Short-term Storage of Encapsulated Zygotic Embryonic Axes of Tea (*Camellia sinensis* (L.) Kuntze) at Low Temperature

T.H. Seran, K. Hirimburegama[1] and M.T.K. Gunasekare[2]

Department of Agronomy, Faculty of Agriculture
Eastern University of Sri Lanka
Chenkalady, Sri Lanka

ABSTRACT. *Studies on cold storage of encapsulated embryonic axes of tea (Camellia sinensis (L.) Kuntze) were carried out to obtain efficient plant recovery. Both encapsulated zygotic embryonic axes (formed in 3% sodium alginate and 100 mM calcium chloride) and natural seeds were stored at 4^0C for 0, 4, 8, 16 and 20 weeks. At the end of each period, the embryonic axes excised from stored natural seeds (control) and encapsulated embryonic axes were cultured on Murashige and Skoog (MS) basal medium supplemented with 3 mg/l Benzyl Aminopurine (BAP) and 0.5 mg/l Indole Butyric Acid (IBA). The results showed efficient germination and plant recovery from stored encapsulated embryonic axes compared to natural seeds. High rates of germination (95%) and plant recovery (58.3%) were achieved from encapsulated embryonic axes after four weeks of storage. Meanwhile, low percentage germination (16.7%) was observed from the embryonic axes isolated from natural seeds stored for the same period as compared to the non-stored embryonic axes (90%). Further, it revealed that there was no significant reduction in germination and plant recovery until 8 weeks of storage of encapsulated embryonic axes. Encapsulated embryonic axes are important in extending the seed viability and for efficient germination than natural seeds of tea.*

INTRODUCTION

Natural seeds of desirable genetic materials of tea (*Camellia sinensis* (L.) Kuntze) are stored at low temperatures (4-5^{0}C) until being used by researchers and breeders. Because of recalcitrant nature, tea seeds are unable to retain their viability during long-term storage (Kato, 1989). Salinero and Silva-Pando (1986) reported that seed viability of tea is relatively short-lived even under moist conditions at 3-5^{0}C. It is clearly implied by Kuranuki and Yoshida (1996) that cotyledons are highly susceptible to desiccation whereas excised embryonic axes were highly tolerant. Therefore, embryonic axes excised from tea seeds play an important role in short-long term storage at low temperature. Studies have been carried out on cryopreservation of embryonic axes of *C. sinenesis* (Chandel *et al*., 1995; Chaudhury *et al*., 1991; Kuranuki and Yoshida, 1996; Wesley-Smith *et al*., 1992). However, there is no evidence on the cold storage of this type of conventional plant material of *Camellia* spp. at low temperature *i.e*. 4-5^{0}C.

[1] Department of Plant Sciences, University of Colombo, Colombo, Sri Lanka.

[2] Division of Plant Breeding, Tea Research Institute, Talawakelle, Sri Lanka.

Encapsulation technique is more advantageous for maintenance of moisture content around the plant material (Friend, 1993; Janeiro *et al*., 1997; Mondal *et al*., 2000). Sebastiampillai and Anandappa (1979) reported that high initial moisture content of tea seeds is an important factor that ensures high germination. Janeiro *et al*. (1997) mentioned that survival and germination percentages of both encapsulated and non-encapsulated somatic embryos stored at 4^0C were significantly low but the reduction was much greater for non-encapsulated embryos. Thus, encapsulation technique would be useful to enhance germination and recovery of plants and also for safe germplasm exchange and storage. However, Mondal *et al*. (2000) recently reported that although there are a few reports on the production and storage of synthetic seeds of *C. reticulata* and *C. japonica*, there is no report on synthetic seeds (formed in commercially used gel alginate) of tea. Somatic embryos and shoot tips were used as embedded materials in those studies. Although, in the past a number of studies have been conducted on the storage of plant material of *C. sinensis*, most of them reported their survival and germination but not plant recovery. Therefore, the present work was aimed at studying the feasibility of cold storage of encapsulated zygotic embryonic axes and to evaluate their *in vitro* germination and conversion to plants.

