nana, *E. superbum*, could successfully be regenerated *in vitro* via somatic embryogenesis. The protocol developed will be useful for rapid *in vitro* propagation of the species and also for the subsequent genetic manipulation studies.

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Propagation of tea (*Camellia sinensis* (L.) O. Kuntze) by shoot proliferation of alginate-encapsulated axillary buds stored at 4°C

Tea or *Camellia sinensis* (L.) O. Kuntze is an important commercial crop generating employment for a large number of people. It is a popular beverage crop having medicinal, anti-oxidative and anti-microbial properties[1]. The three main types of tea, i.e. 'Cambod', 'Chinary' and 'Assamica' cultivars are distributed in widely-distanced regions of southern, northern and northeastern parts of India. Micro-propagation and artificial seed techniques ensure an efficient exchange of germplasm[2] among these regions and also among the tea-growing countries for successful storage, delivery and establishment of tea germplasm. 'Artificial seed technology'[3–7] provides a protective coating of essential nutrients to the encapsulated propagules[8], maintains high adaptability and vigour during their storage[2,3], removes the hurdles of delivery and establishment[9,10] and has the added advantage of facilitating easy handling, storage and shipping[2].

Artificial seed production has been reported in ornamental *Camellia*[10], *Valeriana wallichii*[11], banana[12], spruce[13], mulberry[14,15], sweet potato[16], eucalyptus[17], orchids[18,19], *Ocimum*[20], apple[21], etc. Yet, there is no report till date on tea, except for our earlier report[22]. Tea embryos, as reported earlier, are recalcitrant in nature[23] and have a poor conversion frequency. Hence in the present study, attempts were made to develop a system for the production of artificial seeds in tea employing viable propagules like axillary buds, and also to understand the effect of low-temperature storage on their bud-sprouting efficiency.

Aseptic cultures of nodal segments of *C. sinensis* were initiated on half-strength, 0.8% agar solidified MS medium[24] supplemented with 3% sucrose and 8.88 µM BA, in combination with 0.98 µM IBA (ref. 25) in 250-ml Erlenmeyer flasks (Borosil, Mumbai). After 30 days, the sprouted buds of the nodal segments were transferred to hormone-free MS medium for further multiplication, so as to allow the shoots to attain a height of 3.0 cm. Finally, about 0.2 to 0.5 cm long segments comprising defoliated single nodes were taken as explants after three sub-cultures of four weeks each. Five replicates per treatment with 20 explants in each were used for every experiment which was repeated thrice. All cultures were maintained at laboratory conditions (25 ± 2°C with a 16 h light photoperiod of 52 µmol m^{-2} s^{-1}).

Individual nodal explants were transferred into varying concentrations (i.e. 2, 3, 4, 5 and 6%) of sodium alginate in liquid basal MS medium and also in MS medium supplemented with 5 µM TDZ in combination with 10 µM NAA (MS1). MS1 was selected on the basis of our earlier report[26]. Drops of sodium alginate solution containing one nodal explant each were then slowly dropped into different concentrations of $CaCl_2.2H_2O$

solution (i.e. 25, 50, 75 and 100 mM) and stirred continuously for 30 min on a magnetic stirrer. The different concentrations and combinations of $CaCl_2.2H_2O$ and sodium alginate solutions were tested in order to optimize the size, shape and texture of alginate beads for bud-sprouting. Perfectly-rounded beads obtained, were hydrated in sterile MS1 medium supplemented with 5 µM TDZ and 10 µM NAA, and finally placed horizontally on agar-solidified MS1 medium for sprouting of the axillary buds. Similarly, the beads prepared in basal MS medium were hydrated and placed on hormone-free, basal MS medium. Irrespective of the medium in which they were prepared, the alginate beads were transferred to hormone-free MS medium for further shoot multiplication. Comparative shoot proliferation of the un-encapsulated and encapsulated nodal explants derived from both basal MS and MS1 media were also evaluated with respect to (i) per cent explant response or bud sprouting, (ii) days taken for bud sprouting, and (iii) number of shoots formed per nodal explant.

