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Efficient *in Vitro* Rapid Axillary Bud Proliferation from Mature *Terminalia chebula* Retz. - A Medicinal Tree

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ABSTRACT

Efficient *in vitro* rapid axillary bud proliferation in *Terminalia chebula* Retz., a subtropical Indian medicinal tree using shoot buds from 5 yrs old tree as explants is presented. Removal of high quantities of phenolic compounds by frequent subculturing in the woody plant medium supplemented with 0.8 mg/l 6-benzylamino purine (BAP) for establishment of buds and 1.50 mg/l BAP + 0.05 mg/l naphthalene-1-acetic acid for shoot proliferation proved to be an ideal medium. This was followed by subculturing of nodal segments by removing apical buds on shoot proliferation medium for four times at an interval of four weeks to enhance shoot re-multiplication. Maximum rooting was obtained by pulsing microshoots with indole-3-butyric acid (2 mg/l) under dark for 2 hours and thereafter culturing them on half strength woody plant medium containing 0.1% activated charcoal but without any growth regulators. Roots developed within 15 days and regenerated plantlets were transferred to soil under non-sterile conditions for hardening.

Keywords: micropropagation, *in vitro* propagation, subculturing, rooting
Abbreviations: BAP, 6-benzylamino purine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **NAA-α**, naphthalene-1-acetic acid; **WPM**, Woody plant medium

INTRODUCTION

Terminalia chebula Retz. commonly known as Harad is a subtropical medicinal tree of Western Himalaya belonging to the family Combretaceae. It is also called haritaki because it carries away all diseases or it is sacred to Siva (Hara). Haritaki has several interesting synonyms, portraying its various peculiarities like pathya – since it removes obstructions from the pathways and channels of the body; abhaya – as it promotes fearlessness; amrta – an ambrosia ; divya – a divine herb; medhya – nervine tonic; pranada – life saving; jivaniya – vitalizing herb; vayahstha – one which promotes longevity and maintains youth; rasayana phala – a rejuvenating fruit, etc. It is found in the sub-Himalayan tracks from Ravi to West Bengal, Assam and in all deciduous forests of India, specifically in Madhya Pradesh, Bihar, Assam and Maharashtra. It is found growing at an altitude of 1500 m. It attains a height of 15-24 m and a girth of 0.46-0.73 m or more. The tree is rounded, crowned with spreading branches. The leaves are 10-20 cm long, ovate, acute, in almost opposite pairs. There are two small glands near leaf-base. Flowers dull white in spikes at the ends of branches. The fruits are hard, 2-4 cm long, with 5 ribs on its body, yellowish green in colour. The seeds are single, light yellow, non-adherent to the pulp of the fruit. Its flowering and fruiting season is from April to June. Ripening of seeds takes place from November to March.

In the Indian pharmacopoeia, the fruit of *T. chebula* is extensively used as an adjuvant to other medicines for numerous diseases. The fruit has numerous medicinal and tanning purposes. The mature fruit are collected during January-April by shaking the trees and are then dried in thin layers preferably in shade and graded marketing. The raw myrobalans (the name given to the astringent fruits of several species of *Terminalia*, largely used in India for dyeing and tanning and exported for the same purpose) are graded under different trade names, selection being based upon their solidness, colour and freedom from insect attack (Chadha 1976). The chief kinds are the chebulic or black myrobalan, from *T. chebula*, which are smooth, and the beleric, from *T. belerica*, which are five-angled and covered with a greyish down.

