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Micropropagation of *Buchanania lanzan* spreng.-an endangered medicinal plant of the Western Ghats

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Abstract
Micropropagation protocol was developed for an endangered medicinal plant *Buchanania lanzan* (Family Anacardiaceae) via direct organogenesis from leaf explant. The chironji oil obtained from the seeds used in nutraceuticals and cosmetics and the leaf extract is used as an antioxidant and antidiarrheal drug. A method for rapid *In vitro* propagation of *Buchanania lanzan* (L.) was developed *In vitro* seeds culturing. The seeds sterilized thoroughly and inoculated M.S media. Supplemented with GA_3 and BAP leaf explant obtained from seedling used for *In vitro* shoots and roots development. *In vitro* seed germination show a 79.33% response to concentration on MS+GA_3 200mg/l. Direct organogenesis using the leaf explant treated with thidiazuron (TDZ), and Coconut water at 2 mg/l, 2 ml/l respectively were found to be optimum for direct shoot induction and growth with an average of 3.17 ± 0.37 shoots per explants. Successful *In vitro* rooting was induced from the cut end of the micro shoots. The maximum number of roots was obtained on 3.39 ± 0.22 on MS + NAA 1.5 mg/l. The regenerated shoots along a well-developed root have been effectively acclimatized then established within poly cups containing base rite afterward transferred to earthen pots containing garden soil and manure (2:1), then grown under greenhouse conditions with a 78 percent survival rate.

Keywords: *Buchanania lanzan*, micropropagation, coconut water, direct organogenesis

Introduction
Buchanania lanzan is categorized under the 195 red-listed medicinal plant species of Indian origin, that needs conservation measures as reported by the Foundation of Revitalization of Local Health Tradition (FRLHT), Environmental data system (ENVIS) - Centre on Medicinal Plants, Bangalore, Govt. of India is included within the Red Data Book published by the International Union for Conservation of Nature and Natural Resources (IUCN) [1, 2]. *Buchanania lanzan* grows on yellow sandy-loam soil and is usually found within the dry forests of Jharkhand, Madhya Pradesh, Chhattisgarh, and Uttar Pradesh. Medium-sized deciduous tree, growing to about 50 ft. tall and has high socio-economic value providing livelihood to tribals [3].
The plant Flowering begins from January to March and fruits ripen from April to May. It bears fruits every containing one seed, which is popular as an edible nut, known as chironji. The fruits of chironji mature in two to three months and are harvested manually in April and May. The green-colored skins of harvested chironji fruits flip black on storage which has to be removed before shelling. The chironji nut has a tremendous demand in overseas markets and therefore, to earn trade the government and private organizations have evinced have evinced keen interest in increasing its production and processing capacity [4]. The juice of the leaves is digestive, expectorant, aphrodisiac, and purgative. The gum after mixing with goat milk is used as an analgesic [5]. The kernel contains about 52% oil [6]. The *Buchanania lanzan* seed is also used as a substitute for almonds and the oil extracted from it is used in the oil from the seeds is used to reduce granular swelling of the neck [7]. The ointment is made from the kernel which is used to relieve itch and prickly heat. The gum from the bark is used for treating diarrhea and intercostals pains and leaves are used for promoting wound healing [8, 9].
Buchanania lanzan is conventionally reproduced through seeds (in natural conditions) but germination is often difficult because the hard testa limits the production of a large number of seedlings during a defined time. The propagation via cutting has been reported but the limitation to it is that from selected individuals few propagules are produced [10]. Currently *in vitro* propagation is being viewed as an alternative method of clonal propagation. Over-exploitation destroyed the natural habitat *B.Lanzan* tree warranted an urgent need to develop an alternative technique like tissue culture to accelerate the production of plantation propagules. Sharma *et al*., developed a protocol for somatic embryogenesis and plantlet regeneration of *Buchanania lanzan* by immature zygotic embryos cultured on Murashige and Skoog (MS) medium attempted to improve the regeneration and multiplication of *B. Lanzan* with limited success [11]. However, micropropagation of *B.Lanzan* from mature trees has remained problematic. Poor explant response and phenolic exudates from cut ends of explants taken from plus tree pose great problems for the establishment of *in vitro* cultures of *B.Lanzan* Keeping in view the requirement for longer incubation time for the limited shoot and root regeneration, in the current study an attempt was made to standardize the micropropagation protocol using the seeds and leaf from the *in vitro* raised seedlings.

