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In-Vitro Technology for Genetic Conservation of Banana—A Case Study

NIRMALYA BANERJEE

Department of Botany, University of Calcutta, Calcutta

The present communication deals with the various in-vitro *techniques attempted to conserve the germplasm of banana. The major aspects included are rapid clonal propagation through meristem culture of several cultivars belonging to different genomic constitutions, their medium-term storage under minimal growth condition and finally somatic embryogenesis. Rapid* in-vitro *multiplication of banana has been achieved by culturing the explants in Murashige and Skoog's semi-solid basal medium supplemented with low concentration of indole acetic acid and a fairly high level of benzyl amino purine. The genomic constitution of the cultivar on one hand as well as the concentration of benzyl amino purine in the medium on the other hand seem to control the rate of multiplication of shoot buds. Eventually, the multiplication rate was greatly accelerated by increasing the concentration of benzyl amino purine in the medium. However, the presence of one or two 'B' genomes which came from* Musa balbisiana *causes an increase in the rate of multiplication as compared to the cultivars possessing only 'A' genome, from* Musa acuminata. *Regarding the* in-vitro *conservation of banana germplasm, established shoot tip cultures could be successfully stored for over two years in the same container without any transfer to fresh medium by simply reducing the incubation temperature and light intensity from 30° to 15°C and 1000 lux respectively. The survival percentage of various cultivars at different stages of conservation have been critically analysed. The regeneration of somatic embryos through the induction of callus and their further studies have been briefly described in the last phase of the paper. The somatic embryos, when carefully analysed histologically, exhibited striking similarities with their zygotic counterparts.*

Conservation of germplasm of banana means collection, proper identification of genomic constitution of the accessions, multiplication and finally their maintenance in a disease-free condition at national, international and regional levels. There are some aspects like maintenance of genetic stability and disease indexing which are also closely associated with the term conservation of germplasm.

Both dessert and cooking bananas (*Musa* species) are considered as a major staple food in the tropical and sub-tropical parts of the world, particularly in Africa, Central America and South-East Asia. The commercially grown bananas have evolved by various mechanisms from two species of *Musa* viz., *M. acuminata* and *M. balbisiana* carrying the so called 'A' and 'B' genomes, respectively. The whole new range of edible cultivars are mostly triploids and sterile. As far as the conservation of banana germplasm is concerned, there are two possible approaches to achieve this goal. One approach is through *in-situ* conservation and the other is through *in vitro* conservation. For *in-situ* conservation, a number of attempts have been made to increase the multiplication rates using various conservation techniques (Barker, 1959; Hamilton, 1965; Ascenso, 1967). Barker (1959) obtained an increased rate of multiplication of banana by stripping the older leaf sheaths from the pseudostem, thereby exposing the corresponding buds which were then covered with soil. In the field condition, banana plants show a strong apical dominance which means, as long as the mother plant is present, it will suppress the development of its lateral shoots. Immediately after the removal of the mother plant, the lateral shoots start elongating. Using this property, De Langhe (1961) was able to increase the production of plants simply by destroying the apical bud of the mother plant. All these conventional approaches have some serious limitations and disadvantages. Some of these are: (1) plants kept in the field are fully exposed to diseases like 'bunchy top', 'panama disease', 'black sigatoka' etc. and also to natural calamities like drought, flood, etc; (2) the rate of multiplication is not sufficiently high to meet the demand of the growing world population; and (3) a large number of germplasm requires lot of space and manpower for proper conservation in the field.

To overcome these disadvantages, scientists in the late seventies adopted *in vitro* approaches by which banana germplasm can be conserved with high multiplication rate in a much smaller area (Cronauer and Krikorian, 1984; Banerjee and De Langhe, 1985; Jarret *et al.*, 1985; Wong, 1986; Banerjee *et al.*, 1986; Vuylsteke and De Langhe, 1985; Banerjee *et al.*, 1987, Banerjee and Sharma, 1988).

In the present paper, three different approaches will be discussed regarding the conservation of 15 commercial cultivars of banana and plantain belonging to five genomic constitutions (AA, AAA, AAAA, AAB and ABB). These are :

(a) shoot tip culture at normal temperature,

(b) shoot tip culture at reduced temperature, and

(c) somatic embryogenesis through callus induction.

MATERIALS AND METHODS

The cultivars of banana and plantain belonging to various genomic constitutions which have been used in the present investigation are listed in Table 1.

