Journal of Research & Development, Vol 3 (2003)
ISSN 0972-5407

Embryo Culture of Chestnut (*Castanea sativa* Mill.)

Fayaz A. Shah, Azra N. Kamili and A. M. Shah

Plant Tissue Culture Laboratory
Centre of Research for Development, University of Kashmir
Srinagar, J & K-190006

ABASTRACT

Embryo culture of *Castanea sativa* Mill. was initiated to investigate the morphogenetic potential of the plant species *in vitro* using MS (x1/2) (1962) basal medium and different phytohormonal concentrations. Shoot formation from the embryos of *Castanea sativa* Mill. was induced when excised embryos were cultured on basal medium. Root initiation was observed in 20% of these cultures after eight weeks. Multiple shoot formation was also induced when isolated embryos were cultured on medium fortified with low concentration of BAP. By raising the concentration of BAP multiple shoot induction was reduced and callus formation was enhanced drastically.

. Auxins (NAA) favoured root elongation as compared to shoot elongation in cultured embryos. Sub cultured root and embryonal shoot tips from such cultures favoured only callus formation in presence of BAP and NAA used separately.

Keywords: Chestnut, embryo culture, multiple shoots.

Abbreviations: MS (x1/2)- Murashige & Skoog (half salt strength)
BAP-6-Benzyl amino purine, NAA-naphthalene acetic acid.

INTRODUCTION

Chestnut (*Castanea sativa* Mill) belongs to family fagaceae and its seeds are reported to be very nutritious (Deasmasion & Adrian, 1986, c.f. Jindal and Karkara, 1991). The major sugars of chestnut seeds are raffinose, stachyose, and sucrose (Dey, 1981.c.f. Jindal and Karkara, 1991). The seeds of chesnuts are very tasty and are consumed after roasting.

Chestnuts are mainly propagated by seeds but the established cultivars are raised by vegetative methods. The conventional methods of propagation of chestnuts are very slow and this has resulted in reduction of germplasm frequency of occurrence of the plant in the valley. The valley possesses only one species of *Castanea*, i.e. *Castanea sativa* Mill which

is in less abundance. So there arises a need to incorporate the unconventional method for the quick multiplication of *Castanea sativa*. The technique of *in vitro* culture has gained worldwide importance and has been put to exploitation by a number of workers in the propagation of many important trees. (Abbot, 1977; Jones, 1979; Winton, 1968; Zimmermann, 1978, 1985). The *in vitro* technique is considered superior to conventional methods of propagation, because of quick propagation rate of plants in relatively shorter period of time and is irrespective of season.

Reports of *in vitro* culture of chestnut have been a few despite being one of the first woody species to which *in vitro* techniques were applied. Several workers have considered culture of embryonic axes and nodal explants from embryos, mature and juvenile material for shoot multiplication and plantlet regeneration of *Castanea sativa* (Vieitez and Vieitez, 1980, 1980; Vieitez *et al.*,1983; Biondi *et al.*, 1981; Mecpheeters *et al.*, 1980; Rodriguez, 1982; Chevre *et al.*, 1983; Chauvin and Salesses, 1988). Production of adventitious buds and roots from epicotyl and hypocotyl sections was reported by Sanjose *et al.*, 1984. Till date no such studies have been carried out in J & K State where the germplasm stock of this plant species is dwindling due to propagation problems by conventional methods. An attempt was therefore made to initiate work for quick propagation of *Castanea sativa* using tissue culture technique and also to determine the culture conditions necessary for its in vitro plantlet formation. This paper reports the establishment of cultures from embryos to induce development of adventitious and axillary shoots and their multiplication and to a lesser extent initiation and development of normal embryonic roots.

MATERIAL AND METHODS

Nuts of *C. sativa* Mill, were collected from horticulture garden Theed, Harwan (Distt. Srinagar) in autumn. Before storage nuts were given 0.2% $HgCl_2$ treatment for 10-15 minutes followed by triple rinse with distilled water. This was done in order to avoid fungal contamination during storage. The seeds were finally dried and stored in Polyethylene bags (30-40cm) with 15-20 pinholes at 4°C in refrigerator. The nuts were chilled for minimum period of 25 - 30 days. Before using nuts were soaked in filtered water for 24 Hours. The embryos were dissected out from the surrounding cotyedons under Laminar air flow. The excised embryos were sterlized with $HgCl_2$ 0.1% solution for 8-10 minutes followed by triple rinsing with autoclaved double distilled water. The sterile embryos were then inoculated on MS (x1/2) medium (1962) with or without various growth regulators. The pH of medium was adjusted between 5.5-5.6 by using NaOH (0.1N) or HCl (0.1N) before jelling the medium with 0.8% agar. The medium was boiled and finally dispensed into 100ml. flasks

Journal of Research & Development, Vol.3 (2003)
ISSN 0972-5407

which were plugged and autoclaved for 15-25minutes at 15lb pressure and 121°C temperature. The cultures were maintained at 25 ± 3°C with 16-18 hours photoperiod from cool white fluorescent tube lights (3000 lux) at 50 - 65% R.H.

