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Effect of growth hormones on callus induction and shoot induction in *Crataeva religiosa* var. Nurvula (Buch-Ham)

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Abstract
An efficient protocol for *in vitro* cloning of *Crataeva religiosa.* was developed using leaf segment (LS), nodal segment (NS) and internodal segments (INS) as explants. The technique involves *in vitro* shoot regeneration, rooting of microshoots and transplantation of regenerated plantlets under *in vivo* condition. Sterilized explants were cultured on MS media supplemented with different auxin (IAA, IBA, NAA, & 2,4-D) and cytokinins (Kn & BAP) within a concentration range of 0.5-3.0mg/L used singly or in combination. The best shoot multiplication was obtained from nodal explants on MS medium supplemented with BAP+NAA (1.5+0.5) mg/L along with CW (5% v/v). Excellent rooting of microshoots (4-6cm) was noticed on the medium (1/2 MS salt) fortified with combination of auxins [NAA+IBA, (1+0.5) mg/L]. Compact callus which was hydrated, green and crystalline in appearance was obtained from LS and INS on medium having 1.5mg/L 2,4-D. Nodal explants were superior to internodal as well as leaf explants in response to shoot proliferation. Regenerated plantlets were transferred to pots having mixture of sand: soil: vermicuilite (1:1:1) and little fungicides (Eco fungicide). The survival rate of plantlets was much promising (around 88%) and regenerated plantlets were healthy, green and morphologically identical to mother plants.

Keywords: *Crataeva religiosa*, callus culture, MS medium, 2,4-D, BAP, 5% CM

Introduction
Crataeva religiosa var. *nurvula* Hook. F. (Syn.- *Crataeva tapia* Linn., Family - capparaceae) comprising 500 species (Deng *et al.*1999). *Crataeva* named in honour of the Greek botanist Crataevas, a renowned writer on medicinal plants, at the beginning of the first century B.C. (Raghavan and Venkatasubban 1941) [2] and *religiosa* comes to grow near religious places (Warrier. 1995, Panda 2004, Anonymous 2005) [8]. It is moderate sized deciduous tree commonly called Varuna (Williamson 2002) [5], distributed tropical and sub-tropical region and in India found in Peninsular region, Western region, Gangetic Plains, and Eastern India, up to Tripura and Manipur (Williamson, M. 2002) [5], also found in dry deep boulder formations in sub-Himalayan tract (Kirtikar KR, Basu BD 1995). Flowers are large, hermaphrodite, whitish to milky white in color (Sharma *et al.* 2006, Udaysing and Gaikwad 2011) [6] and abundantly during March- May at optimal level (Sharma *et al* 2011). The bark is used in the urinary disorders including kidney and bladder stones, calculous affections, as lithontipic and also an antidote in snakebite [Malini *et al.* 1995, Bhatachargee 2001] [16]. The bark is contraceptive and cytotoxic and useful in kidney bladder stones, fever vomiting and gastric irritation [Gagandeep and Khadilkar 2006] [10]. rheumatism. Leaves are used in febrifuge and tonic and also in rheumatism [Sanayaima *et al.* 2006, Walia *et al.* 2007] [19], People loved to use the leaves and bark of *C. religiosa* in jaundice, eczema, rabies [Ganesan *et al.* 2009] [20] of Tamilnadu. *C. religiosa* is used as antihepatotoxicity, antitumor, anti-inflammatory (Shirwaikar *et al.* 2004) [24], chemoprotective agents (Surh 2003) [25], antimicrobial (Shai 2008) [26], antiarthritic (Agarwal and Rangari 2003) [1], antihyperglycemic, antioxidant, cytotoxic, hypotensive, antiedemic, and antiperoxidant activities (Sunitha 2001) [27] due to most important bioactive compound lupeol. Another bioactive compound betulinic acid is responsible for inhibition of HIV (Yogeeswari and Sriram 2005, Fujioka *et al.* 1994, Huang *et al.* 2006) [28, 29, 30], also act as antibacterial, antimalarial, anti-inflammatory, anthelmintic and has antioxidant properties (Chandramu *et al.* 2003) [31] and antitumor-activity selective against human melanoma cells (Selzer *et al.* 2000) [32].
Due to floral buds drop, hence seed production is hampered (Anonymous 1948) [34]. Erratic seed germination, destructive harvesting and habitat loss from deforestation have endangered survival of this plant. As a result of this endangerment, in India the plant has been categorized as rare or vulnerable in natural environment (Bopana and Saxena 2008) [35]. Due to huge medicinally demand of this plant might lead to extinction of *Crataeva religiosa* in the near future. These factors demand for conservation and propagation of this medicinal plant by conventionally biotechnological method or Plant Tissue Culture.

