explant, 1.5 cm shoot length). The appropriate concentration of glutamine and AC was prevented pre-mature leaf drop and beneficial for shoot growth respectively. To promote shoot elongation, grown explants were transferred to MS medium supplemented with 1.9 µM Kinetin, 1.8 µM BA, 10 mg/l thiamine HCL, and 0.5% AC (MS-2) both in the liquid and solid forms thus solid MS proved to be better for pre-rooting elongation of shoots. Transfer of rooted plantlets on half strength of white's modified medium (enhancing the level of KCL 845 mg/l, NaH_2PO_4 300mg/l, additional nitrogen source in the form of $(NH_4)_2SO_4$ 790 mg/l and $NaNO_3$ 1800 mg/l, and Iron source was supplied as in MS medium instead of White's medium) noticed further elongation of shoots and also obtained well-developed root system.

Rooting: Elongated shoots were treated with auxins (4.9 µM IBA in combination with 5.7 µM IAA or 5.4 µM NAA) for 24h in the dark condition which proved optimal and all plantlets were transferred to rooting medium (half strength of MS with 0.5% AC and 2% Sucrose (MS-3), and full strength of White's medium in both liquid and solid form) thus MS-3 invoked higher frequency (60%) as compared to the White's media (40%) within 3-4 weeks.

Acclimatization: The rooted plantlets were transferred to pots containing sterile vermiculite: sand (1:1) under culture room conditions. They were covered with an inverted glass beaker to maintain humidity and treated by one fourth strength MS mineral salts twice in a week and daily tap water. In the beginning, beaker removed initially for a few hours daily then removed completely after 2 weeks and transferred to the greenhouse after three weeks when new leaves were grown. Pre-transplantation to the greenhouse, 60% of the plants survived. The Plants, once established in soil showed uniform growth and no physiological abnormalities have been observed [3].

Initiation of Nodal culture and Multiplication of Shoots: Cotyledonary nodes were inoculated on MS media supplemented with 2.68 µM NAA, 4.4 µM BAP, and additives (glutamine 684.20 µM, thiamine 29.65 µM, and 0.3% AC) showing 96.7% bud break response and without adding additives in medium bud break response decreased to 86.7%. Micro-shoot elongation was poor on bud break medium so all micro-shoots were further transferred to elongation media (MS media containing 2.46 µM IBA and 2.22 µM BAP) and was induced higher percentage (70%) of micro-shoots elongation.

Rooting: All proliferated shoots were transferred to MS media supplemented with 4.92 µM IBA for 24 hours. Plant growth regulators free White's Medium with 5% AC was found better treatment for best rooting and maximum rooting (43.33%) was achieved.

Acclimatization: All rooted plantlets were shifted to a glass jar filled with quarter level vermiculite moistened by Hoagland's solution. The plastic cap of glass jar was loosened gradually overtime period of 2-3 days when plantlets showed new growth after 4-5 weeks then all transferred to small cups holding vermiculite moistened by Hoagland's solution and placed in mist chamber. After two weeks plantlets were transferred to soil: FYM (Farm Yard Manure) (1:1) in poly bags in the mist chamber where 1.5 minutes misting at 10 minutes interval was given to maintain 85-95% relative humidity and also the temperature was maintained between

28-30 °C. One month later, all plantlets were shifted under 50% agro-net shade and after two weeks transferred to field conditions where all plantlets were growing well [10].

Initiation of Nodal culture and Multiplication of Shoots: Hundred percent (100%) bud break response with the single shoot (1.7 cm) was observed after 10 days of inoculation when nodal explants inoculated in MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA whereas alone 1.0 mg/l BAP with MS media showed 90% bud break response with the single shoot (1.5 cm) after 9 days of inoculation.

Rooting: Aseptically all proliferated shoots were excised and implanted on half MS media supplemented 2.0 mg/l IBA, was found better treatment for root formation (80%) but thin and long roots were observed after 20 days of implantation while MS media supplemented 2.0 mg/l IAA showed short and healthy root formation (70%) after 23 days of implantation.

