Micropropagation for Production of Quality Banana Planting Material in Asia-Pacific

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FOREWORD

Banana and plantains are the second largest food-fruit crops of the world produced in the tropical and subtropical regions of mostly the developing countries. The two together are positioned fourth in terms of gross value. During recent years, growth of banana cultivation has witnessed great strides reaching 95.60 million tonnes in 2009 from 66.84 million tonnes in 2001. Adoption of Panama disease resistant Cavendish varieties in place of Gros Michel; mechanization in production; efficient water management, harvest and postharvest handlings; and integrated pest and disease management have helped in this growth. Adoption of high quality, disease free planting material developed through tissue culture has been an important addition during recent years.

Asia-Pacific countries, particularly India, China and Philippines, are the major players in banana cultivation, contributing more than 45 % of the total world production. The area expansion has, however, been accompanied by rampant spread of pests and diseases, most important among which are viral diseases transmitted through non-indexed planting material. Thus, the need for production of disease free, quality planting material is being felt more than ever before not only in the Asia-Pacific but in other regions as well. Adoption of clean tissue culture based banana planting material by small holder farmers in Kenya is a well documented success story. Similar successes have been achieved to various degrees in India and Philippines, besides Australia. One of the important examples in India has been the realization of up to 120 t/ha productivity under the FAO and Government of India supported demonstration orchards. However, large scale adoption of tissue culture planting material for banana production is rather limited in the Asia-Pacific region due to several constraints, including high cost and sometimes poor quality of planting material. There is a need to address these issues so that the expected benefits of tissue culture technology reach the small holders farmers.

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB) an important program of APAARI, has the mandate to promote modern technologies and tools for the benefit of agriculture in the region. One of the activities of APCoAB is to bring out status reports of biotechnological applications that have

proved useful in the region. The present publication, "Micropropagation for Production of Quality Banana Planting Material in Asia-Pacific" is one such report in this series after those on potato and sugarcane brought out in recent past. I congratulate the authors for compiling this detailed report on production of disease free planting material of banana with interesting examples from Australia, India, Philippines and Taiwan. I am sure this publication will be useful to the scientists and entrepreneurs interested in establishing banana tissue culture infrastructure as well as to the farmers interested in growing banana as a commercial crop for higher productivity and profitability.

(Raj Paroda)
Executive Secretary
APAARI

CONTENTS

ACRONYMS AND ABBREVIATIONS

APAARI	:	Asia-Pacific Association of Agricultural Research Institutions
APCoAB	:	Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB)
ATL	:	Accredited Test Laboratory
BaMMV	:	*Banana mild mosaic virus*
BAP	:	Benzyl aminopurine
BAPNET	:	Banana and Plantain Network
BBrMV	:	*Banana bract mosaic virus*
BBTV	:	*Banana bunchy top virus*
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
BCIL	:	Biotech Consortium India Limited
BSV	:	*Banana streak virus*
BVX	:	*Banana virus X*
cDNA	:	Complementary deoxyribonucleic acid
CGMV	:	*Cucumber green mottle virus*
CMV	:	*Cucumber mosaic virus*
cRNA	:	Complementary ribonucleic acid
dNTP	:	Deoxynucleotide triphosphate
DAC-ELISA	:	Direct antigen coating - enzyme linked immunosorbent assay
DAS-ELISA	:	Double antibody sandwich - enzyme linked immunosorbent assay
DB-PCR	:	Direct-binding PCR
DBT	:	Department of Biotechnology
DEPC	:	Diethyl pyrocarbonate
DIBA	:	dot-blot immunoassay
DIG	:	Dig-oxigenin
DLDV	:	*Datura leaf distortion virus*
EBIA	:	Electro blot immuno assay
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme linked immunosorbent assay
FAO	:	Food and Agriculture Organization of the United Nations
G_1S	:	First generation suckers
G_3S	:	Third generation suckers
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
ICAR	:	Indian Council of Agricultural Research
IC-PCR	:	Immuno capture polymerase chain reaction
IC-RT-PCR	:	Immuno capture real time polymerase chain reaction

