RESEARCH ARTICLE

STANDARDIZATION OF MICROPROPAGATION TECHNIQUE FOR *IN VITRO* CALLUS GENERATION FROM NODAL EXPLANTS OF *BUCHANANIA COCHINCHINENSIS* (LOUR.) ALMEIDA

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Abstract: The present research entitled "Standardization of micropropagation technique for *in vitro* callus generation from nodal explants of *Buchananiacochinchinensis* (Lour.) Almeida." was conducted in the tissue culture laboratory of Forest Biology and Tree Improvement, Forest College and Research Institute, Mulugu, Siddipet during 2020-22. The main objective of the present study was to generate *in vitro* callus from the nodal explants of *Buchananiacochinchinensis*. Callus was generated from the cut end of nodal explants after 3-4 weeks of inoculation. Maximum callus response (178.00 ± 2.08) was observed in WPM supplemented with 2mg/L TDZ and optimum callus response (155.66 ± 0.88) was observed in MS supplemented with 2,4-D (4mg/L) and followed by MS with 3mg/L 2,4-D and minimum callus response of 34.66 ± 2.60 was observed in MS alone and followed by MS supplemented with 0.5mg/L 2,4-D. In the present study, the protocol was standardized for callus generation from the nodal explants.

Keywords: Callus, Chironji, *In vitro*, Nodal explant, Murashige & Skoog, Woody Plant Medium

INTRODUCTION

Buchananiacochinchinensis(Lour.) Almeida is one of the economically important, valuable and a major Non-Timber Forest Produce (NTFP), which has many ethnomedicinal uses and is famous for its valued fruit. It is a magnificent tree for social forestry and agroforestry. It belongs to the family Anacardiaceae. It is underexploited fruit and life supporting tropical forest tree species for different tribals in north, central, and west India. It is locally known by the names of Chironji, priyal, achar, char, almondette and its telugu name is charumamidi, locally called moori (Malik *et al*., 2012).*B. cochinchinensis* is native from Indian subcontinent to China and is endemic to tropical dry deciduous forest in India and found in Burma, Nepal and few other countries (Chauhan *et al*., 2012). *Buchananiacochinchinensis*is a very hardy tree which can grow in rocky and gravelly red soils and is also common in eroded ravine lands of our forest. It also grows in deteriorated rocky areas, salt-affected areas and prefers the tropical and subtropical climate (Singh *et al*., 2010). Morphological characteristics of *Buchananialanzan*Spreng, the plant reach the height of 12-17m and up to 1.3m in girth and leaves are thick and leathery those are with a blunt tip, broadly oblong and rounded base and 2-10 cm broad, apex obtuse, margin entire, coriaceous, tomentose beneath, straight parallel veins up to 10-20 run along the leaves. Pyramidal panicles of small flowers appear in auxiliary and terminal panicles with the presence of sessile greenish white flowers (Singh *et al*., 2006; Singh and Singh, 2014). It is widely used by many Indian tribes for treating various diseases (Behera *et al*., 2008). It has multifarious uses in terms of timber for match boxes, crates, desks, moulding, agricultural implements, packing cases, furnitures; fodder for goats, sheeps, buffaloes; dry wood is utilized for fuel, medicines, also it is resistant for termite attack and lac of kusumi strain is reared on the chironji trees (Avani*et al*., 2015; Rajput *et al*., 2018).

