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# Induction of *in vivo* somatic embryos from tea (*Camellia sinensis*) cotyledons

**Tapan Kumar Mondal[†,*], Amita Bhattacharya[#], Madhu Sharma[#] and Paramvir Singh Ahuja[#]**

[#]Biotechnology Department, Institute of Himalayan Bioresource Technology, Palampur 176 061, India
[†]Biotechnology Laboratory, Research and Development Department, Tata Tea Ltd, Munnar 685 612, India

**Somatic embryos were obtained *in vivo* from tea seed cotyledons of three important Indian tea cultivars on moist sterile sand. *In vivo* embryogenesis was influenced by the incubation temperature, time of seed collection and genotypes. Out of the three cultivars, UPASI-9 was the most responsive (23.3 ± 0.59) during September, followed by T-78 (16.5 ± 0.84) during October and Kangra Jat (11.7 ± 0.69) during November. However, among the three different temperature regimes (24, 28 and 32°C), embryogenesis was noticed only at 28°C for all the cultivars. Histological evidence confirms somatic embryogenesis.**

TEA (*Camellia sinensis*) is one of the most important plantation crops in the world. Although conventional breeding and vegetative propagation are the only means for plant improvement, they are slow, time consuming and labour intensive. Therefore, improvement through biotechnological means has tremendous potential to overcome the limitations of conventional approaches.

Embryogenesis is the most popular regeneration pathway of plants because of its wide applications[1]. Embryogenesis has been reported for most of the crop species[2,3]. The process has been successfully exploited for clonal propagation[4], artificial seed production[5], cryopreservation for the storage of elite clones[6], embryo rescue[7] and most importantly, *in vitro* manipulations through genetic transformation[8]. In *Camellia* too, somatic embryogenesis has been studied well and used for multiplication of elite clones[9], artificial seed production[10,11], embryo rescue of inter-specific crosses[12] and transgenic plant production[13]. In all the reports of embryogenesis, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. However, induction of *in vivo* embryogenesis for any crop species has not been reported so far.

The present study was undertaken to germinate tea seeds under sterile conditions in steel boxes with moist sand, for the use of protoplast isolation. Although a chance observation, in this paper we report the induction of embryogenesis on cotyledon surface of mature tea seeds under *in vivo* conditions.

Mature fruits of the three cultivars, viz. UPASI-9, T-78 and Kangra Jat were collected from tagged bushes growing at Tea Experimental Station, Palampur, Himachal Pradesh, (1290 msl at 32°N and 78°S) during September–November. The reason for choosing these particular months was because seeds of these cultivars show highest embryogenic potential during these periods[14]. Among the cultivars, UPASI-9 is the most popular in South India, whereas T-78 and Kangra Jat are widely grown cultivars in north-east India and Kangra valley (Himachal Pradesh) respectively.

Seeds were separated from fruits and viability was tested by sinker-floater test[15]. Half of the sinker seeds of each cultivar was shown in field as per conventional procedure for control treatment[15]. The other half was washed thoroughly with Tween-80 for 5 min and then with distilled water for 6–8 times. Seeds were then surface-sterilized with 4% (w/v) calcium hypocholoride solution for 10 min followed by 5–6 washings in sterile de-ionized water. An additional treatment of 10 mg/l streptomycin sulphate was given for 25 min to avoid endogenous bacterial contamination.

A layer of 0.5 cm absorbent cotton was placed at the bottom of a steel box (22 cm diameter × 6 cm height) over-layered by a 4 cm thick sand mix (sand : activated charcoal :: 3 kg : 4 g). This was then moistened with 200 ml of distilled water and autoclaved at 104 kPa and at 121°C for 60 min. Fifteen seeds of each cultivar were inoculated in steel boxes under aseptic condition. Then, seeds of each cultivar were demarcated by placing autoclaved toothpick sticks between them. For identification, the respective position of the seed of each cultivar was marked outside the steel box.

The steel boxes were kept in dark in BOD incubators (Narang Scientific Works Pvt Ltd, New Delhi) at three different temperatures (24, 28 and 32°C) and observations were recorded after every 30 days. The experiments were

*For correspondence. (e-mail: mondaltk@yahoo.com)

repeated for three subsequent years (1994–1995, 1995–1996 and 1996–1997).

For histological studies, tissue at the point of protuberance formed on cotyledon was fixed in FAA (formaldehyde : acetic acid : 50% ethanol, 5 : 5 : 90) and dehydrated in a *n*-butyl alcohol series. Sections (10 μm thick) were stained with safranine-fast green and the slides were mounted in DPX [distrene 8–10 gm (British resin product), dibutylphothalate 5 ml, xylene 35 ml].

