Tree and Forestry Science and Biotechnology ©2011 Global Science Books

High Frequency Shoot Proliferation, Rooting, Acclimatization and Field Establishment of *Terminalia chebula*: A Tree of Pharmaceutical Importance

B. Shyamkumar • Charu C. Giri*

Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad, 500007, A.P. India

Corresponding author: * giriccin@yahoo.co.in

ABSTRACT

High frequency axillary shoot proliferation from cotyledonary nodes was achieved in *Terminalia chebula* Retz. Repeated subculture of cotyledonary node explants to fresh media resulted in formation of enormous new shoots. About 44.33 ± 0.88 first generation shoots could be obtained from a single cotyledonary node explants after 12-14 weeks of culture. Highest rooting percentage of 93.4 ± 0.9, 75.3 ± 0.88 in *in vitro* propagated shoots was obtained on media containing mannitol 1.0% (w/v) and 0.3% (w/v) polyvinyl pyrrolidone (PVP), respectively. Acclimatized plants showed 98.4% survival rate in glasshouse conditions. Histological/anatomical studies clearly indicated the multiple shoot induction pathway regeneration without intervening callus phase from cotyledonary node explants**.** Plants hardened in the poly-bags containing soil and vermi-compost in the portable mist chamber showed 84.4% survival rate in field conditions. Nearly 45% of the plants were finally established in the field conditions at CPMB, Osmania University, Hyderabad, India. Plants were also transferred to different climatic locations and established at SAIRD, Gaddipally, Nalgonda District, A.P., India. The field established plants showed early flowering and fruit setting. The present study describes a rapid method for generating planting stock material for forestry and their subsequent establishment in field conditions.

Keywords: axillary shoots, acclimatization, cotyledon, rooting field establishment
Abbreviations: AC, activated charcoal; **ABA**, abscisic acid; **BA**, 6-benzyladenine; **FM**, formulation media; **GA_3**, gibberellic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MZE**, mature zygotic embryo; **NAA**, α-naphthalene acetic acid, **PVP**, polyvinylpyrrolidone

INTRODUCTION

Forests are one of the most important natural resources of the world and vital for sustaining human life. These valuable resources are on decline due to rapid deforestation and indiscriminate human intervention. Trees are an important and integral component of the forest flora and biodiversity. Many valuable plants in forest vegetation have become vulnerable, and are on the verge of extinction due to over exploitation by aimless removal of trees and lack of systemic programme for their propagation. There is a great need to propagate and conserve the important forest tree genetic resources in particular (Giri *et al.* 2004). Micropropagation of tree species offers a rapid means of producing planting stock for forestation programmes, woody biomass production and conservation of elite and rare germplasm (Ďurkovič 2008; Gonzalez-Arnao *et al.* 2008; Liu and Pijut 2008; Park *et al.* 2008; Prakash and van Staden 2008; Tikat and Onay 2009). Micropropagation can also be used for genetic transformation and also to study the stability of transgenes in long-term micropropagation of plants as in case of transgenic birch (Liu and Pijut 2010; Yevtushenko and Mishra 2010; Zeng *et al*. 2010). Due to the limited success with mature tree explants, different seedling derived explants such as hypocotyls, cotyledon, cotyledonary node were extensively used for micropropagation of trees for developing propagation protocols and generating planting materials. In the recent past, there are many *in vitro* propagation protocols available using seedling explants which is useful for generating planting material for forestry (Pandey and Jaiswal 2002; Walia *et al.* 2003; Giri *et al.* 2004; Cheepala *et al.* 2004; Feyissa *et al.* 2005; Yucesan *et al.* 2007; Park *et al.* 2008; Gomes and Canhoto 2009; Tikat and Onay 2009; Feng *et al*. 2010).

Terminalia chebula Retz. belongs to the family Combretaceae and is an important medicinal tree and a rich source of tannin. The tree is found mostly in mixed dry deciduous forests. *T. chebula* is distributed in the forests of Northern India, Central provinces, Bengal, Orissa, Tamil Nadu, Andhra Pradesh, Mysore and in the southern parts of Maharashtra. Dried fruits of *T. chebula* are commonly known as chebulic myrobalans (Khare 2004). The dried pericarp of the seed is rich in tannins and contains 30-35% of astringent substances-chebulagic acid, chebulinic acid, tannic acid, gallic acid, etc. that belong to hydrolysable tannins (Chen *et al.* 2000; Khare 2004). *T. chebula* is called the "King of Medicines" and is always listed first in the Ayurvedic Materia Medica (Warrier *et al.* 1997). The plant is used to treat many diseases such as digestive diseases, urinary diseases, skin, parasitic infections, heart diseases, ulcers, colic pain and hemorrhoids and also shows effect on wound healing (Suguna *et al.* 2002; Khare 2004). Anti-HIV and antibacterial activity has also been found in *T. chebula* (Sandip 2003; Bonjar 2004). Chebulagic acid from immature seeds of *T. chebula* was found to suppress the onset and progression of collagen-induced arthritis in mice, which may be beneficial for the management of rheumatoid arthritis and other inflammatory diseases (Lee *et al.* 2005). *T. chebula* has also shown a cardioprotective effect against the potent chemical isopreterenol (Suchalatha and Shyamala 2004). Besides its medicinal value, chebulic myrobalan is used widely for tannin industry. The natural regeneration in this tree from seeds is very poor and growth of the tree is also very slow compared to other species of the genus *Terminalia* (Thakur 2000; Shankar 2001). There is a need to develop a micropropagation strategy for large-scale multi-

Received: 7 June, 2009. Accepted: 1 October, 2010.

Original Research Paper

plication of this important medicinal tree. Subsequently, this may become a means for supply of planting material for forestry (Ramesh *et al.* 2003). We reported earlier for the first time the multiple shoot induction from cotyledonary node explants and somatic embryogenesis in *T. chebula* (Shyamkumar *et al.* 2003; Anjaneyulu *et al.* 2004; Anjaneyulu and Giri 2008). In the present communication, we report high frequency axillary shoot proliferation, rooting, acclimatization, establishment and study of *T. chebula* plants in field conditions.

