S. L. J. Tea Sci. **57** (*1*), *20–23, 1988, Printed in Sri Lanka.*

STUDIES ON THE TISSUE CULTURE OF TEA (*CAMELLIA SINENSIS* (L.) O. KUNTZE)
3. Regeneration of plants from cotyledon callus culture

P. V. Arulpragasam, Razia Latiff and Poorna Seneviratne
(Tea Research Institute of Sri Lanka, Talawakele, Sri Lanka)

Studies were undertaken to investigate conditions that would be suitable for high frequency of somatic embryogenesis and plant regeneration in tea using callus cultures derived from cotyledon tissues.

Pieces of cotyledons were first established and made to grow in agitated liquid media and then transferred to solid callusing media, where they showed good growth and callus formation. These cultures when grown under higher light intensity developed embryoid-like structures, which later turned green. In sucrose enriched media the frequency of embryogenesis increased appreciably. However, further development was not observed until they were transferred to the regeneration media, which contained higher levels of cytokinin and low concentrations of auxins. After 8 - 10 weeks in the regeneration media the embryos developed into plantlets with distinct shoot and root. In some instances only root formation was observed. The plantlets have been successfully acclimatized and planted in soil.

INTRODUCTION

Although a number of papers have been published on the regeneration of plants from callus in woody plants, there are only a few reports of successful attempts on tea (*Camellia sinensis* (*L.*) O. Kuntze), and still less on the regeneration of plants from cotyledon callus of tea. Wu (1976) was successful in obtaining few plants from cotyledon callus and Wu *et al.* (1981) succeeded in obtaining new clones using cotyledon callus. Kato (1986) obtained embryoids directly from cotyledons of *C. sinensis* and *C. japonica*. It was only after 1½ years and several sub-cultures later that Wu (1976) was able to obtain the few plants. The number of plants obtained by Wu *et al.* (1981) was necessarily limited. It is now known that the successful establishment and growth of callus and plant regeneration depends on the culture conditions employed and not on the source of the explant (Nabors *et al.*, 1982; Heyser *et al.*, 1983).

The present study was therefore undertaken to determine the conditions required for high frequency regeneration of plants from cotyledon callus. Cotyledon tissue was selected as the explant because it would be free of contamination and it has been found to lend itself to long-term culture, a feature which could be used to advantage in subjecting the callus to prolonged stress conditions in attempts to produce stress tolerant plants.

MATERIALS AND METHODS

Pieces of cotyledon were taken from mature seeds obtained from the bi-clonal tea seed garden. The seeds were sterilized by dipping them in 70% alcohol and flaming before cracking the shell. The cotyledon pieces were transferred to a liquid medium (MS 4, Table 1) in 250 ml conical flasks. These flasks were placed on an orbital shaker and agitated continuously. Once callus formation was induced the cotyledon pieces were transferred to a solid medium (MS 5, Table 1) and incubated in the dark for further callus formation. When sufficient amount of growth has taken place they were dissected out and cultured again for further multiplication. The callus tissues were then transferred to the regeneration medium (MS 6, Table 2) and incubated in the light at 2000 Lux intensity at 26 - 28°C at 16 h photoperiod. The callus was subcultured at monthly intervals for a period of 8 - 12 weeks when embryoid like structures were formed. The embryoids were transferred to another medium (MS 7, Table 2) for further development and growth.

When the embryoids developed into plantlets with about 4 - 5 leaves they were planted into small plastic pots containing a mixture of sterile soil and sand and were placed in a humid chamber under 100% humidity for two weeks. They were gradually exposed to higher light intensity and low humidity and acclimatized to outside conditions.

TABLE 1 — *Composition of the media used for the induction and multiplication of callus obtained from the cotyledon of tea (mg/l)*

Component	MS 4	MS 5
Major elements	MS *	MS *
Minor elements	MS *	MS *
Thiamine-HCl	0.4	1.0
myo-Inositol	100	100
Pyridoxine-HCl	—	5.0
Glycine	—	20
Nicotinic acid	—	5.0
IAA	—	2.0
IBA	0.01	—
2,4-D	—	2.0
Kinetin	—	2.0
BAP	1.0	—
GAs	0.1	—
Yeast extract	—	2000
Sucrose	30,000	30,000
Phytagar	—	8,000
pH	5.6 - 5.8	5.6 - 5.8

*Murashige and Skoog (1962) Mineral salts

TABLE 2—*Composition of media used for the formation of embryoids and their germination (mg/l)*

Components	*MS 6*	*MS 7*
Major elements	MS *	MS *
Minor elements	MS *	MS *
Thiamine-HCl	0.1	0.1
Myo-Inositol	100	100
Pyridoxine-HCl	0.5	0.5
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
BAP	10	—
Yeast extract	2000	—
Sucrose	30,000	20,000
Phytagar	8,000	8,000
pH	5.6 - 5.8	5.6 - 5.8