RESULTS

Different Morphogentic responses of cultured embryos of *C. sativa* on MS (x1/2) medium fortified with different concentrations and combinations of growth regulators are summerized in Table 1.

Table 1. Morphogenetic response of cultured embryos of *Castanea sativa* under different phytohormonal concentrations and combinations.

Growth Medium	Nature of response *	Degree of callus formation	Percentage of response
Ms (x1/2) Basal	Elongation of embryo, shoot formation (4-6cms long) with 2-4 leaves, and 20% root induction.	—	90
MS (x1/2) + BAP (4.4 µm)	Elongation of embryo, shoot formation and multiple adventitious shoot induction in rosette form Compact nodular whitish callus formed at the base of shoots.	+	70
MS (x1/2) + BAP (8 µm)	Shoot growth and axillary shoot induction	+	70
MS (x1/2) NAA (2.6 µm)	Shoot development suppressed, moderated root elongation.	—	70
MS (x1/2) BAP (2.1 µm) + NAA (2.8 µm)	Shoot and root initiation, shoot growth suppressed, moderate root elongation.	+	60

* Data scored after 6 weeks, 10 replicates/treatment

-No callus growth, + low,

The embryos of *Castanea sativa* when cultured on basal medium showed shoot induction, after 4 weeks. After 8 weeks 4-6 cm long shoot bearing 4-6 leaves were produced. (Fig. 1a) A long root also developed in 20% of cultures. (Fig. 1b). Shoot apex and roots of newly formed plantlets were used as primary cultures for further investigations. Embryonal shoot tips and roots favoured compact green callus formation in presence of BAP and NAA used separately. (Fig.1c). Excised embryos when cultured under influence of NAA favoured root elongation but suppressed shoot growth after its initiation. (Fig.1d). Likewise a combination of BAP (2.1 m) and NAA (2.8 m) showed almost similar results. Excised embryos showed multiple shoot regeneration in rosette shape on BAP ((4.4 m) enriched medium (Fig.1e). After 8 weeks the basal zone of multiplying shoots generally formed nodular callus composed of very firm and compact tissue. (Fig.1e). Isolated shoots again showed multiple shoot induction in medium supported with BAP 4.6 m + IBA .01 m /l.

Table 2. Effect of various phytohormones on invitro shoot growth, its multiplication and root growth of *C. sativa*.

Nature of Response*				
Growth Medium	Shoot growth and number	Root growth and number	%age of response Shoot	Root
MS (x1/2) basal	4-6cms long with 2-4 leaves	Single long tap root at base	90	20
MS (x1/2) BAP (4.4 μm)	2-4cms long with 8-10 shoots	—	70	-
MS (x1/2) BAP (8 μm)	2-4cms long with 4-6 shoots	—	70	-
MS (x1/2) + NAA (2.6 μm)	0.5cm long	Single root	40	70
MS (x1/2) BAP (2.1 μm) +NAA (2.8 μm)	0.5 - 1cms long	Single long root	40	60

*Data recorded after 6 weeks, 10 replicates/treatment.

An attempt was also made to see the effect of various phytohormones on shoot growth, its multiplication and root growth in *in vitro* born organs of *C. sativa* (Table 2). Shoot elongation was maximum on basal medium followed by BAP when used alone. Auxin NAA or its combination with BAP showed least shoot growth. Number of regenerated adventitious shoots was maximum with BAP (4.4 m) followed by higher concentration of BAP but basal medium failed to show such response in the same way as NAA + BAP enriched medium and NAA fortified medium did. A single root was found in all the cases except when BAP alone was supplied in the medium which instead favoured multiple shoot regeneration.