MATERIALS AND METHODS

Multiple shoot induction from cotyledonary node explants

Seven to eight day old seedlings with a first pair of primary cotyledonary leaves were used for axillary shoot induction as cotyledonary node (CN) explants. After initial phase of shoot induction from cotyledonary nodes following treatment with basal media and different plant growth regulators, the media promoting optimum numbers of axillary shoot induction from cotyledonary nodes were selected (Shyamkumar *et al.* 2003). The media i.e. {½MS (Murashige and Skoog 1962) + gibberellic acid (GA_3) 0.3 mg/l + indole-3-butyric acid (IBA) 1.0 mg/l + 6-benzyladenine (BA) 10.0 mg/l} of Sigma-Aldrich, St. Louis, MI, USA was referred to as standardized media (SM). The first set of multiple shoots obtained in SM was considered as Initial-I stage (zero). These CN explants along with induced axillary shoots were grown further for duration of 4 weeks *in vitro* for obtaining more number of shoots referred to as Initial-II stage (zero).

The original cotyledonary nodes were repeatedly sub-cultured on fresh standardized media after harvesting of the newly formed shoots for two times. These two subculture passages were referred to a Stage-I (1^{st} subculture) and Stage-II (2^{nd} subculture). The number of multiple shoots produced was scored at different stages of the growth in different culture passages. Each treatment is tested with 20 replicates of the explants.

Rooting of *in vitro* propagated axillary shoots

Shoots of 2.5-3.0 cm in height excised from *in vitro* grown axillary shoots producing cultures. The axillary shoots of different age group i.e. 4 week, 6-7, 8-9 and 10-11 weeks were evaluated for their rooting response. The shoots were transferred to the semi-solid MS (full and half strength) media supplemented with various concentrations of plant growth regulators, antioxidants and media additive. Different auxins such as IBA, NAA of various concentrations were used in the media. Auxins viz. IBA: 1.0, 2.0, 3.0 and 5.0 mg/l and NAA: 1.0, 2.0, 3.0, 5.0 mg/l were used either individually or in combination in the media. Different antioxidants such as AC: 0.5, 1.0 and 2% (w/v), PVP (MW 10, 000: Duchefa, Haarlem, The Netherlands): 0.1, 0.25, 0.3, 0.5% (w/v) and PGL (MW 162.14: Duchefa): 100 mg/l, 200 mg/l were used in the media. Media additive mannitol at 0.5, 1 and 2% (w/v) was used in both half- and full-strength MS media. Rooting efficiency were recorded as the rooting percentage, number of roots formed per shoot and length of the root. The nature of the roots formed in different experiments was recorded.

Histological analysis of organogenesis in *T. chebula*

The cotyledonary node at different stages of growth i.e. 7-8-, 14-, and 21-day old with multiple shoots was fixed in FAA (formalin: glacial acetic acid: ethanol, 5: 5: 90, v/v) for 48 h, dehydrated through a series of ethanol-xylol, and embedded in paraffin (Johansen 1962; Giri and Giri 2007). Tissues were sectioned at a thickness of 6.0 µm, double stained with 1% (w/v) saffranin and 0.1% (w/v) toluidine blue, and observed under a microscope. Chemicals used for the histological study was obtained through Qualigenes-Glaxco Fine Chemicals, Mumbai, India. Microphotographs were taken at 10-20X magnification.

Maintenance of pH, sterilization of media and incubation of cultures

The pH of the MS media was adjusted to 5.8 ± 0.1, as per the required value by adjusting with 0.5 N HCl and 1 N NaOH. The media were dispensed in the tubes, flasks and bottles prior to autoclaving. Powdered agar was used as gelling agent at 0.9% (w/v). The media were autoclaved at 121°C and 15 lb for 15 min. All the cultures were kept in the light conditions under 16 hr photoperiod with a light intensity of 3000 lux provided by cool white fluorescent tubes at 25 ± 2°C and 80% relative humidity.

Statistical analysis

Detailed statistical analysis was done based on the observations and scoring of data on culture responses at different stages of the experiments conducted. Response has been expressed in terms of number of shoots per explant and average length of shoots. Each treatment with 20 replicates and all experiments were repeated three times. In case of rooting response has been expressed in terms of number of percent rooting, number of roots per shoot and average length of root. Each treatment with 25 replicates and all experiments were repeated three times. Statistical analysis was done using Matlab version 5.3, and SPSS version 10.0 Math Works Inc., USA, statistical packages.

Transfer of *in vitro* rooted plants, acclimatization, hardening and field evaluation

An indigenous mist chamber was prepared in the glass house at CPMB, Osmania University using, iron dome rectangular base and polythene sheet to cover the dome to maintain humidity.

Poly-bags of 6 cm diameter and 15 cm length were filled with soil and soil + vermicompost in a 3: 1 ratio before transfer of *in vitro* rooted shoots. Small cut holes were made at base of the poly-bags for easy percolation of excessive water. Vermicompost use din the present study was procured from commercial sources (Nagavellama *et al*. 2006).

Acclimatization protocols

Four methods of transfer were followed for the acclimatization and hardening of *in vitro* rooted plantlets to mist chamber conditions. The procedures used are:

Method I: Transfer of *in vitro* rooted plants free from agar directly to the poly-bags containing soil and kept in the mist chamber.

Method II: Growth of rooted plants in bottles containing autoclaved soil fortified with MS nutrients and IBA (1.0 mg/l) for a period of 2-3 weeks in the culture room conditions and subsequent transfer to poly-bags containing soil and hardened under mist chamber conditions.

Method III: An alternate method included growth of plants in culture vessel containing autoclaved soil for a period of 2-3 weeks in the culture room and transfer to poly-bags containing mixture of soil and vermicompost at 3:1 ratio and subsequent transfer to mist chamber.

Method IV: Growth of rooted plants in bottles for 2-3 weeks and transfer of bottles to mist chamber and acclimatized for another 2-3 weeks and subsequent transfer of plants were to poly-bags containing soil and vermicompost (composting of plant biomass facilitated by earthworms) in a 3: 1 ratio, and acclimatized in mist chamber for another 2-3 weeks.

The plants transferred using all the above methods were watered everyday and after two month of acclimatization in the mist chamber the plants were transferred to field conditions. The observations such as survival percentage of the plants, height and number of leaves per plant were recorded before transferred to the field. Plants were kept outside of the mist chamber in glasshouse conditions under the shade for 4-5 days before transferring to the field. During this period the plants were watered regularly. Minimum of 40-50 plants used in each method for hardening of plants in mist chamber at glass house conditions.