Seed germination in the control treatment was invariably observed in all the genotypes, depending upon the maturity of seeds. Immature and aged seeds did not germinate. Only mature seeds (with black-coloured, firm seed coat) were germinated. Maximum germination was observed in UPASI-9 (98 ± 1.05), followed by T-78 (97 ± 0.23) and Kangra Jat (95 ± 1.34) respectively (Table 1). When seeds were inoculated aseptically in steel boxes, cracking of seed coat or 'testa' was not always followed by germination. Instead, some protuberances were seen to develop from the abaxial surface of the cotyledons. These protuberances were 2–4 mm in size and were pale-yellow to cream in colour. These emerged as small and rounded structures. The extent of induction of such structures in our study was influenced by the genotype, time of seed collection and incubation temperature (Table 1).

Since seeds of different clones mature at different times, the response of seeds collected in different months was variable (Table 1). While maximum response of UPASI-9 was observed in September, that of T-78 was observed in October, whereas November was found to be most effective for Kangra Jat seeds. In a separate study we found a similar type of embryogenic *in vitro* response of these cultivars, where UPASI-9 gave maximum embryogenesis in August, T-78 in October and Kangra Jat in November respectively[14].

The maximum number of seeds showing embryogenic response was observed in UPASI-9 compared with the two other cultivars (Table 1). The response in UPASI-9 seeds collected in September was 23.3 ± 0.59, while that of T-78 collected in October was 16.5 ± 0.84 and Kangra Jat collected in November was 11.7 ± 0.69.

The formation of such somatic embryos per seed was also genotype-specific. While the maximum number of somatic embryos (15–16 per seed) was observed after 90 days of incubation at 28°C in UPASI-9 (Figure 1 *a*), that of Kangra Jat and T-78 showed 10–12 and 6–8 such embryos per seed respectively (data not shown). After 28–42 days, the embryos underwent a typical embryogenic pathway as evident by cup-shaped structures (Figure 1 *b*).

A selected regime of temperature (24, 28 and 32°C) for incubating the seeds was examined so as to determine the effect on embryogenesis, *in vivo*. At 32°C, no response was seen in any of the three genotypes, irrespective of the time of seed collection. Only a very low germination frequency (1.6 ± 1.84) was observed in the Kangra Jat seeds. At 24°C, a small percentage (4.2 ± 1.90) of Kangra Jat seeds germinated, but there was no development of somatic embryos. However, when the seeds were incubated at 28°C, both germination and adventive embryos were observed. The percentage of germination was lower in comparison with that of embryo formation (Table 1). However, a considerable amount of seeds remained unresponsive, irrespective of the treatments.

One important observation was that the seeds which follow normal germination, did not show embryogenesis. However, in some cases embryogenesis was observed coupled with normal root development. (Figure 1 *c*). This may be due to the fact that shoot pole undergoes abnormal proliferation, which leads to the expression of totipotency of the cell in terms of protuberance. Subsequently, these protuberances turn into somatic embryos. In the present study, induction was not dependent upon the external supply of nutrients and growth regulator, but depends on the external environment, which probably shifts the metabolic process of germination. This may be governed by specific interaction of the endogenous hormones in the seeds with that of specific environment within the steel box at 28°C. However, hormone content and ratio may differ from seed to seed, even from the same plant. There-

**Table 1.** Effect of temperature, genotype and time of seed collection on *in vivo* formation of somatic embryos in tea seed*

| Clone | Month of seed collection | Control | | Incubation temperature | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 24°C | | 28°C | | 32°C | |
| | | G | SE | G | SE | G | SE | G | SE |
| Kangra Jat | September | – | – | – | – | – | – | – | – |
| | October | 45 ± 0.67 | – | – | – | – | – | – | – |
| | November | 95 ± 1.34 | – | 4.2 ± 1.9 | – | 9.2 ± 1.2 | 11.7 ± 0.69 | 1.6 ± 1.84 | – |
| T-78 | September | 45 ± 1.74 | – | – | – | 3.2 ± 0.67 | 5.0 ± 1.6 | – | – |
| | October | 97 ± 0.23 | – | 1.7 ± 0.18 | – | – | 16.5 ± 0.84 | – | – |
| | November | 27 ± 0.27 | – | – | – | – | – | – | – |
| UPASI-9 | September | 98 ± 1.05 | – | – | – | 11.2 ± 1.03 | 23.3 ± 0.59 | – | – |
| | October | 47 ± 0.55 | – | – | – | – | – | – | – |
| | November | – | – | – | – | – | – | – | – |

*Data ($\bar{X}$ ± SE) pooled from three consecutive independent years (1994–1995, 1995–1996 and 1996–1997) experiments.
G, germination frequency; SE, somatic embryo formation.

fore, the result of interaction between the endogenous hormonal level and the external environment also differs. The seeds in which the metabolic process has already been determined for the germination due to different hormonal levels, may mask the external environment influence in our study, resulting in normal germination.