Very recently, unique biomaterials and biofilms are being extracted from seeds, which promise to become a major contributor in pharmaceutical industry (Swastik and Abhijit, 2015). In country level, the Chironji's approximate yearly trade is 5000-10000 MT and the price of the seed changes based on the size. On an average, the price of Chironji is Rs. 500 to 750 per kg. Chironji of bastar region is in high demand and recently it has been shifted to a scarce commodity and was priced up to Rs. 1000 per kg (Rajput *et al*., 2018). Problems in regeneration of *B. cochinchinensis* include, it is usually propagated through seeds but it has low germination capacity due to hard seed coat. Fungal contamination (*Fusarium*sps.) is the most common problem associated with its seed storage, an increased humidity also favours the contamination by fungus. Chironji loses its viability after three months

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of harvest and seeds are also recalcitrant in nature. When seeds get exposed to sunlight they lose the ability to germinate. The Chironji trees are prone to attack by the insect pests and stem borer-*Plocaederusobesus*, in the forests of central India (Malik *et al.*, 2012; Meshram and Soni, 2014). Chironji is vegetatively propagated by chip budding and softwood grafting (Tewari and Bajpai, 2001; Singh and Singh, 2014). But, the availability of root stocks is less and hence these propagation methods are considered to be less effective. Moreover, propagation by root cuttings is very slow process. Population of Chironji trees got minimized in the natural forests as well as non-forest areas due to over grazing and excessive felling (Singh *et al*., 2002).

Research on development of various biotechnological tools is required to conserve the rare and vulnerable tree species. Mass propagation and germplasm collection of species need to be done, as it is becoming vulnerable day by day due to overexploitation for commercial purposes. Less research is done on the subsequent stages after the shoot proliferation and its propagation in field after producing the plant from the explants. There is a still lot of scope for improving the *in vitro* propagation methods of Chironji. Keeping all these facts in consideration there is needed to develop many biotechnological tools to conserve the species and there is an urgent need for a technology for its easy multiplication and propagation. Micro propagation is considered to be a best technique, it is boon for conservation of species and huge number of plantlets can be regenerated by using any part of the plant as explant within a short interval and with limited space. Irrespective of seasons, multiplication of plant can be done by using tissue culture technique which creates the availability of commercial, economical and medicinally important forest tree species. Based on the above facts, uses and problems the research is being planned under the following objective.

Objective *In vitro* callus generation from nodal explants of *B. cochinchinensis*.

MATERIALS AND METHODS

Overview of methodology was represented in the following Figure 1.

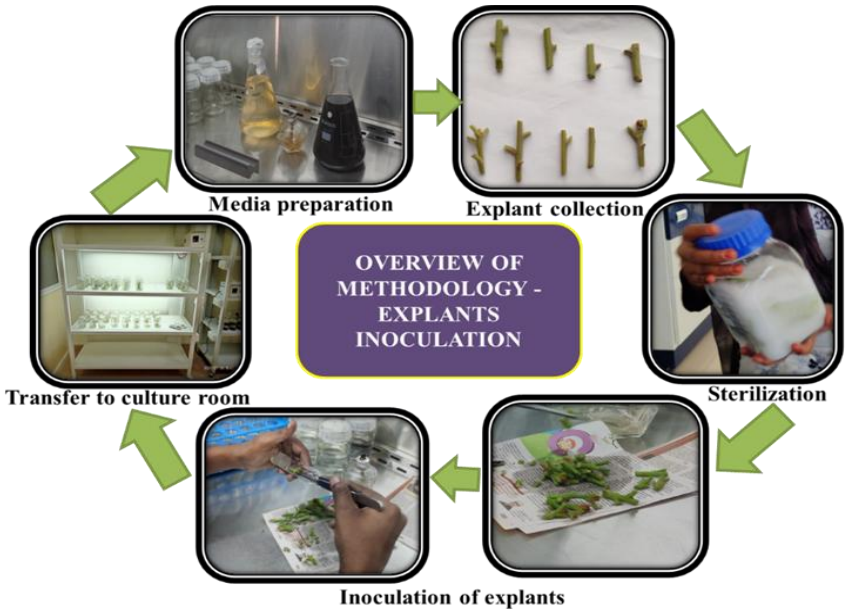


Fig 1. Overview of methodology

Media preparation, collection and sterilization of explants

For indirect organogenesis, the MS/WPM culture media were supplemented with different Plant growth regulators like 2,4-D, TDZ (Thidiazuron)and other additives like Arginine, Adenine sulphate (ADS), Citric acid, Ascorbic acid, Myo-inositol. Plant Preservative Mixture (PPM) (0.5ml/L) was added in the media in order to reduce the fungal contamination (Table 1). The pH of medium was adjusted to 5.5-5.6 by using either 1N NaOH or 1N HCl. 30ml of media was poured into each culture bottle and packed in autoclavable bags. Further, media bottles were sterilized at 15 psi pressure and 121 ºC for twenty minutes.

