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BRIEF COMMUNICATION

Multiple shoot induction from cotyledonary node explants of *Terminalia chebula*

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Abstract

A protocol for multiple shoot induction from cotyledonary node explants of *Terminalia chebula* Retz. has been developed. Germination frequency of embryos (up to 100 %) was obtained on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm^{-3} gibberellic acid (GA_3). Maximum number of shoots (6.4 shoots per cotyledonary node) was obtained on half-strength MS + 0.3 mg dm^{-3} GA_3 + 1.0 mg dm^{-3} indole-3-butyric acid (IBA) + 10.0 mg dm^{-3} benzylaminopurine (BAP) after 4 weeks of culture. When the cotyledonary nodes along with the axillary shoot buds were allowed to grow in the same medium upto 19.2 shoots were obtained after 8 - 9 weeks. Best rooting (100 %, 5.5 roots per shoot) was observed when shoots were excised and transferred to half-strength MS medium containing 1.0 mg dm^{-3} IBA + 1 % mannitol and 1.5 % sucrose. Survival of rooted plants *in vivo* was low (35 - 40 %) when they were directly transferred to soil in glasshouse. However, transfer to soil with MS nutrients and 1.0 mg dm^{-3} IBA in culture room for a minimum duration of 2 weeks increased the survival percentage of plants to 100 %.

Additional key words: acclimatization, *in vitro* propagation.

Terminalia chebula Retz. (*Combretaceae*) is an important tree of pharmaceutical and trade value (Chadha 1989). Direct sowing of seeds in the field results in erratic, inadequate germination and low survival of the seedlings. These factors contribute to high production cost of seedling stock (Bhardwaj and Chakraborty 1994). The natural regeneration of *T. chebula* from seeds *in situ* and *ex situ* is also extremely low (Shankar 2001). *In vitro* propagation has proved as a means for supply of planting material for forestry (Ahuja 1993, Lakshmisita and Raghavaswamy 1998). Further, development of *in vitro* plant regeneration protocol is a pre-requisite for genetic transformation studies. Cotyledonary node explants have been used for multiple shoot induction in tree propagation (Franca *et al.* 1995, Das *et al.* 1996, Distabanjong and Geneve 1997, Pradhan *et al.* 1998, Das *et al.* 1999, Purohit *et al.* 2002, Walia *et al.* 2003). In the present communication, induction of multiple shoots from cotyledonary node explants and efficient rooting of shoots is reported for the first time in *T. chebula*.

Seeds of *Terminalia chebula* Retz. were collected from the elite plant in the Tiger Forest Reserve, Srisailam, Andhra Pradesh, India. The seeds without pulp were washed in tap water containing detergent *Laboline* (*Qualigens Fine Chemicals*, Mumbai, India) and rinsed thoroughly, and surface sterilized with 0.1 % mercuric chloride for 10 min, and rinsed with sterile distilled water 7 - 8 times. The embryos excised from seeds (15 - 17 mm size) were further surface sterilized with 0.1 % *Bavistin* for 3 - 4 min and washed with sterile distilled water. The excised embryos were cultured in 2.5 cm diameter tube containing Murashige and Skoog (1962, MS) medium

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Abbreviations: BAP - benzylaminopurine; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog.