The fruits of this tree species are mainly used as medicine, internally and externally; they are dried and finely powdered. Fruits have antiamoebic (Sohni *et al*. 1995), antitumour (Kashiwad *et al*. 1992), blood purifier (Mehta *et al*. 1993) and cardiotonic properties (Reddy *et al*. 1990; Khanna *et al*. 1993). They arrest the activity of HIV-virus and are also used as one of the ingredients in medicine against AIDS (EI-Mekkaaway *et al*. 1995). Fruits are a laxative, stomachic, tonic, astringent, and or a gargle in stomatitis and alterative. Local application to chronic ulcers and wound has also been reported (Jain 1994). Fruit pulp is an important part of *Triphala* which is traditionally used as a panacea for stomach disorders (Bharadwaj 1995). Externally, the paste applied effectively reduces the swelling, hastens the healing and cleanses the wounds and ulcers. Diseases of eyes like conjunctivitis respond well when eye wash of triphala decoction is given with internal medicament of triphala powder with ghee and honey (http://www.herbalcure india.com/herbs/terminaliachebula.htm). The gargles with its decoction bestow excellent results in stomatitis and diseases of the throat (Venugopal 2002). The decoction or its cream is salutary in cleansing the chronic wounds and from better healing, respectively (Jain 1994). Triphala can be used externally for hair wash, for brushing the teeth in pyorrhea or bleeding gums and its decoction for washing chronic, non-healing wounds and ulcers (Jain 1994). The main purgative part in *Triphala* is Harad. The pulp is used as a dentrifice to cure bleeding and ulceration of gums (Sharma and Sood 1997). The dried fruits may be soaked overnight in water and the water having extracts of the fruit is taken by the patient. Due to the worldwide deviating trends from allopathy to an Ayurvedic system of medicine, the demand for these fruits has increased. Besides its medicinal value, the timber of this species is mainly used for agricultural implements and for manufacturing good quality paper and tannin.

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Short Communication

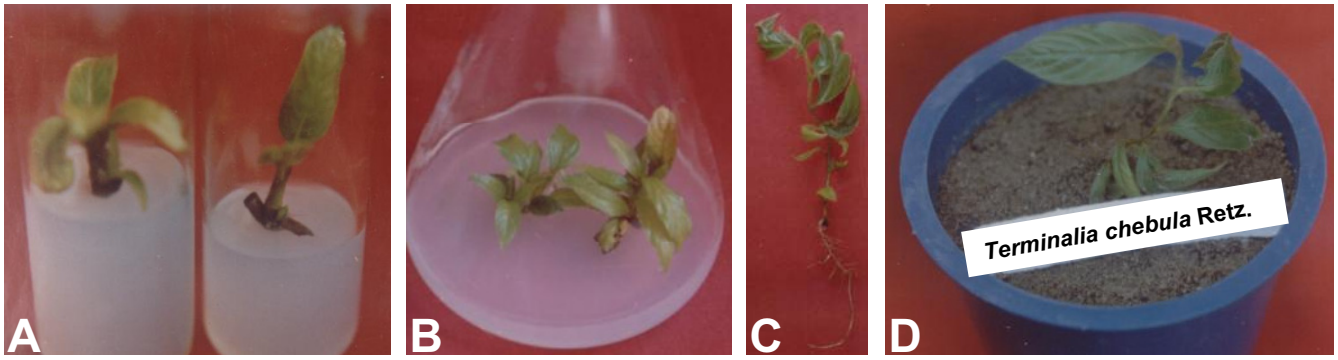

Fig. 1 ***T. chebula in*** **vitro and acclimatization.** (**A**) Establishment of *T. chebula* shoot buds on woody plant medium supplemented with 0.8 mg/l BAP + 0.02 NAA. (**B**) Proliferation of *T. chebula* microshoots on woody plant medium supplemented with 1.50 mg/l BAP + 0.05 mg/l NAA. (**C**) *In vitro* rooted *T. chebula* plantlet on half-strength WPM supplemented with 0.1% charcoal. (**D**) Hardened *T. chebula* plant.

Natural reproduction is generally unsatisfactory. This is attributable to poor germinative capacity of seed (Singh 1995), destruction of seed by insects, rats, squirrels and other rodents. As far as vegetative propagation is concerned, Pande (1997) reported that all the stem cuttings failed to survive irrespective of the time of planting and hormone tried. Similarly, the success rate through grafting is as low as 30% (Pande 1997). Therefore, an *in vitro* plant regeneration system in this species could provide a viable means of conserving the rare genotypes *ex situ*. Development of *in vitro* plant regeneration protocols is not only essential for propagation but it is a pre-requisite for genetic transformation studies.

