Journal of Plantation Crops 20(2): 110–113 *December* 1992

IN VITRO STUDIES ON CACAO (THEOBROMA CACAO L.) —SOMATIC EMBRYOGENESIS FROM COTYLEDON EXPLANTS*

PHILOMINA ABRAHAM,[1] K. V. SAJI and R. D. IYER
Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala, India

ABSTRACT

Attempts to standardize techniques and culture media, for *in vitro* induction and growth of somatic embryoids from cotyledon, leaf and stem segments of cacao, arc described in this paper. With cotyledon explants excised from nearly mature seeds, maximum response in terms of callus induction followed by profuse somatic embryogenesis, was obtained on MS media supplemented with 0.5 mg/l NAA + 0.5 mg/l BAP + coconut water (15%). With regard to leaf nnd stem segments, only a callus type of response could be elicited. The somatic embryoids on separation and culture in liquid media of same composition, using filter paper bridges, developed a distinct tap root with a cap-like covering at the tip, which got sloughed off as the root elongated, on transfer to zeatin (0.5 mg/l) + kinetin (0.1 mg/l) containing media.

INTRODUCTION

Cacao (*Theobroma cacao* L.), a member of the family Sterculiaceae is one of the most important beverage and food crops in the tropics. The seeds or beans of *Theobroma cacao* provide the raw-material for the manufacture of cocoa, chocolate and cocoa butter.

Cacao has a dimorphic growth habit with two kinds of branches — the orthotropic erect shoots and the plagiotropic horizontal branches. Problems are encountered in the vegetative propagation of cacao by conventional method because of the dimorphic branching system and lack of sufficient number of erect shoots for getting scion material for grafting, since these shoots are more suitable.

Hence, tissue culture offers great scope not only for clonal propagation but also for the genetic improvement of cacao by production of isogenic plants through androgenic haploids and their use in hybridization.

Only a limited number of tissue culture studies have been made in *Theobroma cacao*. In general, all attempts to initiate a callus in cacao tissues have been successful but attempts to regenerate plantlets from this callus have met with only limited success.

It was Archibald (1954) who first obtained a callus from stem explants on culture media of White's formula without adding any growth regulators, and increasing growth by addition of coconut water. For callus induction. Hall and Collins (1975) and later Jalal and Collins (1979) used Murashige and Skoog's (1962) medium with the organic supplements used at double' the strength. Pence et al. (1980) used a modified MS medium for the induction and proliferation of accessory embryoids on immature zygotic embryos, but did not get germination

* *M. Phil dissertation submitted to Mangalore University.*
* *Contribution No. 825, Central Plantation Crops Research Institute, Kasaragod.*
1 *Lecturer in Botany, Nirmalagiri College, Cannanore, Kerala.*

from these embryoids. Lee and Rao (1982) observed embryoids being formed in clumps of callus derived from seedling tissues and immature seed embryos cultured on a liquid medium. The present study was undertaken to standardize a suitable tissue culture method for rapid vegetative propagation through somatic embryogenesis.

MATERIALS AND METHODS

Three kinds of explants were used for the present studies conducted at CPCRI, Kasaragod. Explants were taken from the mature trees of Forastero variety available at the campus. Tender leaves, tender orthotropic shoots and cotyledonary segments from the zygotic embryos of mature (140 day old) fruits were cultured.

Tender shoots of cacao were collected from the field in a polythene bag and cut into pieces of 3–5 cm and kept in a beaker covered by double layered cheese cloth under running tap water for an hour. It was found necessary to wash off the mucilage occurring in cacao tissues; after this, stem and leaf segments were sterilized separately with 0.01% Mercuric chloride and Bavistin for 10 minutes and 5 minutes, respectively. They were then rinsed with sterile double glass-distilled water 4–5 times to remove even the last traces of Hg_2Cl_2. After trimming, they were carefully inoculated onto culture media in tubes.

Cotyledons were dissected out from 140-day old seeds after sterilizing them by dipping in 95% Ethyl alcohol and flaming it. Aseptic cotyledons were dissected out by removing the seed coat inside the laminar flow chamber, and were cultured on Murashige and Skoog's (1962) media supplemented with 15% coconut water and different levels of NAA and BAP.

