Environmental and Experimental Biology (2019) 17: 179–183 *Original Paper*
DOI: 10.22364/eeb.17.18

Direct shoot regeneration from excised leaf segments of *Crataeva nurvala*

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Abstract

Crataeva nurvala Buch Ham. (Capparidaceae) is an important medicinal tree. Stem and root bark of the tree are important constituents of various pharmaceutical products. Its natural propagation is hampered due to seed feeding insects. The present work describes a method to obtain plants of *C. nurvala* from excised leaf segments through direct shoot regeneration. An average of six shoots regenerated from single leaf segment on Murashige and Skoog medium supplemented with 2 mg L^{-1} 6-benzyladenine and 0.1 mg L^{-1} indole-3-acetic acid within 45 to 60 days. These shoots were rooted on ½ Murashige and Skoog medium containing 0.5 mg L^{-1} α-naphthaleneacetic acid. About 80% rooted plants were successfully acclimatized in natural conditions. These results will be useful for future studies using leaf segments for genetic transformation, genome editing and mutation breeding with *Crataeva* and related taxa.

Key words: adventitious shoots, *Crataeva nurvala*, micropropagation, tissue culture.
Abbreviations: BA, 6-benzyladenine; KN, kinetin; IAA, indole-3-acetic acid; NAA, α-naphthaleneacetic acid.

Introduction

Crataeva nurvala Buch Ham. (Capparidaceae) is a high value medicinal tree. The medicinal importance of *C. nurvala* has been documented in traditional systems of medicine (Bopana, Saxena 2008). The root and stem bark of the tree is utilized in traditional indigenous systems of medicine as a blood purifier, sedative, anthelmintic and antipyretic (Bhandari et al. 1951; Krishnaiah et al. 2011). The bark powder of *C. nurvala*, *Caesulia axillaries*, and *Morinda citrifolia* cooked in mustard oil is used in treatment of baldness and sores (Poonam, Singh 2009). It is considered as useful source of saponins, triterpenes, alkaloids, tannins, glucosinolates, phytosterols and flavonoid glycosides (Bhattacharjee 2012). Bark of this plant is one of the key ingredient of various pharmaceutical products like Urox® (Schoendorfer et al. 2018), Divya Vrikkdoshhar Vati® and Mahamanjisthadi Kwath Pravahi® of Patanjali Ayurved, Stondab® syrup of Dabur India, Renomet® of Matxin labs™ Purim®, Nefrotec Vet®, Himplasia® and Renalka® of Himalaya Drug company (2019). Therefore, the tree is in high demand for pharmaceutical industries.

The most challenging obstacle in micropropagation of perennial plants is recalcitrance of explants (Benson 2000; McCown 2000). These plants have complex seasonal cycles, which obscure control of their growth during *in vitro* culture (Benson 2000). Habitat disfigurement, changes in microclimate conditions and over-exploitation have drastically diminished its natural population in the forest (Shirin, Maravi 2006; Yadav, Gupta, 2006). The natural propagation of *C. nurvala* is hampered because of poor seed germination and heavy infestation by insects like *Aschistonyx baranii* and *Aschistonyx crataeve* (Nataraj, Padhya 1988; Panwar, Vashistha, 2008; Kher et al 2016; Walia et al. 2003). In natural conditions it is also propagated via root suckers. However, propagation by root suckers is a time-consuming process and a very limited number of plants can be obtained from root suckers (Kher et al 2016; Walia et al. 2003). Keeping the importance in view, the present study was focussed on development of an efficient shoot regeneration protocol for *C. nurvala* from leaf segments.

