Direct and callus mediated regeneration from nodal and internodal segments of *Crataeva religiosa* G. Forst. var. *nurvala* (Buch.-Ham.) Hook. f. & Thomson

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*Crataeva religiosa* G. Forst. var. *nurvala* (Buch.-Ham.) Hook. f. & Thomson (syn. *Crataeva nurvala* Buch.-Ham.), a fast growing medicinal tree species, has been successfully micropropagated under *in vitro* conditions. Direct multiple shoots were regenerated from nodal explants on Murashige and Skoog’s (MS) medium, solidified by agar and supplemented with 1 mg/L 6-benzyl amino purine (BAP) and 0.5 mg/L α-naphthalene acetic acid (NAA). However, the successful regeneration from internodal callus was achieved on 2.0 mg/L BAP and 0.5 mg/L NAA. The frequency of *in vitro* multiplication from culture of nodal segments was higher compared to that of callus mediated regeneration from culture of internodal segments. Further, the best rhizogenesis was achieved on solidified MS medium supplemented with 3 mg/L indole-3-butyric acid (IBA).

**Keywords:** Callus, *Crataeva religiosa* var. *nurvala*, in *vitro* regeneration, rhizogenesis, shoot multiplication

**Introduction**

*Crataeva religiosa* G. Forst. var. *nurvala* (Buch.-Ham.) Hook. f. & Thomson (syn. *Crataeva nurvala* Buch.-Ham.) (Family: Capparaceae) is a fast growing, beautiful tree, closely related to *C. adansonii*1,2. It is distributed in the tropical zone and commonly found throughout India, Myanmar and Sri Lanka3. The tree is planted in the gardens for its ornamental and medicinal purposes. It flowers profusely during March-May with maximum bloom in April. This avenue tree, refers as ‘Veruna’ by Vedic deities, is the best known Ayurvedic medicine for alleviating inflammatory bowel disorders/ulcerative colitis4. The stem-bark is extensively used as an anti-inflammatory, anti-arthritis and antilithic agent due to a chemical, lupeol. The chemical has been found to decrease oxalate and peroxidase levels but increase antioxidant status in rat kidney5. The tree is normally propagated by root suckers, which restrict its distribution to very limited area. Thus, clonal propagation could be important for the preservation and propagation of this plant species6.

Despite of high flowering, the natural fruit production in var. *nurvala* has been low because of floral gall formation, which corresponds to its low productivity7. Poor seed germination and seedling establishment are also responsible for its limited prevalence. But for accentuating quantity of plant, nodal and callus cultures are desirable, among which nodal culture mediated plants are more in number per subculture and their maintenance is easier as compared to callus-mediated multiplied plants8. Enhancement of extremely low population is of deep concern. Although both nodal and callus cultures were suitable for multiplication but the present work was targeted to search the explants which could be most effective for rapid multiplication. It was found that direct regeneration of plants as compared to indirect mode yielded clonal plants for large scale propagation as well as for genetic transformation study. Therefore, a study on the clonal propagation is needed to preserve characters of different elite plants6.

**Materials and Methods**

**Plant Material and Sterilization**

The plant *C. religiosa* var. *nurvala* was maintained in the SAP Garden of DRS Department of Botany, BRA Bihar University, Muzaffarpur for the last 2 yr. Healthy nodal and intermodal segments (2 cm) were excised and washed thoroughly in water and Tween 20 (5 drops in 100 mL) for 10 min under constant agitation. The explants were then immersed for 2-3 min in 0.2% (w/v) HgCl₂ (E-Merck, India). These nodal and internodal explants were again washed 3 times with sterilized double distilled water. Under aseptic conditions, they were then placed vertically onto the culture medium.
Media and Incubation Conditions
Murashige and Skoog’s (MS) medium with 3% sucrose (w/v), 0.8% agar and supplemented with different concentrations of α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylamino purine (BAP) either alone or in combination was used in culture tubes/bottles for callus induction and shoot proliferation. The MS medium was also fortified with different concentrations of IAA or IBA for rooting. The pH of each medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before addition of agar and autoclaved at 121°C for 15 min. The cultures were maintained at 25±2°C and 12 h photoperiod with a light intensity of 6000 lux supplied by cool-white fluorescent lamps (Philips, India). These cultures were routinely transferred to fresh medium after 4th wk of inoculation.