Materials and Methods
Collection of seeds and raising of aseptic seedlings
The seeds were collected from the fruits and separated outer layer from the fruit were surface sterilized by several consecutive chemical treatments started with 4–5 times of washing by double-distilled water, treatment 5 % Bavastin and then washed with tween 20 for 5 min. Under laminar airflow, the seeds were washed 3–4 times with sterilized distilled water and 1 % (w/v) aqueous mercuric chloride for 3 min. then were immersed in 70 % alcohol for 30sec seeds were rinsed thoroughly with sterilized distilled water and inoculated in tissue culture bottles containing 25 ml of semisolid Murashige and Skoog's (MS) medium [12]

Culture conditions
The MS medium supplemented with 3 % (w/v) sucrose 0.8 % (w/v) agar (Hi-Media, Mumbai, India), The pH of the medium was adjusted to 5.8 ± 0.03 before autoclaving for 20 min at 120 ^{0}C. Seeds were inoculated per 100-ml culture bottle containing 25 ml semisolid media for initiating the *in vitro* seedling. *In vitro* raised explants were subcultures in the semisolid medium for shoot multiplication or rooting. The cultures were incubated at a temperature of 25 ± 3^{0}C under 12 h daily illumination with white fluorescent tube light.

Callogenesis and Caulogenesis
In vitro-raised seedling leaf were subcultured individually as an explant for callus-mediated shoot organogenesis. Supplementation of BAP, TDZ, and coconut water at the range of 1to 3 mg/l was effective in induced shoot organogenesis from the callus.

Rooting in microshoots and acclimatization
The micro shoots measuring 2–2.5 cm in length were transplanted on the MS medium as well as medium supplemented with different concentrations of auxins *viz*. NAA and IBA investigated their effects for rooting. The percentage of rooting, number of roots per shoot, were recorded. The hardening and acclimatization of *In vitro*-raised plantlet.

Statistical Analysis
Three replicates of each treatment were made and studied and the experiment was laid both on the systematic and randomized pattern. Analyzed with ANOVA (Analysis of Variance) using the statistical program SPSS 17.0 at P ≤ 0.05, according to Duncan's multiple range test ± standard errors (S.E.) in order significant differences among treatments to be established [13].

Results
Establishment of aseptic seedling and explants
In vitro seed germination has been proved as an effective strategy to provide aseptic germplasm as a source of explants and may inevitably reduce the chances of contamination. Usually, the explants derived from the *In vitro* raised plantlets are more resistant to microbial contamination during plant tissue culture studies, as compared with explants taken from the field-grown plant's seeds extracted from the mature greenish fruits of *B. Lanzan* exposing the seeds to sterilization put in the MS media with different hormonal concentration GA_3 and BAP. Seeds showed germination 79.33 % at the concentration GA_3 200 mg /l. Average 16 days to seeds germination (Fig 1). On increasing concentration of GA_3 the germination percentage was reduced. BAP hormonal concentration shows a lower germination rate 22.60% at 2.5mg/l compared to GA_3 (Table 1)

Table 1: Effect of different concentrations of cytokinins and their combinations on plant growth in multiplication stage of *Buchanania lanzan* seeds germination

MS + GA3 Mg/l	MS + BAP Mg/l	Days in seeds germination	Seed germination rate
50		22.17 ± 0.93	10.16 ± 0.58
100		21.07 ± 0.97	17.33 ± 0.88
150		19.70 ± 1.54	38.32 ± 0.88
200		16.47 ± 1.56	79.33 ± 3.51
250		17.67 ± 2.46	53.33 ± 2.91
300		17.50 ± 0.35	39.67 ± 2.03
	0.5	22.17 ± 0.93	9.33 ± 0.54
	1	18.40 ± 0.69	13.33 ± 1.13
	1.5	19.07 ± 0.94	16.20 ± 1.46
	2	18.83 ± 0.28	18.30 ± 1.12
	2.5	19.93 ± 1.27	22.60 ± 2.82
	3	18.83 ± 0.71	21.90 ± 0.40

Data presented in the table are Mean ± SE (standard error) scored At 30 days of inoculation of 5 seeds each and repeated thrice.