Materials and methods

Explant source and aseptic culture

Young and fully expanded mature leaves (Fig. 1 A – D) from one-year-old seedlings of *C. nurvala* were collected from the P.G. Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar during December 2016 to March 2017 (only single season). Leaves were cleaned in mild tap water-flow for 10 min to remove adhering dust. Later, leaves were soaked in 5% (v/v) aqueous Neutral Rankleen® liquid detergent (RFCL, India) solution for 5 min. Subsequently these leaves were rinsed under mild tap water flow for 5 min to eliminate remnant detergent. Then

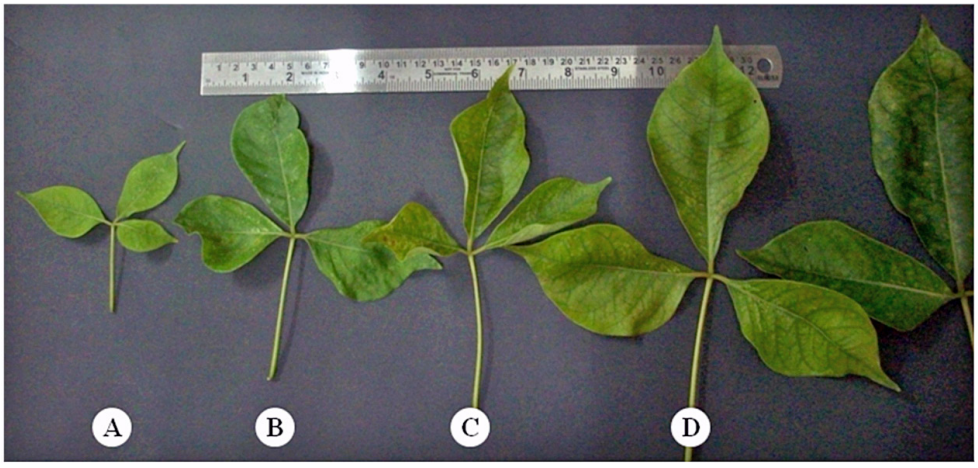

Fig. 1. A – D, sequential developmental stages of leaves of *Crataeva nurvala*. Plant is having trifoliate leaf, hence, leaflets from young to mature leaves were used as explants.

leaves were aseptically disinfected with aqueous 0.1% w/v mercuric chloride solution (SRL, India) for 2 min with mild shaking and the disinfected leaves then were finally four times cleaned with autoclaved distilled water. After surface disinfection, leaf segments were transferred to media containing plant growth regulators (Table 1).

Culture medium

The culture medium consistsed of Murashige and Skoog (MS; Murashige, Skoog 1962) medium with 3% (w/v) sucrose (Himedia, Mumbai, India). The pH level of all media was calibrated to 5.8 by 0.1 N HCl or NaOH prior to adding 0.8% (w/v) agar (bacteriological grade, Merck, Mumbai, India). Medium was autoclaved at 121 °C for 15 min. For culture initiation, borosilicate glass test tube (15 × 150 mm, Borosil, Ahmedabad, India) was used with 20 mL medium and one explant per tube, plugged with non-absorbent cotton enfolded in a sole layer of cheesecloth.

Direct shoot regeneration from excised leaf

Intact young leaflet (size not determined) or leaflet segments from mature and expanded leaves (size not determined) with midrib were cultured on MS medium with combinations of 1, 2 and 3 mg L^{-1} cytokinin 6-benzyladenine (BA) or kinetin (KN) with 0.1 mg L^{-1} auxin either α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA). For further elongation and multiplication, in vitro regenerated shoot clumps were separated from leaves and subcultured on the MS medium supplemented with 0.1 mg L^{-1} BA. All plant growth regulators were procured from HiMedia.

Rooting and acclimatization

For *in vitro* rooting shoots (length > 3 cm) were transferred to ½ MS medium containing 0.50 mg L^{-1} NAA and cultivated for 30 days. Rooted shoots were cautiously taken out from the culture vessels and cleaned with sterile water to eliminate traces of adhering medium. The plantlets were then transferred to plastic pots containing cocopeat and moss (1:1) for 15 days and kept in a plant tissue culture cabinet. The plantlets were enveloped with clear transparent polythene cover (100 micron thick) to maintain high humidity, and irrigated with 15 mL of ¼ MS at a consistent interval of seven days. Following 15 days, the polythene cover was removed and plantlets were cultivated in pots with soil/sand/cocopeat (1:1:1) for the next 15 days in a plant tissue culture cabinet. Afterwards, plantlets were transferred to pots with garden soil in natural environmental conditions.