Material and Methods
Explants [Nodal segment (NS), internodal segment (INS) & leaf segment (LS)] collected from 9-12 months old *in vivo* grown *Crataeva religiosa* plant (from Campus of B R A Bihar University, Muzaffarpur, Fig.1) were washed thoroughly under running tap water for 15-20 minutes and then treated with 1% Savlon along with 4-5 drops of Tween20 for about 20-25 minutes with constant shaking followed by 3-4 times washing with double distilled water (DDW) to eliminate remnant detergent. Explants were immersed in 0.1% HgCl2 ((SRL, India) (Kher *et al.* 2019) [11] for 3-5 minutes and finally washed 3 times with sterilized DDW.

Sterilized explants (NS, 8-12mm, INS, 8-12mm, LS-6x8mm) were aseptically cut and inoculated singly in culture tubes (25x150mm) containing MS (Murashige & Skoog, 1962) medium with 3% sucrose 0.8% agar (bacteriological grade, Merck, Mumbai, India) and different combination and concentration of auxin and cytokinin (Table- 1, 2 & 3). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Cultures were maintained at 25 ± 2°C with light intensity of 2000 lux (Cool, white) under 16/8 hours photoperiod.

Ten replicates were maintained for each experiment and were repeated twice. Shoot proliferation and elongation lasted for 4-5 weeks, callus induction lasted 5-6 weeks and rooting for 6-7 weeks, the time of each stage was fixed. Percentages of explants showing shoot proliferation, number of shoots/explants and lengths of shoots were taken as parameters for evaluating the morphogenic potentialities of explants in the present experimental system. Calli were maintained for a long term (about 2.25 years) by sub-culturing every 5 weeks on suitable medium.

Result and Discussion

Callus induction

After 12 and 14 days of inoculation on LS and INS explants respectively, callus started to form from sharp cut ends on MS media having different combination and concentration of PGRs (Table 1), the best callus biomass (687±25.10)mg was obtained on 1.0mg/L 2,4-D on 25 days old culture while INS biomass (603±26.51)mg was obtained on 1.5mg/L 2,4-D after 21 days of culture and % response was also optimum for bpth on the above concentration and the % response of explants was also optimum on the above combination of hormone (Table 1). Callogenesis in explants (LS, INS) was encountered in all the combination of hormones tested (Table 1), promising results were also noted on NAA+2,4-D+BAP (0.5+0.5+3.0) mg/L in combination (Table 1). Callus induction in NS was not encouraging on any combination of hormones and hormones above 3 mg/L had adverse effect on callus initiation and explants finally turned brown. These findings are compatible with the observations of Basu *et al.* (2009),

Table 1: Callus induction from leaf and internodal segments of *Crataeva religiosa* under effective concentration and combination of phytohormones (30 Days old culture) *