The shoot proliferation efficiency of the encapsulated nodal explants stored for different time periods was tested. Encapsulated nodal explants were stored both at culture lab conditions as well as at low temperature (4°C) for 15, 30, 45 and 60 days on MS1, after which the alginate beads were transferred to hormone-free MS medium. The effect was evaluated with respect to (i) days taken for sprouting of nodal explants, (ii) per

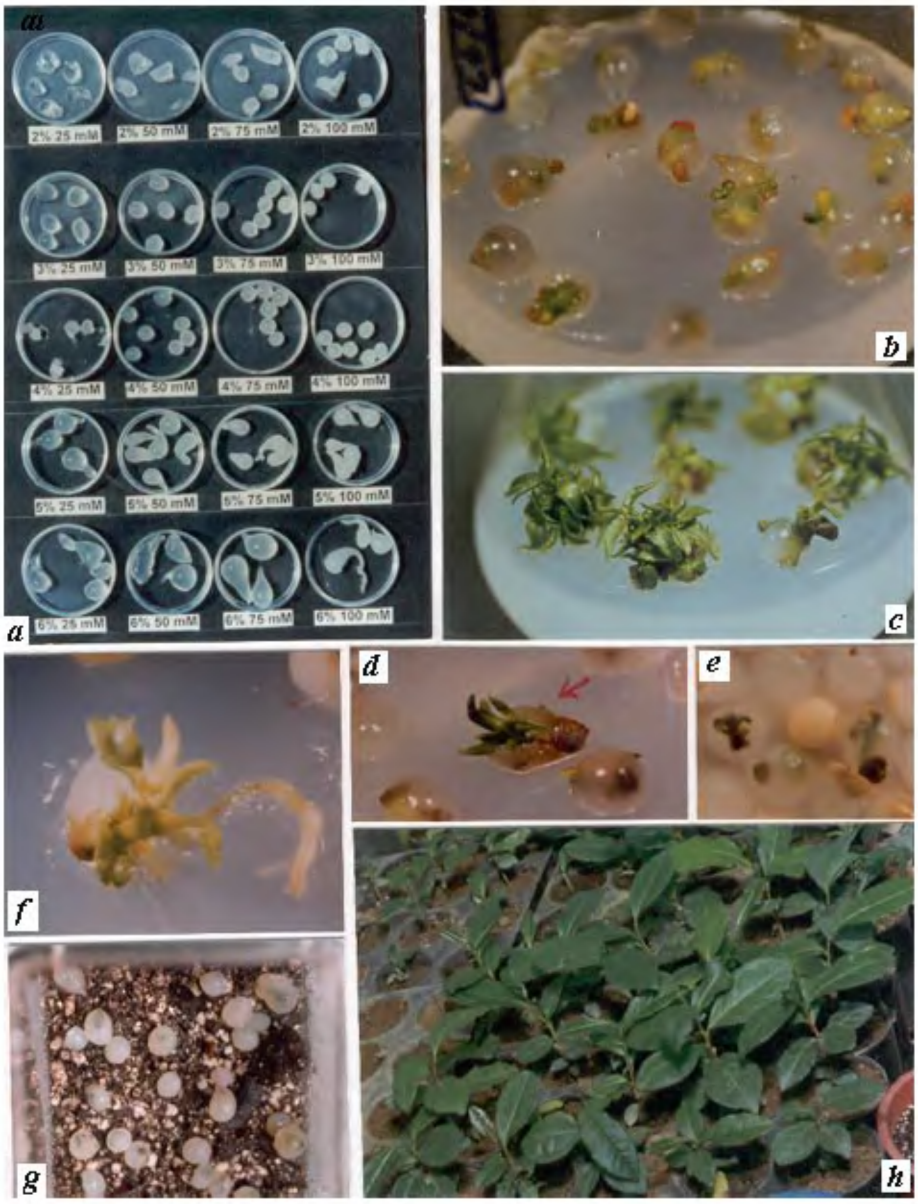

Figure 1. ***a***, Polymerization using sodium alginate (4%) and $CaCl_2$ (75 mM); ***b***, Swelling of alginate beads and sprouting of axillary buds of nodal explants; ***c*** and ***d***, Multiplication of shoots sprouted from alginate beads; ***e***, Alginate beads encapsulating nodal explants; ***f***, Rooting of micro-shoots encapsulated in alginate beads; ***g***, Alginate beads on sterile soilrite mix moistened with liquid MS1 medium; and ***h***, Healthy shoots growing in pots.

cent response of alginate beads or artificial seeds with respect to bud sprouting, and (iii) number of shoots formed per bead at intervals of 15 days for a period of 60 days.

