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Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli

V. M. Kulkarni*, P. Suprasanna and V. A. Bapat

Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

Banana is India's premier fruit crop having great socio-economic significance. A range of *Musa* genotypes exists in local pockets, either cultivated or wild. Rajeli, an economically valuable genotype of the Western coast of Maharashtra, is traditionally grown for making Sukeli (dried bananas) of desired quality. At present, this unique cultivar is under threat of extinction due to fungal and viral diseases. We have successfully regenerated Rajeli plants via shoot-tip culturing and somatic embryogenesis. Prolific multiple shoot formation and elongation were induced in 72% of the cultures. Embryogenic callus was also obtained from male flower buds cultured on callus-inducing medium with 2,4-D. Somatic embryos transferred to the medium with BAP showed plumule development, followed by complete plantlet formation on MS basal medium without any growth regulators. A large number of plants have so far been regenerated. The tissue culture system reported herein demonstrates its potential for use in genetic manipulation studies. Also, since the fruits can be dehydrated and stored for extended periods, Rajeli appears to be a suitable candidate cultivar for expressing therapeutic proteins.

Keywords: Banana, multiple shoots, *Musa*, Rajeli, Sukeli, somatic embryogenesis.

BANANA is the world's fourth most important food crop after rice, wheat and maize[1]. It is a staple food, and an export commodity, which contributes to the food security of millions of people in the developing world, and when traded in local markets provides income and employment to rural populations[1]. India is a leading country in global banana production; however, exports have not been substantial[1,2]. Post-harvest problems such as short shelf life after ripening and cosmetic attractiveness[3] are faced by banana growers for exporting fresh bananas. New and innovative strategies for enhancing banana export must be sought, so as to earn a place among the banana-exporting countries.

It would be most appropriate if the genotypes in which either the bananas or other plant parts can be processed

*For correspondence. (e-mail: vmk@apsara.barc.ernet.in)

for making a 'value-added' marketable product, are given focused attention. We have identified one such promising traditional banana cultivar, Rajeli (AAB), in which the harvested bananas can be air-dried in a few sequential steps. These 'value-added' dried bananas, referred to as 'Sukeli' (Figure 1 *e*), are ideal for marketing to distant locations within and outside India, for the following reasons.

The dehydrated Rajeli bananas or Sukeli taste like dry figs and can be stored for a long period under dry and appropriate conditions. Further, these have high consumer acceptance and fetch about Rs 120–170/kg, the prices approximately ten times higher than the fresh bananas in the domestic market. Transportation costs for international and domestic trading can be significantly less due to reduced weight upon drying. Additionally, the conventional drying process uses no chemical treatments or additives and the product is absolutely safe for human consumption.

Sukeli-making has been a traditional small-scale business in the Vasai–Virar region of Maharashtra and farmers opine that desired high quality Sukeli can be made only from Rajeli. Unfortunately, due to fungal and viral diseases, commercial banana plantations, including valuable banana genotypes like Rajeli, are on the verge of extinction. A few enthusiastic farmers in the Agashi and Kofrad villages near Virar have the germplasm of this cultivar in their small land holdings.

There is an urgent need for conservation of Rajeli and promoting farmer's traditional practice of Sukeli-making. It is also imperative that adequate disease-free planting material needs to be produced for restoration of the area under cultivation as well as its subsequent expansion. One way to achieve this goal is the establishment and scale-up of an efficient tissue-culture protocol, either using the shoot apices[4] or the inflorescences as explants[5,6]. This communication describes our successful efforts in regenerating plants in banana cv. Rajeli.

The side-suckers of banana cv. Rajeli were collected from farmers in village Kofrad and used as source material