Hormonal concentration (mg/L)				*Biomass of callus*		*% of responding explants*		*Nature of Callus*		*Other response*
IBA	***NAA***	***2,4-D***	***BAP***	**LS**	**INS**	**LS**	**INS**	LS	INS	-
0.5	--	--	--	226±12.56	201±13.70	35±4.0	28±2.0	WF		-
1.0	--	--	--	480±30.15		78±4.4		CC		-
2.0	--	--	--	71±08.00		28±3.4		BC		-
--	0.5	--	--	405±42.80	160±05.48	49±4.2	32±3.1	WC	WC	-
--	1.5	--	--	307±25.80	106±09.73	55±6.1	35±2.8	WF	WC	-
--	--	0.5	--	470±20.30	478±18.60	65±5.6	60±4.3	HC	WC	-
--	--	1.0	--	387±25.10		68±4.6		GC		-
--	--	2.0	--	260±29.20		55±3.2		HC		-
--	--	--	0.5	295±22.40	408±12.65	46±7.3	47±7.3	CC	CC	-
--	--	--	1.5	428±44.51	580±15.24	44±5.5	80±4.5	CC	GC	-
--	--	1.5	0.5	652±25.60	460±17.10	78±5.6	46±3.6	WC	BC	-
--	0.5	--	0.5	435±13.40	435±13.40	72±7.1	72±7.1	WC	WC	-
--	0.5	--	1.5	426±20.60	426±20.60	65±3.0	65±3.0	CC	GC	-

Prakash *et al.* (2014) and Kher *et al.* 2019 [12]. The callus in general was crystalline, hydrated, green and compact in texture, however, calli were white & friable in some combination of hormones (Table 1 2,4-D is usually the choice of auxin for callus induction in the present experiment system but 2,4-D alone or in combination with BAP was not suitable for long term culture and callus mediated regeneration, as also reported by Bhaskaran & Smith (1990), Naseem & Jha (1994) and Chaudhury & Qu (2000). The callus was conserved for a period of about 2 years by regular sub-culturing at interval of 30-35 days on MS medium supplemented with BAP+NAA (0.5+0.5) mg/L was also supported by Kumari (2016). Callus induction was limited by several factors, when these requirements were adequate (temperature 25+2°C, pH- 5.8 and 2000 lux light), the culture response was maximum.

Table 2: Direct shoot regeneration from nodal explants of *Crataeva religiosa* on effective concentration and combination of growth regulators (28 Days old culture) *

Hormonal concentration (mg/L]					*Total no. of shoots*	*Mean length of shoot(cm)*
Kn	***BAP***	***NAA***	***IBA***	**CW(v/v)**		
0.5	0.0	0.0	0.0	0%	1.19±0.23	0.11±0.19
1.5	0.0	0.0	0.0	0%	2.10±0.27	0.58±0.26
2.5	0.0	0 0	0.0	0%	1.65±0.16	1.15±0.15
0.0	0.5	0.0	0.0	0%	2.20±0.34	1.70±0.10
.0	1.5	0.0	0.0	0%	3.89±0.12	4.20±0.20
0.0	2.5	0.0	0.0	0%	3.35±0.28	2.90±0.34
0.0	1.0	0.5	0.0	0%	3.92±0.54	4.60±0.49
0.0	1.5	0.5	0.0	0%	6.20±0.42	6.50±0.15
0.0	2.5	2.5	0.0	0%	4.30±0.42	4.95±0.40
0.0	1.5	0.0	0.5	0%	3.40±0.12	3.80±0.45
0.0	2.5	0.0	1.5	0%	1.50±0.12	1.30±0.11
0.0	1.5	0.5	0.0	5%	6.38±0.40	6.55±0.21
0.0	1.5	0.5	0.0	10%	5.95±0.19	2.82±0.25

Table 3: Rooting of microshoots of *Crataeva religiosa* on media/RM having effective combination and concentrations of phytohormones*

