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SHORT RESEARCH ARTICLE

In vitro mass multiplication of economically endangered forest plant Chironji (*Buchanania lanzan* Spreng.)

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Abstract

A protocol was developed for organogenesis and *in vitro* multiplication of chironji using young leaf and nodal segments. Maximum 41 callus were induced in MS containing 2.5mg/l 2,4-D after 3 weeks of inoculations of leaf explants. Further, callus and nodal segments were inoculated alone and combination of Thidiazuron (TDZ), BAP, and Kinetin and the maximum shoot induction was obtained in a wood plant medium 2.5mg/L TDZ alone enriched with 0.1% of activated charcoal. In comparison, the maximum shoot proliferation (78%) was observed in ½WPM containing 2.5mg/L TDZ and 0.5mg/L GA_3 with 5.7 shoots per explants. Maximum 8 roots were observed in *vitro* regenerated shoots with WPM supplemented with 2.0 mg/l IBA enriched with 0.2% activated charcoal. Plants with 3-4cm in root length were acclimatized and transferred to pots containing an autoclaved mixture of soil, sand and manure in 2:1:1 ratio. Approximately 70% survival rate was recorded from *in vitro* grown plantlets on transfer to pots.

Keywords: Callus, chironji, hardening, nodal segments, shoot induction, root induction

Chironji (*Buchanania lanzan* Spreng.) a medium-sized deciduous forest tree native to the Indian subcontinent and belongs to the family of Anacardiaceae. It is widely distributed in the states of Madhya Pradesh, Chhattisgarh, Jharkhand, South East UP, Gujarat, and Rajasthan. It is an excellent agroforestry plant with the ability to withstand adverse climatic conditions and gained great importance due to its multifaceted uses, which are being exploited economically by many tribal communities (Rajput et al. 2018). Chironji kernels are a natural source of nutrition and are known to retain various metabolites with high pharmaceutical value.

Besides vegetative propagation, the most common method of propagation of Chironji is through seeds that are lentil-sized, slightly flattened, and composed of hard seed coat rendering it the nature of recalcitrance (Rajput et al. 2018). Associations of fungal contamination of *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. with seeds are responsible for lower germination rate (Sharma 1 et al. 1998). Various unconventional cultivation practices, heavy biotic pressure, and overexploitation have resulted in the depletion of its population from forested as well as non-forested areas, which has pushed the genus to be on the verge of extinction. This accelerated overexploitation has landed it under the 195 red-listed medicinal plant species of Indian origin (Rajput et al., 2018). Vegetative propagation is less effective due to the low availability of rootstocks and dependency on seasonal conditions. Therefore, applying the plant tissue culture approach is extremely important for the multiplication and conservation of the Chironji plants. Consequently, the objective of our present study was to

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develop an efficient *in vitro* mass multiplication protocol that could be used for large-scale multiplication of the Chironji.

Sterilization, explants preparation and inoculation of explants for callus initiation

The young leaves and nodal segments were hand-plucked from the Plant Tissue Culture Nursery, Biotechnology Centre, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur. Collected explants were thoroughly washed with tap water and surface sterilized with 0.15 (v/v) Tween-20 for 15 minutes, followed by 0.1% mercuric chloride for 3-6 minutes. Surface sterilized leaf explants (2-3cm) were culture in MS medium supplemented with different concentration of 2-4,D (0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) alone. After three weeks, callus was induced and further transferred to Woody Plant Medium (WPM) enriched with activated charcoal for shoot induction.

Shoot and root initiation from callus and nodal segments

For shoot induction from callus and nodal segments, WPM with different concentrations of TDZ, BAP, and Kinetin was used alone or in combinations, and WPM medium without growth regulator as a control. After the 45 days, the regenerated shoots were transferred into shoot multiplication medium consisted of TDZ (2.5 mg/L) and GA_3 (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) enriched with activated charcoal. *In vitro* regenerated shoots were transferred for root initiation in WPM consisting of different concentrations of IBA (0.5, 1.0, 1.5, 2.0, and 3mg/l) enriched with activated charcoal. For acclimatization, soil, sand, and vermicompost were mixed in a combination of 2:1:1 and standardized the conditions. The experiments were carried out completely randomized design with three replicates. Data were analyzed by determining significant differences among variable concentrations of phytohormones, and the analysis was done using JMP software version 15 (SAS, 2019) using Turkey-Kramer HSD test at $p \leq 0.05$.