Acclimatization: After root development, all plantlets were taken to transfer in the sterilized soil and sand mixture (3:1) in pots. Each pot covered with a polythene bag to maintain humidity and irrigated (alternative days) with half MS salts solution after that all plantlets were gradually transferred to natural conditions where sixty percent (60%) of plants survived or acclimatized well [38].

Initiation of Nodal culture and Multiplication of Shoots: During flowering stage of mother plants, nodal explants were collected and inoculated on MS media supplemented with 0.5 mg/l Kn, 3.0 mg/l BAP, and 0.5 mg/l IBA, induced multiple shoot formation and the number of shoot bud induction increased when explants were treated with the same medium but 0.5 mg/l IBA replaced with 0.5 mg/l IAA.

Rooting: All sprouted shoots were transferred to MS media added with 0.5 mg/l IAA and 1.0 mg/l NAA, showed effective root initiation.

Acclimatization: Proliferated plantlets with the developed root system (5-6-week-old) were shifted in plastic pots containing sterilized soil and soilrite (a mixture of exfoliated vermiculite, peat moss, and horticulture grade perlite) in the ration of 1:3. All pots having plantlets were covered with polythene bags to maintain humidity and placed in the culture room for 10 days after that they were transferred to a plastic bag having soil and manure (1:3) under 50% agro-net. Only 5% of plants were survived in the field conditions [40].

Initiation of Nodal culture and Multiplication of Shoots: Nodes were inoculated in the MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l IBA, showed shoot development response (6.6%) followed by 6.6% response in MS medium supplemented with 3.0 mg/l BAP and 0.3 mg/l IBA while all other combinations of auxin and cytokinin failed to show any response from the nodal explant of *C. wightii*. Location of explants on the mother plant, Apical dominance and size of explants are the major factors to assess regeneration ability from the explant.

Location of explant on mother plant: Shoot tips and its two successive nodes (1^{St} and 2^{nd} nodes) of each twig inoculated on MS media containing 3.0 mg/l BAP, 0.05 mg/l IBA, and 0.5 mg/l GA_3, showed highest shoot development response (16%) in shoot tips followed by 1^{St} nodes (9.2%) and 2^{nd} nodes (6.0%).

Apical dominance: Best percentage response (27%) of node-ADs (nodes from branches with shoot tip and leaves removed 2-3 days before inoculation) were observed on MS media supplemented with 2 mg/l BAP and 0.5 mg/l GA_3. **Size of explants:** Node-ADs (1. Single node (0.5-0.8 cm) and 2. Node-ADs with two nodes and two internodes were inoculated on MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l GA_3, showed shoot development response (28%) in Single node-ADs and 26.92% response in two nodes with two internodes-ADs. However, the leaves sprouted from single nodes were dropped and callus initiation observed from the same position while 2^{nd} node-ADs with two nodes with two internodes were survived for more than 6 weeks on the same medium.

Rooting: Regenerated shoots were dipped in auxin solutions (200ppm) (IAA+IBA, IAA+NAA, and IBA+NAA) and then transferred to the MS media supplemented with AC. The highest root initiation response (26.22%) reported when shoots were treated with IBA+NAA solution followed by 25.55% response when dipped in IAA+IBA solution and 24.55% response when treated IAA+NAA solution within 20 days of culture.

Acclimatization: All rooted plantlets were further transferred to wick culture (nutrients solution moves up the wick from the solution reservoir through capillary movement and reach to plant root system) and successfully acclimatized in the filed pots [42].

Initiation of Nodal culture and Multiplication of Shoots: Nodal segments were excised from the new juvenile branches of *C. wightii* and inoculated in MS media supplemented with 8.88 µM BAP, 0.57 µM IAA and other additives (50 mg/l ascorbic acid, 25 mg/l citric acid, and 25 mg/l adenine sulphate) induced highest bud break response (84.5%) within 2 weeks of inoculation. For further elongation and shoot multiplication, these micro-shoots were subcultured and maintained on the same media for 4 weeks. The same experiments were repeated on B5 medium and WPM. The highest percentage of bud break response (53.8%) reported on B5 medium and 65.4% response reported on WPM. MS media with various PGRs and other additives was found a better medium for initiation and multiplication of nodal explants and mean shoot length (0.65 cm) was also higher than the explants which were inoculated in B5 (0.35 cm) and WPM (0.28 cm).