Culture conditions

Cultures were maintained at 25 ± 2 °C, 16 h photoperiod

Table 1. Effects of plant growth regulators on shoot bud regeneration from leaves of *Crataeva nurvala*. Values represent mean ± SE. Means followed by the same letter within each column are not significantly different according to Duncan's multiple range test at $P < 0.05$. Data were recorded after second subculture

Plant growth regulator (mg L^{-1})	Number of shoot buds per explant
Control	0 f
BA (1.0) + NAA (0.1)	2.83 ± 0.31 cd
BA (2.0) + NAA (0.1)	4.50 ± 0.43 b
BA (3.0) + NAA (0.1)	4.33 ± 0.21 b
BA (1.0) + IAA (0.1)	3.50 ± 0.22 c
BA (2.0) + IAA (0.1)	6.83 ± 0.17 a
BA (3.0) + IAA (0.1)	4.67 ± 0.21 b
KN (1.0) + NAA (0.1)	1.67 ± 0.21 e
KN (2.0) + NAA (0.1)	2.00 ± 0.26 d
KN (3.0) + NAA (0.1)	2.67 ± 0.21 cd
KN (1.0) + IAA (0.1)	2.33 ± 0.21 d
KN (3.0) + IAA (0.1)	2.83 ± 0.40 c
KN (3.0) + IAA (0.1)	3.33 ± 0.21 cd

at a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (36 W Philips, India).

Statistical analysis

Each treatment consisted of at least five replicates and experiments were repeated thrice. Results were subjected to analysis of variance using SPSS ver.19. The mean values were calculated and compared by Duncan's multiple range tests ($P < 0.05$).

Results

In the present study effects of combinations of cytokinin and auxin on intact young leaflet (Fig. 1A) and excised leaf segments from mature leaf (Fig. 1B – D) of *C. nurvala* were investigated. The first visible changes in cultured leaf explants were a slight bulging towards the cut end of leaf veins within the first week after transfer to shoot induction medium. Explants on plant growth-free MS medium did not induce adventitious shoots. However, MS medium supplemented with 1, 2 and 3 mg L^{-1} BA or KN with 0.1 mg L^{-1} IAA or NAA induced adventitious shoot bud formation within 30 days (Table 1). MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} IAA was most effective, and produced a mean value of 6.83 shoot buds per explant, after 45 to 60 days when compared to other plant growth regulator combinations (Table 1). There were no significant differences in shoot regeneration from leaf segments or intact leaflets (data not shown). However, the frequency of shoot regeneration was greatly influenced by plant growth regulator concentration and combination (Table 1). Shoot buds protruded from the basal, apical and peripheral parts of intact leaflets from young leaves (Fig. 2 A – C, 3 A – D) and leaf segments from mature leaves (Fig. 3 A – D) within 30 to 35 days. When the culture period was extended to 60 days on the same medium, some adventitious shoots developed fully and formed a multiple shoot clusters. In the present study, all adventitious shoots buds formed were connected with side veins of the midrib (Fig. 2 A, 3 C – D, 4 A – B).

It was also observed that more shoot buds were formed from the region which was connected towards the medium, irrespective of leaf position and age, as based on morphological observation (Fig. 2 A – C, Fig. 3 A – B). After shoot bud differentiation, shoot clusters were subcultured on the MS medium supplemented with 0.1 mg L^{-1} BA for shoot elongation.

About 70% *in vitro* raised shoots were successfully rooted on ½ MS medium supplemented with 0.5 mg L^{-1} NAA. About 80% rooted plants successfully survived in natural conditions.