The results on shoot multiplication and root induction are presented in Tables 1 and 2. For callus induction, fresh young leaf explants (2-3cm) were inoculated on an MS medium consisting of varying concentrations of 2,4-D

Table 1. Effect of different plant growth regulators with WPM on *in-vitro* shoot multiplication

S. No.	Culture media	Plant growth hormones		Morphogenic explant (%)	No. of shoots per explants
		TDZ	GA_3		
1	½ WPM	2.5	0.5	78.00	5.7
2	½ WPM	2.5	1.0	74.20	5.2
3	½ WPM	2.5	1.5	70.90	5.1
4	½ WPM	2.5	2.0	62.00	4.2
5	½ WPM	2.5	2.5	40.20	4.40
6	½ WPM	2.5	3.0	36.00	2.0
7	Control	0	0	0	0
	SEm±				0.82
	CD (1%)				1.55
	CD (5%)				1.11

Table 2. Effect of different treatment on root initiation of Chironji plants

Treatments	Total shoot inoculated	Total root initiated	Root initiation%	Days taken for root initiation%
T_1 – ½WPM	15	2	13.33	45.0
T_2 – ½WPM + 0.2% activated charcoal	12	4	33.33	49.0
T_3–½WPM+0.2% activated charcoal + 1.5 mg/L IBA	10	4	40.00	50.0
T_4–½WPM + 0.2% activated charcoal + 2.0 mg/L IBA	13	8	61.53	46.0
T_5–½WPM+0.2% activated charcoal + 3.0 mg/L IBA	15	7	46.66	47.0
SEm ±				0.91
CD (1%)				0.89
CD (5%)				0.67
SD	2.12	2.44	0.36	2.04

(0.5 to 3.0mg/L). The responses of callus induction were varied with respect to changing the concentration of plant growth regulators. The maximum 41 callus induction was observed in MS+2,4-D (2.5mg/l) followed by 38 callus in MS + 2,4-D (2.0mg/L) after three weeks of inoculation. Whereas the lowest callus induction 25 was observed in MS+2,4-D (3.0mg/l). The average mean of the percent callus induction was 31.66 on different concentrations of 2,4-D. The callus formed was distinguishable based on texture and color, into type I–white compact callus, granular in texture, and type II-greenish white and compact, nodular calli (Fig. 1). The role of 2,4-D for callus induction different plant and tree species such as *Trachyspermum* Nasab (2018), *Valenaria jatamansi* (Gautam et al. 2021), and *Moringa oleifera* Lam. (Chand et al. 2019). Only 2,4-D was used to induce callus in Chironji explants in the present study. In culture medium, 2,4-D plays a critical role in the induction of callus in cereal crops. Similarly, in culture medium for woody plants, auxin, especially 2,4-D, is usually added for callus induction and cell growth, somatic embryogenesis, shoot, root initiation, and stimulation of growth from shoot apices and shoot tip cultures.

Significant effect of different concentration plant growth regulators such as TDZ, BAP, and Kinetin alone or in combination was observed on shoot initiation from the callus of chironji enriched with 0.1% of activated charcoal. The maximum shoot initiation (30%) was observed in ½ WPM supplemented with 2.5mg/L TDZ, whereas the minimum shoot initiation (4%) was observed in 0.5mg/l. Similarly, in another experiment, callus was treated with different concentrations of BAP, and a maximum number of shoot initiation (9%) was observed in ½ WPM supplemented by 2.5mg/L BAP and the lowest (3%) was observed in 0.5mg/L. In the case of Kinetin, the maximum shoot initiation (9%) was observed in ½ WPM supplemented by 2.0mg/L Kinetin, and the minimum rate of shoot initiation (2%) was observed in 0.5mg/l. The callus cultured on ½ WPM medium fortified with TDZ performed better than ½ WPM medium fortified with BAP and Kinetin. Likewise, Huetteman and Preece (1993) stated that TDZ is highly effective for inducing axillary shoot formation in several woody plants. Earlier, alone or a combination of hormones were reported by Ling et al. (2013) in *Labisia* spp. The high cytokinin activity and positive response of woody species to TDZ have established it as among the most active cytokinins for *in vitro* manipulation of many woody species.