Even though *in vitro* micropropagation of *T. chebula* (Deshmukh *et al*. 2005), somatic embryogenesis (Anjaneyulu *et al*. 2004) and formation of shoots from cotyledonary explants (ShyamKumar *et al*. 2004) has been reported, there is no report, however, devising an efficient and reproducible regeneration method best suited for achieving higher multiplication of this species. Keeping in view the scant natural regeneration, low seed germination response *ex situ* and difficulty in producing nursery planting material for *T. chebula*, the present study sets out to achieve these objectives.

MATERIALS AND METHODS

The axillary and terminal shoot buds 7-8 mm in size from a 5-year-old tree of *Terminalia chebula* Retz. were used as explants and washed for 3-5 min under running tap water, thereafter treated with 2% detergent (Teepol, Central Drug House Pvt. Ltd., New Delhi) for 3-5 min and again washed with sterile distilled water. These explants were surface sterilized using 0.1% $HgCl_2$ for 2 min followed by 4-5 rinses with sterile distilled water under aseptic conditions.

During establishment a serious problem associated with the tissue culture of this tree is the leaching of phenolic compounds produced by the wounded tissues, which interfere with the development of the explant and gradually turns brown. The frequent subculturing to fresh medium at an interval of 24 hours three times and thereafter incubating for 4 weeks removed the problem of high quantities of phenolic compounds.

Surface-sterilized shoot buds were established on McCown and Lloyds (1981) woody plant medium (Deshmukh *et al*. 2005) containing 0.8% agar, 3% sucrose and supplemented with 0.8 mg/l 6-benzylamino purine (BAP) with 0.02 naphthalene-1-acetic acid (NAA) All the constituents of media were procured from SISCO Research Laboratories Pvt. Ltd., Mumbai. The established shoots were isolated and cut into 1.0-1.5 cm segments and transfer to multiplication medium (WPM + 1.5 mg/l BAP + 0.05 mg/l NAA). All the cultures were kept in the culture room at 26 ± 2°C under 16 hours photoperiod at 20 µmol m^{-2} s^{-1}.

Microshoots 1.5-2.0 cm long were taken after four weeks from multiplication medium and subjected to four subsequent subcultures on the same medium to see the effect on *in vitro* shoot proliferation. Also, shoots from each subculture were subjected to root induction. The number of shoots, length of shoots, percentage rooting, number and length of roots were recorded for each subcultured shoot.

In vitro rooting of microshoots was done by dipping them in pre-autoclaved IBA (2 mg/l) solution for 2 hours under dark aseptic conditions and thereafter subculturing in half-strength WPM supplemented with 0.0-0.2% activated charcoal.

Rooted *in vitro* plants were washed gently under tap water, treated with 0.3% Bavistin (Carbendazim, United Phosphorous Ltd., Gujarat) to avoid fungal attack and transferred to pots containing sand. Pots were initially covered with jam jars to maintain humidity and acclimated slowly to the natural environment.

All the experiments were laid out in a completely randomized Design (CRD). The observations recorded were subjected to analysis through CRD as described by Cockran and Cox (1992). The level of significance for the F-test was 5%.

RESULTS AND DISCUSSION

The results presented in this paper show that there was an increase in the number of shoots with an advancement of subculturing. However, the increase was statistically insignificant in different subcultures (**Fig. 1A, 1B**). The number of shoots formed per explant progressively increase up to the fourth successive subculture. At the first subculture the number of shoots formed per explant were 3.66 but 4.66 in the fourth subculture. It may be seen that there was slight decrease in the length of shoots. Maximum average shoot length (4.13 cm) was observed in first

Table 1 Effect of subculture on shoot number and average shoot length (cm).

Subculture	Days	№ shoots	Shoot length
S1	30	3.66	4.13
S2	60	4.06	4.06
S3	90	4.33	3.93
S4	120	4.66	3.80
Mean		3.82	3.98
Standard error		1.51	0.11
CD (0.05)		3.49 (NS*)	0.25

*Not significant
10 explants/treatment; replicated thrice; Completely Randomised Design.

Table 2 Effect of subculture on root number and average root length (cm).