Medium	*[Hormonal concentration (mg/) L]*			*Total no. of roots/culture*	*% of response*
	NAA	*IBA*	*IAA*		
MS	0.5-2.0	0.5-2.0	0.0	---	---
	0.5-3.0	0.5-3.0	0.0		
1/2MS	0.5	0.0	0.0	2.9±0.11	52±2.81
	1.0	0.0	0.0	3.5±0.15	60±4.30
	1.5	0.0	0.0	2.4±0.12	47±3.52
	0.0	0.5	0.0	5.3±0.14	70±2.40-
	0.0	1.0	0.0	3.8±0.13	66±2.10
	0.0	1.5	0.0	2.6±0.15	56±2.23
	0.0	0.5	0.5	1.8±0.28	46±2.27
	0.0	1.0	0.5	2.5±0.21	49±3.20
	0.0	1.5	0.5	1.3±0.17	30±2.11
	0.0	0.5	0.5	2.0±0.24	42±2.80
	0.0	1.5	0.5	1.6±0.32	35±3.32
	0.0	2.5	1.0	0.7±0.28	36±2.85
	0.5	0.5	0.0	3.8±0.20	65±2.23
	1.0	0.5	0.0	6.2±0.25	81±3.92
	1.5	0.5	0.0	3.3±0.12	60±4.49
	2.5	1.5	0.0	2.7±0.54	53±3.24

*Data scored (Mean ± SE) from 10 replicates of microshoots which were repeated twice.
Period Days: 25 Growth; RM- Rooting media (1/2 MS salts)

Callus mediated shoot regeneration

reen calli were sole responsible for shoot regeneration on subculture medium (MS) containing NAA and BAP within a concentration range of 0.5-3.0mg/L, the best shoot regeneration from callus in bunch was obtained on BAP+NAA (1.5+0.5) mg/L (Fig. C) after 15 days of subculture and percentage response on this combination was also much promising (about 82%). Non-green calli could not respond to the medium and have not capabilities of differentiation and regeneration even on different media and hormonal combinations, this was also supported by Narayanswamy (1977) and Naseem & Jha (1994). Tiny shoots (about 2cm) were isolated and sub-cultured on MS medium containing only 0.5mg/L Kn for shoot elongation (Fig. D). From the present findings, it is evident that cytokinin alone as well as high cytokinin & low auxin ratio promotes shoot regeneration and elongation (Singh *et al.* 2009, Kumar *et al*.2010, Mallick *et al.* 2012 and Sushila *et al*. 2013, Kumari *et al.* 2015).

Shoot regeneration

Nodal excised part is predominant explants for direct regeneration of shoots was obtained from supported with different auxin (NAA & IBA) and cytokinin (BAP& Kn) in various combination and concentration within a diverse range of 0.5-3.0mg/L (Table 2), multiple shoot induction as gradely shoot length was recorded on MS medium fortified with BAP+NAA (1.5+0.5) mg/L in addition with 5% v/v CW (Fig. E). Bulging with/or hypertrophy in nodal explants was prominent before initiation of shoot buds. Regeneration of shoots on suitable medium was noticed after 10 days of inoculation. Table 2 shows that promising number of shoots was also obtained on MS medium containing BAP+NAA (1.5+0.5) mg/L along with 10% v/v CW and BAP+NAA (1.5+0.5) mg/L without CW. Growth regulators [BAP+NAA (1.5+0.5) mg/L] and CW (5%v/v) together exhibited synergistic effect and induced better shoot regeneration (Table 2). Such an effect of CW and growth hormone was also recorded by Naseem & Jha (1994) and Kumari *et al.* 2015. Direct shoot bud regeneration in the present system depends on quantitative interaction of auxin and cytokinin, this was also reported in *Delbergia lenceoleria* (Dwari & Chand, 1996) and *Aloe vera* (Khanam *et al*, 2014, Kumari and Naseem, 2015). Tissue culture studies on a number of medicinal plants including *Crataeva religiosa* (Naseem & Jha, 1997, Singh *et al*. 2009, Kumar *et al*. 2010, Mallick *et al.* 2012, Khanam *et al.* 2014. Kher *et al.* 2015, Kumari and Naseem 2015, Kumari *et al.* 2015, Sharma *et al.* 2017and Kher *et al.* 2019) [11, 12] suggest that a fine balance of exogenous auxin and cytokinin is necessary before successful regeneration can occur, hormones above 3 mg/L and CW above 10% v/v had adverse effect on shoot multiplication. *In vitro* florigenesis and optimum shoot elongation (6.5±0.15) cm were recorded after 28 days of culture on suitable combination of hormones (Table 2, Fig. F). Florigenesis in culture, an event of biological interest was also reported by Tran Tanh van (1973), Naseem &Jha (1994) and Patil & Jayanti (1997).