Direct shoot induction from callus and nodal segments

For direct shoot induction from nodal segments, WPM medium was used with different concentrations of TDZ, BAP, and Kinetin and WPM medium without growth regulator as a control. Sterilized explants were placed vertically on the medium and incubated at 25±2°C with a photosynthetic photon of 50 µmol^{-2} s^{-1} under 16/18hr photoperiod and 60-70% humidity for 21-24 days. After 21-24 days of incubation, cultures were transferred to the same medium for further growth. The number of shoots per explant was counted after 45 days of incubation. Nodal segments showed an initial response of swelling after 15 to 20 days incubation. Maximum shoot initiation response (34%) was observed in ½ WPM fortified with 2.5mg/l TDZ, while minimum shoot initiation response (3%) was observed in ½ WPM fortified with 0.5mg/L TDZ (Fig. 2 A-B). In Kinetin, the maximum shoot initiation response (9.2%) was obtained on ½ WPM supplemented with 2.0mg/l, whereas the minimum shoot initiation response (2.1%) was obtained on ½ WPM supplemented with 0.5mg/l. Similarly, in the case of BAP the maximum shoot initiation response (9.3%) was obtained on ½ WPM supplemented with 3.0mg/L, whereas the minimum shoot initiation response (3.4%) was obtained on ½ WPM supplemented with 0.5mg/L. The nodal segments inoculated on basal medium without any growth regulator exhibited a 0% regeneration response. Activated charcoal

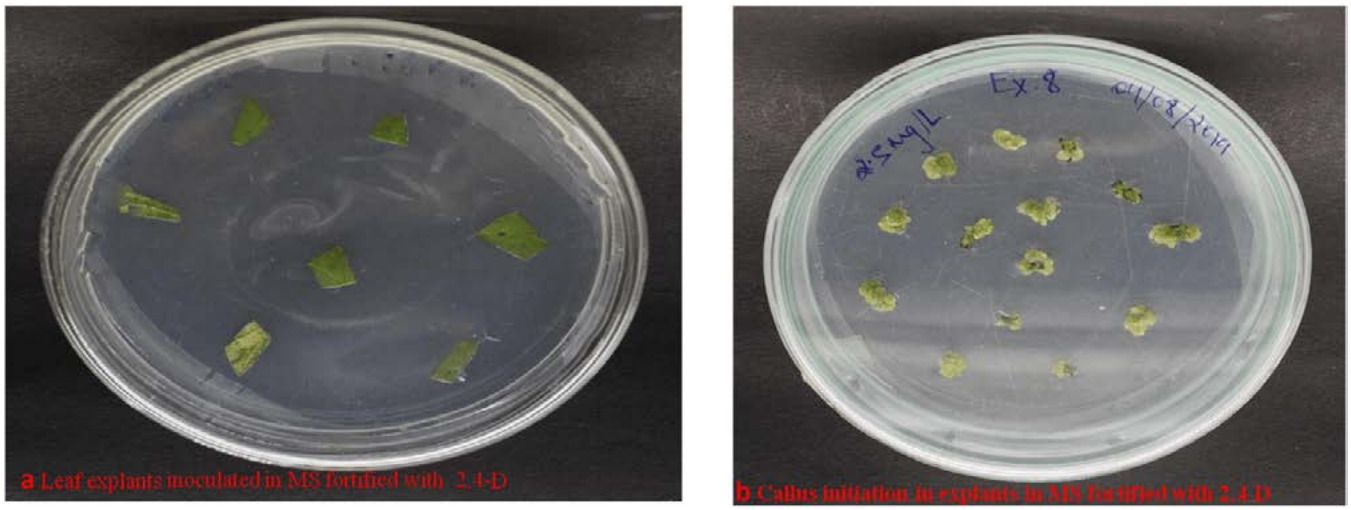

Fig. 1. Callus induction from leaf disc explants of chironji in MS fortified with different concentration of 2,4-D