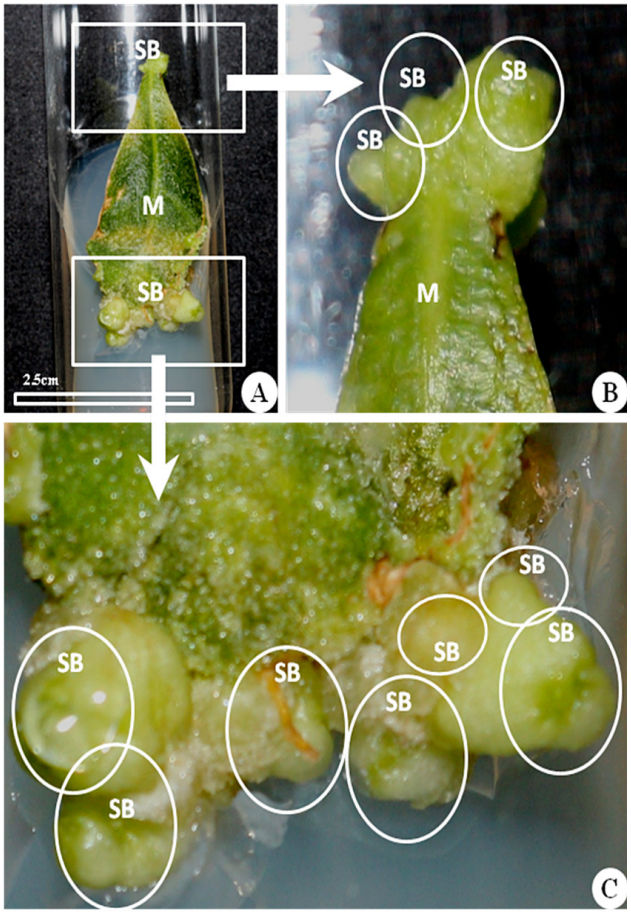

Fig. 2. Adventitious shoot regeneration from small size intact leaflet of *Crataeva nurvala* on MS medium containing 2 mg L^{-1} BA and 0.1 mg L^{-1} IAA, 3 % sucrose and 0.8 % agar. A, full leaflet; B, basal region; C, apical region. SB, shoot bud; M, midrib.

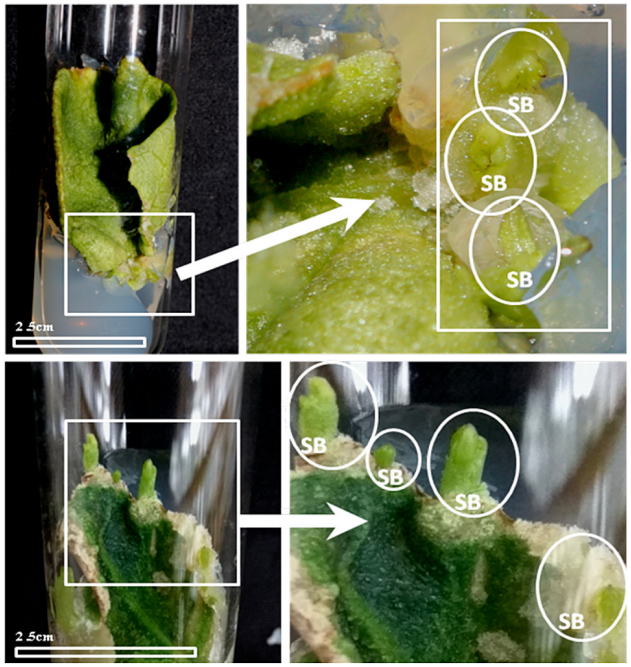

Fig. 3. Adventitious shoot regeneration from leaf segments from mature leaflet of *Crataeva nurvala*. Culture medium and conditions the same as for Fig. 2. Upper part, basal region; lower part, apical region. SB, shoot bud; M, midrib.