Subculture	Days	Root formation (%)	№ shoots	Shoot length
S1	30	00.00 (00.00)	0.00	0.00
S2	60	00.00 (00.00)	0.00	0.00
S3	90	50.50 (45.72)	3.33	3.50
S4	120	70.50 (58.40)	3.62	3.16
Mean		30.25 (26.03)	1.74	1.67
Standard error		1.16	0.33	0.23
CD (0.05)		(2.55)	0.77	0.54

10 explants/treatment; replicated thrice; Completely Randomised Design.
Values expressed in the parentheses are arcsine transformation of percentage.

Table 3 Effect of different concentrations of activated charcoal supplemented in hormone-free half-strength WPM on rooting after four weeks.

Activated charcoal (%, w/v)	Days to initiate rooting	Rooting (%)	№ roots/ shoot	Length of roots (cm)	Types of roots
0.00	8	53.75	1.50	2.12	Callused roots
0.05	8	61.25	3.25	2.62	Healthy roots
0.10	6	72.50	3.62	4.12	Healthy and vigorous roots
0.20	6	67.50	3.37	3.37	Healthy roots
Mean		63.50	2.93	3.06	
Standard error		2.97	0.34	0.32	
CD (0.05)		(6.85)	(0.78)	(0.73)	

10 explants/treatment; replicated thrice; Completely Randomised Design.

subculture while least (3.80 cm) in the fourth subculture (**Table 1**). Similarly, San-Jose *et al*. (1990) reported a significant increase in the number of shoots of *Quercus petraea* following reculture. *Anogeissus sericea* cultures could be maintained up to sixteen passages without a decline in the multiplication rate (Kaur *et al*. 1992). Shoot multiplication generally increased with progressive subcultures, probably due to the shoots becoming more juvenile (Rughava Swamy *et al*. 1992).

Table 2 shows that *in vitro* raised microshoots did not root at all in first as well as in second subcultures. Thereafter, 50.50% shoots rooted in the third subculture, which further increased to 70.50% in the fourth subculture. The number of roots increased with subsequent subculture while their length decreased non-significantly in the same period. It is evident that there is no root induction in the first and second subculture, only developing from the third subculture, possibly due to physiological juvenility of the microshoots. There is no effect of increased subculture on root number and length per explant.

In bud culture from a 20-year old *Eucalyptus citriodora* tree (Gupta *et al*. 1981) none of the rooting experiments resulted in root formation in either the initial explant or in the first three subcultures and 35-40% of shoots rooted at the fourth subculture. However, juvenility of microshoots increased until the ninth subculture in *Robinia pseudoacacia* (Kanwar *et al*. 2000).

Table 3 shows that roots in the control callused and did not develop into a well developed root system. However, activated charcoal at 0.1% produced maximum (**Fig. 1C, 1D**), healthy and vigorous roots compared to 0.05 and 0.2% activated charcoal. Similarly Mackay *et al*. (1995) observed that the best shoots of *Cercis candensis in vitro* formed on WPM containing 6.70 µM NAA and 0.1% activated charcoal.

In the present study, the medium used was WPM supplemented with various concentrations of NAA and BAP for establishment of shoot buds, shoot proliferation and multiplication. The suitability of WPM may be attributed to its low salt concentration of salts and better interaction with the genotypes. ShyamKumar *et al*. (2004) reported the induction of multiple shoots from cotyledonary explants using half strength MS medium supplemented with various concentrations of GA_3, IBA and BAP. Somatic embryogenesis has also been reported in *T. chebula* by Anjaneyulu *et al*. (2004) using cotyledon and mature zygotic embryo callus cultures. The callus cultures of cotyledons and mature zygotic embryo were initiated on MS medium containing 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) with either 0.01 or 0.1 mg/l Kinetin. Induction, proliferation and development of somatic embryogenesis was obtained through different culture passages. Embryogenic cotyledon callus with globular somatic embryos was obtained on MS basal medium supplemented only with 50 g/l sucrose. The highest frequency of somatic embryo germination occurred on MS basal medium containing BAP (0.5 mg/l) with 30 g/l sucrose.

We conclude that the subculture of *in vitro*-shoots shows a progressive increase in the multiplication rate so commercial micropropagation of superior or elite *T. chebula* is possible.

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