* Murashige and Skoog (1962) Mineral Salts

RESULTS

The explants used in this study were cotyledons from mature seeds and were initiated in a liquid medium. Increase in size of the explant was observed within one week of culture though no callus formation was observed during this period. Callus growth was observed after 2 - 3 weeks and when transferred to the callusing medium containing 2, 4-D showed good growth within 6 - 8 weeks when incubated in the dark (Fig. 1). Different callus types were recognisable in the growth. Most of the calli were yellow or tan coloured while some were white and transparent (Fig. 2). They were mostly loosely packed and friable. When transferred to fresh callusing medium and incubated in the light, most of the calli turned from friable to the firm type in 8 - 10 weeks (Fig. 3). Embryoid like structures began to develop when transferred to a medium (MS 6) containing BAP as the only growth regulator. Of the different concentrations of BAP tested, 8 - 10 mg/l gave the best results. Some of the embryoids turned green and some developed roots. Some of these embryoids when grown in a medium enriched with sucrose (60 g/l) turned brown. The embryoids (Fig. 4) developed shoots and roots within one month of transfering to a medium (MS 7) with less sucrose (20 g/l) and containing no growth regulators (Fig. 5). After one month in the regeneration medium these plants (Fig. 6) were successfully acclimatized to field conditions.

S. L. J. Tea Sci. 57 (1), 20–23, 1988. Printed in Sri Lanka.

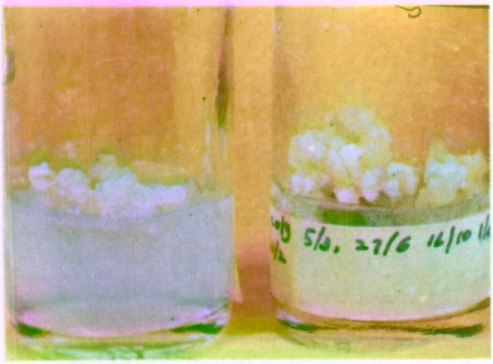

Fig. 1 — Development of callus in cotyledon pieces

Fig. 4 — An embryoid developed from callus

Fig. 2 — Different callus types in the growth

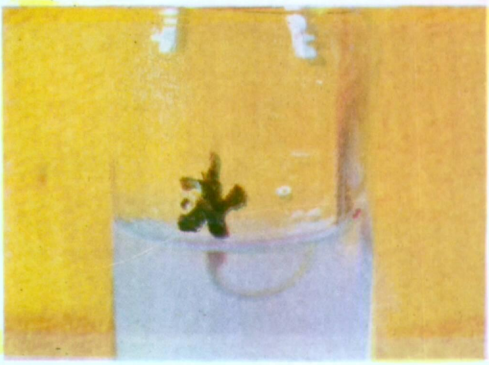

Fig. 5— Growth of The Embryoid in Culture

Fig. 3 — Development of firm type of callus with embryoid formation

Fig. 6 — A regenerated plantlet

P. V. Arulpragasam, Razia Latiff and Poorna Seneviratne

DISCUSSION

A method for the culture of callus derived from cotyledons and the method for regenerating plants from these have been successfully worked out. Plant cell and tissue cultures are ideal systems for selecting variants. In this study cotyledon tissues were used as explants and it has been found that it is free of contamination, a major problem in establishing explants in culture, and it can be multiplied rapidly and plants could be regenerated from these without much difficulty. In our study it has been found that cotyledon callus lends itself to long term culture. This characteristic could be used for selecting plants for stress tolerance. The length of selection is an important criterion, since selection agents that are used allow for the preferential growth of variants. Several generation times are required for the few variant or mutant cells to become dominant cell types in the population, before attempts at regeneration are made. Thus cotyledon callus culture offers a useful system for screening and selecting plants for stress tolerance.

The frequency of regeneration of plants from callus cultures depends very much on culture conditions employed. It has been reported that the frequency of regeneration of plants would be much higher if one is able to differentiate between the 'embryogenic' and the 'non-embryogenic' callus and culture them seperately (Nabors *et al.*, 1982). Histological studies are being carried out at the moment to differentiate between these two types of callus so that we could achieve a high frequency of somatic embryogenesis.

ACKNOWLEDGEMENT

We are grateful to Dr P. Sivapalan, Director, TRI for his keen interest in this work and his constant encouragement and to our colleagues for their assistance.

REFERENCES

KATO, M. (1986). Results of organ culture on *Camellia japonica* and *C. sinensis*. *Japan J. Breed.* **36**, 31-33.

MURASHIGHE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**, 473-497.

NABORS, M.W., KROSKEY, C.S. and MCHUGH, D.M. (1982). Green spots are predictors of high callus growth rates and shoot formation in normal and salt stressed tissue cultures of oats (*Avena sativa* L.). *Z. Pflanzenp.* **105**, 341-349.

WU, C.T. (1976). Studies on the tissue culture of tea plant. *Bull. Taiwan Tea Exp. Stn.* **72**, 30-42.

WU, C.T., HUANG, T.K., CHEN, G. and CHEN, S.Y. (1981). In *Proc.* COSTED *Symp. on Tissue Culture of Economically Important Plants*, Singapore, ed. A.N. Rao. 104-106.

HEYSER, J.W., DYKES, T.A., DEMOTT, K.J. and NABORS, M.W. (1983). High frequency, long term regeneration of rice from tissue culture. *Plant Sci. Lett.* **29**, 175-182.