Nodal explants were collected from the plus trees of Chironji from FCRI, Mulugu. Both young and mature nodal explants of 5cm size were collected in the early morning hours and next process was carried out in the laboratory. Further they were treated with different chemicals for different time durations (Table 2, Figure 3). Antioxidant treatment was given to the explants with the 0.1% of ascorbic acid and citric acid. After carbendazim treatment, the next step was carried out completely in the bio safety cabinet. After surface sterilization the ends of nodal

explants which were subjected to sterilant were cut by using autoclaved scalpel to avoid the effect of sterilant which might have entered into the cells of explants during the process of sterilization.

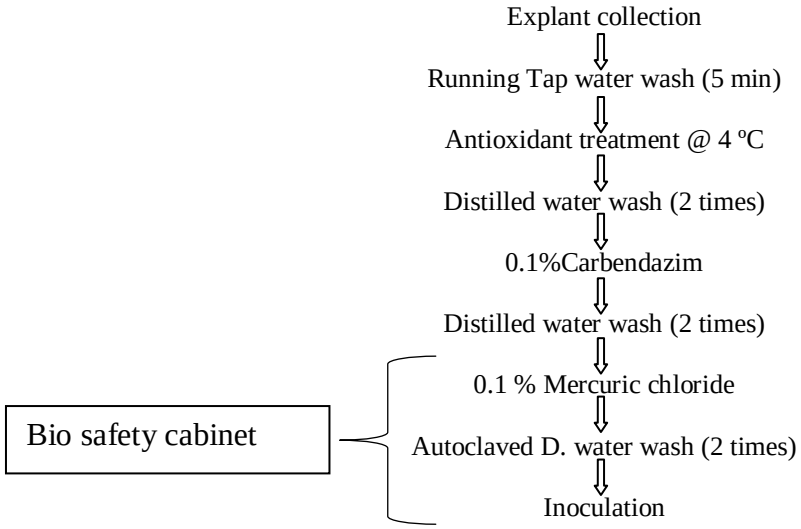
Table 1. MS/WPM media along with different PGRs Concentration for callus generation from nodal explants

S. no	Culture media + PGRs	Other additives	Antibiotics
1	Control	Arginine-25mg/L Ascorbic acid-25mg/L Citric acid-25mg/L Adenine sulphate - 50mg/L Myo-Inositol – 100mg/L	Plant Preservative Mixture (PPM)-0.5ml/L
2	MS + 0.5 mg/L 2,4-D		
3	MS + 1 mg/L 2,4-D		
4	MS + 2 mg/L 2,4-D		
5	MS + 3 mg/L 2,4-D		
6	MS + 4 mg/L 2,4-D		
7	MS + 5 mg/L 2,4-D		
8	WPM + 2mg/L TDZ		

Table 2. Surface sterilization treatments - Chironji explants (nodal cuttings)

S. No	Treatments
T_1	Carbendazim (0.2%)- 15mins + $HgCl_2$ 0.1%-5mins
T_2	Carbendazim (0.2%)- 20mins + $HgCl_2$ 0.1% - 4mins
T_3	Antioxidant treatment@4ºC (5 mins) + Carbendazim (0.2%)- 20mins + $HgCl_2$ 0.1% - 3mins
T_4	Antioxidant treatment@4ºC (10 mins) + Carbendazim (0.2%)- 20mins + $HgCl_2$ 0.1% - 3mins