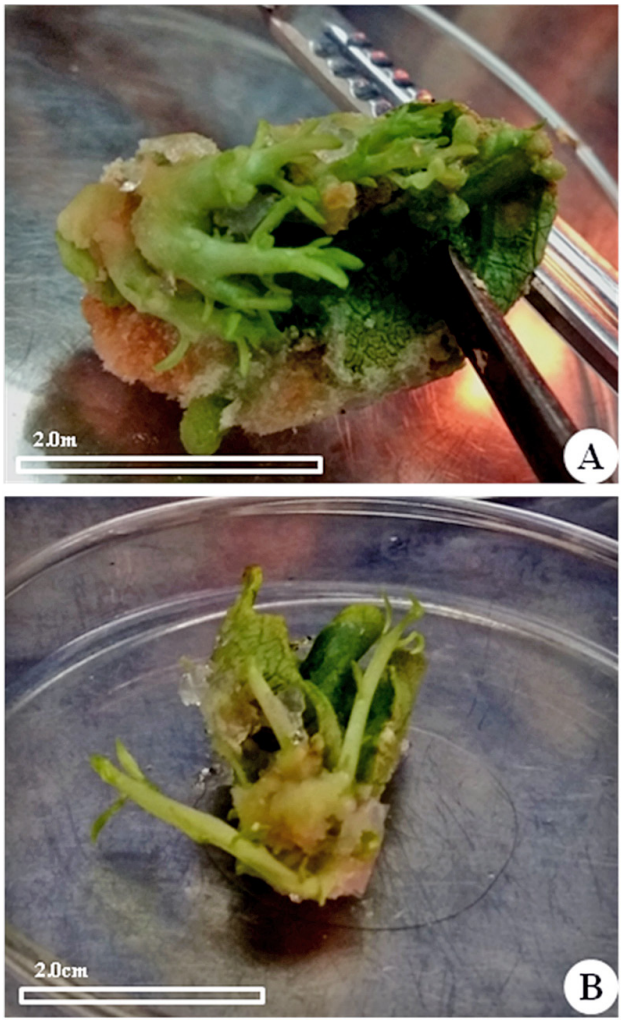

Fig. 4. Elongated shoots of *Crataeva nurvala*. Culture medium and conditions the same as for Fig. 2.

Discussion

It is well established that *in vitro* plant morphogenesis is an important step for plant regeneration in tissue culture, and an important tool for developmental biology study, and serves as an model experimental system for plant genetic transformation research *in vitro* (Li et al. 2019). Although woody perennial plants are known to be recalcitrant, *C. nurvala* is more amenable for *in vitro* cultures (Inamdar et al. 1990; Nataraj 1992). Direct adventitious shoot bud formation from leaf explants has been reported in *Sedum sarmentosum* (Kim, Sivanessan, 2016), *Chirita swinglei* (Chen et al. 2016), and *Withania coagulans* (Rathore et al. 2016). Both cytokinin and auxin have been recognized for a long time to act either antagonistically or synergistically to regulate a number of important developmental processes, such as the formation and maintenance of meristem (Su et al. 2011). A key advance in leaf evolution is the acquirement of a flat lamina by means of adaxial–abaxial polarity, which optimizes the most important task of photosynthesis (Yamaguchi et al. 2012). *De novo* organogenesis can be easily accomplished in plants by appropriate hormonal regulation (Pulianmackal et al. 2014). The significant role of low concentration of auxin in combination with high concentration of cytokinin on shoot regeneration has previously been reported in *Althaea officinalis* (Naz et al. 2015).

Further investigation is required to check effect of polarity and leaf orientation on shoot regeneration potential from leaves. Leaves can have differential maturation potential, and cells near leaf bases are more juvenile and thus exhibit higher regeneration potential (Karam, Al-Majathoub 2000). Also, increased density of vascular tissues, levels of plant growth regulators and metabolites near the petiole base are reported to be positive attributes for regeneration (Rathore et al. 2016).

Lower concentration of cytokinin has been utilized for shoot elongation and multiplication of *Tinospora cordifolia* (Panwar et al. 2018), and *Hybanthus enneaspermus* (Revathi et al. 2018). In contrast, lower concentration of gibberelic acid also has been used for elongation of shoots (Gharari et al. 2019). Hence, a comparative study on effects of lower concentration of cytokinin and gibberelic acid on shoot elongation and multiplication can be useful for multiplication and maintenance of cultures of *C. nurvala*.

Earlier reports (Babbar et al. 2009; Basu et al. 2009; Bopana, Saxena 2009; Walia et al. 2007) also suggested the use of ½ MS medium for *Crataeva* sp. Walia et al. (2007) used ½ MS or Woody Plant Medium supplemented with 0, 0.11, 0.54, 2.69 and 5.37 μM NAA and found that ½ MS medium supplemented with 0.11 μM NAA was the most effective medium for *in vitro* rooting. Micropropagated plants were very delicate and adapted for controlled conditions like uniform supply of light, temperature, humidity, and nutrients. Therefore, transfer of *in vitro* raised plants to the field condition requires proper care.