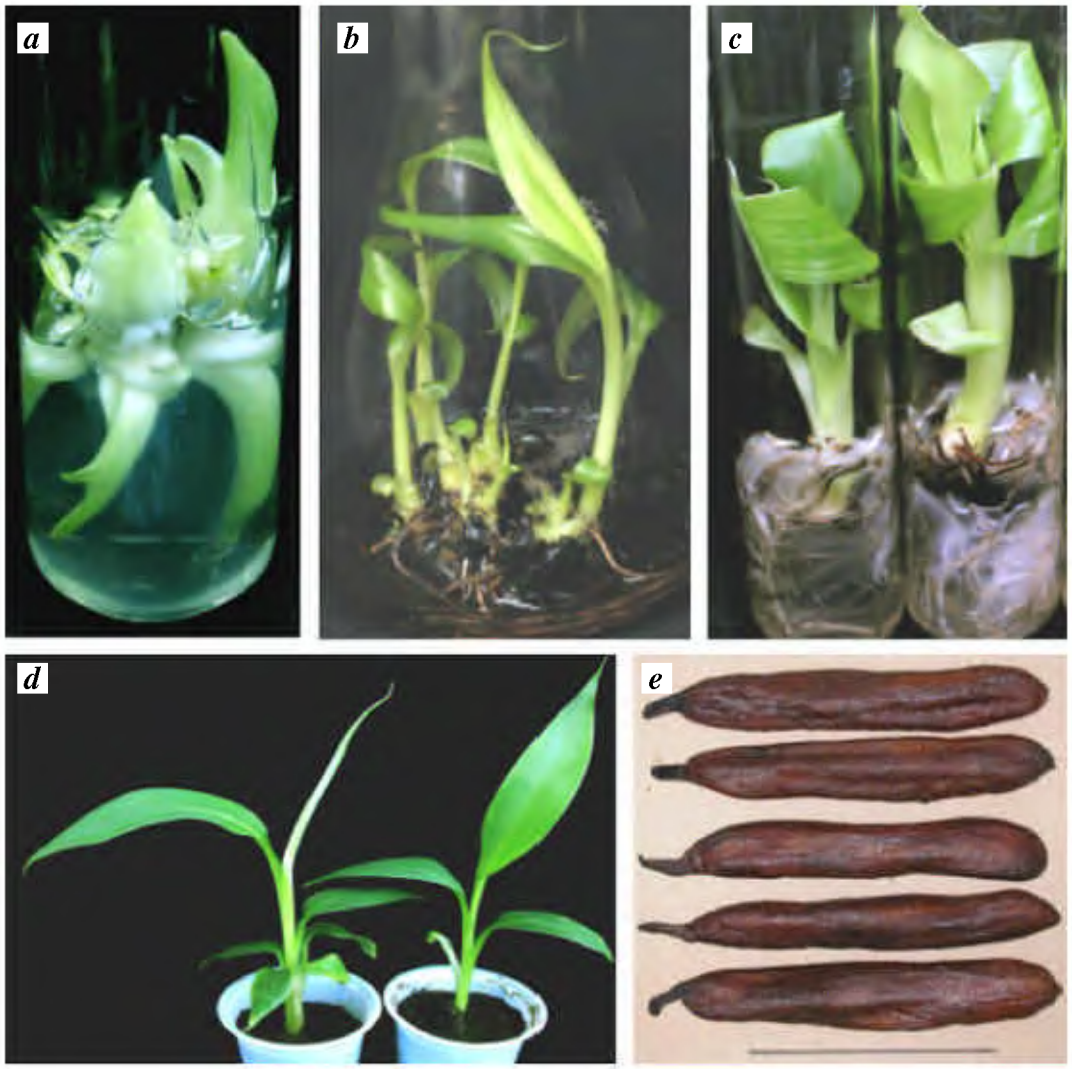

Figure 1. Regeneration of cv. Rajeli through multiple shoot formation. ***a***, Multiple shoots; ***b*** & ***c***, Rooted plants on liquid and Gelrite medium respectively; ***d***, Hardening of rooted plantlets; ***e***, Dried bananas (Sukeli). Bar = 10 cm.

Table 1. Response of cv. Rajeli to plant regeneration through multiple shoot formation and somatic embryogenesis

Explant	Cultured explant (no.)	Responding explant (No.)	Plantlets generated		Survival in greenhouse (%)
			No.	Days	
Shoot tip	25	18 (72%)	227	100	100
Male inflorescence	288[a]	128[b] (44%)	406	120	95

[a]Sixteen male cones, 18 explants from each. [b]Responding explants that have yielded nodular/compact/embryogenic calluses.