IEM	:	Immuno electron microscopy
INIBAP	:	International Network for the Improvement of Banana and Plantains
ISEM	:	Immunosorbent electron microscopy
Mabs	:	Monoclonal antibodies
M-IC-PCR	:	Multiplex immuno capture polymerase chain reaction
MoA	:	Ministry of Agriculture
MOPS	:	Morpholinopropansulfonic acid
MoST	:	Ministry of Science and Technology
MS	:	Murashige and Skoog medium
NAA	:	Naphthalene acetic acid
NARS	:	National Agricultural Research System
NASH	:	Nucleic acid spot hybridization
NBT	:	Nitro blue tetrazolium chloride
NCM	:	Nitro cellulose membrane
NCS-TCP	:	National Certification System for Tissue Culture Raised Plants
NRCB	:	National Research Centre for Banana
PAS-ELISA	:	Protein-A coated antibody sandwich trapped enzyme linked immunosorbent assay
PBS-T	:	Phosphate buffered saline Tween 20
PBS	:	Phosphate buffered saline
PBS-TPO	:	PBS - T polyvinyl pyrrolidone and ovalbumin
PCR	:	Polymerase chain reaction
PDO	:	Polyvalent degenerate oligonucleotide
PDORT-PCR	:	Polyvalent degenerate oligonucleotide polymerase chain reaction
pNPP	:	p-nitrophenyl phosphate
PTA-ELISA	:	Plate trapped antigen enzyme linked immunosorbent assay
PVP	:	Polyvinyl pyrollidone
QBAN	:	Quality Banana Accredited Nursery
RT-PCR	:	Real time polymerase chain reaction
SDM	:	Spray dried milk
SDS	:	Sodium dodecyl sulphate
SFAC	:	Small Farmers' Agri-Business Consortium
SSC	:	Standard saline citrate
STMS	:	Sequence tagged microsatelite site
TAE	:	Tris acetic acid
TAS-ELISA	:	Triple antibody sandwich enzyme linked immunosorbent assay
TBRI	:	Taiwan Banana Research Institute
TERI NEH	:	The Energy Research Institute – North Eastern Hill
TNAU	:	Tamil Nadu Agricultural University

1. INTRODUCTION

Banana and Plantains (*Musa* spp.) are some of the earliest crop plants having been domesticated by humans. Bananas are consumed as ripe fruit, whereas plantains, which remain starchy even when fully ripe, need cooking for palatability and consumption. Originally crops from humid tropics, they have acclimatized to a broad range of climatic conditions. While bananas have come to occupy the status of a high value, commercial crop, plantains have remained a staple food of many ethnic groups. Irrespective of their commercial status, banana and plantains are referred as 'Poor man's apple'.

Banana is globally ranked fourth, next to rice, wheat and maize in terms of gross value of production. It is a major staple food crop for millions of people as well as provides income through local and international trade. Among the starchy staple food crops, banana ranks third with respect to the total production. Though cassava and sweet potato are positioned as first and second, banana and plantain have almost equal importance in all the tropical regions of the world. Traditional bananas and other species of family *Musaceae* have been the major calorie source of many ethnic tribes of Africa and Pacific Islands. High consumption of bananas has been reported in small countries of Pacific Islands like Samoa (132 Kcal) and Vanuatu (92 Kcal). Bananas also find importance in the diet of Caribbean (Haiti and Dominican Republic) and Latin American countries (Ecuador and Brazil).

Presently, banana is grown in around 150 countries across the world on an area of 4.84 million ha producing 95.6 million tonnes (Table 1). Asia, Africa and Latin America are the major banana producing continents (Fig. 1). Among the major producers, India alone accounts for 27.43 % (26.2 million tonnes) followed by Philippines, producing 9.01 million tonnes and China, Brazil and Ecuador, with production ranging from 7.19 to 8.21 million tonnes (Table 1). Production in India, China and Indonesia has witnessed giant leaps in the last eight years. In the last five decades, the per cent contribution of the African continent has witnessed marginal reduction from 12 % to 9 % while the decline has been prominent in Latin American countries, from 40 % to 30 %. This is mainly due to the Asian countries achieving substantial increase from 38 to 60 % (Fig. 1). Plantains are mainly grown in Cameroon, Columbia, Costa Rica and Ecuador with the total world production in 2009 being 34.32 million tonnes (FAO STAT 2001). In

banana trade, Ecuador, Costa Rica, Philippines and Columbia alone contribute 64% of the world's banana export. While Philippines along with Taiwan derive substantial earnings from banana export, the great bulk of bananas produced in Asian region is traded and consumed in domestic markets.