Rooting: Proliferated micro- shoots were dipped in liquid MS and White's medium supplemented with 4.92 µM IBA and 5.71 µM IAA for 24 hours under the dark condition and further transfer to semi-solid half-strength MS and White's medium added with 2% Sucrose and 0.5% AC. The highest root development response (86.7%) was recorded on half-strength hormone-free MS media after 4-5 weeks with multiple adventitious roots (2.85) of 6.46 cm length whereas only 46.7% root response was reported on White's medium.

Acclimatization: *In vitro* hardening in the culture room, well-rooted plants were removed gently and then washed for 5-10 min by autoclaved distilled water to remove traces of medium. These plantlets gained 3-5 cm height within 4-5 weeks and during *In vitro* hardening, plantlets showed a 62% survival rate. *Ex Vitro* hardening in the plastic cup at the mist chamber and polythene bags in greenhouse shade showed 100% survival of plantlets. Finally, healthy and robust plants were transferred to field conditions after five months [13].

Initiation of Nodal culture and Multiplication of Shoots: Nodal segments having 1-2 nodes form young juvenile branches were inoculated on modified MS media supplemented with 3.0 mg/l BAP, 0.5 mg/l IAA and other additives (50 mg/l ascorbic acid, 25 mg/l citric acid, 25 mg/l arginine and 0.3% AC), showed maximum bud break response (82%) with the mean number of shoots (2.40) per explant and mean shoot length (1.82 cm). Further multiplication, initial proliferated shoots with mother explants were subcultured to modified MS media supplemented with 3.0 mg/l BAP, increased 3-fold multiplication of shoots within 30 days. **Rooting:** All elongated shoots were dipped in liquid modified MS medium and White's medium added with 0.5 mg/l IBA under the dark condition for 2 days and further transfer to semi-solid half-strength MS and White's medium added with 2% Sucrose and 1% AC. The highest root development response (85%) was recorded on half-strength hormone-free MS media within 40 days with multiple adventitious roots (3.21) of 6.04 cm length whereas only 54% root development response with adventitious roots (2.76) of 5.87 cm length was reported on White's medium.

Acclimatization: Hardening in culture room, well-rooted plants were removed gently and then washed for 5-10 min by autoclaved distilled water to remove traces of medium and placed in small plastic pots having sterile soilrite. To maintain humidity, all pots were covered with polythene bags and all plantlets were irrigated with half MS salts solution on alternate days. Polythene bags were removed after 2 weeks. Acclimatize all the plantlets to the field conditions (greenhouse) gradually and then transfer to the natural condition [9].

Initiation of Nodal culture and Multiplication of Shoots: Maximum shoot bud induction response (90%) was obtained from the nodal explants on MS media supplemented with only cytokinin 3.0 mg/l BAP [34]. While a hundred percentage bud break (100%) response reported when explants inoculated on MS media supplemented with 1.5 mg/l BAP [47].

2.1.4.1.2 Callus Induction (Indirect regeneration) from the nodal explant

Callus induction (90%) initiated from the nodal explants when they were inoculated in MS media containing 2.0 mg/l NAA while inoculation of explants in MS media supplemented with 2.0 mg/l Kn and 1.0 mg/l NAA was found best for callus induction as well as callus growth [38].

Nodal explants inoculated on the MS media supplemented with 5.0 mg/l 2,4-D and 0.5 mg/l Kn, showed better treatment for callus induction, and reported maximum guggulsterone (0.062%) content from the callus [20].

Callus induction reported from the nodal explants when they were inoculated in MS media supplemented with various concentrations of BAP (1, 2 mg/l) and the combination of BAP and GA_3 (1+0.5, 1+1, 1+1.5, 2+0.5, 2+1, and 2+1.5 mg/l) [42].