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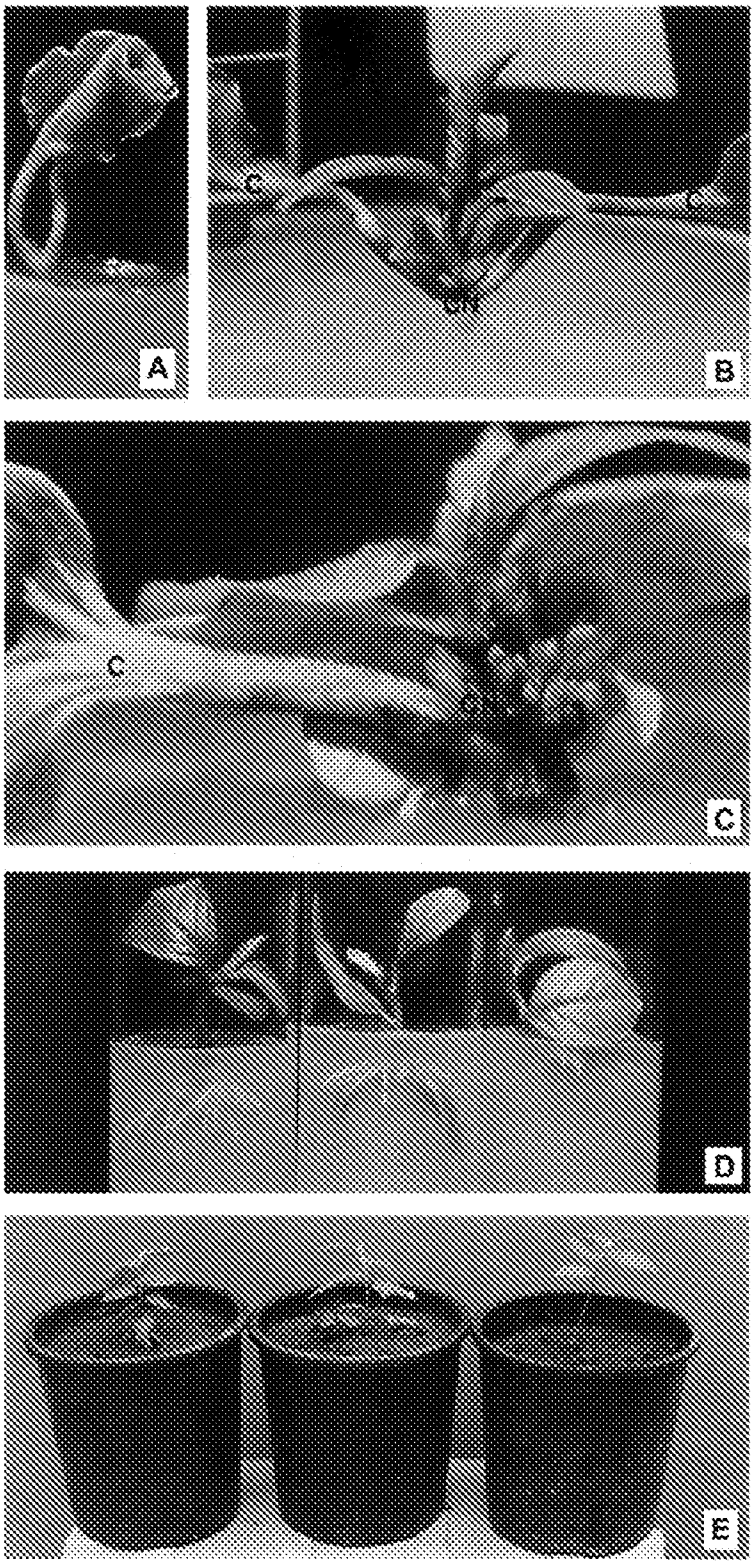

Fig. 1. *In vitro* propagation of *T. chebula* from cotyledonary node explants. *A* - 7 to 8-d-old seedlings used as cotyledonary node explants; *B* - induction of axillary shoots at the cotyledonary node after four weeks of culture (C - cotyledon, CN - cotyledonary node); *C* - induction of new shoots adjacent to the axillary shoots after 8 - 9 weeks of culture. *D* - rooting of first generation shoots in half-strength MS semisolid agar medium with 1 % mannitol; *E* - *in vitro* propagated plants established in soil in glasshouse conditions.

supplemented with 0.9 % agar, 3 % sucrose, and different concentrations of GA_3. The pH of the media was adjusted to 5.8 prior to autoclaving. All the cultures were incubated in the dark at 25 ± 2 °C and 80 % relative humidity. To circumvent the browning problem the excised embryos were transferred to fresh medium at regular intervals. Subsequent to germination the cultures were grown under 16-h photoperiod with an irradiance of 100 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

One-week old seedlings with a first pair of primary leaves were used as cotyledonary node explants. These were cultured on full and half strength MS medium and different plant growth regulators (BAP, GA_3, IBA) were tested for the initiation of axillary shoots. Shoots initiated from cotyledonary nodes were selected after 4 week of culture. These cotyledonary nodes along with the induced axillary buds were grown for further duration (8 - 9 weeks) *in vitro* for obtaining more number of shoots.

Excised shoots (2.5 - 3.0 cm in height) were transferred to semisolid full and half strength MS media supplemented with different concentrations of IBA (1.0, 2.0, 3.0 and 5.0 mg dm^{-3}) for root induction. Acclimatization of the rooted plants was carried out in a glasshouse on soil mixture in pots at relative humidity of 90 %, and temperature of 26 ± 2 °C. To achieve a higher rate of survival, *in vitro* rooted plants were transferred to soil mixture in culture vessels containing MS nutrients with IBA and grown in culture room at relative humidity of 80 %, and temperature of 25 ± 2 °C. All the experiments were repeated for a minimum three times with similar culture parameters. Statistical analysis of the data was done using *Statistical Package Matlab Version 5.3*, *Math Works* Inc., USA.