Fig 1: (A) *Buchanania lanzan* tree growing in the forest of Bhadra Wild Life Sanctuary, (B)-A Flowering twig, (C) Fruits, (D) seed germination in *In vitro*, (E) Hardened plant derived from seed culture

Callus induction and differentiation of shoots from leaf culture

Leaf explants excised from 28day-old aseptic seedlings of *B. Lanzan*, were used for callogenesis and caulogenesis. The response of different concentrations of cytokinins TDZ, BAP, and coconut water for shoot regeneration from leaf explants are shown in Table 2, the leaf calli cultured on MS medium without growth regulators (control) did not show caulogenic response. However, the addition of plant growth regulators enhanced the multiplication rate and the number of shoots per explants. The percentage response varied with the type of growth regulator used TDZ, BAP, and coconut water concentration (0.5, 1.0, 1.5, 2.0, and 2.5, mg/l), resulted in direct shoot bud differentiation from the explants within 2 and 3 weeks of incubation, The initial response was noticed in the form of swellings of the explants within 4–5 days of incubation and then differentiation into direct shoot buds in successive days. Among the two cytokinins tested TDZ was found to be more efficient than others for initiation and subsequent proliferation of shoots Of the various concentrations of TDZ tested, 2 mg/l and 2ml of coconut water proved to be most effective as on this medium an average of 80% regeneration of shoot on leaf explant and also 3.17 ± 0.37 shoots per explant were developed (Fig. 2). An increase in TDZ concentration along with coconut water 2ml resulted in a decrease in regeneration potential and the number of shoots per explants as well. At a lower concentration of TDZ the number of shoot regeneration and the frequency was lower (15%). Shoot induction was also observed in the explants cultured on MS medium fortified with BAP. (3 mg/l) was found to be less effective as it induced no shoots growths per explants (Table 2). When the concentration of TDZ and BAP was increased beyond optimum level a gradual decrease was observed in regeneration frequency, the number of shoots per explants, and average shoot

Table 2: Effect of various concentrations of BAP, TDZ, and Coconut water on shoot organogenesis from leaf calli of *Buchanania lanzan*

Plant growth regulators mg/l			Frequency of shoot Regeneration per leaf explant %	Mean number of shoots/explant
BAP mg/l	TDZ mg/l	Coconut water ml/l		
0.5		2	7	1.13 ± 0.03
1.0		2	5	1.20 ± 0.06
1.5		2	10	1.43 ± 0.23
2.0		2	12	1.40 ± 0.20
2.5		2	10	1.70 ± 0.12
3.0		2	5	1.50 ± 0.06
	0.5	2	10	1.80 ± 0.12
	1.0	2	17	2.10 ± 0.20
	1.5	2	52	2.97 ± 0.15
	2.0	2	80	3.17 ± 0.37
	2.5	2	40	2.1 ± 0.21
	3.0	2	15	1.9 ± 0.06

All treatments had 3 replicates and were repeated thrice. Each value represents Mean ± SE.