TABLE 1. LIST OF BANANA CULTIVARS WITH THEIR GENOMIC CONSTITUTIONS

Cultivar	Genomic constitution
Pisang Lilin	AA
Pisang Tongat	AAA
Musa acuminata	AAA
Dwarf Cavendish	AAA
Silk	AAB
Valeri	AAB
Mululou	AAB
Asamiensa	AAB
Ntanga	AAB
Agbagba	AAB
Pisang Abu Perak	ABB
Espermo	ABB
Saba	ABB
Monthan	ABB
IC-2	AAAA

Meristem culture technique

For isolation of shoot meristems of banana, all possible meristem bearing plant parts e.g. suckers, peepers, dormant eyes as well as the parental pseudostems were used. Cubes of tissues measuring 2-4 cm were first surface sterilised with 0.1 per cent mercuric chloride solution for 15 min. Tissues were then thoroughly washed with sterile distilled water and 1-2 mm^3 small shoot meristems with 1-3 leaf primordia were excised aseptically from the sterilised tissue. Explants were cultured on Murashige and Skoog's basal medium (Murashige and Skoog, 1962) containing 3 per cent sucrose and 10 mg/l ascorbic acid which was added to reduce the tissue and media blackening possibly caused by oxidation of polyphenols.

For shoot outgrowth, the basal medium was supplemented with 0.2 mg/l indole acetic acid (IAA) and 0.2 mg/l benzyl amino purine (BAP) and, for the proliferation of shoot buds, BAP level was raised to 2 mg/l keeping the IAA level constant. For the induction of roots, 0.2 mg of indole butyric acid (IBA) was used in the medium with half strength of macrosalts. The cultures were incubated at 25°C under 16 h photoperiod of 3000 lux light intensity.

Low temperature storage

For low temperature, rapidly proliferating established shoot tip cultures were used. Tissues were kept in MS semi-solid medium supplemented with 0.2 mg IAA and 2 mg BAP. Three different low temperatures (5°, 10°, and 15°C) were tried to find out an optimum temperature suitable for medium-term storage. A constant illumination of 1000 lux intensity was given to the cultures.

Somatic embryogenesis

For the induction of callus, thin meristematic layers from the rapidly proliferating meristem cultures were carefully excised and cultured on MS basal medium

supplemented with various concentrations of 2, 4-D and 2, 4, 5-T. After induction, the callus was periodically transferred to liquid medium supplemented with different concentrations of NAA and BAP, IAA and BAP or no hormone for somatic embryogenesis. Bipolar embryos were histologically analysed and compared with their zygotic counterparts following standard microtome technique.

RESULTS AND DISCUSSION

Regeneration of plants

Small explants consisting of 2-3 leaf primordia turned green within 2-3 weeks of inoculation. Depending upon the concentration of BAP used in the medium, two distinct types of growth responses were noticed. With 0.2 mg/l IAA and BAP, single shoot outgrowth from excised tip was observed in all the tested cultivars. In this medium, the outermost leaf primordium slowly opened and unfurled the lamina within four weeks. Gradually, the inner leaf primordia emerged. Rooting of these young shoots could be achieved within two weeks in root induction medium where IBA (0.2 mg/l) was added as the only growth regulator. After root induction, small plantlets were transferred to flasks containing MS semi-solid medium with half concentration of inorganic salts supplemented with no growth hormones. Plantlets grew fast in this last phase of development (Plate I Fig. 1). Finally they were transferred to soil with 70-80 per cent survival (Plate I Fig. 2).

Proliferation of shoot meristem

Proliferation of meristems could be achieved by culturing the isolated explants on MS semi-solid medium supplemented with IAA (0.2 mg/l) and BAP (2 mg/l). A sharp difference in the rate of proliferation as well as in the proliferative growth was noticed in different cultivars depending upon the genomic constitution. In the diploid (Pisang Lilin), triploid (Dwarf Cavendish) and tetraploid (IC-2) cultivars possessing only A genome, the rate of meristem proliferation was rather low which was manifested by the formation of a bunch of leafy shoots (Figs. 3 and 4) while the hybrid types with AAB (Asamiensa) and ABB (Bluggoe), the proliferation rates were high with the formation of fleshy, white bulbous structures each in turn, bearing a number of tiny meristems on their surface (Fig. 5). From the rates of proliferation recorded in each cultivar from first to fifteenth subculture, the cultivars with ABB genomic constitution exhibited very high rates which went upto 30-35 tips/explant. On the contrary, diploid, tetraploid and triploid *acuminata* types showed low rates ranging from 5-10 tips/explant. The AAB cultivars showed intermediate rates, usually ranging from 10-25 tips/explant. It was proved statistically by two-way Anova test that a high level of significant difference exists in the rates of proliferation between different cultivars in the same subculture and between subcultures of the same cultivar (Banerjee and Sharma, 1988).