Encapsulated buds were also placed on sterile soilrite moistened with MS1 medium under culture lab conditions, to test the emergence of shoot buds from them. Freshly excised cut-ends of individual three-cm long micro-shoots obtained from stored un-encapsulated and encapsulated axillary buds were treated with 500 mg l^{-1} IBA for 30 min and transferred directly to a potting mix containing garden soil : river bed sand:farmyard manure :: 9 : 1 : 1 (pH 5.4) in hikkotrays, and kept in the hardening chamber for rooting and acclimatization. Data were recorded on percent rooting, growth and percent survival of the rooted plants under poly-house conditions.

Artificial seeds having smooth texture appropriate for the sprouting of axillary buds, were produced when 75 mM $CaCl_2.2H_2O$ solution in combination with 4% sodium alginate was used (Figure 1 *a*, *e*). While the alginate beads prepared and placed on MS1 showed swelling after 30 days followed by bud sprouting (Figure 1 *b*), the ones prepared in hormone-free, basal MS medium and cultured on basal MS medium failed to respond. Further multiplication of the sprouted shoot buds from MS1 beads occurred on hormone-free MS medium (Figure 1 *c–f*).

Encapsulated and un-encapsulated axillary buds of nodal explants sprouted after 30 days of culture on MS1 medium and sprouting was only slightly higher (90.2%) in un-encapsulated buds compared to the encapsulated ones (88.1%). On transfer to hormone-free MS medium, the number of shoots formed per nodal explant was lower (5.2) compared to the alginate-encapsulated ones (8.0), during the initial stages. However, the number of shoots formed after 60 days was higher (17.3) in the un-encapsulated explants compared to their alginate-encapsulated counterparts (12.0). The encapsulated explants failed to respond on hormone-free, basal MS medium.

The bud-sprouting efficiency (90.2 to 92%) of both un-encapsulated and encapsulated nodal explants was maintained up to 45 days, but declined to 45.9 and 50% when stored up to 60 days at culture lab conditions (Table 1). Although storage at 4°C did not affect the bud-sprouting efficiency adversely in either un-encapsulated or encapsulated nodal explants, low-temperature storage helped in reducing the time taken for sprouting by 15 days (Table 1). Moreover, the buds stored at 4°C sprouted 15 days earlier than the ones stored at 25 ± 2°C; the number of shoots produced per subculture from either conditions was at par (Table 1).

A very low percent of encapsulated buds prepared in MS1 (Figure 1 *g*) sprouted after 70 days, when placed on sterile soilrite moistened with MS1 and kept under culture lab conditions, but turned necrotic thereafter.

Roots were initiated in 3.0 cm long micro-shoots derived from encapsulated and un-encapsulated axillary buds after 60 days of IBA treatment. No differences in rooting efficiency were observed in the micro-shoots obtained from either encapsulated or un-encapsulated buds, and the rooting behaviour and survival were similar to our earlier observation[27]. No adverse effect was observed on the rooting of micro-shoots derived from buds stored at 4°C. The healthy, rooted micro-shoots from alginate-encapsulated beads grew vigorously in pots (Figure 1 *h*).