The media were prepared by dissolving the chemicals in double distilled water, 15% coconut water was added and made upto the final volume, and pH adjusted to 5.8 using N/10 NaOH before adding agar. After adding 0.6% agar to the medium it was dissolved in a domestic pressure cooker for 5 minutes. Molten medium was dispensed into 25 x 150 mm Borosil culture vials (15–20ml/tube) and plugged with non-absorbent cotton or aluminium. foil and autoclaved for 20 minutes at 17 pounds sq. $inch^{-1}$ at 121°C.

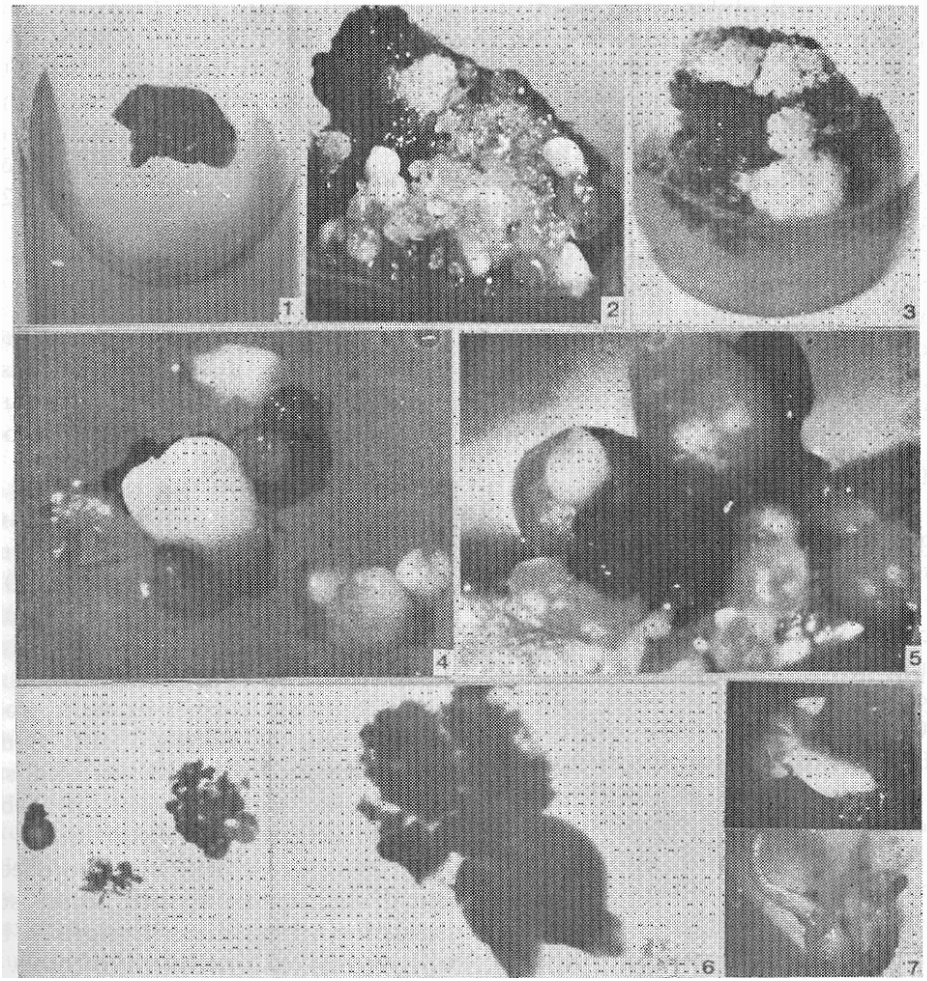
RESULTS AND DISCUSSION

Cotyledonary explants were the most responsive among the three different explants tried (Fig. 1). All the three explants produced very good amount of callus but only the callus from the cotyledons produced somatic embryos (Fig. 2).

The initiation of callus started within 10-15 days from the vascular region and attained profuse dimension in 40–50 days (Fig. 3). When the callus was subcultured in a medium with low auxin and cytokinin levels (NAA 0.5 mg/l; BAP 0.5 mg/l) although somatic embryos were produced (Figs. 4 and 5) they did not germinate to become a plantlet. They as usual started callusing on semi-solid medium (Table I). When the embryos of different stages (Fig. 6) were separated and transferred to a liquid MS medium with same supplementation, each of them developed a brown covering on its base like a root-cap. The liquid MS medium was then modified with the addition of Zeatin (0.5 mg/l) and Kinetin (0.1 mg/l). It was observed that the tap root developed within a week by rupturing the brown root-cap like covering (Fig. 7).