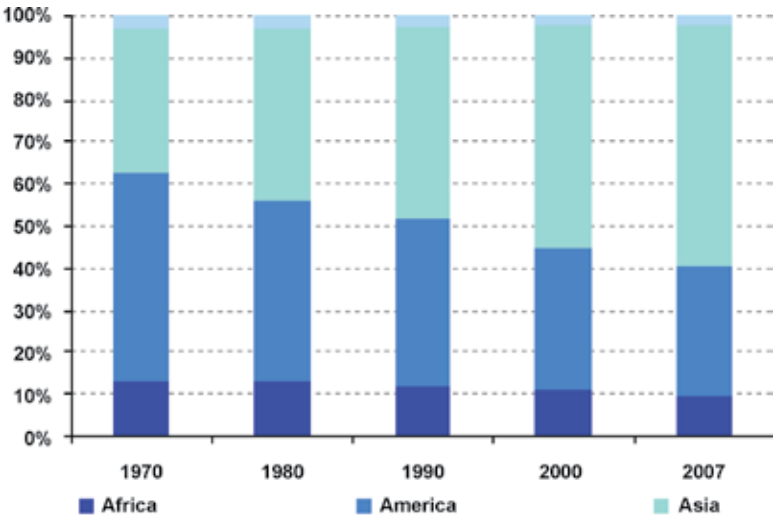


Fig. 1. Distributors of the world banana production by the continent. Source: www.unctad.org

Unlike in temperate countries, where Cavendish clones predominate, Asian and Pacific consumers are offered a wide

Table 1. Area, production and productivity of banana in major banana producing countries

Country	Area (000 ha)		Production (million tonnes)		Productivity (t/ha)		% global Production
	2001	2009	2001	2009	2001	2009	2009
Brazil	510.29	511.64	6.18	7.19	12.10	14.06	7.52
Cameroon	66.35	86.00	0.63	0.82	9.52	9.53	0.87
China	253.95	311.11	5.48	8.21	21.56	26.38	8.59
Columbia	51.06	74.11	1.46	2.02	28.78	27.26	2.11
Costa Rica	44.52	42.59	2.06	2.13	46.30	49.94	2.23
Ecuador	228.99	216.12	6.08	7.64	26.54	35.34	7.99
India	466.20	709.00	14.21	26.22	30.48	37.00	27.43
Indonesia	277.00	105.00	4.30	6.27	15.52	59.74	6.56
Mexico	71.05	-	2.02	1.80	28.54	27.52	1.88
Philippines	386.50	446.40	5.06	9.01	13.09	20.19	9.43
World	4317.03	4843	66.84	95.60	15.48	19.74	

Source: FAOSTAT (2011); Kumar *et al.* (2010)

choice of banana and plantain cultivars of varying colors (yellow, red, orange and green), flavors, textures, sizes and shapes (Annexure I). Many cultivars are consumed

fresh as dessert fruit, while a great number of culinary varieties are used in hundreds of recipes based on cooking bananas and plantains.

Problems of Banana Production in Asia and the Pacific

Traditionally, banana is grown as a perennial crop where the plant is allowed to produce continuous shoots from a subterranean stem. But, the yields fall after three to five years and decline rapidly after ten to fifteen years. The need to shift to cyclic replacement with a new plantation comprising cycles of one crop and one ratoon has been realized only recently in most Asian countries. Natural calamities such as typhoons, floods, droughts and occasional volcanic eruptions cause devastating losses in banana production. The consequent need for fresh seedlings at regular intervals has led to very large increase in the demand for clean planting material.

Banana is vulnerable to a number of biotic and abiotic stresses which limit its production, particularly among small and marginal farmers with limited resources. BBTV (*Banana bunchy top virus*), CMV (*Cucumber mosaic virus*), BSV (*Banana streak virus*) and BBMV (*Banana bract mosaic virus*) are the four most important virus diseases affecting bananas. In addition, banana is an attractive host for nematodes, particularly *Pratylenchus coffeae, Meloidogyne incognita, Helicotylenchus multicinctus* and *Radopholus similis*. The pests also spread through transportation of non-quarantined planting material. Insect pests like, banana weevils (*Odoiporus longicollis,* and *Cosmpolites sordidus*) which till recently had a limited presence in some states of India have now spread to Bangladesh and beyond. Disease and pest pressure on Asian bananas is unlikely to lessen in the foreseeable future.