for establishment of multiple shoot cultures. The suckers were thoroughly washed with tap water and the shoot apical region (about 2 cm in diameter) was excised and washed with liquid detergent followed by several washes with distilled water. The explants were surface-sterilized by sequential treatment with commercial chlorine bleach (5% w/v, 15 min), 70% ethanol (8 min) and 0.1% $HgCl_2$ (7 min) under aseptic conditions. Each treatment was followed by minimum four rinses with sterile distilled water and excision of few sheathing leaves and part of corm tissue. Finally, the shoot apices (trimmed to a size of 1.0–1.5 cm, with minimum basal corm tissue) were cultured on a filter paper support on MS[8] liquid medium supplemented with 8.9 µM benzylaminopurine (BAP), 222.06 µM adeninehemisulphate (AH) and 3% sucrose. After 10 days of initiation, the shoot tips were sectioned vertically and subcultured on proliferation medium (MS + 8.9 µM BAP + 222.06 µM AH + 3% sucrose). Multiple shoots that formed were maintained by subculturing at an interval of 3–4 weeks, on the medium of same composition, either gelled (Figure 1 *a*) or in liquid (Figure 1 *b*). Rooting of the shoots (Figure 1 *c*) was readily achieved by placing the excised individual shoots on MS medium supplemented with 5.37 µM naphthaleneacetic acid (NAA) and 1% sucrose.

Exuberant multiple shoot formation (3–4 shoots per culture) and elongation were noticed in 72% of the cultures initiated (Table 1). Excision of maximum possible corm tissue was essential for controlling excessive release of phenolic compounds, thus obtaining better response from explants during initiation. The surviving cultures were required to be transferred to fresh medium frequently (every 10–12 days) during the initial phase. Alternatively, these could also be grown in liquid medium for reducing browning due to phenolic compounds. Once established, the multiplication ratio was fairly stable and varied between 3.0 and 3.5. At this stage, the subculturing interval was increased to 3–4 weeks. During all the stages, viz. initiation, multiplication and rooting, Gelrite was found to be a suitable gelling agent.

The fresh male flower cones were collected from Rajeli plants at the time of complete bunch emergence and cultured according to procedures described in a previous report[5]. Outer enveloping bracts were removed and surface-cleaned with absolute alcohol. Under laminar airflow, the remaining outer enveloping bracts were removed until the inner part (2–3 cm in length) containing male flower primordia (from 1 to 18, 0 being the meristem) was isolated. Individual male flower primordia were excised and cultured on MS medium supplemented with 18.10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5.37 µM NAA, 5.71 µM indole 3-acetic acid (IAA), 4.09 µM *d*-biotin and 3% sucrose for initiation of callus. Proliferation of callus and somatic embryo induction was induced on MS medium supplemented with 1 mg/l *d*-biotin, 4.52 µM 2,4-D, 100 mg/l malt extract and 4.5% sucrose, at pH 5.3. For somatic embryo conversion, MS medium supplemented with 2.22 or 8.9 µM BAP and 3% sucrose was employed. Subsequent plant development was achieved on the medium supplemented either with 5.37 µM NAA or no growth regulators, and 3% sucrose.

The pH of all the media was adjusted to 5.7. The media were gelled with 0.2% Gelrite and autoclaved at 15 lb pressure for 20 min. All the cultures for initial callus induction were maintained under dark conditions and others were incubated under light (12 h photo-period with 1000 lux) at 25 ± 2°C.

Male flower buds cultured on induction medium exhibited swelling of the floral primordia within the first 3–4 weeks, followed by the development of whitish callus after another 6–8 weeks (Figure 2 *a*). A total of 288 explants derived from 16 floral buds yielded 44% callus induction (Table 1). The callus comprised compact, nodular, embryogenic regions and non-embryogenic, loose, cellular mass. The embryogenic callus often showed somatic embryos on the surface, mostly at the early stages of embryo development (Figure 2 *b*). The embryogenic tissues showed good proliferation during subcultures on MS medium supplemented with 1 mg/l *d*-biotin, 4.52 µM 2,4-D and 100 mg/l malt extract. Somatic embryos transferred to conversion medium added with 2.22 µM or 8.9 µM BAP developed further into plantlets (Figure 2 *c*, *d*) at the frequency of 15% compared to medium without BAP (2–3%). Subsequent transfer of these plantlets to medium without any growth regulators or with NAA showed complete plantlet development (Figure 2 *e*).