Transfer of plants to field conditions and evaluation of plants in field conditions

Pits of 30.0 cm × 30.0 cm × 60 cm were dug before 3 days of transfer of the hardened plants from the glass house conditions. The pits were burnt with all dried leaf material and kept open for one day. Later pits were filled with vermicompost and soil in a 1: 3 ratio and kept it open for one to two days before transfer of the plants. Poly-bags were cut with a blade and plants were taken out with intact soil and kept in the pit without disturbing the soil. The plants were watered for every day and care was taken.

RESULTS

Multiple shoot induction from cotyledonary node explants of *T. chebula*

Repeated sub-culturing at regular intervals of original cotyledonary node (CN) explants on standardized media was done after harvesting of the newly formed shoots to obtain higher number of multiple shoots. The shoots obtained initially on ½MS + BA 10.0 mg/l + GA_3 (0.3 mg/l) + IBA (1.0 mg/l) were about 6.4 ± 0.4/CN after 4 weeks of culture and is referred to as Initial-I stage (zero) (**Table 1, Fig. 1A, 1B**). Further these cultures with multiple shoots were allowed to grow for another four weeks and are referred to as Initial-II stage zero. At a maximum of 19.2 ± 1.5shoots/CN were obtained from such individual cotyledonary node cultures after 8 weeks. First subculture was given to cotyledonary node explants after removing newly formed axillary shoots and those shoots used for rooting. These cotyledonary nodes on first subculture (Initial-II stage zero) i.e. after 8 weeks, actively growing multiple shoots were obtained (**Fig. 1 D**).

From the onset of first subculture passage (8 weeks) a maximum of 13.2 ± 0.2 shoots/CN was obtained by 11 weeks of culture. The length (3.2 ± 0.1) of the shoots formed was less compared to the initially formed shoots. The shoots formed were normal, green and healthy with reduced shoot length (**Fig 1E, 1F**). Further subculture of the same CN after 11 weeks showed a decline in the number of shoots (5.50 ± 0.25) at Stage-II after 14 weeks of culture (**Table 1**). The base of the cotyledonary node was also darkly blackened. The shoots formed were pale and later turned white a clean indication of reduction in the chlorophyll content. Keeping in view the decline in the number of shoots, further subculture of cotyledonary node to a fresh medium was not further carried out. Therefore, overall in 12-14 weeks about 44.33 ± 0.88 shoots were obtained from a single cotyledonary node from different experiments conducted.

Histological/anatomical analysis to study the ontogeny of multiple shoot induction from cotyledonary node

The study was conducted by taking sections of the cotyledonary node explants of 7-9-, 14- and 21-day old cultures inoculated on multiple shoot media. From the study it reveals that, the axillary bud initiated and began to elongate after 7-9 d of CN. Direct multiple shoot formation from the cotyledonary node explants was observed. The initiation of new shoot buds was formed successively from actively dividing meristematic cells with dense cytoplasm at the base of emerging shoots of CN after 14 d of culture. Histological study revealed that multiple shoot formation occurred without intervening callus phase (**Fig. 1C**).

Rooting of *in vitro* propagated shoots of *T. chebula*

The rooting of the first generation shoots were studied using MS media with different reduced constituents of nutrients along with PGRs IBA and NAA. The rooting of shoots was observed on ½MS + IBA (1.0 mg/l) and ½MS + IBA (2.0 mg/l) within 15-20 days of culture. The highest rooting percentage (53.6 ± 0.66) was observed on ½MS + IBA (1.0 mg/l) with 3.2 ± 0.2 roots/shoot (**Table 2, Fig. 2A, 2B**). As maximum rooting response was obtained in ½MS + IBA (1.0 mg/l) media, it was considered as formulated media (FM) for further treatments such as effect of media additives and antioxidants on induction of rooting. Enhance rooting response was observed using various concentration of mannitol in the MS (full and half strength) containing IBA (1.0 mg/l) showed varied response. The rooting of shoots was observed on half strength MS supplemented with IBA (1.0 mg/l), mannitol (0.5, 1%) and sucrose either 3 or 1.5% (**Table 3, Fig. 2B**). Highest rooting percentage (93.4 ± 0.9) was observed on ½MS + IBA (1.0 mg/l) + mannitol (1%) with a reduced concentration of sucrose at 1.5%. A maximum of 5.2 ± 0.2 roots/shoot with lateral roots

Table 1 Effect of different culture passages and duration of culture on multiple shoot induction from cotyledonary node (CN) explants of *Terminalia chebula*.*

Culture passage	Duration of culture** (weeks)	No. of shoots/CN Mean ± SE	Average shoot length (cm) Mean ± SE
Initial I (Zero)	4.0	6.45 ± 0.44	3.6 ± 0.1
Initial II (Zero)	8.0	19.2 ± 1.50	3.4 ± 0.10
Stage I (After1st subculture)	11.0	13.2 ± 0.20	3.2 ± 0.10
Stage II (After2nd subculture)	14.0	5.5 ± 0.25	2.6 ± 0.10

*Standardized medium (½ MS + BA (10.0 mg/l) + GA_3 (0.3 mg/l) + IBA (1.0 mg/l) was used for the present study. ** Duration of subculture and the observed multiple shoot induction/cotyledonary node was calculated from the initiation of culture. The 1st subculture took 8 weeks and the 2nd subculture took 12 weeks. Observations after the 1st and 2nd subculture were made with 11- and 14-weeks-old cultures.