DISCUSSION

The micropropagation technique applied to *C. sativa* seems to be helpful in solving the problem of vegetative propagation by conventional methods. Present findings under different phytohormonal regimes resulted in various morphogenetic responses and depicts that embryo possesses the potentiality for multiple shoot formation which is an important step in micropropagation. Complete seedling formation was accomplished in 20% cultures without the use of plant growth regulators by using embryo culture technique. Embryos produced an apical shoot with two nodal buds after four weeks on MS basal medium. The shoots after transferring to fresh basal medium produced a long and healthy embroynal shoot. These findings show conformity with Strultu, *et al*., 1986. In present findings multiple shoot formation was obtained by using BAP 4.4 m/l. in the cultures of excised embryos. Multiple shoot development as a response to cytokinin is reported on shoot culture of woody species of horticulture importance (Abbott, 1977; Jones, 1979; Zimmermann, 1978, 1985). Present findings in chestnut reveal that cytokinin BAP induced the most intense development of adventitious and axillary shoots. The maximum number of multiple shoots has also been reported with BAP 4.4 µm or 8.8 µm/l by Vieitez and Vieitez (1980) which is thus in agreement with present studies where BAP 4.4 µm/l was also found to be the best for multiple shoot formation (8-10 shoots) in excised embryos. The Multiple adventitious shoots assumed the form of a rosette especially at BAP 4.4 µm/l which is contrary to that of Vieitez 1980 and Vieitez, who reported rosette formation especially at BAP 22 µm/l although axillary shoot development was reported by these workers to be favoured by BAP 4.4 or 8.8 µm/l which is in conformity with present studies. Higher concentration of BAP resulted in development of vigorous callusing in embryonal axis and at the base of shoot which is similar to the records of Rodriguez (1982). Present data also reveals that maximum shoot elongation was achieved on basal medium which is in accordance with the findings of Vieitez and Vieitez (1980). Contradictory to the reports of these workers our findings also

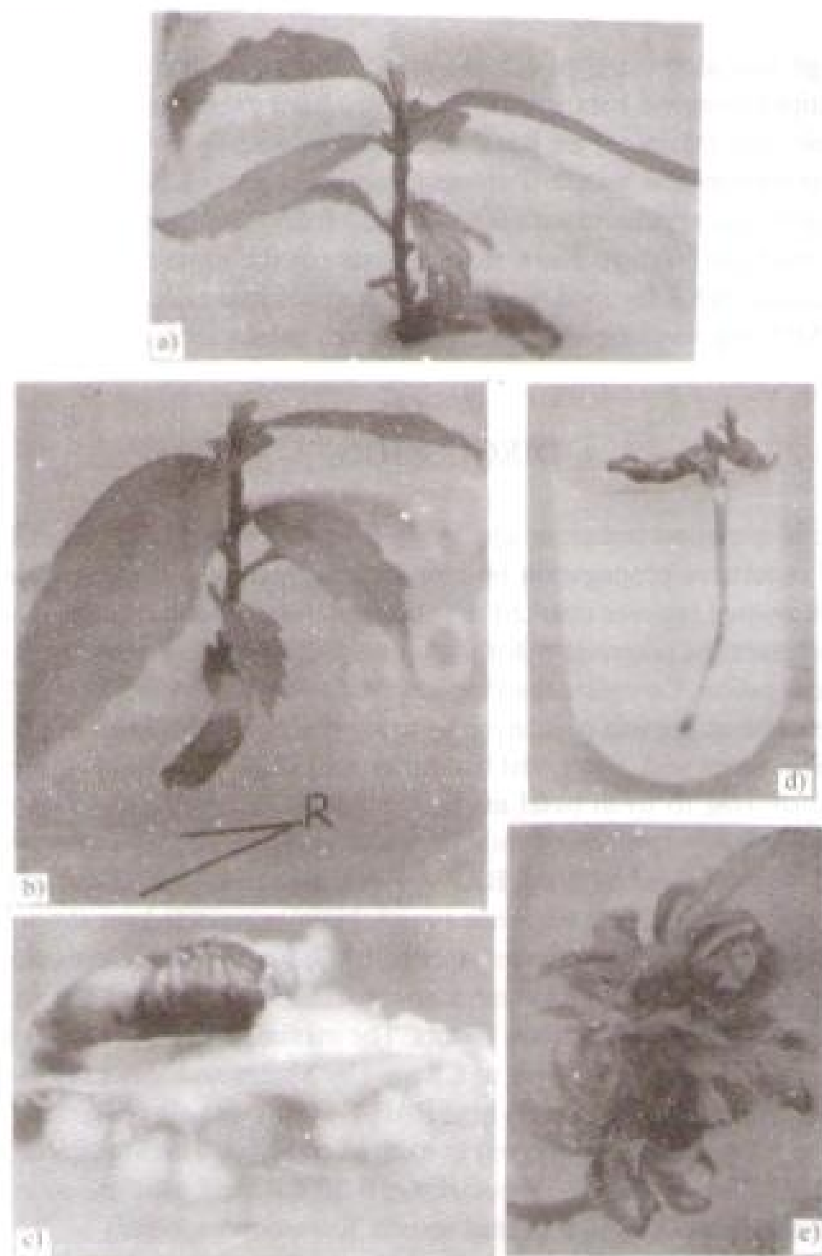

Fig 1(a - e): Morphogenetic response of embryos of *Castenea sativa* to various concentrations and combination of phytohormones.