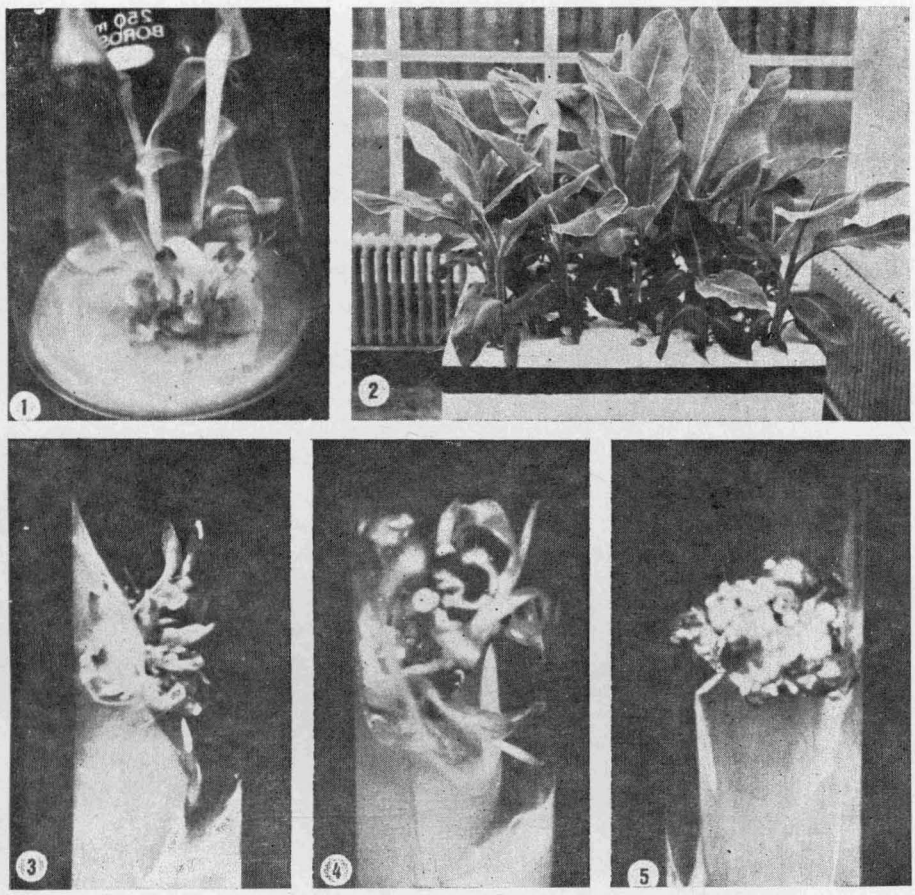

Plate I Fig. 1. Regeneration of rooted plantlets of *Musa* cv. Espermo (ABB).

Fig. 2. *In-vitro* plantlets transferred to soil.

Fig. 3. Proliferation of shoot-tips in *Musa* cv. Dwarf Cavendish (AAA) possessing only 'A' genome :

Fig. 4. Proliferation of shoot-tips in *Musa* cv. Pisang Linn (AA) possessing only 'A' genome

Fig. 5. Proliferation of shoot meristems in *Musa* cv. Espermo (ABB).

In spite of the significant difference in the proliferation rates between various genotypes, the regeneration potential for each cultivar did not show any significant decrease with increase in the age of culture. In other words, complete plantlets can be readily regenerated from each of these tiny meristem tips regardless of the age of the culture.

One very interesting feature that has been noticed regarding the influence of B genome on the acceleration of proliferative capacity was that the increase in dosage of B genome as in 'Bluggoe' leads to further acceleration of proliferative

capacity as compared to that of the cultivar 'Silk' (AAB), whereas in cultivars with A genome, the addition of A does neither retard nor accelerate the rates (Banerjee and Sharma, 1988).

As far as the ontogenesis of shoot meristem proliferation is concerned, these multiple buds developed from a uniform meristematic layer of cells which originated through rapid extension and subsequent flatening of the initial meristematic dome. These newly generated shoot primordia repeated the same ontogenetic pathway and ultimately gave rise to a number of primordia. This process continued as long as the tissues were kept in shoot proliferation medium (Benerjee *et al.*, 1986).