MATERIALS AND METHODS

Plant materials

Mature fruits of tea cultivar TRI 2043 were harvested from the seed bearing plants at the Tea Research Institute (TRI), St. Coombs estate, Sri Lanka and seeds were separated from the collected fruits. After removal of the cracked or damaged seeds, uniform-sized seed stock was tested for viability by the sinker-floater method (Barua, 1989) to discard floaters. Half of the 'sinker' seed stock was used as a control in this study. Seed coats were removed from the other half of the 'sinker' seed stock and zygotic embryonic axes were carefully isolated from these decoated seeds; subsequently stored in distilled water until being surface sterilized. Thereafter, the embryonic axes were washed thoroughly in sterilized distilled water and surface sterilized in 2% Clorox (sodium hypochloride - active ingredient 5.25%) with 2-3 drops of Tween 20 for 10 min. They were then rinsed three times with sterilized distilled water under aseptic conditions.

Encapsulation of zygotic embryonic axes

Sodium alginate (3%) was prepared in MS (Murashige and Skoog, 1962) basal medium containing 3% sucrose, 3 mg/l Benzyl Aminopurine (BAP) and 0.5 mg/l Indole Butyric Acid (IBA) (germination medium devoid of agar). Solution of sodium alginate (3%) in combination with calcium chloride dihydrate (100 mM) was separately autoclaved at 121^0C for 20 min. The sterilized embryonic axes were immersed well in the sterilized sodium alginate solution using forceps. They were then picked up and dropped individually into a sterilized calcium chloride solution using a pre-sterilized Pasteur pipette (3 mm internal diameter) and kept for 20 min with occasional agitation to form gel beads. Size of the resulting capsules was 4 to 5 mm. These beads were rinsed three times with sterilized distilled water and placed in sterilized petri dishes (90 mm × 15 mm), which were kept at an angle (10-20^o) to remove the excess water.

Storage of encapsulated embryonic axes and natural seeds

Encapsulated embryonic axes (Plate 1) were carefully cultured in each culture bottle (125 ml capacity) containing 10 ml of distilled water, solidified with 0.8% (w/v) agar (pH 5.8 prior to autoclaving). Each culture bottle with five encapsulated embryonic axes was then sealed with parafilm. The method to prepare storage substrate used in this study was the same as Maruyama *et al*. (1998) where 1% (w/v) agar was used to solidify the substrate. 'Sinker' natural seeds (control) were enclosed in clean sealed polythene bags. These bags with natural seeds and the culture bottles with encapsulated zygotic embryonic axes were stored for 0, 4, 8, 16 and 20 weeks at 4-5^{0}C in a refrigerator. Each treatment had 90 samples in an experiment, which was repeated once.

Plate 1. Encapsulated zygotic embryonic axes.

***In vitro* germination**

Zygotic embryonic axes were carefully isolated from stored seeds and sterilized as described earlier. Encapsulated zygotic embryonic axes and sterilized naked embryonic axes stored for different periods were cultured on the germination medium (0.8% agar). The culture bottles (125 ml capacity), each containing 35 ml of the germination medium and five explants, were then incubated at 22±2^{0}C under white fluorescent light (16 h of photoperiod at 25 μmol/m^2/s radiation). Orientation of the germinating plantlets was corrected at fourth week of culture. Lack of necrosis or absence of contamination was taken as criteria for survival. Germination response of plant materials following cold storage at 4-5^{0}C was recorded at 8th week of culture and growth of plantlets (having no shoot clusters) was also recorded.

Statistical analysis

Data were analysed using the SAS software. All data were first subjected to the Shapiro-Wilk test (p=0.05) for normality before subjecting them to analysis of variance (ANOVA). Arcsine and log transformation techniques were used for values expressed as percentages and for length of root, respectively. The significant differences between the means were estimated at the 5% level using Duncan's Multiple Range Test (DMRT).