a) Shoot formation from embryo on MS (x1/2) basal. (after 4 weeks)
b) Root induction (R)on MS (x1/2) basal. (after 8 weeks)
c) Formation of green, compact and nodular callus on MS (x1/2) + BAP (15 µm). (after 4 weeks)
d) Shoot initiation and root elongation on MS (x1/2) + NAA (2.6 µm). (after 4 weeks)
e) Multiple shoot induction on MS (x1/2) + BAP (4.4 µm). (after 8 weeks)

show elongation of shoots, although to a lesser extent, on BAP enriched medium.

Present studies form an important step for micropropagation of chestnut. Extension of these studies for complete micropopagation will prove highly beneficial for the J & K State where horticulturists face an important problem of propagation by conventional methods.

ACKNOWLEDGEMENT

The authors wish to thank Director, CORD for providing facilities to carry out these investigations.

REFERENCES

Abbot, A.J., 1977. Propagation of woody species in tissue culture. *Scientia Horticulture* **28**: 155.

Biondi, S., Canciani, L.DE. Paoli, G. and Bagnin, N., 1981. Shoot formation from bud cultures of mature chestnut. pp. 181-185. In: *Colloque international Surla culture in vitro Des Essence Forestieries*. A FOCEL, Iufro, Fontain Bleau.

Chauvin, J.E. and Salesses, G. 1988. Advances in chestnut micropropagation *Castanea* species. *Acta horticulturae* **227**: 340-345.

Chevere, A.M., Gill, S.S., Mouras, A. and Salesses, G. 1983. *In vitro* vegetative multiplication of chestnut. *J. of Horticultural Science* **58**: 23-29.

Jindal, K.K and Karkara,B.K. 1991. Chestnuts. pp 498-518. In: *Temperate fruits*. (S.K. Mitra, D.S. Rathore and T.K. Bose, Eds.). Horticulture and Allied Publishers, Calcutta, India.

Jones, O.P. 1979. Propagation in vitro of apple trees and other plants. Methods and applications. *Scientific Horticulture Science*. **54** (2): 155-158.

Mecpheeters, K. Skirvin, R.M. and Bly-Momen, C.A. 1980. Culture of chestnut (*Castanea* Spp.) *in vitro*. *Hortscience* **15**: 147-418

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**: 475-497.

Rodriguez, R. 1982. *In vitro* propagation of *Castanea sativa* Mill. through meristem culture. *Hort. Sci.*, **17**: 6, 880-888.

Sanjose, M.C. Vieitez, A.M. and Vieitez, E. 1984. *In vitro* plantlet regeneration from adventitious buds of chestnut. *Journal of Hort. Sci.* **59**: 359-365.

Strultu, D.G., Grellier, B., Marciniak, D. and Letouze, R. 1986. Micropropagation of chestnut and condition of mycorrhizal Synthesis *in vitro*. *New phytol.* **102**: 95-101.

Vieitez, A. M. and Vietiez, M.L. 1980: Culture of chestnut shoots from buds *in vitro*. *J. Hort. Sci.* **55**: 83-84.

Vieitez, A.M. and Vieitez, E. 1980. Plantlet formation from embryonic tissue of chestnut grown *in vitro*. *Physiol. Plant.* **50**: 127-130.

Vieitez, A.M. Ballester, A. Vieitez, M.L. and Vieitez, E. 1983. *In vitro* plantlet regeneration of mature chestnut. *J. of Hort. Sci.* **58**: 457-463.

Winton, L. 1968. Morphogenesis in clonal propagation of woody plants. pp. 243-264. In: *Frontiers of Plant Tissue Culture* (T.A. Thorpe, Ed.). Calgray.

Zimmermann, R.H. 1978. Tissue culture of fruit trees and other fruit plants. *Proc. Int. Plant Prop. Soc.* **28**: 539-545.

Zimmermann, R.H. 1985. Tissue Culture Propagation of woody plants —large scale production. pp. 165-177. In: *Tissue culture in forestry and agriculture*. (K.W. Henke, M.J. Hughes, Constaintin, and A. Hollander, Eds.). Plenum Press, New York.