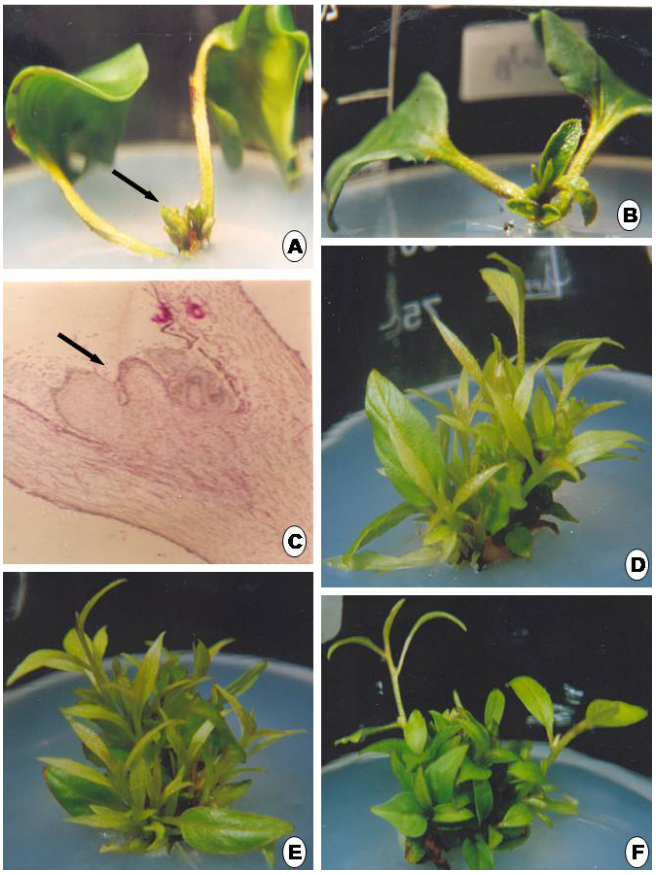

Fig. 1 Multiple shoot induction from cotyledonary explants of *T. chebula*. (**A, B**) Initiation of multiple shoots on cotyledonary node. (**C**) Histological section depicting multiple shoot induction through organogenesis pathway without intervening callus. (**D, E**) Profuse multiple shoot induction on first subculture after 8 weeks (stage I). (**F**) Further growth of multiple shoots after 11 weeks (stage II).

Table 2 Effect of different concentrations of auxins on root formation *in vitro* in the first generation of *Terminalia chebula* shoots.*

Media	PGR (mg/l)		No of shoots showing a rooting response % Mean ± SE	No of roots or shoots/ explant Mean ± SE	Average root length (cm) Mean ± SE
	IBA	NAA			
MS	1.0	0.0	-	-	-
MS	2.0	0.0	-	-	-
MS	3.0	0.0	-	-	-
MS	5.0	0.0	-	-	-
½ MS	1.0	0.0	53.6 ± 0.66	3.2 ± 0.2	5.1 ± 0.7
½ MS	2.0	0.0	21.3 ± 0.66	3.0 ± 0.2	5.0 ± 0.1
½ MS	3.0	0.0	-	-	-
½ MS	5.0	0.0	-	-	-
MS	0.0	1.0	-	-	-
MS	0.0	2.0	-	-	-
MS	0.0	3.0	-	-	-
MS	0.0	5.0	-	-	-
½ MS	0.0	1.0	-	-	-
½ MS	0.0	2.0	-	-	-
½ MS	0.0	3.0	-	-	-
½ MS	0.0	5.0	-	-	-
MS	1.0	0.5	-	-	-
MS	2.0	1.0	-	-	-
½ MS	1.0	0.5	-	-	-
½ MS	2.0	1.0	-	-	-

* Observations were made after 4 weeks of culture. -: No response. n = 25. Values represent mean ± SE and each experiment was repeated in triplicate.

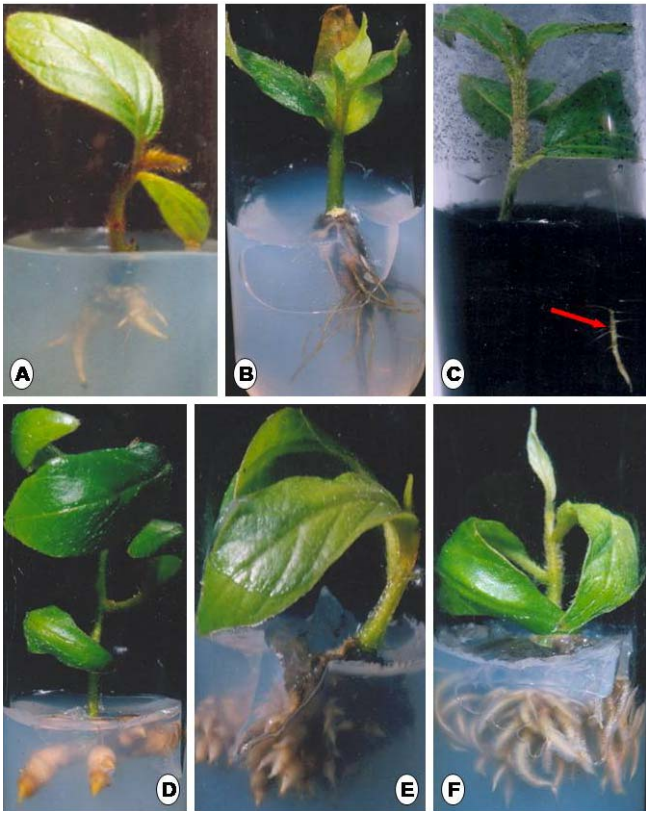

Fig. 2 Rooting of shoots on media containing IBA and antioxidants. (**A, B**) Rooting of shoots containing IBA. (**C**) Rooting of shoots and the arrow indicates the growth of roots in charcoal containing media. (**D**) Induction of roots from shoots in antioxidant (PVP) containing medium. (**E, F**) Profuse growth and high number of roots per shoot with green leaves on antioxidant (PVP)-containing medium.

showing vigorous growth was observed. The rooting response was not observed on full strength MS media irrespective of mannitol concentrations. There was no rooting response observed on MS (full and half strength) supplemented with 2% mannitol.

Various concentrations of antioxidants viz. AC, PVP and phloroglucinol supplemented to standardize rooting media showed varied rooting responses. Rooting response 54.3 ± 0.33 and 52.3 ± 0.33 was obtained on media containing activated charcoal 0.5 and 1.0%, respectively (**Table 4, Fig. 2C**). The number of roots induced per shoot was (2.3 ± 0.2). Increased rooting percentage (67.0 ± 0.4 and 75.3 ± 0.88) was observed in media containing PVP (0.25 and 0.3%, respectively). The antioxidant not only promoted higher rooting but also the rooted plants were healthy and green compared to other antioxidants (**Fig. 2D**). The number of roots/shoot excluding lateral roots (7.3 ± 0.4 and 7.3 ± 0.2) was also high in PVP media compared to media containing activated charcoal and phloroglucinol (**Fig. 2E, 2F**). Media supplemented with phloroglucinol (100 and 200 mg/l) did not promote rooting response (38.6 ± 0.66 and 34.6 ± 0.66) rather there was a decrease compared to Activated charcoal and PVP. There was no significant difference observed among length of the roots formed in the media containing different antioxidants.