In vitro propagation in *Crataeva* from auxillary buds has proved to be the most acceptable and reliable method (Sharma and Padhya 1996, Sharma *et al.* 2003 and Shirin and Maravi 2006) [21, 23] for regeneration of plants, nodal explants as a means of micropropagation have been reported in a number of texa including *C. religiosa* as an ideal explant for direct shoot multiplication (Shirin and Maravi 2006, Walia *et al.* 2007, Panwar and Vashistha 2008 and Prakash *et al.* 2014) [23, 18, 17]. Direct regeneration of multiple shoots/ shoots from nodal culture is highly desirable since the regenerants are genetically identical to the mother plant (Naseem and Jha, 1997, Mallick *et al*. 2012, Prakash *et al.* 2014 and Kumari *et al.* 2015).

Rooting and plantlet formation

Regenerated microshoots (4-6cm) obtained in 3.2 and 3.3 were excised and individually implanted on MS as well as rooting media(RM) *i.e* ½ MS+PGR (Plant growth regulator) for rhizogenesis, rooting was obtained on ½ MS medium in presence of different auxin (IAA, NAA and IBA) either used singly or in combination within a different concentration range of 0.5-2.0mg/L and optimum rooting of microshoots (Fig. G & H) was achieved on ½ MS supplemented with NAA+IBA(1+0.5)mg/L within 20 days.

Low salt medium with combination of auxin (NAA+IBA) have been found to have stimulatory effect on root induction in many plant species (Laxmi-Sita *et al*. 1986 and Naseem & Jha, 1994). No rhizogenesis was recorded on MS medium with/without PGR (Table 3). Physical growth conditions described under callus induction were also optimal for rhizogenesis. Regenerated plantlets were transferred to plastic.

Fig A: Showing curling of leaf NAA ON 0.5mg/L

Fig B: LEAF explants showing callus induction on 2,4D 1.0mg/

Fig C: Callus induction with shoots on 2,4D+BAP 1.5+0.5mg/L

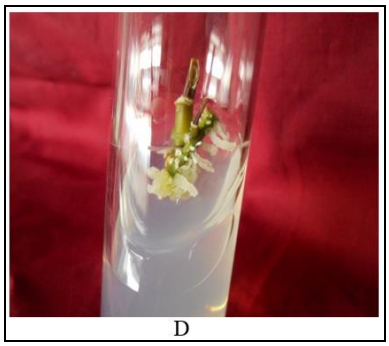

Fig D: Callus with multiple shoots on NAA+BAP (0.5+1.5mg/L

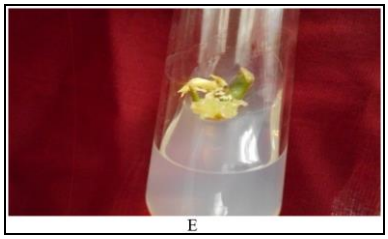

Fig E: Callus with multiple shoots on NAA+BAP+5%CW (0.5+1.5mg/L

Pots (Fig. I) having sterilised soil mixture (vermiculites+ sand+ soil, 1:1:1) and little fungicide (Eco fungicide), plantlets were acclimatized for a week in culture room and finally transferred to shade house. *In vitro* raised plantlets were healthy, green and morphologically identical to mother plants and the survival rate of plantlets was also encouraging (around 90%).

Conclusion

In the present experiment, multiple shoots/shoots developed directly [NS] and indirectly *via* Callus formation [INS, LS] can be used as ideal explants for *in vitro* cloninig of *Crataeva religiosa* and mass propagation achieved by this method is highly efficient & productive.

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