After 30 days of incubation, the number of relatively undifferentiated cells had increased in the epidermis and few layers of the cortex (sub-epidermis; Figure 2 *a*). The sub-epidermal cells became densely cytoplasmic and indentation on the cotyledon surface was formed as a result of repeated anticlinal and periclinal divisions of the same. The majority of the sub-epidermal cells divide periclinally, although other types of plane are also observed. Their further growth led to the development of globular protuberances at 90 days of incubation (Figure 2 *b*). Subsequently, repeated divisions and dedifferention of proembryogenic cells had given rise to what were considered as true embryogenic cells that contained a large nucleus. The embryogenic cells developed into a superficial meristem that after repeated cell division, formed a pattern of indention and protuberance of embryogenic tissue (Figure 2 *c*). The protuberances which arose from the abaxial surface of the tea cotyledon,

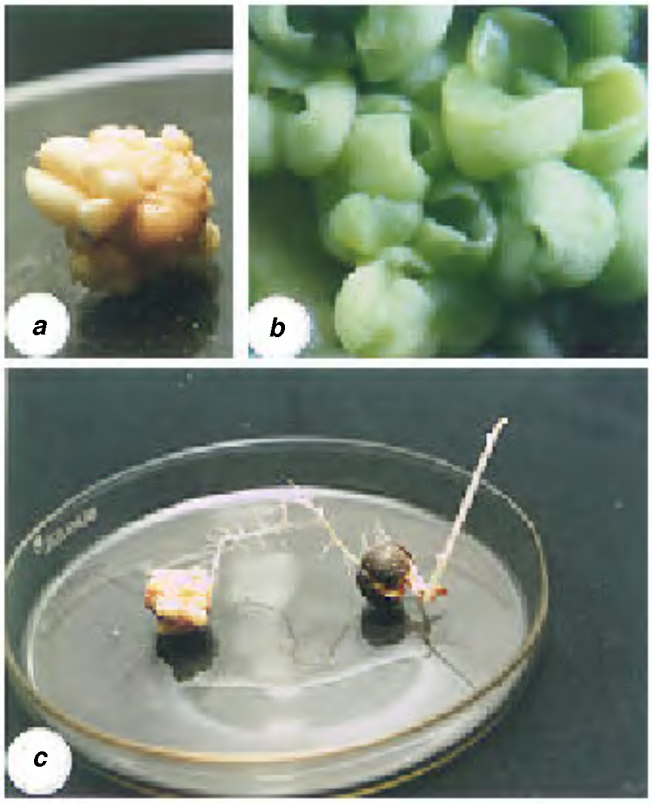


**Figure 1.** ***a***, UPASI-9 seed harbouring 15–16 somatic embryos on the cotyledon surface after 90 days of incubation at 28°C on moist sterile sand; ***b***, A close-up view of cup-shaped structure of somatic embryos on the cotyledon of UPASI-9 after 112 days of incubation at 28°C on moist sterile sand; ***c***, Induction of somatic embryo (left) and normal germination (right) observed on the same seed while incubating at 28°C on moist sand.

