Somatic embryogenesis and plant regeneration in a seeded banana [*Ensete superbum* (Roxb.) Cheesman]

Ensets (*Ensete* spp.), belonging to the family Musaceae, are the wild bananas, cultivated in tropical and subtropical regions of Africa and Asia. These are the staple food of almost 10 million people[1], and have also been used to help ward-off famine in Ethiopia[2]. This genus consists of 7–8 species[3] and is known for its broad genetic base and possession of several desirable genes[4]. Such wild genepool could be utilized for improvement of cultivated bananas by employing various *in vitro* techniques[5].

In nature, ensets propagate sexually by means of seeds and do not produce vegetative suckers. However, these can be induced to undergo clonal propagation by hollowing out an old corm and packing the hole with soil and dung before flowering[6]. Since this conventional method of enset propagation is time-consuming and at the expense of a matured plant every time, the need for further optimizing and commercially utilizing micropropagation techniques for mass multiplication is evident. Further, in this regard, there have been very few reports on *in vitro* propagation of ensets through multiple shoots[7–9] and there has

been only one report[10] on somatic embryogenesis, but in *Ensete ventricosum*. Herein we describe a protocol for the *in vitro* regeneration of an Indian enset, *Ensete superbum* (Roxb.) Cheesman, through somatic embryogenesis.

The male floral apices of *E. superbum* were cultured *in vitro* to obtain multiple shoots as described earlier[7], which were maintained on MS[11] supplemented with 6-benzylaminopurine (BAP) (2 mg/l), adeninehemisulphate (AH) (30 mg/l) and gibberellic acid (GA$_3$) (1 mg/l), gelled with agar (0.8%). The multiple shoots were subcultured on MS + BAP (5 mg/l) + AH (30 mg/l), gelled with Gelrite (0.2%) (the control medium) to obtain callus.

The calluses obtained were subjected to various media for the induction of embryogenicity (Table 1). The embryogenic calluses developed were cultured on MS medium alone or supplemented with glutamine (400 mg/l) for somatic embryo development. Germinated somatic embryos were transferred to half-strength MS medium for further growth. After 3–4 weeks, the test-tube plants (5–6 cm in height and with 2–3 leaves) de-

rived through somatic embryogenesis were gently washed in running tap water to remove all the adhering pieces of gelled medium. These were subsequently transferred to perforated polythene bags filled with autoclaved mixture of soil and Soilrite (Chowgule Industries Ltd, Mumbai) and weaned in the greenhouse for three months, under natural light with relative humidity of 90–100% at ambient temperature of 25°C. Approximately 40–60 cm tall plants were field-planted.

Multiple shoot cultures initially exhibited a high level of vitrification (Figure 1 *a*) in the range 40–50% on MS + BAP (5 mg/l) and AH (30 mg/l) + Gelrite (0.2%). The vitrified tissue subsequently developed into heterogenous and gelatinous/watery callus (Figure 1 *b*) in 60–70 days, which could be maintained by regular transfer to fresh medium.

These calluses were further transferred to various media (Table 1) with a view to obtain compact and embryogenic callus. Although the morphology of all the cultures (100%) within each treatment was uniform, it varied among treatments. Control medium supplemented with naphthaleneacetic acid (NAA)

**Table 1.** Response of *Ensete superbum* callus cultures to different media

Medium	Response
MS + BAP (5 mg/l) + AH (30 mg/l) (control)	Heterogenous and gelatinous/watery callus
MS + BAP (5 mg/l) + AH (30 mg/l) + 2,4-D (2.5 mg/l)	Mixed callus with structures which could not be identified either as embryogenic globules or shoot meristematic clusters
MS + BAP (5 mg/l) + AH (30 mg/l) + Picloram (1 mg/l)	Compact and nodular callus
MS + BAP (5 mg/l) +AH (30 mg/l) + 2,4-D (2.5 mg/l) + Picloram (1 mg/l)	Compact and nodular callus
MS + BAP (5 mg/l) + AH (30 mg/l) + NAA (1 mg/l)	Fast-growing mucilagenous/watery callus with few shoot primordia which did not grow after subculturing
MS + NAA (1 mg/l) + Picloram (1 mg/l)	Fast-growing mucilagenous/watery callus with few shoot primordia which did not grow after subculturing
MS + 2,4-D (2.5 mg/l) + NAA (1 mg/l)	Mixed callus with structures which could not be identified either as embryogenic globules or shoot meristematic clusters

12 cultures per treatment; Data scored after 45 days.