In-vitro storage

There are several possible approaches to achieve minimal growth of cultures. The most important ones are manipulation of culture medium, addition of growth retardants like ABA and incubation of cultures at reduced temperature and low light intensity. One of the principal objectives of storage experiments would be to slow down the growth rate and extend the subculturing intervals from normal 4-6 weeks to a much-longer period, for example, once a year or more.

In the present investigation, attempts have been made to store the meristem cultures of several cultivars of banana having various genomic constitutions at different reduced temperatures, e.g. 5°, 10°, 15°C. The cultures were kept in the shoot proliferation medium in presence of 1000 lux light intensity. The problem of medium dessication has been overcome by sealing the plugs with parafilm without any harmful effect.

One of the three reduced temperatures, (5°C) was found too cold for banana and as a result none of the cultivars survived beyond 6-8 weeks. At 10°C, the cultures survived longer but even this temperature was not very suitable for medium-term storage. The most satisfactory result was achieved at 15°C where the cultures remained healthy for 15-18 months depending on the genomic constitutions of the cultivars. Thus, it was observed that AAB and ABB cultivars were more tolerant to low temperatures than the cultivars with only A genome. After 14-16 months of continuous storage at 15°C, AAB and ABB cultivars showed a survival percentage of 66-100 per cent while the survival percentage of A type cultivars came down to 25-35 per cent.

In the last two decades, a number of research papers have been published on the progress of *in-vitro* storage of several plants, mostly temperate species, using different storage techniques. Medium term storage has been successfully achieved using low temperature incubation technique in various plant species, such as strawberry 6 years (Mullin and Schlegel, 1976), apple 12 months (Lundergan and Janick, 1979), potato (Westcott, 1981), *Lolium multiflorum* (Dale, 1980), *Trifolium pratens* (Cheyne and Dale, 1980), *Trifolium repens* (Bhojwani, 1981). Although reduced temperature in the range of 5°-10°C have been found suitable for temperate species, meristem cultures of tropical species like sweet potato

(*Ipomoea batatas*) show considerable chilling injury at these temperatures. However, satisfactory reduction in growth rate has been achieved at 15°-18°C (Alan, 1979). These results are in agreement with our results where meristem cultures of different genotypes of *Musa* suffer from chilling damage during incubation at 5°-10°C. However, some other tropical crops like *Colocasia esculantum* have been successfully stored *in-vitro* through meristem culture at 9°-10°C for three years (Staristsky *et al.*, 1986).

Somatic embryogenesis

In banana, the induction of callus is extremely difficult, simply because the explant tissue which may be leaf, shoot, root or even flower bud turns black when cultured on semisolid or liquid medium containing growth regulators. The blackening and subsequent killing of the explant is presumably caused by heavy amount of oxidised polyphenols released by the tissue itself. Addition of antioxidants in the medium does not help.

To overcome this problem of blackening, thin meristematic layers of cells were taken from the meristem cultures as explant for the induction of callus since meristem cultures after few passages show much less blackening. Several concentrations of 2, 4-D were tested for the induction of callus and the best results were obtained with the concentration 1.25 mg/1. The calli induced after 4-6 weeks exhibited slow growth initially. On prolonged incubation in the same medium, numerous globular proembryo-like structures were formed. The proembryos were sloughed off from the rest of the callus mass when transferred to liquid medium. After 2-3 weeks, these proembryos turned green but no leaf development was observed.

On the other hand, explants when cultured on MS semi-solid medium supplemented with 2, 4, 5-T (1.25 mg/1) produced numerous globular proembryoid on the surface of the callus after 7-9 weeks. Transferring them in liquid culture medium containing NAA (0.02 mg/l) and BAP (0.25 mg/l) yielded bipolar embryos within 2-3 weeks. In these somatic embryoids, both shoot and root development could be observed. Histological analysis of the somatic and zygotic embryos revealed some interesting results. The seed embryos were characteristically mushroom shaped where the cap-like structure was the haustorium which constituted the major part of the cotyledon. The stalk-like part represents the epicotyl-hypocotyl-radicle axis. The most interesting thing is that the shoot and root apical meristems always lie at an angle of more or less 90° which is a characteristic feature of the family *Musaceae*. The somatic embryos also revealed bipolarity with distinct root and shoot meristems connected by strands of vascular tissues. They are characteristically enclosed by an epidermal layer of cells. Moreover, the hook-like formation of shoot and root meristem axis was clearly observed in these embryoids (Banerjee *et al.*, 1987).

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