The media combinations namely FM: ½MS + 1.0 mg/l IBA, MM: ½MS + 1.0 mg/l IBA + 1% mannitol + 1.5% sucrose and PVPM: ½ MS + mg/l IBA 1.0 + 0.3% PVP promoting highest rooting response obtained from previous experiments were used to study the effect of axillary shoot age on multiple shoot induction. In case of FM first generation shoots of different age groups showed a variation in the rooting percentage of the shoots. Axillary shoots with age groups 4 and 6-7 week showed higher rooting percentage 53.6 ± 0.6 and 45.3 ± 0.6, respectively. Further there was a decline in the other age group of axillary shoots (**Table 5**). The first generation shoots of beyond 11 weeks axillary showed no rooting response. Axillary shoots in 4 and 6-7 weeks age groups cultured on mannitol media showed 93.4 ± 0.9 and 67.5 ± 0.4 rooting response, respectively. There was a decline in the rooting percentage i.e. 41.7 ± 0.2 and 28.6 ± 0.8 in 8-9 and 10-11weeks old shoots, respectively.

Table 3 Effect of various concentrations of mannitol and sucrose in full- and half-strength MS medium for root induction in *Terminalia chebula* shoots.*

Media	Concentration of mannitol % (w/v)	Concentration of sucrose % (w/v)	No of shoots showing a rooting response % Mean ± SE	No of roots or shoots/explant Mean ± SE	Average root length (cm) Mean ± SE
MS	0.5	3.0	-	-	-
½ MS	0.5	3.0	41.3 ± 0.6	3.2 ± 0.2	4.7 ± 0.1
MS	1.0	3.0	-	-	-
½ MS	1.0	3.0	76.0 ± 0.4	3.8 ± 0.1	5.1 ± 0.7
MS	2.0	3.0	-	-	-
½ MS	2.0	3.0	-	-	-
MS	0.5	1.5	-	-	-
½ MS	0.5	1.5	57.3 ± 0.6	3.7 ± 0.2	5.2 ± 0.1
MS	1.0	1.5	-	-	-
½ MS	1.0	1.5	93.4 ± 0.9	5.5 ± 0.1	5.2 ± 0.2
MS	2.0	1.5	-	-	-
½ MS	2.0	1.5	-	-	-

* Observations were made after 4 weeks of culture. -: No response. n = 25. Values represent mean ± SE and each experiment was repeated in triplicate.

Table 4 Effect of different antioxidants and their concentrations on rooting of *in vitro* shoots of *Terminalia chebula* using formulated medium (FM).

Antioxidant	Concentration % (w/v)	No of shoots showing a rooting response % Mean ± SE	No of roots or shoots/explant Mean ± SE	Average root length (cm) Mean ± SE
Activated charcoal	0.5	54.3 ± 0.33	2.3 ± 0.2	5.5 ± 0.2
	1.0	52.3 ± 0.33	2.3 ± 0.2	5.5 ± 0.2
	2.0	21.3 ± 0.66	2.7 ± 0.2	5.2 ± 0.1
Polyvinylpyrrolidone	0.1	60.6 ± 0.66	7.0 ± 0.3	5.8 ± 0.1
	0.25	67.0 ± 0.40	7.3 ± 0.4	5.5 ± 0.2
	0.3	75.3 ± 0.88	7.3 ± 0.2	5.1 ± 0.7
	0.5	50.6 ± 0.66	7.0 ± 0.1	5.1 ± 0.4
Phloroglucinol	100 mg/l	38.6 ± 0.66	2.3 ± 0.2	5.5 ± 0.2
	200 mg/l	34.6 ± 0.66	2.3 ± 0.2	5.5 ± 0.2

FM: ½ MS + 1.0 mg/l IBA. * Observations were made after 4 weeks of culture. n = 25. Values represent mean ± SE and each experiment was repeated in triplicate.

Table 5 Effect of *in vitro* propagated *Terminalia chebula* axillary shoot age on rooting using different media.†

Age of shoot (weeks)	No of shoots showing a rooting response % Mean ± SE	No of roots or shoots/explant Mean ± SE	Average root length (cm) Mean ± SE
*4	53.6 ± 0.6	3.75 ± 0.25	5.5 ± 0.7
*6-7	45.3 ± 0.6	3.25 ± 0.25	5.1 ± 0.2
*8-9	26.8 ± 0.4	2.7 ± 0.25	5.1 ± 0.2
*10-11	15.4 ± 0.3	2.3 ± 0.25	3.8 ± 0.1
*12-14	-	-	-
**4	93.4 ± 0.9	5.5 ± 0.1	5.2 ± 0.2
**6-7	67.5 ± 0.4	5.2 ± 0.2	5.1 ± 0.2
**8-9	41.7 ± 0.2	4.7 ± 0.2	4.8 ± 0.2
**10-11	28.6 ± 0.8	3.5 ± 0.2	4.7 ± 0.2
**12-14	-	-	-
***4	75.3 ± 0.8	7.3 ± 0.2	5.8 ± 0.1
***6-7	61.2 ± 0.6	6.1 ± 0.2	5.5 ± 0.2
***8-9	32.3 ± 0.8	5.8 ± 0.2	5.1 ± 0.7
***10-11	16.3 ± 0.6	5.3 ± 0.2	5.1 ± 0.4
***12-14	-	-	-

*FM: Formulated media (½ MS + 1.0 mg/l IBA). **MM: Mannitol medium (½ MS + 1.0 mg/l IBA + 1% mannitol + 1.5% sucrose. ***PVPM: Medium with PVP (½ MS + 1.0 mg/l IBA + 0.3% PVP. †In each treatment, observations were made after 4 weeks of culture. n = 25. Values represent mean ± SE and each experiment was repeated in triplicate.