Table 2. Conversion and germination of *E. superbum* somatic embryos

Medium used	*Frequency of responding cultures (%)	*Number of embryoids per culture	Hardening success (%)
MS (half-strength)	41.7	0.92 ± 0.38	0.0
MS (half-strength) + glutamine (400 mg/l)	83.3	2.42 ± 0.49	80.0

12 cultures per treatment; *Data scored after 30 days.
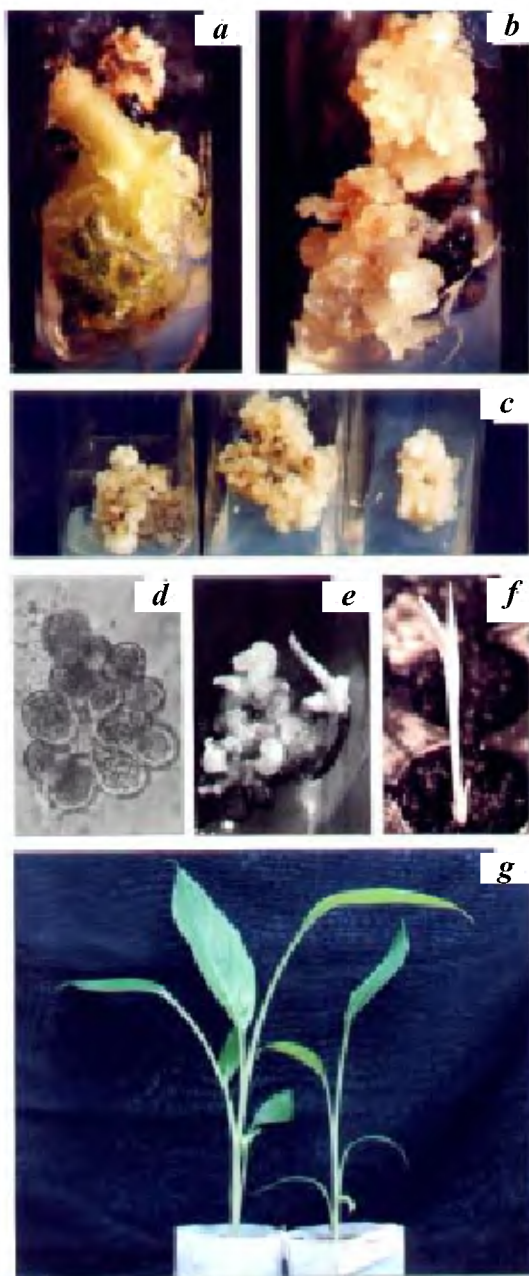
Figure 1. *a*, Vitrification of cultured shoot tips of *Ensete superbum*; *b*, Callus formation from vitrified shoots of *E. superbum*; *c*, Nodular and embryogenic callus of *E. superbum*; *d*, A cluster of globular and densely-filled cells from *E. superbum* callus; *e*, Fully developed somatic embryo of *E. superbum*; *f*, Weaning of *E. superbum* plantlet obtained through somatic embryogenesis; and *g*, Normally growing hardened plants of *E. superbum* in polybags.

(1 mg/l) produced vigorously proliferating mucilagenous and watery callus, occasionally mixed with few shoot primordia (Table 1). Highly embryogenic, nodular and compact white callus (Figure 1 *c*) was observed on MS + BAP (5 mg/l) + AH (30 mg/l) + Picloram (1 mg/l). Although the control medium supplemented with only 2,4-dichlorophenoxy-acetic acid (2,4-D) (2.5 mg/l) produced non-embryogenic callus, it did not interfere with the activity of Picloram in combination. Squash preparations from the embryogenic calluses showed densely filled, round embryogenic cells (Figure 1 *d*).

The transfer of embryogenic calluses growing for 45 days on MS + BAP (5 mg/l) + AH (30 mg/l) + Picloram (1 mg/l) with or without 2,4-D (2.5 mg/l) to MS (half-strength) + glutamine (400 mg/l) resulted in the formation of fully-developed embryos and was superior to MS (half-strength) alone (Table 2). The frequency of responding cultures exhibiting somatic embryos on MS (half-strength) + glutamine (400 mg/l) was exactly double compared to that on MS (half-strength). It was also observed that MS (half-strength) induced rhizogenesis and did not support shoot development. In contrast, the medium supplemented with glutamine (400 mg/l) exhibited formation and development of complete somatic embryos (Figure 1 *e*) which subsequently developed into normal plantlets. The plantlets were hardened in the green-house (Figure 1 *f* and *g*), with about 80% success, for three months and subsequently field-transferred in June 1998. These plants flowered after another two years and exhibited no morphological aberrations.

The studies showed that in the case of *E. superbum*, the regeneration process started with the induction of vitrification using high levels of BAP followed by callusing, and the finding was in close conformity with an earlier report[10] in the case of *E. ventricosum*. It was also observed that Picloram played a critical role for obtaining embryogenic callus. Similarly, Picloram has also been observed to exert beneficial effects in the callus cultures of wheat[12], onion[13], along with BAP in soybean shoot apices[14]. In contrast, it has also been reported that while stimulating callus growth, Picloram decreased embryogenicity and regenerability compared to 2,4-D[15].

The results also demonstrated that glutamine was an essential factor for the conversion of the somatic embryos to complete plants with well-developed shoot and root. The usefulness of inclusion of glutamine in the regeneration medium for maturation and conversion was noticed in mango[16] and in *Medicago sativa*[17]. This could be due to the accumulation of storage reserves during the maturation phase[18] which are required for further growth of the embryo, and in support, such accumulation of storage proteins has also been reported to be significantly increased by glutamine application[19].

Present investigations have thus shown that the economically valuable wild ba-

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 Erlennmeyer 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 $CaCl_2.2H_2O$