The present paper reports successful implementation of artificial seeds of tea, wherein *in vitro*-grown axillary buds can be used as micro-propagules for clonal propagation of elite and desirable tea clones. It also demonstrates the advantages of artificial seeds in effective storage of axillary buds as viable micro-propagules of tea for periods as long as

Table 1. Bud-sprouting efficiency and shoot-multiplication rate of alginate-encapsulated and un-encapsulated nodal explants stored for different periods of time at 4°C and at culture lab conditions

				Number of shoots per bead/explant			
Time on MS1	Stored at (°C)	Explant	Germination (%)	15 days	30 days	45 days	60 days
0 day	25 ± 2	UE	90.2	–	5.2 ± 0.7	11.5 ± 0.9	17.3 ± 0.8
		AE	88.1	–	8.04 ± 0.4	9.8 ± 0.7	12.0 ± 0.9
15 days	4	UE	90.1	4.2 ± 0.4	9.7 ± 0.5	12.9 ± 0.2	18.9 ± 0.7
	25 ± 2		89.1	–	5.3 ± 0.2	12.0 ± 0.3	17.6 ± 0.2
	4	AE	94.3	–	11.0 ± 0.4	12.7 ± 0.2	20.8 ± 0.8
	25 ± 2		90.2	4.0 ± 0.2	9.9 ± 0.5	11.9 ± 1.0	18.5 ± 2.0
30 days	4	UE	92.5	5.1 ± 0.4	10.8 ± 1.6	12.2 ± 0.8	19.3 ± 0.4
	25 ± 2		90.5		5.0 ± 0.2	10.8 ± 0.5	13.0 ± 1.8
	4	AE	95.0	3.5 ± 0.6	10.5 ± 0.6	13.2 ± 0.6	21.7 ± 0.4
	25 ± 2			–	12.0 ± 0.5	13.0 ± 1.7	19.0 ± 0.5
45 days	4	UE	89.7	3.7 ± 0.7	9.5 ± 0.7	13.9 ± 2.9	18.5 ± 0.6
	25 ± 2		91.0	–	9.5 ± 0.3	12.5 ± 1.5	19.1 ± 0.2
	4	AE	91.0	5.0 ± 0.4	11.7 ± 0.4	13.7 ± 0.9	19.3 ± 0.8
	25 ± 2		92.0	–	11.3 ± 0.4	12.2 ± 0.7	17.3 ± 1.0
60 days	4	UE	45.0	3.7 ± 1.4	10.5 ± 0.7	12.7 ± 0.2	14.3 ± 1.5
	25 ± 2		40.4	2.7 ± 0.1	9.0 ± 1.0	11.4 ± 0.9	12.0 ± 0.6
	4	AE	50.0	2.7 ± 0.4	7.7 ± 0.7	10.3 ± 0.5	12.5 ± 0.6
	25 ± 2		45.9	–	5.9 ± 0.5	10.9 ± 1.2	11.8 ± 1.0

Data (mean ± SE) pooled from three independent experiments, each with 100 samples per treatment. –, No response; UE, Un-encapsulated; AE, Alginate-encapsulated.

60 days (Table 1) as in mulberry[14], *Santalum*, *Quercus* and *Betula*[28].

A solution of 4% sodium alginate and 75 mM $CaCl_2$ yielded perfectly rounded alginate beads with nodal explants. An initial treatment of 5 µM MTDZ and 10 µM NAA followed by transfer to hormone-free, basal MS medium was shown to enhance shoot proliferation[26]. Thus, the per cent bud-sprouting response was better when axillary buds were cultured on MS1 or basal MS medium supplemented with TDZ and NAA. However, transfer to hormone-free, basal MS medium was essential for further multiplication, as in our earlier report on the effect of TDZ on shoot proliferation from nodal explants[26]. This is probably due to the fact that continued presence of TDZ in the culture medium has a deleterious effect on tissues growing under *in vitro*-grown conditions[26,29]. Since TDZ has the potential to stimulate endogenous cytokinin biosynthesis or in altering cytokinin metabolism[26,30,31], continued presence of TDZ in the culture medium was not required in the present study.

Storage of artificial seeds of tea at 4°C reduced the time taken for bud sprouting in both the encapsulated and unencapsulated axillary buds by 15 days. Apart from retaining the viability of the micro-propagules[9,10,32], low temperature is also known to improve bud sprouting as well as proliferation efficiency of shoot buds of many species, viz. kiwifruit[33], apple[34], poplar[35] and ornamentals[10]. This study opens the possibilities of effective storage of axillary buds, thereby enabling transportation and exchange of valuable germplasm of tea across the globe.

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