Development of tissue culture technology has been the foundation of high quality, disease free planting material production at a mass scale, particularly in vegetatively propagated crops. This publication provides detailed information on macro- and micropropagation procedures in banana along with disease detection and elimination techniques with the objective of facilitating banana growers and other stakeholders access to same useful technologies of healthy banana seedling production. It also analyses the current staus and future prospects of micropropagation for production of quality banana planting material in the Asia-Pacific region.

2. BANANA PROPAGATION

Banana is a crop with dual propagation abilities, sexual through seeds and asexual through suckers. Seed propagation is common in wild species which are diploid and undergo normal meiosis, fertilization and seed set (Figs. 2, 3). The extent of seed set, germinability and dormancy depends on the species. In *Ensete*, the only other genus of Musaceae, seed propagation is the only means of perpetuation since sucker production is absent. Seeds are generally brown to black in colour, 2-6 cm in diameter, round or triangular in shape, and mostly compressed in appearance (Fig. 3). Fruits of wild species are inedible, being full of seeds that are enveloped in thin mucilaginous pulp (Fig. 2). All cultivated commercial bananas are triploid and sterile, excepting a few parthenocarpic AA and AB diploids. Sucker propagation is the only natural means of their perpetuation; artificial methods of propagation include macropropagation and micropropagation. Sucker propagation and macropropagation are dealt in this chapter while micropropagation is detailed in Chapter 3.

Figs. 2 & 3. Wild banana fruit (Fig. 2) and seeds (Fig. 3).

2.1 Sucker propagation

There are two types of suckers, sword suckers – with a well developed base, pointed tip and narrow leaf blades, and water suckers, which are small, less vigorous, broad leaved and emerge in clumps (Fig. 4).

Natural regeneration of cultivated bananas through suckers is very slow due to hormone-mediated apical dominance of the mother plant. A plant produces only

Fig. 4. Sword (left) and water sucker (right)

5-20 suckers during its life time of 12-14 months. For accelerating the propagation rate, suckers with growing buds or cut rhizomes called 'bits' and 'peepers' are used. Several good bits, each with a centrally placed germinating eye can be cut from an unbunched rhizome after trimming the roots. Selection of appropriate mother plant for raising new propagules either through *in vivo* or *in vitro* methods is important.

About one kilogram uniformly sized rhizomes or bits, well-trimmed around the growing sprout are the best starting material. Although immediate planting is preferred, in some parts of India rhizomes are sun dried for 2-3 days after paring and pralinage treatment (trimmed of all roots, dipped in mud slurry and sprinkled with nematicide) and stored in shade for a week before planting.

Mother nursery block and selection of mother plants

- Mother plant should be healthy, true to type and free from diseases and pests, especially virus diseases.
- The male flowers buds should be retained to check the presence of virus diseases (male flower buds exhibit symptoms of late infection of viruses like BBTV and BBrMV).
- Mother plants should be raised under roofless insect proof shade net with sufficient height.
- Mother nursery must be located away from other banana plantations with an isolation distance of 500 m to maintain purity and to avoid spread of virus diseases.
- Mother plants should be grown under very good management conditions so as to facilitate the true expression of traits.
- Individual plants should be tagged with a master code number so that the plantlets developed could be traced back to the mother plant.
- Pedigree record and source of each mother plant should be maintained and catalogued.
- Once indexed, the mother suckers can be maintained in field or concrete rings with frequent decapitation to facilitate production of more axillary buds. They also serve as explants for culture initiation.

2.2 Macropropagation

Macropropagation is an excellent option for producing low cost quality planting material. This is a simple method because of the ease of multiplication, saves cost of producing planting material and has the potential of producing 50-60 shoots per sucker in 4-5 months. Macropropagation is achieved by two methods and could be adopted either in the field conditions (*in situ*) or in the nursery (*ex situ*). It involves, decapitation, decortication and hardening.

2.2.1 Decapitation

4-6 month old plant is headed back (Fig. 5), the pseudostem is cut down and cross cuts/ incisions are made on the growing meristem so as to stimulate the production of lateral buds. This method results in the production of 9-15 uniform shoots per plant in a short span of time and is highly suitable for small and marginal farmers whose requirements of planting material are relatively small. Suckers of choice varieties can be maintained in a nursery either in sawdust bed or in a big, bottomless concrete pot. The initial planting material should preferably be certified as virus free and multiplied at farm level under an insect proof net house.