Maximum rooting response (75.3 ± 0.8 and 61.2 ± 0.6) was observed in 4 and 6-7 week age group axillary shoots cultured on media containing PVP. Similar to the other media there was a decline in the rooting response of axillary shoots (**Table 5**). There was no rooting response observed in 12-14 weeks age group axillary shoots. The 12-14 weeks old shoots were not suitable for rooting because the leaves were not healthy and green started becoming yellow.

Acclimatization and hardening of the micropropagated of *T. chebula* plants

Acclimatization of the *in vitro* propagated plants was accomplished in mist chamber in glasshouse conditions indigenously developed at CPMB, O.U. The survival rate was 37.5% when rooted plants transferred directly from agar to polybags containing soil in glass house conditions. However, transfer of rooted plants from agar medium to soil fortified with full strength MS nutrients and 1.0 mg/l IBA in bottles under culture room conditions for 2 weeks helped to increase the survival rate to 87.7%. There was an enhancement in the survival percentage i.e. 93.9% when similar plants transferred to polybags containing soil and vermicompost in a 3: 1 ratio. Survival percentage of plants increased up to 98.4%, when bottles incubated in mist chamber for 2-3weeks before transfer to the polybags (**Fig. 3A**). The height and number of the leaves of the plants were recorded after 6-8 weeks of transfer in the glass house conditions (**Table 6**). After 6-8 weeks in the mist chamber the plants were transferred to the field conditions. The hardened plants were kept outside the mist chamber in glasshouse conditions under shade for 2-3-days and later they were transferred to the field conditions. This protocol has helped in the establishment of mist chamber acclimatized plants in field conditions.

Fig. 3 Acclimatization, hardening of *in vitro* propagated rooted plants under mist chamber conditions in poly bags and establishment in field conditions for plantation. (**A**) Acclimatization and growth of *in vitro* rooted plants in poly bags using a portable mist chamber. (**B**) Establishment of acclimatized plant removed from poly-bags in field soil conditions. (**C**) Growth of established plants field conditions, Growth of a 7-year-old plant in field conditions. (**D**) Early flowering (arrow) in 7-year-old plant. (**E**) Healthy fruits in eight year old established plant in field conditions.

Acclimatization and establishment of micropropagated plants in field conditions

The field evaluation of the hardened plants was carried out after one month of the transfer to the field conditions. The plants hardened in poly-bags containing soil showed survival percentage (47.9%) in the field conditions (**Table 7**). Whereas plants hardened in the poly-bags containing soil and vermicompost showed 84.4% survival rate in the field

Table 6 Acclimatization and hardening of micropropagated *Terminalia chebula* plants in mist chamber in glasshouse conditions.

Growth conditions /No of plantlets transferred	Transferred to substrate for growth	No of plants survived	% survival	*Shoot height (cm) Mean ± SE	No of leaves/plant Mean ± SE
Plantlets in agar medium/(40)	Poly-bags with soil	15	37.5	8.6 ± 0.1	4.05 ± 0.25
Soil in bottles (*in vitro*) culture room 2-3 weeks/(45)	Poly-bags with soil	35	87.5	11.5 ± 0.2	5.75 ± 0.57
Soil in bottles (*in vitro*)culture room 2-3 weeks/(55)	Poly-bags with soil and vermicompost 3: 1	50	93.9	12.5 ± 0.28	5.75 ± 0.25
Soil in bottles (*in vitro*) culture room 2-3 weeks. Incubation in mist chamber for 2 weeks/(50)	Poly-bags with soil and vermicompost 3: 1	48	98.4	13.5 ± 0.28	5.45 ± 0.25

*Data for shoot height and no. of leaves were recorded after 8 weeks of transfer of *in vitro* propagated plantlets to soil in glasshouse conditions.

Table 7 Acclimatization and establishment of hardened micropropagated *Terminalia chebula* plants in field conditions.*

Method of acclimatization	No. of plants transferred	No. of plants that survived	% Plant survival
Hardened* Plant + planted in field, CPMB, O.U	48	23	47.9
Hardened** Plant + planted in field, CPMB, O.U	45	38	84.4
Hardened***plant (2-3 weeks) + planted in field, SAIRD, Gadddipally Nalgonda District, A.P., India	65	50	76.9

* Hardened plant (polybag + soil - vermicompost). ** Hardened plant (polybag + soil + vermicompost). *** Hardened plant (polybag + soil + vermicompost). Observations were made after one month of transfer to the field.

(**Fig. 3B**). The hardened plants acclimatized once again for another 2 weeks in the mist chamber at Gaddipally, Nalgonda District, after transport from Hyderabad and later transfer to the field conditions showed 76.9% survival rate. Field evaluation of the micropropagated plants were studied in the field at CPMB, O.U, Hyderabad (**Fig. 3C**).

Evaluation and study of the hardened plants in field conditions at CPMB, O.U was carried out. Early flowering was observed from in vitro propagated field established plants during the sixth and seventh year however no fruit setting was obtained. Flowering and fruit setting was observed in eighth year (**Fig. 3D, 3E**).

DISCUSSION

Multiple shoot induction from cotyledonary node explants of *T. chebula*

Formation of the new shoots was observed through repeated subculture of the original explants to the fresh media. But the number of shoots formed was less compared to the initial formed shoots. This may be due to the highest meristematic actively growing cells at the initiation of axillary bud and cells surrounding the first formed shoot were more competent for shoot formation. These observations show that regions of the axillary bud can be induced to become meristematic and initiate multiple shoots. Similarly in many of the reports the highest axillary shoot induction was obtained from cotyledonary explants in *Terminalia arjuna*, *Quercus leucotricophora*, *Sesbania rostrata* (Pandey and Jaiswal 2002; Purohit *et al.* 2002; Jha *et al.* 2004). Further subculture (II) of the initial explant showed decline in the number of axillary shoots. This may be due to the loss in the meristematic activity of the cells and maturity of the explant also counts one of the factors. Similar type of response was observed in the decline of number of shoots in *Sesbania rostrata* (Jha *et al.* 2004). In the present study the combination of cytokinin and two auxins showed best in induction of the multiple shoots. This attributes to the synergistic interaction between cytokinin and auxin and this promoted induction of more number of shoots. Similarly the combination of cytokinin and auxin showed optimal response in *Quercus leucotricophora* and *Pinus virgiana* (Purohit *et al.* 2002; Tang *et al.* 2004).