Hundred percentage frequency of callus induction was observed when nodal explants were inoculated on MS media supplemented with 5.0 mg/l BAP and 5.0 mg/l NAA, showed compact and light brown callus (0.80g) proliferation. Shoot bud induction (90%) with profuse callus (100%) at the end of explants was observed when treated with 1.0 mg/l 2,4-D on MS media [17].

2.1.4.2. Internode culture

Inter nodal segments were used as explants and inoculated on MS media supplemented with various PGRs but they were failed to respond as direct organogenesis and indirect organogenesis (Callus induction) [38].

2.1.4.3. Leaf culture

There is no evidence of direct organogenesis from the leaf culture but callus induction (indirect organogenesis) was reported.

2.1.4.3.1. Indirect organogenesis from the leaves

Callus formation from the leaves reported when leaves explant were inoculated in MS media supplemented with Kn and 2, 4-D individually and in combinations. Initially, green callus was observed and later on turned yellowish to brownish with maturity [20].

Hundred percentage (100%) callus induction was observed in leaf segments inoculated on MS MS media supplemented with 2.0 mg/l NAA and 2.0 mg/l 2, 4-D individually. Both PGRs were equally effective in the term of callus growth. Callus was brownish (2.0 mg/l NAA) and white (2.0 mg/l 2, 4-D) in colour. Callus initiation started from the cut ends of the leaf explants and finally cover the whole surface of the explants [38].

Leaf explant was inoculated in MS media supplemented with 2.0 mg/l 2,4-D, showed maximum callus (semi-compact and light green) proliferation (0.90g) followed by Friable and light green callus proliferation (0.87g) on MS media added with 5.0 mg/l Kn and 0.5 mg/l NAA [17].

(Table 3)

Table 3: List of different Mediums supplemented with Cytokinins, Auxins, and other additives used for the micropropagation of *C. wightii*

Explants	Medium	Cytokinin	Auxin	Other additives	Reference
Apical and Axillary Meristems	MS Media	Kinetin, BAP	NAA	Ascorbic acid, Citric acid, Adenine sulphate, AC	Bhardwaj and Alia [4]
Nodes, shoot Apexes, and leaves	MS Media	Kinetin, BAP	2,4-D	No Additives	Verma *et al.* [47]
Nodes, shoot Apexes, and leaves	MS Media	Kinetin, BAP	2,4-D	Ascorbic acid, Citric acid, AC, Polyvinyl-pyrrolidone	Jakhar *et al.* [8]
Nodes and Shoot Apexes	MS Media	Kinetin, BAP	NAA, IAA	No Additives	Shekhawat *et al.* [34]
Nodes and Leaves	MS Media	Kinetin, BAP	NAA	No Additives	Kumavat *et al.*