Fig. 5. Decapitated sucker

2.2.2 Decortication

Fig. 6. Decorticated sucker

The pseudostem of the mother corm or sword sucker is cut transversely 2 cm above the collar region and then the apical meristem is removed leaving a cavity of 2 cm diameter and 4 cm depth (Fig. 6). This is done to overcome the apical dominance. Decapitation and decortication activate the lateral buds giving rise to more side shoots. Generally, the corms that have already flowered give better results than corms that have not yet flowered. Hence, healthy corms left in the field after harvesting are also a potential explant both for *in situ* and *ex situ* mass multiplication.

2.2.3 Ex situ mass multiplication

Sword suckers are pared partially (trimmed of all roots and the outer surface scraped) and the growing points are excised out with a sharp knife. These corms are surface sterilized by dipping in 0.3 % bavistin for 15 min, allowed to dry for a day and then planted in the initiation medium, usually comprising rice husk or sawdust, though the latter is much preferred. The initiation medium should essentially provide anchorage, moisture supply and proper aeration to the roots. Before use, the medium is moistened and decomposed for a period of 2-3 weeks to allow dissipation of the build up heat during decomposition. The medium could be enriched with sterilized vermicompost, biofertilizers and rooting hormones like IBA (2,500 ppm). 200 g of VAM/*Azospirillum* is mixed with 10 kg of vermicompost and 500 g of the foresaid mixture is applied per corm. This treatment induces the production of lateral buds through an enhancement in the population of *Pseudomonas* and *Aspergillus* species, while IBA induces rooting in the developing multiple shoots.

2.2.3.1 Secondary decortication

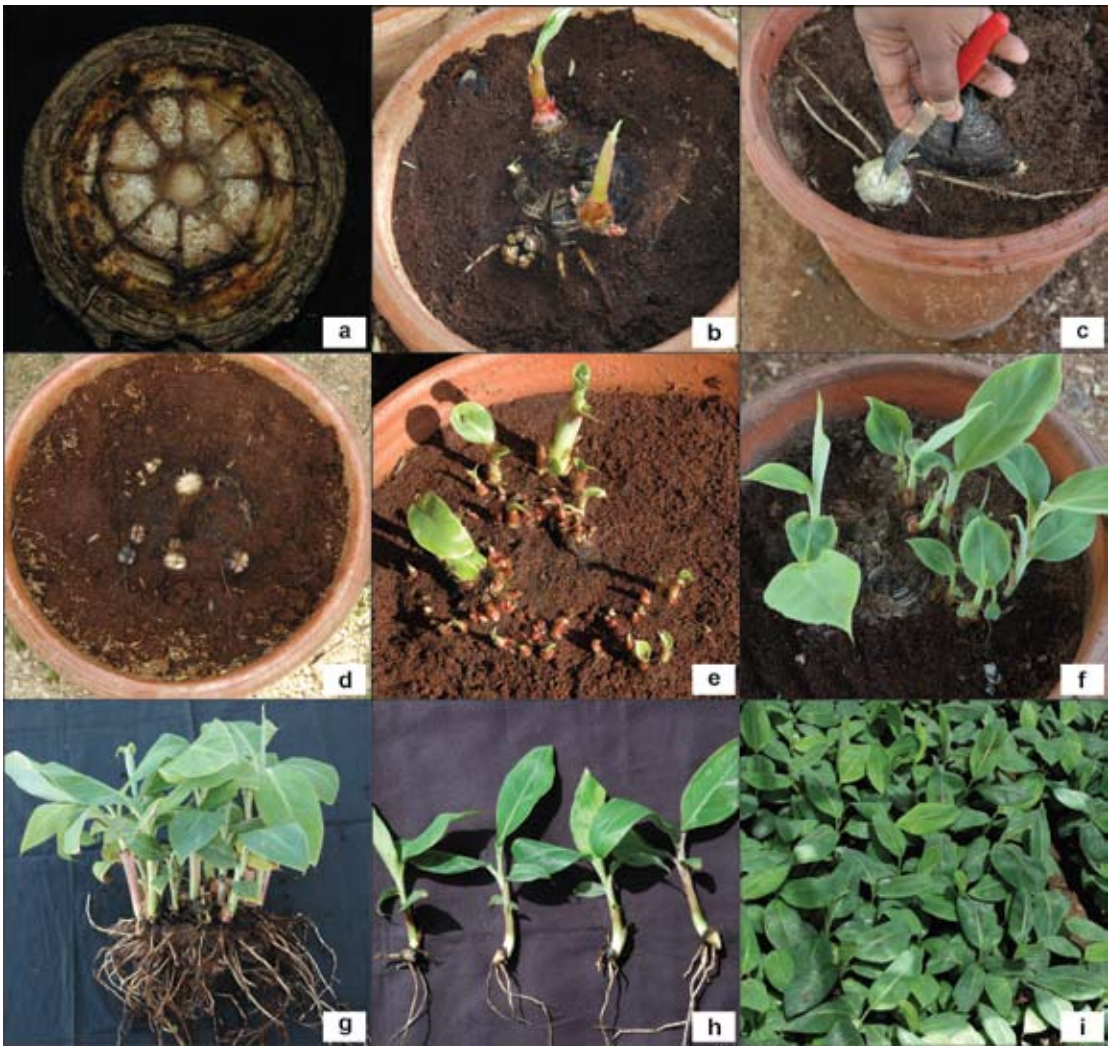
After 30-35 days of first decortication, 3-4 buds emerge from the mother corm, depending on the variety. When the side shoots attain a height of 15-20 cm with 3-4 leaves, secondary phase decortication is done by heading back with a sharp knife followed by 3-4 transverse cuts. This encourages production of multiple shoots. Third phase decortication is again attempted after 20-25 days but with greater care as the shoot buds are located very close. Thus by the end of 140-150 days, a total of 50-60 shoots are produced from a single sucker (Fig. 7).