Figure 2. Steps followed in sterilization process:



Inoculation, culture conditions and subculture

For callus generation, the nodal explants were cut into small slices of 0.5 cm size. The explants were inoculated in media jars containing MS or WPM medium along with different plant growth regulators within the bio safety cabinet. Further, the culture bottles were labelled properly and shifted to culture room. Seven treatments and one control (MS along with other additives) were followed for callus generation. Each treatment was carried out with three replications (Table 1). In culture room, the explants were kept in relative humidity of 60-70% and temperature of 22-28ºC. For callus initiation, the inoculated culture bottles were kept under dark conditions. Callus generated from the explants was sub-cultured after every twenty one days of inoculation and callus was dissected from the nodal explants and dried cuttings were removed by using sterile scalpel blade and forceps in the bio safety cabinet and the generated callus was inoculated in the fresh media.

Observations and data collection

The number of days was counted from the inoculation day to the appearance of callus on the

explants and also, the number of explants with callus response was counted. Calli generated from each treatment was expressed in terms of mean number of explants responded with calli generation for each treatment with three replications.

Statistical analysis

The experimental data related to the number of nodal explants responded for callus was analyzed by taking the three replications for each treatment by laying the experiment in randomized pattern. Analysis of data was done with ANOVA (Analysis of Variance) using the statistical program SPSS 23.0 at $P < 0.05$, according to the Duncan's multiple range test ± standard error (S.E) in order to establish the significant differences among the treatments (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Surface sterilization of explants is a crucial step in establishing the contamination free cultures and the use of fungicides for contamination reduction was reported by Shields *et al*. (1984). Various treatments were followed to avoid the microbial contamination in the cultures as per (Table 2). With increase in the carbendazim concentration and duration of exposure there was decrease in contamination. Whereas for mercuric chloride treatment, initially the nodal cuttings had dried but on decreasing the time duration of mercuric chloride up to 3 mins, the explants did not dry and callus initiation was observed. Then, the nodal cuttings had initiated the callus without contamination and without drying with the treatment 4 (T_4) (Table 2). Therefore, T_4 was found to be effective not only in reducing the contamination but it also contributed to the callus initiation.

The results of the present study were in accordance with the study conducted by Davis (2022) in *Acacia* hybrid by using Ethanol and Mercuric chloride and Minipara*et al*., (2019) had done surface sterilization for removing both external and internal contaminants of plant material. Less contamination was reported in treating the hypocotyls explants with 200 ppm cefotaxime, 500 ppm carbendazim along with 0.1% mercuric chloride with good survival of explants. Mercuric chloride used in surface sterilization, acts on the protein sulfhydryl group and destroys the function of enzyme in contaminants like bacteria and fungi was reported in *Annona squamosa*. According to the study conducted by Indrapal (2018) on Chironji, Surface sterilization of explants by using the Carbendazim (1%) for 10 min + $HgCl_2$ (0.1%) for 5mins + Ethanol (70%) for 10 sec was able to reduce contamination and supported the survival of explants. When compared with the above study, the present experiment was found contradictory, where the surface sterilization of explants with low concentration of Carbendazim for longer duration and $HgCl_2$ with same concentration but for more duration was able to reduce the contamination with successful survival of the explants. In the present study, antioxidant treatment was done for removing the phenols during the sterilization of explants and same study was reported by Nathar*et al*., (2015) in *Semecarpusanacardium.*

For callus induction, the mature and young nodal explants were inoculated in eight different media combinations of MS media with different concentrations of (0.5-5mg/L) 2,4-D and WPM along with 2mg/L TDZ and MS media without PGRs as control. Calli response was varied with respect to the plant growth regulator used and also with different concentrations of 2,4-D. There was no indication of callus response for 2 weeks after inoculation.