RESULTS

Viability and *in vitro* germination of stored seeds

Zygotic embryonic axes coated in 3% gel alginate stored at different time intervals turned yellow and exhibited 100% survival at the end of each storage period. Meanwhile, development of fungi was observed visually in most natural seeds stored for 8, 16 and 20 weeks. Therefore, these seeds were not used for further work. The survival and germination rates of embryonic axes (the control) and encapsulated embryonic axes cultured on the germination medium after each storage periods are shown in Table 1. The highest survival (95.0%) was obtained in cultured encapsulated embryonic axes stored for 4 weeks, but lower (16.7%) in naked embryonic axes (control). On the other hand, 75% of cultured naked embryonic axes, stored for 4 weeks showed bacterial infestation during the first two weeks of culture whereas this phenomenon was noted during the fourth week only in 1.7% of encapsulated embryonic axes stored for the same period. Even though these infested embryonic axes germinated, they were considered as non-surviving tissues.

Table 1. The frequencies of survival and germination (at 8th week) of naked zygotic embryonic axes and encapsulated zygotic embryonic axes cultured on culture medium following cold storage at different time intervals.

Storage period (weeks)	Survival (%)		Germination (%)	
	Control	Encapsulated embryonic axes	Control	Encapsulated embryonic axes
0	98.3	93.3	90.0	86.7
4	16.7	95.0	16.7	95.0
8	-	73.3	-	70.0
16	-	23.3	-	23.3
20	-	8.3	-	8.3

Note: Percentages were calculated from 180 cultured naked embryonic axes or encapsulated embryonic axes. Naked embryonic axes isolated from the stored natural seeds were used as a control at each period, where the seeds stored for 8, 16 and 20 weeks were not used for these experiments due to the development of fungi (indicated by dash).

In vitro plant conversion

Efficient plant conversions from cultured encapsulated zygotic embryonic axes stored for 0, 4 and 8 weeks and also non-stored naked embryonic axes cultured on the germination medium were achieved (Table 2). There were highly significant (P<0.001) differences on plant conversion among those tested treatments. The vigorous normal plantlets, having single shoot, were obtained from cultured encapsulated embryonic axes stored for 0 and 4 weeks (Plates 2A, B). Growth of plantlets (having no shoot clusters) obtained from both cultured encapsulated embryonic axes and naked embryonic axes (control) cultured on germination medium at 8th week are given in Table 3. Although, a high rate (56.7%) of plant recovery was achieved from encapsulated embryonic axes following 8 weeks of storage, 1/3 of *in vitro* plantlets had shoot clusters.

Table 2. The percentages of plant conversion or shoot development (at 8th week) from encapsulated and non-encapsulated zygotic embryonic axes cultured under *in vitro* conditions following cold storage at different periods.

Storage period (weeks)	Plant conversion (%)		Shoot development only (%)	
	Control	Encapsulated embryonic axes	Control	Encapsulated embryonic axes
0	68.3[a]	48.3[a]	21.7[ab]	38.3[a]
4	13.3[b]	58.3[a]	3.3[cd]	35.0[a]
8	-	56.7[a]	-	10.0[bc]
16	-	13.3[b]	-	6.7[cd]
20	-	6.7[b]	-	1.7[d]

Note: Values represent the means of two independent experiments, each with 90 samples per treatment. Natural seeds (control) stored for 8, 16 and 20 weeks were not used for the experiments due to the development of fungi (indicated by dash). Means followed by the same letter in each column (plant conversion or shoot development only) are not significantly different at 5% level by DMRT test.

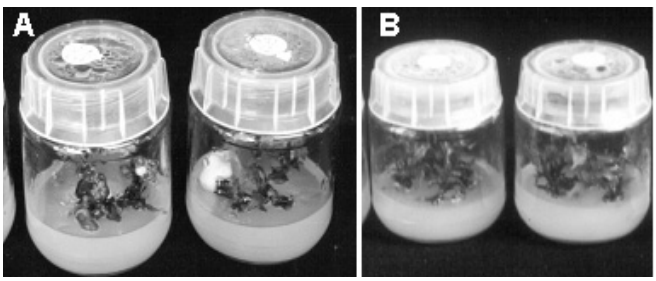

Plate 2. *In vitro* germination response of non-stored (A) and 4 weeks-stored (B) encapsulated embryonic axes.

Table 3. Growth of plantlets (at 8th week) from cultured non-stored naked zygotic embryonic axes (control) and encapsulated zygotic embryonic axes on germination medium following storage at different periods.