2.2.3.2 Hardening

The lateral sprouts of 8-10 cm length are shifted to pro-trays containing equal parts of cocopeat and vermiculite and after sufficient watering left in a shade net (70 % shade) at 80-90 % humidity. High humidity is achieved by intermittent misting. Sprouts are usually maintained in the pro-trays for a period of 15-20 days and then shifted to polythene bags of size 6'x 4' and thickness of 120 gauge for secondary hardening. At this stage, the plants are maintained at 50 % shade and 40-50 % humidity. Watering is done on alternate days and the plants are ready for field planting in 30-45 days.

2.2.4 In situ mass multiplication

I*n situ* production of suckers is induced chemically by pouring 4 ml of 40 ppm BAP into the decorticated cavity and covering the individual mats with a mixture containing equal parts of sandy loam and poultry manure to 5 cm above the ground level. Such chemical induction of lateral buds could be done on the first generation



Figs. 7. Development of multiple shoots through macropropagation.

suckers and continued up to third generation suckers. This method leads to the production of 45-50 shoots in a short span months. The suckers are separated from the mother corm and subsequently rooted in sterile soil medium under intermittent misting.

3. MICROPROPAGATION FOR QUALITY BANANA PLANTING MATERIAL

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants under aseptic conditions using modern plant tissue culture methods. Application of micropropagation in banana has the following advantages:

a. Rapid multiplication

The rate of multiplication in banana is restricted to 5-20 suckers per plant during its growth period, which makes it difficult to obtain sufficient amount of planting material of a clone of choice. Micropropagation facilitates production of large number of plantlets/unit time, thus helping in rapid introduction and dissemination of new varieties.

b. Requirement of limited mother stock

The rapid multiplication technology ensures that limited number of mother plants are required for raising large number of progeny plants. These few mother plants can be maintained with required care at a limited cost.

c. Product uniformity

Being a vegetative reproduction method, micropropagation results a high degree of genotypic and phenotypic uniformity of the progeny plants. The limited variation seen sometimes can be overcome by following appropriate micropropagation, genetic fedility testing and rouging protocols.

d. Season independent production

In conventional field propagation, the production of suckers is highly season dependent and, hence, availability of planting material in a given season is often a limiting factor. The planting season in most of the banana production areas starts with the onset of monsoon, which creates a heavy demand for the planting material often leading to supply of substandard material. Using micropropagation, the production of planting material can be achieved as per needs.