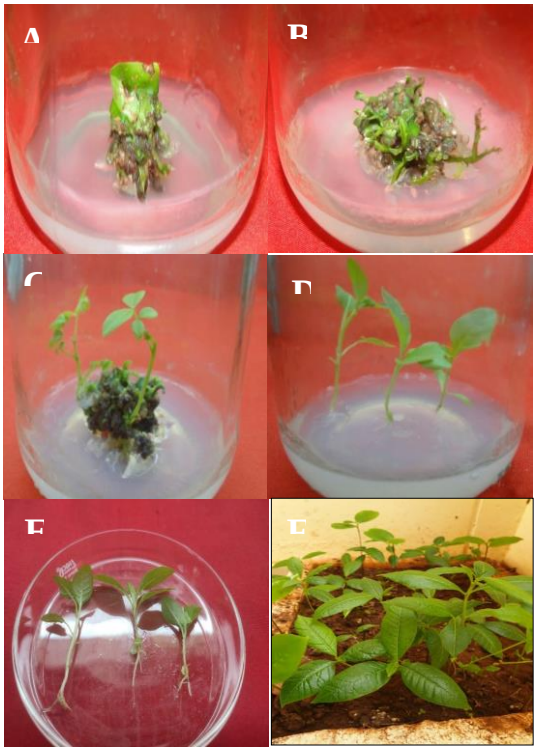

Fig 2: *In vitro* propagation of *B.Lanzan* using leaf explants. (A) Curling of leaf (B) Initiation shoot from the leaf callus (C) Formation of multiple shoots (D) Regenerated plantlets (E)Well developed roots (F) Acclimatized plants.

Rooting of microshoots

In vitro-raised micro shoots (3–4 cm) were transferred to rooting media composed of MS media supplied with various concentrations of IBA, and NAA (0.5, 1.0, 1.5, and 2.0, mg/l). Different concentrations of NAA proved better in inducing the rooting compared to IBA from the basal cut end of micro shoots within 1 week of transfer (Fig. 2). The best rooting in terms of rooting percentage and root length was achieved in the medium 1.5mg/l NAA (Table 3), giving a maximum of roots (3.39 ± 0.22), per shoot with a root length of (3.2 ± 0.20 cm). The roots produced were thick and well developed (Fig.2). A comparatively lesser number of roots was produced on the IBA

Hardening and acclimatization of regenerants

The plantlets were then transferred for hardening (Fig. 4) and were shown a 78 % survival rate in cocopeat trays after hardening by recommended methodologies. The rooted shoots were taken out from the culture vials after attaining a considerable length of roots and washed gently in running tap water to remove the adhered medium and were successfully hardened off inside the growth room in sterile planting substrate (soilrite) for 4 weeks and irrigated with one-quarter strength of MS in the first 2 weeks (Fig. 2). About 78 % of plantlets survived after transferring from soilrite to soil that contained a mixture of manure. All plants were exhibiting normal growth when compared with natural ones.

Table 3: Effect of different auxins at various concentrations on *In vitro* root induction of *Buchanania lanzan*

Auxins (mg/l)		Average no. of roots/ Plantlet	Average root NAA IBA length (cm)
IBA	NAA		
0.5		0	0
1.0		2.41 ± 0.18	1.2.±0.13
1.5		2.05 ± 0.12	2.3±0.12
2.0		1.8 ± 0.18	0.1±0.12
	0.5	2.41 ± 0.18	1.3±0.14
	1.0	2.78 ± 0.19	2.9±0.15
	1.5	3.39 ± 0.22	3.2±0.20
	2.0	2.52 ± 0.19	2.0±0.32

All treatments had 3 replicates and were repeated thrice. Each value represents Mean ± SE

Discussion

In the recent past, due to excessive felling of trees and overgrazing, a considerable reduction in the population of *B. lanzan* in the forest and non-forest areas has been recorded [14]. The population of this species is depleted in the Western Ghats due to the nutritional value of the seeds and the timber and this species is considered as threatening taxa [15]. In nature, seeds are the major source of regeneration but, *In vitro* germination of seed has been proved as an effective strategy to provide aseptic germplasm as a source of explants and may inevitably reduce the chances of contamination [16]. In the present research work also regenerants were produced from the seeds of *B. lanzan*. Supplementation of GA_3 has been found to enhance the rate of seed germination as well as healthy growth in the seedling. The maximum germination percentage recorded for seeds followed by sterilization using. The highest germination percentage (79.33 ± 3.51%) was recorded MS + GA_3 200mg/l shows the improvement of seed germination. GA_3 is known to break the dormancy of several types of seed germination via the synthesis of a-amylase and other hydrolases [17]. The same results show in *Cassia siamea. Clitoria ternatea* [18, 19], suggested that the presence of GA_3 obviate the requirement of seeds for various environment clues as it promotes germination with the inhibition of the ABA effect. But increase the concentration of GA_3 decrease the percentage of seed germination