Treatment Explant	Storage (weeks)	Shoot length (cm)	Root length (cm)	Number of leaves
Naked axes	0	3.12±0.36[a]	2.08±0.29	4.90±0.57
Encapsulated axes	0	3.03±0.42[a]	2.02±0.18	4.40±0.34
Encapsulated axes	4	3.04±0.29[a]	1.59±0.18	5.60±0.31
Encapsulated axes	8	1.58±0.13[b]	1.72±0.14	4.30±0.21
*Encapsulated axes	16	1.09±0.06	1.27±0.09	3.00±0.21
*Encapsulated axes	20	0.84±0.07	1.08±0.09	2.27±0.29
F test		**	ns	-

Note: Values are means ± standard errors of two independent experiments, each with 30 plantlets. Naked axes - naked zygotic embryonic axes; Encapsulated axes - encapsulated zygotic embryonic axes.
Means followed by the same letter in each column are not significantly different ($P<0.05$) by DMRT test.
F test: ** $P = 0.01$; ns - not significant.
* Statistical analysis was not used because of the very low rate (6-13%) of plant conversion (Table 2) and also plantlets have mostly shoot clusters.

DISCUSSION

During storage, visible fungi were observed in most natural seeds stored for 8 weeks or more. Sebastiampillai and Anandappa (1979) reported visible signs of bacterial and fungal infections from about the second week after storage of seeds, sealed in the polythene bags at 22-27^{0}C. They further suggested that high relative humidity and high storage temperature promote the activity of microorganisms resulting in a rapid deterioration of the seeds. It is therefore evident that the microbial infestation in the stored tea seeds and their activity might be slowed down during the storage of plant material at low temperature (4-5^{0}C).

In general, there is an association between seed viability and storage. Intact tea seeds are so sensitive to desiccation (Kuranuki and Yoshida, 1996). Thus, moisture loss from seeds during storage may be attributed to formation of airspace between the cotyledons and the shell (seed coat) as well as between cotyledons. It suggests that microbes may have penetrated through these pathways and infested the zygotic embryonic axes. Therefore, they exhibited poor rate of survival than encapsulated embryonic axes cultured in the germination medium. This finding clearly showed that stored whole seeds are more susceptible to microbial infestation in embryonic axes. In contrast, the successful storage of tea seeds in the sealed polythene bags at 5-7^{0}C for a period of more than three months was achieved, where the stored materials were germinated in sand bed (Sebastiampillai and Anandappa, 1979). In these instances, microbial infestation may not be seen visually as seeds were sown in the sand bed.

The highest frequencies of survival (95%) and germination (95%) of encapsulated zygotic embryonic axes following storage for four weeks were higher than in non-stored encapsulated embryonic axes. Gunasekare (1998) reported that *in vitro* germination rates of encapsulated zygotic embryos coated with agar (5% w/v) and stored in sterilized dry petridishes at 25 and 10^{0}C for 30 days were 46.4 and 43.0%, respectively. The present results are in agreement with other research reports. In those papers, no loss of germination capacity was reported for stored encapsulated embryos of *C. sinensis* (Gunasekare, 1998), *C. japonica* (Janeiro *et al*., 1995) and sandalwood (Rao and Bapat, 1993). Critical moisture content in tea seeds is generally required to obtain higher rates of germination and recovery of plants. Certain amount of desiccation is therefore needed in fresh seeds for efficient germination. Janeiro *et al*. (1996) observed that survival rates increased when both cryopreserved and uncryopreserved embryonic axes of *C. japonica* were desiccated by a current of sterile air in a laminar flow cabinet (26±1^{0}C) for 1.5 or 3.0 h than the control. Similar findings have been obtained with embryonic axes of tea (Chandel *et al*., 1995; Chaudhury *et al*., 1991; Kuranuki and Yoshida, 1996; Wesley- Smith *et al*., 1992).