e. Agronomic advantages

Micropropagated plants exhibit uniform growth and maturity enabling one time harvesting. The once over harvest provides a gain of 60-70 days which allows the farmers to take a short duration legume crop that adds to the income and soil fertility. It also saves on labour and energy for transportation. These are major concerns of the growers.

f. Production of disease free planting material

Using tissue culture, it is possible to develop planting material which is free from sucker borne diseases and pests. Use of healthy planting material complemented with integrated pest management program is the key to a good crop stand in field.

g. Plant exchange

Production of plants in test tubes facilitates safe movement and easy handling of germplasm between laboratories within and across countries.

h. High returns

Since the micropropagation based progeny is genotypically and phenotypically similar to the mother plant, which is often a superior selection, the yield and returns are expectedly higher.

3.1 Shoot tip culture

The earliest reports of *in vitro* culture of bananas came from Taiwan in the 70's (Ma and Shii, 1974; Ma *et al.,* 1978). Till date, protocols have been standardized for *in vitro* propagation of a wide range of *Musa* species and cultivars belonging to various ploidies and genomes (Sathiamoorthy *et al.,* 1998).

Shoot tips can be extracted from the pseudostem, suckers, peepers, lateral buds or even small eyes which contain a shoot meristem (Jarret *et al.,* 1985; Vuylsteke and De Langhe, 1985). Though all of them behave similarly under *in vitro* conditions, peepers and sword suckers are preferred because of their ease of handling and the minimum damage caused to the parent stool during their removal. It is always better to collect the explants from flowering plants so as to ascertain their trueness to type.

The steps followed for production of micropropagation based banana planting material are:

- Selection of mother plant and establishment of mother block nursery,
- Virus indexing of mother plant nursery,
- Culture initiation,

- Culture proliferation,
- Rouging at various stages of proliferation,
- Rooting and primary hardening accompanied by rouging,
- Secondary hardening accompanied by rouging,
- Fidelity testing and virus indexing at various stages of mass multiplication.

3.1.1. Selection of mother plants and establishment of mother block nursery

Selection of proper mother plants and standard requirements for a mother block nursery have been detailed in Chapter 2.

3.1.2 Virus indexing of mother plant nursery

Detailed indexing procedures for banana viruses are presented in Chapter 4. Indexing should be carried out primarily for four viruses, namely, *BBTV, BSV, BBrMV and CMV,* and should be done twice during crop duration, at 6 months stage and at fruiting. If found infected, the entire clump comprising, suckers along with underground mother corm should be removed and destroyed.

3.1.3 Selection of superior initial planting material

Choice of explant is vital for which purpose well maintained mother plants should be selected. Sword suckers should be healthy and not less than 60-80 days of age while the growing meristem should be of 1.0 cm^3 in size.

3.2 Culture medium

Success of *in vitro* culture depends largely on the choice of nutrient medium, including its chemical composition and physical form (Murashige, 1974). Several media formulations has been reported for banana shoot tip culture but nearly half of them are modified MS media (Brown *et al.,* 1995). Other popular media include B_5 (Gamborg *et al*., 1968), SH (Schenk and Hildebrant, 1972), N_6 (Chu *et al*., 1975), and Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1975) media. The culture media vary in both type and concentration of the components, but all have similar basic components of growth regulators, nitrogen, carbohydrates, inorganic macro and micronutrients, vitamins and organic additives.

Generally, the cultures are established on a separate initiation medium, which has a lower concentration of cytokinin than the multiplication medium to which the cultures are subsequently transferred (Jarret *et al.,* 1985, Novak *et al.,* 1989).

The composition of initiation, multiplication and rooting media used at the National Research Centre for Banana, India (NRCB) is given in Annexure II.

After autoclaving, the culture medium is stored in a clean dust free chamber for 1-2 days before use in order to check for any contamination. Bacterial contamination may be observed, particularly during the rainy season. Use of Cefatoxime in the initiation and subsequent subcultures helps to overcome even latent bacterial contaminations.