The optimum and best response of shoot induction and regeneration was from excised from 28-day-old aseptic seedlings leaf used for the *in vitro* regeneration. The highest number of shoot regeneration was recorded through leaf explants on a medium comprised of MS +2mg/l TDZ, and 2ml coconut water shows a maximum of 80% of shoot regeneration and also shows the average 3.17 ± 0.37 shoot per leaf explant When the concentration of TDZ was increased beyond the optimum level a gradual decrease was observed in regeneration frequency, the number of shoots per explant. Similar inhibitory effects of TDZ on growth and elongation at higher concentrations have also been observed on another woody tree *Albizia Chinensis* and *Vitex negundo* [20, 21]. Benzyladenine and thidiazuron were also found to be good in the initiation and subsequent regeneration, and their results coincide with that of earlier reported by *P. marsupium* by Hussain *et al.* [22]. The BAP shows minimum shoot regeneration potential and shoots multiplication rate as shown in Table 2. BAP and TDZ beyond 2.5 mg/ml resulted in a decrease in regeneration potential as it leads to huge callus, which might be one of the reasons for the reduction of multiple shoot regeneration.

Coconut water is composed of many amino acids, nitrogenous compounds, inorganic compounds, organic acids, carbon sources, vitamins, and growth regulators such as cytokinin and auxin. Research conducted by Yong *et al* [23, 24]. Confirms that 94% of coconut water contains growth-inducing compounds as specified by George (1984) [25] can influence *In vitro* cultures. In many plant species, regeneration improvement was achieved by augmenting the culture medium with coconut water. [26]

Rooting of micro shoots, transplantation, and acclimatization of the plantlets to the natural conditions has been considered to be the most important step but difficult task by micropropagation (Murashige 1972) [12]. In the present study, it has been observed that plantlets of *B lanzan* need various types of auxin supplements for the induction of successful rooting. It has been observed that 1.5mg/l concentration of NAA proves best for successful rooting about 3.39 ± 0.22 roots per explant and root length 3.2±0.20 cm giving maximum number rooted in plantlets compared to IBA. In general, it is preferred that the callusing hampers the vascular connection between the shoot and root system leading to the abnormal growth of the plantlets as well as a very high frequency of casualty during the acclimatization process. Several studies in this regard suggested avoiding callusing for healthy shoot and root system development in plantlets [27]. Regenerated rooted plantlets were successfully transferred to pots containing soil, sand, and manure in a 2:1:1 ratio. The pots containing plantlets were kept in the growth room for 2 weeks and then transferred to the greenhouse.

Conclusion

Keeping in view the importance of threatening status, economical value, and earlier drawbacks of the tissue culture techniques applied on *B. lanzan*, the present work describes an efficient protocol of regeneration through the seeds and the leaf explants of *B. lanzan.* Thus, the protocol developed in the present work may be utilized for obtaining a large number of plants of this valuable plant species *B. lanzan* an important medicinal tree.

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References

1. Kirtikar KR, Basu BD. Indian medicinal plants. Dehradun: International Book Distributors, 2006, 1324-1345
2. Anonymous. 'The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products.' Publication and Information Directorate, CSIR; New Delhi, 1952, 78-79.
3. Pandey GP. Effects of gaseous hydrogen fluoride on leaves of *Terminalia tomentosa* and *Buchanania lanzan* trees. Environ Pollution Ser A, Ecol Biol. 1985; 37(4):323-334.
4. Kumar J, Vangaiah PC, Srivastav PP, Bhowmika PK. Chirnji nut processing present practice and scope. Indian journal of traditional knowledge 2012; 11(1):202-204