In the present study, the callus induction was observed mostly from the ends of mature nodal explants within 25-28 days of inoculation. Callus response was observed in less concentration i.e, 4mg/L 2,4-D when compared to the results of Nathar*et al*., (2015), wherein, the nodal explants with callus induction was observed in media combination of MS along with 5mg/L 2,4-D in *Semecarpusanacardium.* In the leaf explants, the callus got initiated within three weeks of inoculation in media combination of MS along with 2.5mg/L of 2,4-D in Chironji species according to the study carried out by Singh *et al*., (2022).

The cultures in the present study shown better callus generation at the ends of explants after a period of 40-45 days in media containing WPM along with 2mg/L TDZ. Nodal explants inoculated in higher concentration of 2,4-D took less time for callus initiation. Whereas, the nodal explants inoculated in the lower concentration like 0.5-2 mg/L 2,4-D, has took more time for calli response. The mean number of explants responded for the callus was found to be highest (178.00 ± 2.08) in the culture media combination of WPM supplemented with 2mg/L Thidiazuron (TDZ) followed by MS along with 4 mg/L 2,4-D with calli response was (155.66 ± 0.88) and the lowest mean number of calli response was observed in Control and MS along with 0.5 mg/L 2,4-D. The optimum level of calli induction occurred in culture media of MS along with 4 mg/L 2,4-D, MS along with 3mg/L as represented in the (Table 4& Figure 3).

The bulged appearance of callus with dark greenish colour callus was observed in the WPM media supplemented with TDZ. Whereas, MS media along with 2,4-D induced small amount of callus with light greenish colour and in further stages of development the calli turned to light brownish colour. Among all the culture media combinations, the concentration of 2,4-D was increased from the 0.5 to 5mg/L, the mean number calli response was also increased as represented in (Table 4).

Table 4. Effect of different PGRs concentration on callus generation from nodal explants

Culture media + PGRs	Mean no of calli response (Mean ± S.E)
Control (MS media)	34.66 ± 2.60^{g}
MS + 0.5mg/L 2,4-D	103.66 ± 2.40^{e}
MS + 1mg/L 2,4-D	114.67 ± 1.76^{d}
MS + 2mg/L 2,4-D	125.67 ± 1.45^{c}
MS + 3mg/L 2,4-D	153.00 ± 0.78^{b}
MS + 4mg/L 2,4-D	155.66 ± 0.88^{b}
MS + 5mg/L 2,4-D	61.00 ± 3.21^{f}
WPM + 2mg/L TDZ	178.00 ± 2.08^{a}