Rooting of first generation *in vitro* propagated shoots of *T. chebula*

In the present study the half strength MS nutrient were found suitable for rooting of the *in vitro* propagated shoots. In the present study highest rooting response was observed on media containing IBA at lower concentration. It is clearly known that naturally occurring auxins shows positive effect on root induction and elongation compared to the synthetic auxins e.g., NAA (Kollarova *et al.* 2004). In the present study the media containing IBA not only induced roots and length of the root was also more. Similarly in maximum of the reports IBA induced highest percentage of rooting compared to the NAA in *Phellodendron amurense* (Azad *et al.* 2005). But at the higher concentration of IBA shoots did not show rooting this may be because of the hindrance effect at that concentration.

In the present study the addition of mannitol increased the rooting percentage of the shoots. This may be due to the stress conditions created and during this stress the shoots initiated rooting to uptake the nutrients. The media containing mannitol not only induced highest percentage of rooting but also the number of roots induced was more. In earlier reports the rooting response was also maximum in *Prunus* when shoots were grown in medium containing sorbitol (Harada and Murai 1996).

In the present study the addition of antioxidants in the media also increased percentage of rooting. Highest percentage of rooting was observed on media containing PVP and number roots formed were also more compared to other antioxidants. This may be due to the complete adsorption of phenolics released by the stems. Further it was also found that the rooted plants were healthier and green compared to the plants grown in medium without PVP for rooting. In the present study highest rooting percentage of 93.4 ± 0.9, 75.3 ± 0.88 was obtained on media containing either mannitol or PVP, respectively.

Acclimatization and hardening of the micropropagated plants

Survival percentage of the plants was increased when rooted plants incubated in culture room conditions for 2-3weeks and subsequent transfer to the mist chamber conditions. This intermediate manipulation not only increased the survival rate but also the length of the roots was increased. Similarly this type of approach showed optimal survival percentage in *Paeonia suffruticosa* (Beruto *et al.* 2004). Addition of vermicompost in the poly-bag along with the soil also increased the survival percentage and number of new leaves formed was high. Similarly, the transfer of plants to soil containing organic compost increased survival rate in *Sesbania sesban* (Jha *et al.* 2003).

Evaluation and study of the hardened plants in field conditions

About 45% of the hardened plants transferred to the field at CPMB were established. The growth rates recorded in the

field transferred showed that plant height; number of leaves, shoot diameter were comparable in the micropropagated as well as in the field transferred plants. Similarly among different these parameters were considered for the plant evaluation in the field conditions in *Morus* spp. and *Quercus leucotricophora* (Zaman *et al.* 1997; Purohit *et al.* 2002).

CONCLUSION

In conclusion, in the present work, high frequency axillary shoot production from cotyledonary node explants, axillary shoots rooted, acclimatization and transfer to the field conditions in *T. chebula*. Maximum of 44.33 ± 0.88 first generation shoots could be obtained from single cotyledonary node after 12-14 weeks of culture. In the present study hardened plants showed 98.4% survival rate in the glasshouse conditions. Nearly 45% of the plants establishment in the field conditions at CPMB, O.U, Hyderabad. Plants were also transferred and established at SAIRD, Gaddipally, Nalgonda District, A.P. The trees established in field conditions showed early flowering and fruit formation. The protocol developed may be beneficial for generating large number of shoots, which will accelerate the *in vitro* propagation process of this important plant.

ACKNOWLEDGEMENTS

The authors would like to thank the AP Netherlands Biotechnology Programme, A.P. of The Netherlands, Ministry for Development Co-operation (presently Agribiotech Foundation) for the financial support. B. Shyamkumar would also like to thank CSIR, New Delhi for the award of Senior Research Fellowship.

REFERENCES

Anjaneyulu C, Shyamkumar B, Giri CC (2004) Somatic embryogenesis from callus cultures of *Terminalia chebula* Retz. an important medicinal tree. *Trees – Structure and Function* **18**, 547-552

Anjaneyulu C, Giri CC (2008) Factors influencing somatic embryo maturation, high frequency germination and plantlet formation in *Terminalia chebula* Retz. *Plant Biotechnology Reports* **2 (2)**, 153-161

Azad MAK, Yokota S, Ohkubo T, Andoh Y, Yahara S, Yoshizawa N (2005) *In vitro* regeneration of the medicinally woody plant *Phellodendron amurense* Rupr. through excised leaves. *Plant Cell, Tissue and Organ Culture* **80**, 43-50

Beruto M, Lanteri L, Portagallo C (2004) Micropropagation of tree peony (*Paeonia suffruticosa*). *Plant Cell, Tissue and Organ Culture* **79**, 249-255

Bonjar GHS (2004) Evaluation of antibacterial properties of Iranian medicinal plants against *Micrococcus luteus*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Bordetella bronchoseptica. Asian Journal of Plant Sciences* **3**, 82-86

Cheepala SB, Sharma NC, Sahi NV (2004) Rapid *in vitro* regeneration of *Sesbania drummundii. Biologia Plantarum* **48**, 13-18

Chen LT, Chen CS, Fen CH, Lin HF (2000) Tannins and related compounds from Combretaceae plants. *Chinese Pharmaceutical Journal* **52**, 1-26

Ďurkovič J (2008) Micropropagation of mature *Cornus mas* 'Macrocarpa'. *Trees – Structure and Function* **22**, 597-602

Feng J-C, Yu XM, Shang XL, Li JD, Wu YX (2010) Factors influencing efficiency of shoot regeneration in *Ziziphus jujuba* Mill. 'Huizao'. *Plant Cell, Tissue and Organ Culture* **101**, 111-117

Feyissa T, Welander M, Negash L (2005) Micropropagation of *Hagenia abyssinica*: a multipurpose tree. *Plant Cell, Tissue and Organ Culture* **80**, 119-127

Gill AS, Handa AK, Neelam K (2004) Medicinal value of important tree species in agroforestry. *Indian Forester* **130**, 615-629

Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: An overview. *Trees – Structure and Function* **18**, 115-135

Giri CC, Giri A (2007) Anatomical and histological techniques for plant tissue cultured *in vitro*. In: *Plant Biotechnology: Practical Manual*, I. K. International Publishing House Pvt. Ltd. New Delhi, pp 93-99