Fig. 2. Multiple shoots formation from the nodal segments of chironji. (A-B) *In vitro* shoots regeneration from the nodal segments on WPM containing 2.5 mg/l TDZ; (C-D) *In vitro* shoot multiplication on WPM containing 2.5 mg/l TDZ and 0.5 GA_3 anfer 45 days of inoculation

Fig. 3. Root induction from the shoot of Chironji (A-B) Root initation in 1/2 on WPM contaning with 2 mg/l IBA after 46 days of inoculation (C-D) Hardening of plants

(0.1%) was also added to the medium to prevent the phenolic secretions from the explants. The nodal segments cultured on ½ WPM medium fortified with TDZ performed better than ½ WPM medium fortified with BAP and Kinetin. Rani et al. (2006) used 2mg/l BAP with 1-mg/L NAA to induce shoots from nodal segments in *Coleus blumei,* whereas Sharma et al. (1991) used 2mg/L kinetin in combination with 1mg/l IAA in *Coleus forskolii*.

Shoots proliferation, root formation, and acclimatization

For shoot, multiplication results show the significant effect of different combinations of growth hormone concentration such as TDZ and GA_3 shoot multiplication with 0.1% activated charcoal (Table 1). The maximum shoot multiplication (78%) was observed in ½WPM supplemented with 2.5mg/L TDZ and 0.5mg/L GA_3 with 5.7 shoots per explants, whereas a minimum rate of shoot multiplication (36%) was observed in ½WPM supplemented with 2.5mg/L TDZ and 3.0mg/l GA_3 with 2 shoots per explants (Fig. 2 C and D). The results indicate that with the increase in the concentration of GA_3, the rate of shoot multiplication decreases gradually. Similarly, Ling et al. (2013) developed an improved protocol for micro-propagation in *Labisia pumila,* using nodal segments with different combinations of hormones. Similarly, an improved protocol for somatic embryogenesis and organogenesis from nodal segments and shoot-apices of *Moringa oleifera* Lam has also been developed (Chand et al. 2019; Pandey et al. 2019).

WPM with various concentrations of IBA (0-3mg/L) enriched with activated charcoal for root formation was used. Maximum 8 roots were observed in 13 inoculated in vitro regenerated shoots with ½WPM supplemented with 2.0mg/l IBA enriched with 0.2% activated charcoal. However, the lowest number of roots initiated *i.e.,* 4 out of 13 shoots inoculated, was observed in ½WPM with 1.5 mg/l IBA supplemented with 0.2% activated charcoal (Fig. 3 A-B). The effect of media and growth regulators on roots formation from *in vitro* generated shoot on enriched WPM is summarized in Table 2. Auxins such as NAA and IBA are the most commonly used for promoting rooting of *in vitro* regenerated shoots, as reported by Naik et al. (1999) in pomegranate and Ling et al. (2013) in *Labisia pumila* var. *alata*. Plants with roots (3-4cm in length) were acclimatized and transferred to pots containing an autoclaved mixture of soil, sand, and manure (2:1:1). Approximately 70% survival rate was recorded from *in vitro* grown plantlets on transfer to pots (Fig. 3 C-D).

Authors' contribution

Conceptualization of research (SWS, RS); Designing of the experiments (SWS, ND, RS); Contribution of experimental materials (RS); Execution of field/lab experiments and data collection (RSR, SS); Analysis of data and interpretation (AK, VKG); Preparation of a manuscript (RS, SVS).

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