In Vitro Culture Conditions and Shoot Regeneration
The explants (nodal, intermodal segments and compact calli) were inoculated on MS medium, supplemented with different concentrations and combinations of hormones (0.2-3 mg/L NAA, 0.5-2 mg/L 2,4-D & 0.5-4 mg/L BAP). The effects of these combinations of growth regulators on callus formation and regeneration were recorded at regular intervals. High percentage of shoot regeneration was detected on MS medium containing 1 mg/L BAP+0.5 mg/L NAA in case of nodal explant and 2 mg/L BAP+0.5 mg/L NAA in case of callus explants from internodal segment (Table 1). The brown, compact calli and their degree of better response in respective days were too recorded at 2 mg/L BAP+3 mg/L NAA (Table 2). The cultures were evaluated for percentage of regenerated shoots, mean number and length of shoots after 4 wk of culture.

Root Regeneration
The regenerants, obtained after 4 wk of culture were separated into pieces and placed on MS medium containing 1-3 mg/L IAA or IBA to induce rooting (Table 3). The culture tubes were provided with 25 mL of medium with 0.75% of agar for easy proliferation of the emerging roots. The duration for root appearance was found to be 25-30 d of inoculation. The data on percentage of root response and number of roots per explants were recorded after 4 wk of subculture.

Hardening and Acclimatization
Rooted shoots of almost 5 cm of 4-wk-old cultures were taken out from the culture tubes gently, washed with distilled water to remove the adhering agar medium, and then transferred to tray-beds containing sterilized soil and vermiculite in 1: 1 ratio. Plantlets were maintained at 25±2°C in the acclimatized culture chamber having 85% humidity for 35 d for hardening. The regenerants were then shifted in the field for exposure to the natural environment.

Statistical Analysis
The percentage response of shoot regeneration, number of multiple shoot, height of shoot and regenerants from callus explants were monitored as growth parameters. Data of each experiment represented by 6 replicates were subjected to statistical analysis (mean±SD) according to new Duncan’s multiple range tests (Gomez & Gomez, 1976).

Results and Discussion
Plant micropropagation via direct nodal regeneration allows large multiplication of plantlets in vitro by preventing clonal variation as compared to regenerated ones from calli alone, which often leads to somaclonal variation. In the present study, direct nodal and callus mediated regenerations were taken into consideration. Nodal and intermodal segments as explants (2-3cm) were excised from 1½-yr-old plant of C. religiosa var. nurvala (Fig. 1a) and used for initial establishment of cultures in combination with different growth regulators at different concentrations. Induction of shoot buds from nodal segments occurred with either BAP alone or BAP in combination with NAA. BAP (1-3 mg/L) alone was reliable concentration for initiating shoot induction in the range of 33-83% from nodal explants (Fig. 1b). But 1.0 mg/L BAP and 0.5 mg/L NAA was very effective (100%) for shoot induction and multiplication in vitro culture (Table 1; Fig. 1c). However, the shoot regeneration from callus explants of intermodal segments at 2 mg/L BAP and 0.5 mg/L NAA was lower (70%). The number of shoots per explants and length of shoot (in cm) among regenerants either from nodal or calli of intermodal explants at the respective concentrations were also recorded in all the combinations after 4 wk. The mean number of shoots ranged between 0.4-4.1 in case of nodal explants and between 0.2-3.4 in case of callus explants. The mean length of shoots in these cases also ranged between 1.4 cm and 0.6-3.1 cm, respectively (Table 1). Thus, the rate of in vitro multiplication as well as their mean number and length from nodal culture was always found to be
higher as compared to that of callus mediated regeneration from internodal culture. Nodal explants were always recorded to be superior for in vitro rapid production of plantlets.\textsuperscript{12,13}.