Gomes F, Canhoto J M (2009) Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants. *In Vitro Cell and Developmental Biology – Plant* **45**, 72-82

Gonzalez-Arnao MT, Panta A, William MR, Roosevelt HE, Engelmann F (2008) Development and large-scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. *Plant Cell, Tissue and Organ Culture* **92**, 1-13

Harada H, Murai Y (1996) Micropropagation of *Prunus mume*. *Plant Cell, Tissue and Organ Culture* **46**, 265-267

Jensen WA (1962) *Botanical Histochemistry*, Freeman, San Francisco, 408 pp

Jha AK, Prakash S, Jain N, Nanda K, Gupta SC (2003) Micropropagation of *Sesbania sesban* from the mature tree-derived explants. *Biologia Plantarum* **47**, 121-124

Jha AK, Prakash S, Jain N, Nanda K, Gupta SC (2004) Micropropagation of *Sesbania rostrata* from cotyledonary node. *Biologia Plantarum* **48**, 289-292

Khare CP (2004) *Terminalia chebula* Retz. In: Khare CP (Ed) *Encyclopedia of Indian Medicinal Plants*, Springer-Verlag, Berlin, Germany, pp 451-453

Kollarova K, Liskova D, Kakaniova D, Lux A (2004) Effect of auxins on *Karwinskia humboldtiana* root cultures. *Plant Cell, Tissue and Organ Culture* **79**, 213-221

Lee SI, Hyun PM, Kim SH, Lee SK, Kim BS, Maeng PJ, Lim JS (2005) Suppression of the onset and progression of collagen induced arthritis by chebulagic acid screened from a natural product library. *Arthritis and Rheumatism* **52 (1)**, 345-353

Liu X, Pijut PM (2008) Plant regeneration from *in vitro* leaves of mature black cherry (*Prunus serotina*). *Plant Cell, Tissue and Organ Culture* **94**, 113-123

Liu X, Pijut PM (2010) *Agrobacterium*-mediated transformation of mature *Prunus serotina* (black cherry) and regeneration of transgenic shoots. *Plant Cell, Tissue and Organ Culture* **101**, 49-57

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Nagavallemma KP, Wani SP, Lacroix S, Padmaja VV, Vineela C, Babu Rao M, Sahrawat KL (2006) Vermicomposting: Recycling wastes into valuable organic fertilizer. *Journal of SAT Agricultural Research* **2**, 1-17 Available online: www.ejournal.icrisat.org

Pandey S, Jaiswal VS (2002) Micropropagation of *Terminalia arjuna* Roxb. from cotyledonary node. *Indian Journal of Experimental Biology* **40**, 950-953

Park SY, Kim YW, Moon HK, Murthy HN, Choi YH, Cho HM (2008) Micropropagation of *Salix pseudolasiogyne* from nodal explants. *Plant Cell, Tissue and Organ Culture* **93**, 341-346

Prakash S, van Staden J (2008) Micropropagation of *Searsia dentata*. *In Vitro Cellular and Developmental Biology – Plant* **44**, 338-341

Purohit VK, Tamta S, Chandra S, Vyas P, Palni LMS, Nandi SK (2002) *In vitro* multiplication of *Quercus leucotricophora* and *Q. glauca*: Important Himalayan oaks. *Plant Cell, Tissue and Organ Culture* **69**, 121-133

Ramesh SDR, Dhir KK, Vij SP, Nayyar H, Kamaljit S (2003) Study of genetic improvement techniques of *Terminalia chebula* Retz: An important multipurpose medicinal tree species of India. *Indian Forester* **129**, 154-168

Sandip B (2003) Medicinal plants with anti-HIV potential. *Journal of Medicinal and Aromatic Plant Science* **25**, 427-440

Shankar U (2001) A case of high tree diversity in a sal (*Shorea robusta*) dominated lowland forest of eastern Himalaya: Floristic composition, regeneration and conservation. *Current Science* **81**, 776-786

Shyamkumar B, Anjaneyulu C, Giri CC (2003) Multiple shoot induction from cotyledonary node explants of *Terminalia chebula*. *Biologia Plantarum* **47**, 585-588

Suchalatha S, Shyamala DCS (2004) Protective effect of *Terminalia chebula* against experimental myocardial injury induced by isoproterenol. *Indian Journal of Experimental Biology* **42**, 174-178

Suguna L, Singh S, Sivakumar P, Sampath P (2002) Influence of *Terminalia chebula* Retz. on dermal wound healing in rats. *Phytotherapy Research* **16**, 227-231

Tang W, Harris LC, Outhavong V, Newton RJ (2004) The effect of different plant growth regulators on adventitious shoot formation from Virginia *Pine* (*Pinus virgiana*) zygotic embryo explants. *Plant Cell, Tissue and Organ Culture* **78**, 237-240

Thakur PS (2000) Comparative performance of multipurpose tree species growing at degraded site. *Indian Forester* **126**, 895-900

Tilkat E, Onay A (2009) Direct shoot organogenesis from *in vitro*-derived mature leaf explants of pistachio. *In Vitro Cellular and Developmental Biology – Plant* **45**, 92-98

Walia N, Sinha S, Babbar SB (2003) Micropropagation of *Crataeva nurvalar*. *Biologia Plantarum* **46**, 181-185

Warrier PK, Nambiar VPK, Ramankutty C (1997) Indian medicinal plants a compendium of 500 species In: Warrier PK, Nambiar VPK, Ramankutty C (Eds) Arya Vaidyasala, Orient Longman, Hyderabad, pp 263-274

Yevtushenko DP, Misra S (2010) Efficient *Agrobacterium*-mediated transformation of commercial hybrid poplar *Populus nigra* L. 3 *P. maximowiczii* A. Henry. *Plant Cell Reports* **29**, 211-221

Yucesan B, Ucar TA, Ekrem G (2007) TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). *Plant Cell, Tissue and Organ Culture* **91**, 243-250

Zaman A, Islam R, Joarder OI (1997) Field performance and biochemical evaluation of micropropagated mulberry plants. *Plant Cell, Tissue and Organ Culture* **51**, 61-64

Zeng F, Qian J, Luo W, Zhan Y, Xin Y, Yang C (2010) Stability of transgenes in long-term micropropagation of plants of transgenic birch (*Betula platyphylla*). *Biotechnology Letters* **32**, 151-156