Plantlets were carefully removed from the culture tubes and gently washed under running tap water to remove adhering pieces of gelled medium. These were then transferred to polythene bags filled with autoclaved mixture of soil and Soilrite (commercial hardening mixture) and were maintained in the greenhouse for 3–4 months, under natu-

ral light with relative humidity of 90–100% at ambient temperature of 25°C. The plants derived through multiple shoots (Figure 1 *d*) exhibited no mortality, while those coming from somatic embryogenesis (Figure 2 *e*) showed < 5% mortality during the hardening phase (Table 1). A total of 633 plants (227 and 406 each coming from multiple shoots and somatic embryogenesis respectively) have been regenerated and hardened. Tissue-culture plants of 50 cm height were field-planted in the experimental block with 100% survival. Plant development was observed to be normal during the hardening phase and no morphological aberrations have been detected till date.

The development of an *in vitro* regeneration system is an integral part and an essential prerequisite for studies related to propagation, conservation and genetic improvement. In the present study, *in vitro* regenerable systems were developed for banana cv. Rajeli. The rate of multiplication of shoots was comparable to that of the genotypes with B genome earlier reported from this laboratory[9]. Culture initiation and subsequent shoot proliferation, however, were not comparable to that of the Cavendish types. This could be explained on the basis of presence of the B genome in cv. Rajeli. Cultivars with A genome respond better to *in vitro* shoot proliferation[9] and, B genomes for callus formation and somatic embryogenesis (unpublished data).

Somatic embryogenesis is a well-established[6,10–12] method in case of banana. However, a large number of banana genotypes still need to be screened for exploring their embryogenic potential for mass production and genetic improvement. Developing protocols for *in vitro* plant regeneration for cultivars like Rajeli will support efforts in conservation and for generating transgenic plants with desirable traits aimed at the improvement of Rajeli. The inflorescence culture system reported herein demonstrates the potential for use in raising highly proliferative, embryogenic cultures for banana cv. Rajeli.

Conceptually, bananas have been thought to be suitable for the expression of therapeutic proteins to serve as edible vaccines[13,14], and transgenic banana plants containing the Hepatitis-B surface antigen coding *s* gene have already been reported from our laboratory[15]. A short post-harvest