Although all stored encapsulated embryonic axes were alive at the end of each storage period lasting for 20 weeks, they failed to survive in the germination medium as exhibited in storage substrate. Ballester *et al*. (1997) reported that for shoot tips of *C. japonica* encapsulated in alginate beads and stored at 2-4^{0}C for 30, 60 and 75 days, the survival rates were 75, 50 and 10%, respectively. In the present study, low frequencies of survival were recorded in the materials stored for 16 and 20 weeks. This may be due to dehydration of *in vitro* encapsulated embryonic axes causing certain amount of shrinkage in the cells during the storage. Thus, some changes in plasma membranes due to osmotic effect result in progressively losing their viability after culturing these materials in the germination medium. Loss of viability in stored tea seeds has been reported (Kato, 1989;

Salinero and Sebastiampillai and Anandappa, 1979; Silva-Pando, 1986). In the present work, *in vitro* encapsulated embryonic axes were continuously stored in the storage substrate for 20 weeks without transfer to fresh substrate. The results indicated that encapsulated embryos could remain viable on the same storage substrate for 8 weeks. Further, the rate of survival was reduced in the cultured encapsulated embryonic axes on the germination medium when stored for 8 weeks than for 4 weeks. This indicates the necessity for transferring of these materials to fresh substrate to maintain moisture content in encapsulated embryonic axes for successful storage without loosing viability.

The germination percentage of encapsulated embryonic axes (70%) stored for 8 weeks was comparatively lower than in 4 weeks non-stored and stored gel capsules (86.7-95%). However, recovery rate of plants was not statistically different. Further, it was noted that rate of plant conversion from non-stored naked embryonic axes was not significantly different from non-stored encapsulated embryonic axes. This result is supported by the report of Janeiro *et al*. (1997) where *Camellia* encapsulation caused no reduction in *in vitro* complete germination as long as the capsule matrix included nutrients and growth regulators.

In the present studies on *in vitro* storage of encapsulated embryonic axes, 68.3% (Table 2) conversion of plants and 1.7% callus formation were achieved in non-stored naked embryonic axes. In contrast, Janeiro *et al*. (1996) indicated 8.3% plant conversion and 45.9% callus formation after 4 weeks of inoculation of non-cryopreserved embryonic axes of *C. japonica* where embryonic axes were cultured in MS basal medium supplemented with 4.44 µM BA, 0.49 µM IBA, 3% sucrose and 0.7% agar. The differences in conversion of plant species within the same genera (*Camellia*) may be due to the different concentrations of growth regulators added to the germination medium. Further, a considerable amount of incomplete germination (in the form of shoot or root only) was observed in all tested materials. Percentage of shoot development only (incomplete germination) was significantly higher (38.3-35%) during the earlier storage period. But it was significantly reduced (10%) in the encapsulated embryonic axes when they were stored for 8 weeks, where gradual decreases in the frequencies of shoot formations (incomplete germination) was noticed thereafter (Table 2). Thus, gel capsules responded differently according to the storage periods. It may be due to desiccation of the gel beads as mentioned earlier. Tendency of shoot cluster formations increased when storage period is increased. This may suggest that reduction of moisture content below the critical level induced dormancy of terminal bud resulting in the production of shoot clusters. This occurrence was observed in encapsulated embryonic axes following 8 weeks of storage where the mean shoot length of plantlets obtained from these *in vitro* encapsulated embryonic axes were significantly lower than in encapsulated embryonic axes stored for 0 and 4 weeks and non-stored naked embryonic axes. However, root lengths were not significantly affected by cold storage among those tested treatments.

CONCLUSIONS

As seeds of *C. sinensis* do not tolerate desiccation, encapsulated embryonic axes play an important role in cold storage than natural seeds. Coating materials in gel beads act as artificial endosperm in embedded genetic materials in order to reduce the risk of contamination for safe storage and transportation, and also to retain their viability to obtain

efficient germination and recovery of plants. Further, it suggests that storage of whole seeds at 4-5^{0}C is not advisable although it may be possible for very short periods if they had been surface sterilized properly before and after storage to reduce the risk of contamination.

The protocol developed in this study would be useful for successful cold storage of hybrid seed material or even somatic embryos at 4-5^{0}C for clonal propagation. Viable seeds are the prime consideration for successful storage and subsequent germination. Future studies are needed to determine the effect of subculture intervals, which are required to improve the efficiency of this protocol for long-term storage of encapsulated embryonic axes.

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