					[17]
Nodes	MS Media and Gamborg's B5 Media	BAP	IAA, IBA	Ascorbic acid, Citric acid, Arginine, AC	Joshi and Mathur [9]
Nodes	MS Media	Kinetin, BAP	2,4-D, IAA, IBA, NAA	Polyvinyl-pyrrolidone, AC	Shweta.N [35]
Nodes	MS Media, Gamborg's B5 Media, and WPM (Woody Plant Medium)	Kinetin, BAP	NAA, IAA, IBA	Glutamine, Ascorbic acid, Citric acid, Adenine sulphate, AC, Thiamine-HCL	Kumar and Kant [13]
Nodes, shoot tips, and axillary buds	MS Media	Kinetin, BAP	IAA, IBA, NAA	Gibberellic acid (GA_3)	Tejovathi *et al.* [42]
Nodes	MS Media	Kinetin, BAP	IAA, IBA, NAA	No Additives	Soni.V [40]
Nodes, inter-nodes, and leaves	MS Media	Kinetin, BAP	IAA, NAA, 2,4-D, IBA	No Additives	Singh *et al.* [38]
Fruits (Seeds), Nodes and Micro shoots	MS Media, Gamborg's B5 Media, and White's Media	Kinetin, BAP	IAA, NAA, 2,4-D, IBA	Glutamine, AC, thiamine	Kant *et al.* [10]
Nodes, Leaves and embryos	MS Media	Kinetin	2,4-D	No Additives	Mishra and Kumar [20]
Immature fruits	MS Media and Gamborg's B5 Media	Kinetin	2,4,5-T	ABA, AC, Mannitol	Kumar *et al.* [14]
Immature fruits	MS Media and Gamborg's B5 Media	Kinetin, BA	2,4,5-T, IBA, IAA	ABA, AC, Gibberellic acid	Kumar *et al.* [15]
Immature fruits	MS Media and Gamborg's B5 Media	Kinetin, BA, TDZ, 2iP,	2,4,5-T, IBA, 2,4-D, Picloram, IAA	ABA, AC, Gibberellic acid	Kumar *et al.* [16]
Seeds, Nodes	MS Media, White's Media, and White's modified Media	BA, Kinetin, 2iP, Zeatin	IAA, NAA, IBA	Glutamine, AC, Adenine, PG, Thiamine-HCL	Brave and Mehta [3]

3. The success of any culture for this plant largely depends on the following factors

- Purpose of study (*In vitro* multiplication, Secondary metabolites production, etc.).
- Selection of elite mother plants as described earlier.
- Selection of Explants as described earlier (Quality of explants, Genotype, Physiological state of the tissue, Time of the collection in a year, Seasonal changes, apical dominance, Location of the explants, and types of explants).
- Appropriate sterilization methods (Different for each explant type).
- Selection of growth-promoting medium.
- Cytokinins and Auxins concentration requirements are different for each type of culture.
- Other additives addition in the medium for promoting the growth of explants.
- The light intensity requirement is different for each type of explants.
- After sprouting, transfer to another medium for multiplication.
- Selection Rooting methods (*In vitro or Ex vitro*) with different growth regulators requirements.
- Hardening methods (mist chamber, greenhouse, and field conditions), Appropriate method selection is very important at this stage because all works which you have done nicely, this will not be worked if your hardening steps are not best.

4. Problems associated with *in vitro* propagation of *C. wightii*

1. Limited resources are available to tackle issues related to the *in vitro* propagation of *C. wightii* and it is highly difficult because the plant is recalcitrant.
2. All resources which are available right now, are talking about the regions which they belong to because of seasonal variations, and locations of the mother plants are crucial for propagation of *C. wightii*.
3. All protocols which are developed earlier, will not affect the same on your experiments for sure.
4. All concentration of Auxins, Cytokinins, and other additives will not work the same as they worked earlier even unpredictable results you will get if you follow the same experimental work.
5. *C. wightii* shows strong apical dominance so consideration must have to give at the time of explant selection.
6. The same sterilization process will not give the same results as reported earlier. Modification is needed.
7. Explants selection methods described earlier will serve your purpose but as mentioned earlier it is solely depending on the nature of explants.
8. Premature leaf drop is a big hurdle but it can resolve using other additives as mentioned earlier.
9. Browning of the callus is not a major issue; you can resolve by adding different additives in the medium.
10. Rooting of the sprouted plantlets is a major problem for this plant because very short and thin roots developed so when you try to out the plant from the vessels, it will break.
11. Inappropriate hardening will lead to the failure of whole experiments. You have to maintain all the things very sophistically includes nutritious, humidity, soil texture, and temperature.
12. MS media supplemented with various PGRs and other additives is the only medium that can serve the purpose of the study. No other mediums are effective on *C. wightii*.
13. You have to make your protocol for the propagation of

this plant because previously published protocols will not be worked effectively but after an extensive review of this concern, admitted some points, use BAP for sprouting, 2, 4-D for callus induction, IAA, IBA, and NAA for rooting, these will help you at beginning of the experiments but the same concentration value of this PGRs will not work at all so you have to modify the developed protocol according to the nature of explants.

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