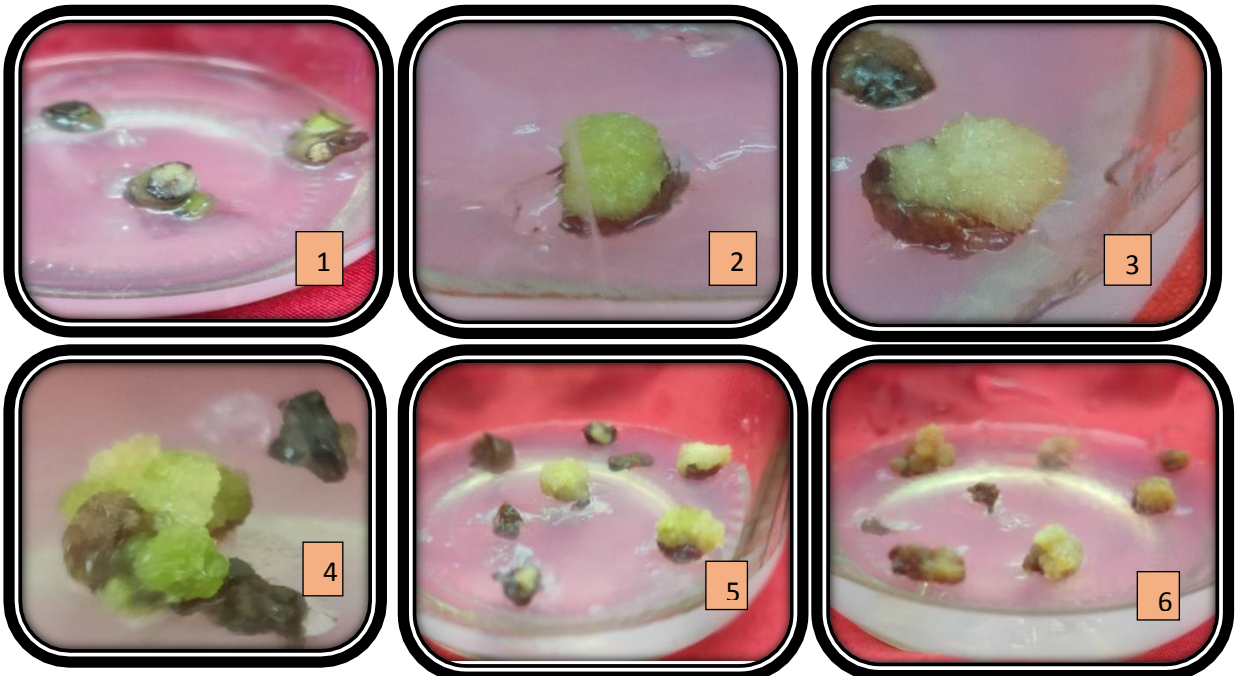
Per each treatment - 3 replications

Note: Data are presented as means ± standard error. Values followed by different superscript alphabets in the same column are significantly different (P <0.05 Duncan's multiple range test).

Adding of absorbents and antioxidants like PVP and Ascorbic acid in the media, was found to be effective in preventing the oxidation. As exudation of phenols was a common phenomenon in Chironji. In present study, organic additives like antioxidants (Ascorbic acid and Citric acid) were added in the media in order to avoid the browning of media and also to avoid damage to regeneration. Similarly, phenolic exudation was prevented in *Acacia* species by addition of antioxidants and in *Elaeocarpus blascoi* by adding PVP according to the study conducted by Das *et al*., (1996); Siva*et al*., (2015).

The results of present study were in harmony with the study reported by Singh *et al*., (2013) in giving the higher frequency of callus on using plant growth regulator TDZ in *Santalum album*. Siva*et al*., (2015) reported similar results in *Elaeocarpus blascoi* with compact callus formation from nodal explants by using media combination of WPM along with 0.45 µM TDZ. Callus formation has also reported by Ndoye*et al*., (2003) in *Balanitesaegyptiaca*and Rajeswari and Kailash (2008) had found that the mean percentage of petiole, cotyledon, epicotyl explants with callus response in range of 80-85% was found in media combination of MS along with BAP and IAA in *Albizzia odoratisimma*, which were lower percentage when compared with the present study, wherein, WPM along with TDZ showed highest mean no of calli response approximately 178.00, which was equal to the 89% of calli response.

Figure 3. Callus generation occurred from nodal explants



1. Nodal explants before inoculation 2. Callus- MS + 3mg/L 2,4-D 3. Callus- MS + 4mg/L 2,4-D 4. Callus- WPM + 2mg/L TDZ 5 & 6. Callus in further stages of development

CONCLUSION

Based on the results of the present study, it was concluded that the mature nodal explants responded well over the young explants. Different surface sterilization treatments were followed for getting the contamination free explants along with healthy cultures. The maximum number of explants free from the contamination and maximum survival percentage was observed when the surface sterilization was done by using antioxidant treatment at 4ºC for 10 mins + Carbendazim (0.2%) for 20mins and $HgCl_2$ 0.1% for 3mins.Maximum number of callus generation occurred from the mature nodal explants in WPM supplemented with the 2mg/L TDZ, the appearance of callus was greenish in colour and followed by the calli response of (155.66 ± 0.88) was observed in media of MS augmented with 4mg/L 2,4-D and the least response of the calli (34.66 ± 2.60) was observed in control (alone MS media).

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