5. Singh J, Patra AK, Nandeshwar DL, Meshram PB, Negi KS. Effect of growth regulators on the rooting of root cuttings of Chironji (*Buchanania lanzan* Spreng). Proceedings of National Workshop on Conservation of Medicinal Plants, 2002, 128
6. Wealth of India- A dictionary of Indian Raw materials and Industrial Products, Vol. I, (PID New Delhi), 1948, 233.
7. Kumar J, Prabhakar P, Srivastav P, Bhowmick P. Physical characterization of chironji (*Buchanania lanzan*) nut and kernels, Food Sci. Res. J. 2014; (5):148-153.
8. Horio T. A phenolic amide from roots of C*henopodium album*. Phytochemistry. 1993; 33(4):807-808.
9. Gohar AA, Elmazar MMA. Isolation of hypotensive flavonoid from *Chenopodium* species growing in Egypt. *Phyto. Res*, 1997; 11(8):564-567
10. Singh Jagdish, Banerjee SK, Francis Alfred. Vegetaive propagation of *Buchanania lanzan* Spreng. Root cuttings. Indian Forester. 2002; 128(6):700-704.
11. Sharma P, Koche V, Quraishi A, Mishra SK. Somatic embryogenesis in *Buchanania lanzan* Spreng. *In vitro* Cell Dev Biol. 2005; 41:645-7
12. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiol. Plant*. 1962; 15:473-479
13. Gomez KA, Gomez AA. Statistical Procedures for Agricultural Research. *2nd*, *John Wiley and Sons* USA, 1984.
14. Shende S, Rai M. Multiple shoot formation and plant regeneration of a commercially-useful tropical plant, *Buchanania lanzan* (Spreng). Plant Biotechnology. 2005; 22(1):59-61.
15. Ajith S, Krishna V, Sudhesh LS. Dormancy breaking of stored seeds of *Buchanania lanzan* Spreng.-An endangered medicinal plant of the Western Ghats. Jetir. 2018; 5:9.
16. Lynch PT. Tissue culture techniques in *in vitro* plant conservation. Plant conservation biotechnology. 1999; 24:41-62.
17. Vieira AR, Vieira MD, Fraga AC, Oliveira JA, Santos CD. Action of gibberellic acid (GA_3) on dormancy and activity of alpha-amylase in rice seeds. Revista Brasileira de Sementes. 2002; 24(2):43-8.
18. Shastri S, Venkatarangaiah K, Sheshagiri A. Seed Germination and Micropropagation Studies of *Mammea Suriga* Kosterm. J Microbiol Biotechnol Food Sci, 2020, 10(2)
19. Parveen S, Shahzad A, Saema S. *In vitro* plant regeneration system for Cassia siamea Lam., a leguminous tree of economic importance. Agrofor. Syst. 2010; 80:109-116.
20. Ahmad N, Anis M. Rapid clonal multiplication of a woody tree, *Vitex negundo* L. through axillary shoots proliferation. Agrofor Syst, 2007, 71(3)
21. Sinha RK, Majumdar K, Sinha S. *In vitro* differentiation and plant regeneration of *Albizia chinensis* (Osb.) Merr. Vitr Cell Dev Biol - Plant. 2000; 36(5):370-373.
22. Husain MK, Anis M, Shahzad A. *In vitro* propagation of Indian Kino (*Pterocarpus marsupium* Roxb.) using Thidiazuron. Vitr Cell Dev Biol – Plant, 2007, 43(1)
23. Yong JW, Ge L, Ng YF, Tan SN. The chemical composition and biological properties of coconut (*Cocos nucifera L.*) water. Molecules. 2009; 14(12):5144-64.
24. Bhowmik TK, Rahman MM. *In vitro* seed germination and rhizome based micropropagation of *Calanthe densiflora* Lindl: An indigenous terristerial orchid of Bangladesh. International J of Botany Studies. 2017; 2(1):110-116
25. George EF, Sherrington PD. Plant Propagation by Tissue Culture-Handbook and Directory of Commercial Laboratories. Exegetics Ltd; Edington, UK, 1984.
26. Nasib A, Ali K, khan S. An optimized and improved method for the *in vitro* propagation of kiwifruit (*Actinidia Deliciosa*) using coconut water, 2008, 40.
27. Kozgar MI, Shahzad A. An improved protocol for micropropagation of teak tree (*Tectona grandis* L.). Rendiconti Lincei. 2012; 23(2):195-202.