In conclusion, the present work describes adventitious shoot regeneration from leaves of *C. nurvala*. This protocol could be viable option for large-scale propagation of other Capparidaceae genera. Direct regeneration is very important because it can be used later for genetic transformation studies. Based on the present study, *C. nurvala* is not a recalcitrant plant for *in vitro* regeneration and propagation.

Author contribution and conflict of interest

MMK and MN conceived and designed the research and analyzed the data. MKK, DBR, DD, DK performed the experiments. MMK, DBR, and MN wrote the manuscript. MN supervised the experimental tasks. All authors have no conflict of interest to declare.

References

Babbar S.B., Walia N., Kaur A. 2009. Large-scale *in vitro* multiplication of *Crataeva nurvala*. In: Jain S. M., Saxena P. K. (eds) *Protocols for* in Vitro *Cultures and Secondary Metabolite*

Analysis of Aromatic and Medicinal Plants. Methods in Molecular Biology. Vol. 547. Humana Press, Totowa, NJ, pp. 61–70.

Basu M.J., Ramanathan R., Yogananth N., Baburaj S. 2009. Micropropagation of *Crataeva religiosa* Hook. f. & Thoms. *Curr. Trends Biotechnol. Pharm.* 3: 287–290.

Benson E.E. 2000. *In vitro* plant recalcitrance: an introduction. *In Vitro Cell. Devel. Biol. Plant* 36: 141–148.

Bhandari P., Dhar M., Sharma V. 1951. Chemical constituents of the root bark of *Crataeva nurvala* Ham. *J. Sci. Ind. Res.* 10: 195–196.

Bhattacharjee S. 2012. The language of reactive oxygen species signaling in plants. *J. Bot.* 2012: 1–22.

Bopana N., Saxena, S. 2009. *In vitro* regeneration of clonally uniform plants of *Crataeva magna*: a high value medicinal tree by axillary branching method. *New For.* 38: 53–65.

Bopana N.A., Saxena S.B. 2008. *Crataeva nurvala*: a valuable medicinal plant. *J. Herbs Spices Med. Plants* 14: 107–127.

Chen Y., Zhang Y., Cheng Q., Niu M., Liang H., Yan H., Zhang X., Teixeira da Silva J.A., Ma G. 2016. Plant regeneration via direct and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) W. T. Wang. *In Vitro Cell. Dev. Biol. Plant* 52: 521–529.

Gharari Z., Bagheri K., Sharafi A., Danafar H. 2019. Thidiazuron induced efficient in vitro organogenesis and regeneration of *Scutellaria bornmuelleri*: an important medicinal plant. *In Vitro Cell. Dev. Biol. Plant* 55: 133–138.

Himalaya Drug company, 2019. Three Leaved Caper. URL http://www.himalayawellness.com/herbfinder/crataeva-nurvala. (accessed 10-10-2019)

Inamdar J.A., Nataraj M., Mohan J.S.S., Subramanian R.B. 1990. Somatic embryogenesis from callus cultures of *Crataeva nurvala* Buch. Ham. *Phytomorphology* 40: 319–322.

Karam N.S., Al-Majathoub M. 2000. Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue. *Sci. Hortic.* 86: 235–246.

Kher M.M., Nataraj M., Teixeira da Silva J.A. 2016. Micropropagation of *Crataeva* L. species. *Rend. Lincei* 27: 157–167

Kim D.H., Sivanessan I. 2016. Influence of benzyladenine and thidiazuron on shoot regeneration from leaf and shoot tip explants of *Sedum sarmentosum* Bunge. *Brazilian Arch. Biol. Technol.* 59: 1–6.

Krishnaiah D., Sarbatly R., Nithyanandam R. 2011. A review of the antioxidant potential of medicinal plant species. *Food Bioprod. Process.* 89: 217–233.