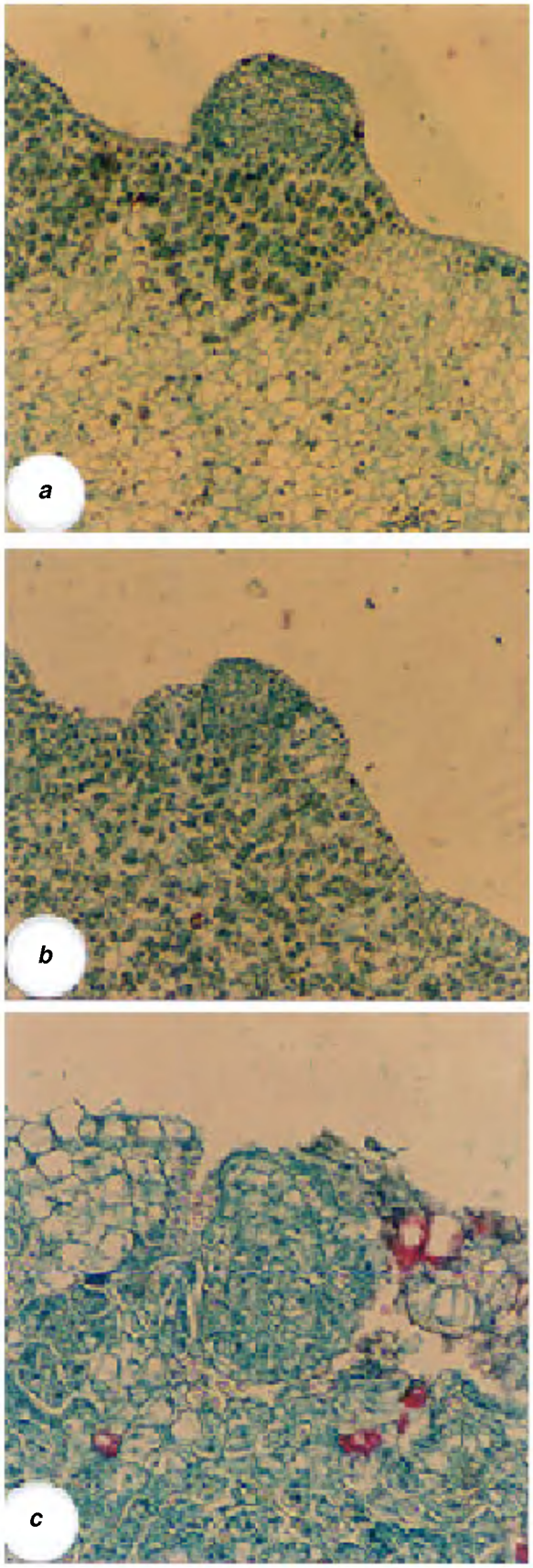


**Figure 2.** Histological study of *in vivo* somatic embryogenesis shows the different developmental stages (***a–c***) of induction (1 cm bar = 50 μm).

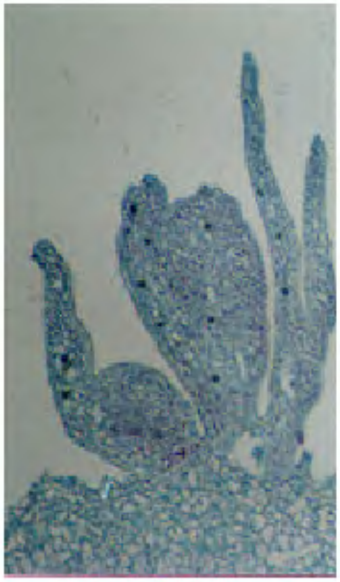

**Figure 3.** Longitudinal section of cup-shaped somatic embryo differentiated on tea cotyledons (1 cm bar = 260 μm).

developed further to cup-shaped structures as evident from histological studies (Figure 3). Although, further development, multiplication or conversion was not observed with these structures on their mother tissue, their morphological nature was found to be very similar (Figure 1 *a*) to the *Camellia japonica* somatic embryos, which were reported to develop only from the abaxial surface of the cotyledons[16]. The abaxial surface of the *Camellia* cotyledons has been reported to be morphologically competent, with high embryogenic potential[16] and this was found to be true even in this study under *in vivo* conditions. Thus our histological findings show that the embryogenesis occurs on the surface of the cotyledon without differentiation of intermediate callus phase. The morphogenic events observed during this developmental process were similar to direct embryogenesis from the cotyledon surface.

The present study indicates that induction of embryogenic response is not only dependent upon the external supply of nutrients and growth regulators, but also upon temperature. At a particular temperature, mature tea seeds are capable of expressing their somatic embryogenic potential even under *in vivo* conditions. Such observations in tea have not been reported so far. Bhatia *et al.*[17], observed *in vivo* response of callusing and adventitious shoot formation from de-embryonated *Arachis hypogea* cotyledons inoculated on moist sand and cotton wool in enamel trays. However, the greater influence of temperature on embryogenesis, forced us to propose a hypothesis that, in *Camellia* the induction of *in vivo* embryogenesis must be regulated by some temperature-dependent gene. Although the reason for this observation is not clear presently, seeds of *Camellia* appear to have a considerable inherent capacity for embryogenesis[9]. Thus at the right stage, with certain levels of internal hormone of tea seed along with the interaction of appropriate moisture level of the substrate and sterile conditions, the seeds are able to produce embryos without any exogenous nutrient. However, we failed to establish any culture *in vitro*, due to the heavy endogenous bacterial contamination.

We conclude that induction of embryogenesis in tea can be possible under *in vivo* condition, without the conventional plant tissue culture method. The technique has potential, as it can be used economically for mass-scale production of embryos. However, further attention is required to circumvent some of the basic questions on embryogenesis, specially with regard to the role of temperature. Studies are underway to establish the culture *in vitro*, by controlling contamination and also to test the effect of temperature for increasing the embryogenesis frequency under *in vitro* condition of these cultivars.


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