Callus induction occurred in about 25 d. Callus induction and growth started with the swelling of epithelial cells of nodal explants (Fig. 1d). No callus induction was observed in MS medium supplemented with 2,4-D alone. However, on the other hand, best callus initiation and growth were observed in combination of NAA (0.5 mg/L)+BAP (0.5–2.0 mg/L) as well as in combination of NAA (0.5 mg/L)+BAP (2.0–4.0 mg/L)+2,4-D (0.5 mg/L) as
compared to other combinations of phytohormones (Table 2; Figs. 1e-g). Thus obtained calli were further transferred on medium with BAP (2.0 mg/L) and NAA (0.5 mg/L), which resulted in high frequency (70%) of shoot regeneration (Table 1).

Callus induction was poor on MS medium supplemented with auxin alone, though NAA supplementation resulted in fragile green to brown callus. With cytokinin alone in MS medium, compact brown callus was formed. Interestingly high concentration of BAP (2.0 mg/L) changed the colour of callus from green to brown and showed low response. The best response was achieved on 4.0 mg/L BAP in combination with 0.5 mg/L NAA and 0.5 mg/L 2, 4-D within 20 d and callus became fragile white from compact white (Table 2). The callus after 2-3 wk of subculture in MS medium with all concentrations of BAP+NAA exhibited shoot bud formation but highest percentage of shoot regeneration (70%) was observed at 2.0 mg/L BAP+0.5 mg/L NAA (Table 1). Generally, BAP is employed for shoot regeneration

However, in several studies, culture media supplemented with NAA and BAP have also been useful for production of shoots.

The in vitro regenerated shoots from both explants (nodal & callus of internodal segments) were transferred to root induction medium comprising of MS salts supplemented with 1-3 mg/L IBA or 1-3 mg/L IAA, which induced in vitro rooting at the basal end of shoots after 4 wk of culture (Table 3; Fig. 1h). But IAA was found to induce rooting at low frequency (45-65%) as compared to IBA (50-75%). The comparative frequency of number of roots per explants for both hormones was also found to be different. It was 3-6.5 in case of IBA and 2-4.7 in case of IAA. IBA showed similar effect for in vitro rooting in Aegle marmelos and Citrus reticulate but it was reverse in case of Cichorium intybus, another potent medicinal plant. After the complete plantlet regeneration, these were hardened and acclimatized in a culture chamber and then transferred to the field conditions (Fig. 1i). A similar procedure for the hardening of in vitro raised plants was adopted in curry leaf tree (Murraya koenigii L.).

Fig. 1 (a-i)—(a) C. religiosa var. nurvala plant in SAP Garden; (b) Development of shoots from nodal explant on MS+3.0 mg/L BAP (mark green healthy shoots in 25-d-old culture); (c) Nodal explant showing development of direct multiple shoots on MS+1 mg/L BAP+0.5 mg/L NAA; (d) Induction of calli and growth of shoots with the swelling of the basal surface of nodal explants on MS+2 mg/L BAP+0.5 mg/L NAA; (e) Induction of green compact callus on MS+1.0 mg/L NAA+0.5 mg/L BAP from internodal segment; (f) Initiation of multiple shoots from white compact callus on MS+0.5 mg/L NAA+2.0 mg/L BAP+0.5 mg/L 2, 4-D; (g) Development of multiple shoot from callus on MS+0.5 mg/L NAA+2.0 mg/L BAP (mark green shoots and swelling of basal part); (h) Rooting on MS+3 mg/L IBA; & (i) 25-d-old plantlet of C. religiosa var. nurvala on transfer in tray bed bag.
In conclusion, the present study reveals an effective regeneration and multiplication protocol for in vitro propagation of *C. religiosa* var. *nurvala*, which can successfully be used for large scale multiplication and propagation of this medicinally important tree.

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