The small leaf bits enlarged to double their initial size within 3-4 days of inoculation.

Fig. 1. Cotyledon explant from 140 days old seed, after 7 days in *vitro*, showing only size increase a MS medium containing 0.5 mg/l NAA + 0.5 mg/l BAP + CW 15%. Fig. 2. Callus development observed from the cut ends of above within 5-8 weeks, followed by somatic embryogenesis on same medium. Fig. 3. Profuse growth of callus from cotyledon explant a subculture in same medium. Fig. 4. Separated somatic embryoids on a m medium. Fig. 5. Profuse somatic embryoid development on subculture in same medium. Fig. 6. Different developmental stages of somatic embryoids - the one on far right showing distinct dicot structure. Fig. 7. Somatic embryo developing a distinct tap root (bottom). Note the darkish root-cap sloughing off (top).



Initiation of callus occurred within a week from the vein region. Profuse callus development was observed on MS supplemented with' NAA (5.0 mg/l) and BAP (1.0 mg/l). Friable callus became compact when it was subcultured on NAA (1.0 mg/l) and BAP (1.0 mg/l). No somatic embryogenesis was evident in the leaf callus.

Tender stem explants also started callusing within 7–10 days after inoculation. The best responding combination was MS supplemented with NAA 1.0 mg/l and BAP (0.1 mg/l). No differentiation was observed in the stem culture.

Table I. *Response of cacao cotyledon segments* in vitro

Sl.No.	Medium + supplements (mg/l)	Type and degree of response
1.	MS + NAA 0.5 + BAP 0.1	C^{+}
2.	MS + NAA 0.5 + BAP 0.2	$C^{++}E^{+}$
3.	MS + NAA 0.5 + BAP 0.5	$C^{++++}E^{+++}$
4.	MS + NAA 0.5 + BAP 1.0	C^{++++}
5.	MS + NAA 0.5 + BAP 0.2 + 2.4-D 1.0	$C^{+++}R^{++}$
6.	MS + NAA 0.5 + BAP 6.0	$C^{+++}L^{+++}$

Index

C^{+} Callusing
C^{++} Good friable callus
C^{++++} Profuse growth of friable and compact callus
E^{+} 2–10 embryoid formation
E^{+++} 10–50 embryoid formation
R^{++} Root formation
L^{+++} Thalloid leaf-like green organ development

With cacao leaf and stem culture, no distinct embryogenesis has been reported until recently, although Litz (1986) did report a limited extent of embryogenic callus from Amelonado cacao leaf tissue culture on MS medium containing 140 mg/l BA and 10 mg/l 2,4-D. This may be because a medium with the right proportion of hormonal supplements has yet to be evolved. Attempts are in progress to induce further developments in these embryogenic tissues of cacao.

ACKNOWLEDGEMENTS

We are grateful to Dr. B. C. Viraktamath for his help in collection of explants and for useful discussions. The first author is grateful to the University Grants Commission for the award of a Research Fellowship to conduct this work towards the M. Phil. degree of Mangalore University.

REFERENCES

ARCHIBALD, J. F. 1954. Culture *in vitro* of cambial tissue of cacao. *Nature* (Lond.) 173: 351–352.

HALL, T. H. R. and COLLINS. H. A. 1975. Initiation and growth of tissue cultures of *Theobroma cacao*. *Ann. Bor.* **39** : 555–570.

JALAL, M. A. F. and COLLINS, H. A. 1979. Secondary metabolism in tissue culture of *Theobroma cacao*. *New Phytol.* **83** : 343–349.

LEE, S. K. and RAO, A. N. 1982. Callus growth and organogenesis in cacao. In A. N. Rao (ed.) *Proc. Symp. on Tissue culture of Economically Important Crops*, Natl. Univ., Singapore. pp. 107–112.

LITZ, R. E. 1986. Tissue culture studies with *Theobroma cacao*. In P. S. Dimick (ed), *Proc. Cacao Biotechnology Symposium*. PP. 111–120. Dept. of Food Science. The Pennsylvania State University.

MURASHIGE. T. and SKOOG. F. 1962. A revised medium for rapid growth and bioarsays with tobacco tissue cultures. *Physiol. Plant.* 15 : 473–497.

PENCE, V. C., HASEGAWA. P. M. and JANICK, J. 1980. Initiation and development of sexual embryos of *Theobroma cacao* L. *in vitro*. *Pflanzenphysiol.* 98: 1–14.