Li T., Cai H., Wang T., Fu Y., Yang W., Zhao A., Cui Z., Wang J., 2019. Plant regeneration in *Ixora chinensis* from young leaves. *Plant Cell Tissue Organ Cult.* In press. doi:10.1007/s11240-019-01691-7

McCown B.H., 2000. Recalcitrance of woody and herbaceous parennial plants: dealing with genetic predeterminism. *In Vitro Cell. Dev. Biol. Plant* 36: 149–154.

Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.

Nataraj, M. 1992. *Crataeva nurvala* Buch. Ham.: *in vitro* studies in three angiosperm taxa. PhD Thesis. Sardar Patel University, Vallabh Vidyanagar, pp. 14–58.

Nataraj M., Padhya M.A. 1988. Insect gall formation in *Crataeva nurvala* Buch Ham. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 58: 87–88.

Naz R., Anis M., Aref I.M. 2015. Management of cytokinin–auxin interactions for *in vitro* shoot proliferation of *Althaea officinalis* L.: a valuable medicinal plant. *Rend. Lincei* 26: 323–334.

Panwar D., Patel A.K., Shekhawat N.S. 2018. An improvised shoot amplification and *ex vitro* rooting method for offsite propagation of *Tinospora cordifolia* (Willd.) Miers: a multi-valued medicinal climber. *Indian J. Plant Physiol.* 23: 169–178.

Panwar S., Vashistha B.D., 2008. Effect of some auxins on regenerative potential of various parts of *Crataeva nurvala* Buch.-Ham. *Ann. Agri Bio Res.* 13: 103–113.

Poonam K., Singh G.S. 2009. Ethnobotanical study of medicinal plants used by the Taungya community in Terai Arc Landscape, India. *J. Ethnopharmacol.* 123: 167–176.

Pulianmackal A.J., Kareem A.V.K., Durgaprasad K., Trivedi Z.B., Prasad K., 2014. Competence and regulatory interactions during regeneration in plants. *Front. Plant Sci.* 5: 142.

Rathore M.S., Mastan S.G., Yadav P., Bhatt V.D., Shekhawat N.S., Chikara J. 2016. Shoot regeneration from leaf explants of *Withania coagulans* (Stocks) Dunal and genetic stability evaluation of regenerates with RAPD and ISSR markers. *South African J. Bot.* 102: 12–17.

Revathi J., Manokari M., Shekhawat M.S. 2018. Optimization of factors affecting in vitro regeneration, flowering, ex vitro rooting and foliar micromorphological studies of *Oldenlandia corymbosa* L.: a multipotent herb. *Plant Cell, Tissue Organ Cult.* 134: 1–13.

Schoendorfer N., Sharp N., Seipel T., Schauss A.G., Ahuja K.D.K., 2018. Urox containing concentrated extracts of *Crataeva nurvala* stem bark, *Equisetum arvense* stem and *Lindera aggregata* root, in the treatment of symptoms of overactive bladder and urinary incontinence: A phase 2, randomised, double-blind placebo controlled trial. *BMC Complement. Altern. Med.* 18: 1–11.

Shirin F., Maravi S. 2006. Clonal propagation of an important medicinal tree *Crataeva nurvala* through enhanced axillary branching. *J. Herbs Spices Med. Plants* 12: 165–174.

Su Y.H., Liu Y.B., Zhang X.S. 2011. Auxin-cytokinin interaction regulates meristem development. *Mol. Plant* 4: 616–625.

Walia N., Kaur A., Babbar S.B. 2007. An efficient, *in vitro* cyclic production of shoots from adult trees of *Crataeva nurvala* Buch. Ham. *Plant Cell Rep.* 26: 277–284.

Walia N., Sinha S., Babbar S.B. 2003. Micropropagation of *Crataeva nurvala*. *Biol. Plant.* 46: 181–185.

Yadav A.S., Gupta S.K. 2006. Effect of micro-environment and human disturbance on the diversity of woody species in the Sariska tiger project in India. *For. Ecol. Manage.* 225: 178–189.

Yamaguchi T., Nukazuka A., Tsukaya H. 2012. Leaf adaxial-abaxial polarity specification and lamina outgrowth: Evolution and development. *Plant Cell Physiol.* 53: 1180–1194.

Received 13 August 2019; received in revised form 12 October 2019; accepted 20 November 2019