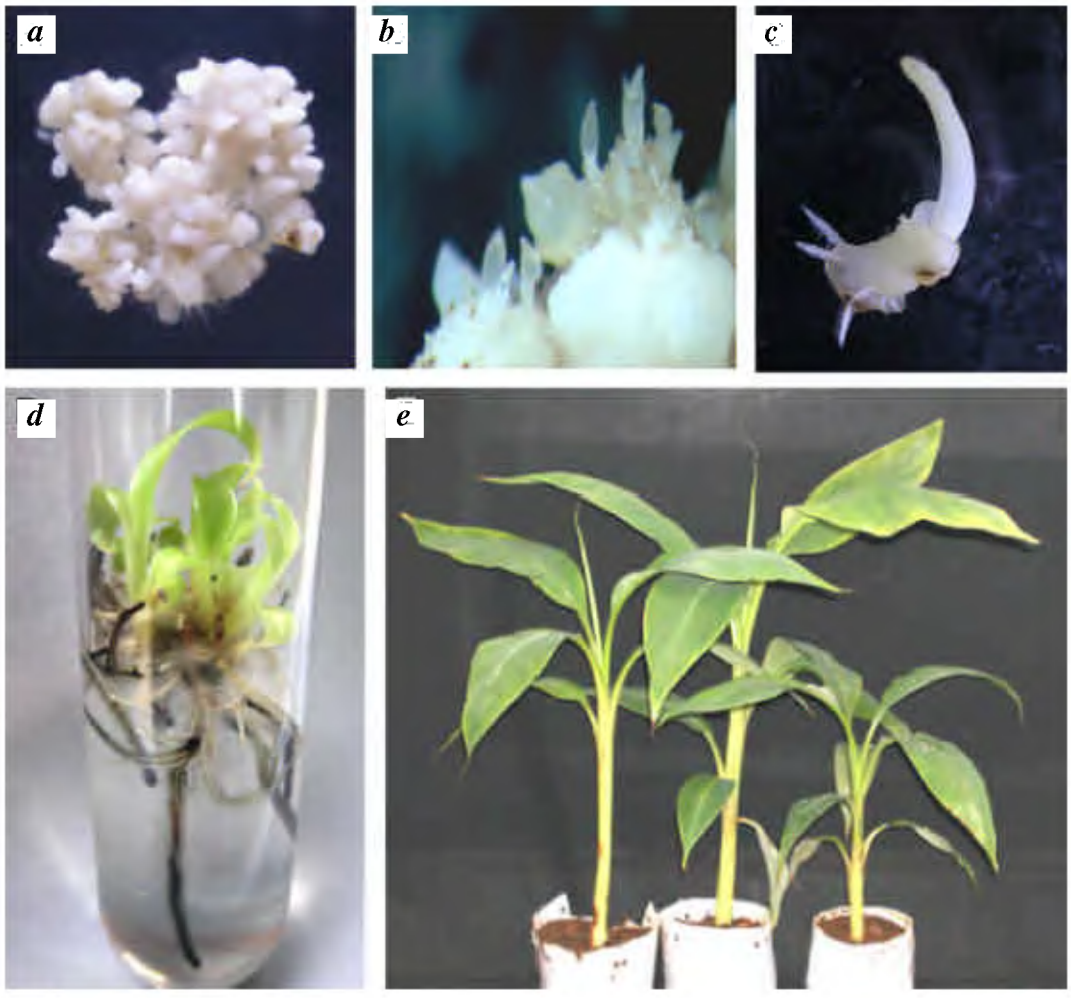

Figure 2. Somatic embryogenesis and plant regeneration from male flower buds of cv. Rajeli. ***a***, Embryogenic callus; ***b***, Somatic embryo formation; ***c***, Single well-developed somatic embryo; ***d***, Plant conversion from somatic embryos; ***e***, Hardened plants.

shelf-life of bananas may, however, pose a major constraint in practically launching transgenic bananas as edible vaccines. The banana varieties which abundantly regenerate *in vitro* and in which the 'transgene products' can be stored for long, are an ideal system for the expression of therapeutic protein genes. It is well known that proteins retain structural integrity and functionality during the dehydration process. Further, the therapeutic protein 'Hepatitis-B surface antigen', is reported to be thermostable[16]. Rajeli seems to be a suitable choice from both these points of view, since the therapeutic proteins can possibly be specifically expressed in maturing bananas and shelved for long in dried form. Such a facility of extended storage either in the dried form or via any other suitable method such as banana juice[17], would offer benefit to researchers and manufacturers for assaying, processing and distributing the therapeutic proteins to remote destinations at convenient time.

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Characterization of oviposition attractants of *Helicoverpa armigera* in two solanaceous plants, *Solanum viarum* and *Lycopersicon esculentum*

R. Srinivasan[1,2], S. Uthamasamy[2] and N. S. Talekar[1,*]

[1]AVRDC – The World Vegetable Center, Shanhua, Tainan 74151, Taiwan, Republic of China
[2]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore 641 003, India

The role of host plant chemicals in oviposition of *Helicoverpa armigera* Hübner (Lepidoptera : Noctuidae) was studied in two solanaceous plants, *Solanum viarum* Dunal. and *Lycopersicon esculentum* Mill. Plant volatiles as well as chemicals extracted from leaves were bioassayed for oviposition attraction in a two-choice olfactometer, where mated adults were given equal opportunity for oviposition either on plant extract side or solvent check side. Two fractions of microwave-assisted extracts from leaves of both plants elicited strong oviposition response. Normal alkanes, 13,17,21-trimethylheptatriacontane and octacosane were the only chemicals present in these fractions of *S. viarum*, whereas besides these chemicals, few other *n*-alkanes and related primary alcohols and aldehydes were present in tomato foliage. Oviposition attractants were also present in volatiles of both plants. Two fractions from *S. viarum* containing several small molecular weight alkanes elicited strong ovipositional response. One of the two fractions of tomato volatiles that elicited moderate oviposition response contained predominantly 3-nitrobenzyl alcohol and minor amounts of 3-nitrobenzaldehyde, whereas the other that showed strong oviposition deterrent activity contained 3-nitrobenzyl alcohol and small amounts of docosane and trimethyldecane, in addition to an unknown compound.

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