Excised embryos incubated in dark showed germination within 2 - 3 d of culture. Germination frequency up to 100 % was observed within 1 week when excised embryos were cultured on MS medium supplemented with 3 % sucrose, 100 mg dm^{-3} myo-inositol and 0.5 mg dm^{-3} GA_3. The germination of excised embryos of *Gentiana* was also enhanced by the incorporation of GA_3 (Moncilovic *et al.* 1997).

Seven to eight days old seedlings with well developed cotyledonary leaves without initiation of primary shoots were found suitable as a source of nodal explants (Fig. 1*A*). The first axillary bud emerged at the cotyledonary nodes after 7 d of culture. Media containing half-strength MS nutrients with either 0.3 or 0.5 mg dm^{-3} GA_3 promoted shoot induction. IBA (1.0 mg dm^{-3}) in half-strength MS medium was essential for induction of high number of axillary shoots (data not shown). Axillary shoots (2.0 ± 0.5 shoots per cotyledonary node) were obtained on half strength MS medium supplemented with 0.3 mg dm^{-3} GA_3 and 1.0 mg dm^{-3} IBA after 4 weeks of culture (Table 1). BAP at 1.0, 5.0, 10.0, 15.0 mg dm^{-3} was used to enhance axillary bud initiation. The highest number of shoots (6.4 ± 0.5 shoots per cotyledonary node) was obtained on half strength MS medium supplemented with 0.3 mg dm^{-3} GA_3, 1.0 mg dm^{-3} IBA and 10.0 mg dm^{-3} BAP after 4 week (Fig. 1*B*, Table 1). The BAP concentration beyond 10.0 mg dm^{-3} did not enhance the number of axillary shoot bud induction rather there was a decline in the number of shoots (Table 1). However, in other woody species it was found that increased number of axillary shoot buds were obtained by manipulating even higher concentrations of BAP in the media (Das *et al.* 1999). It has also been reported, tree species where lower concentration of BAP was optimal to induce maximum number of axillary shoots (Barcelo-Muñoz *et al.* 1999). These observations may be attributed to the different endogenous concentrations of cytokinin in the explants used for induction of axillary shoots. In the earlier reports, cotyledonary node explants has been used for multiple shoot induction in tree species. Cultures with best response in terms of maximum number of axillary shoots were allowed to grow beyond 4 weeks. At a maximum 19.2 ± 1.5 shoots were obtained from such individual cultures after 8 - 9 week of *in vitro* growth (Fig. 1*C*). In a similar finding, cotyledonary node explants of Himalayan oaks were used for multiple shoot induction using a combination of GA_3, IBA and BAP. In their study, addition of GA_3 to the medium enhanced the shoot number (Purohit *et al.* 2002).

Table 1. Effect of plant growth regulators [mg dm^{-3}] added to half-strength MS medium on the induction of shoots from cotyledonary nodes of *T. chebula*. Observation was taken after 4 weeks of culture. Means ± SE, $n = 18$.

GA_3	IBA	BAP	Number of shoots [$node^{-1}$]
0.3	1.0	-	2.0 ± 0.5
0.3	1.0	1.0	2.2 ± 0.4
0.3	1.0	5.0	3.8 ± 0.4
0.3	1.0	10.0	6.4 ± 0.5
0.3	1.0	15.0	2.4 ± 0.2

Although MS media along with an array of concentrations of IBA (1, 2, 3, 5 mg dm^{-3}) and NAA (0.5, 1, 2 mg dm^{-3}) were tested, maximum rooting response (100 %) was observed in half-strenght MS medium supplemented with 1.0 mg dm^{-3} IBA + 1 % mannitol and 1.5 % sucrose (Fig. 1*D*). This combination not only gave high frequency of rooting but it also promoted highest number of roots per shoot *i.e.* 5.5 ± 0.2. In earlier reports, the rooting response was at maximum in *Prunus* when shoots were grown in medium containing sorbitol (Harada and Murai 1996).

Acclimatization of the *in vitro* micropropagated plants was accomplished in glasshouse. A low survival frequency (35 - 40 %) was observed when rooted plants

after 4 weeks of culture were transferred directly from agar medium to soil. However, transfer of rooted plants from agar medium to soil fortified with full strength MS nutrients and 1.0 mg dm^{-3} IBA in culture bottles in culture room for 2 weeks helped to increase the survival to 100 %. This intermediate manipulation not only increased the survival percentage but also increased the length of roots. *In vitro* propagated plants were then successfully transferred to soil in glasshouse (Fig. 1*E*).

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