3.3 Culture initiation

The sword suckers of 2-3 months are removed from healthy disease free mother plants for shoot tip culture (Fig. 8a). The suckers are cut to expose the shoot tip of 10 cm^3 and cut further to about 3 cm diameter and 5 cm length. The explant should be carefully cut to avoid injury to the growing meristem. The shoot tips are washed in tap water and transferred to a container with 0.1 % mercuric chloride for 10 min and then to 0.1 % cetrimide. Then the shoot tips are washed thoroughly under running tap water to remove all traces of the chemicals. Using sharp sterile blade, one or two outer juvenile leaves and the corm base are trimmed out. Afterwards, the shoot tips are washed three times in sterile water in aseptic condition (under laminar air flow) disinfected with 5 % sodium hypochlorite and later with 0.1 % mercuric chloride each for 15 minutes. To avoid bacterial contamination, use of Cefataxime (0.1 %) in the initiation medium is in vogue in some laboratories.

Surface sterilized shoot tips are washed three times using sterile water. The outer surface of explant exposed to sterilizing agent is removed and the explants trimmed using surgical blade (No. 22) to bring the final size to about 3-4 cm length and 1-2 cm diameter (Fig. 8a). The explants are inoculated under sterile conditions in 30 ml of initiation medium in a 250 ml glass jar container.

pH is usually maintained at 5.8, which is prone to changes over culture duration. The optimum incubation temperature should be in the range of 24-26 °C. Generally the light intensity is maintained at 1,500-3,000 lux. Higher levels of 3,000-10,000 lux during later stages improve the survival rate of plantlets upon transfer to soil. Initially, the cultures are maintained at 16 h light/8 h dark cycle and once after rooting they are shifted 14 h light/10 h dark cycle.

Decapitation and wounding of shoot tips are carried out to overcome apical dominance and to encourage axillary bud proliferation. But injuring the apical bud through transverse sections, either four or eight cuts, is a much preferred method. Injuring the explant encourages more production of phenols, but it can be kept at minimum using antioxidants like ascorbic acid.

3.4 Culture proliferation

First subculture is done after 20-25 days of initiation when the explants turn green in colour. The cultures are first checked for contamination, in general symptoms of fungal contamination appear within one week and bacterial contamination symptoms like change of medium colour and texture or visible colonies appear within one week to one month. For subculturing, the outer dead tissue from the base of explant is removed and one or two leaf bases are peeled till the fresh meristematic tip gets exposed. The apical meristem is cut with two gentle cross incisions and the explant is transferred to subculture medium. During 20-25 days after the first subculture, the central meristem produces clusters of proliferating buds and one to three axillary buds get regenerated from the basal parts of explants around the central apical meristem (Fig. 8b). The number of axiliary buds developed during first and second subculture range from 1 to 5 depending on genomic constitution of the variety. In general, diploids like Matti, Anaikomban and Senna Chenkadali produce more buds than commercial cultivars. Among the latter, the number of buds produced during subculture is high in Cavendish (Robusta, Grand Naine – AAA genome) group followed by Plantain (Nendran – AAB genome) and Monthan (ABB genome) types.

Subsequent subculture is done by trimming the tip of emerging axillary buds and removal of dead tissue at the base of explant by gentle scratching. Clusters of proliferating buds develop during third and fourth subculture (Fig. 8c). For further subculturing, the explant is cut into three to four pieces and each slice with two to three proliferating clusters is inoculated to individual culture bottles. This subculture cycle is repeated at 3-4 weeks interval to increase the proliferation rate. During fourth and fifth subcultures, a single clump contains about 15-25 proliferating shoots. After 5-6 subculture cycles, the proliferated buds (Fig. 8d) are transferred to rooting medium containing IBA and activated charcoal (Annexure II). After a month, the rooted plantlets are ready for hardening (Fig. 8e). To minimize somatic variation, the subculturing is restricted to a maximum of seven cycles when each bottle contains 25-30 plantlets with well developed shoots and roots.

Experiments have demonstrated that proliferating shoots can be transferred to polybags (10-20 cm size) having rooting media under green house. This reduces cost and enhances better establishment. Polybag provides enough space for plant growth and natural light enhances the process of hardening.

3.5 Hardening

Once the plantlets are ready for shifting outside the laboratory, they are carefully acclimatized to adapt to the green house and later to least protected field conditions (Fig. 8f). During hardening, the plantlets undergo physiological adaptation to changing external factors like water, temperature, relative humidity and nutrient supply.

Fig 8. Shoot tip culture for banana micropropagation: a. sword sucker and explant; b. shooting after apical disabling; c. proliferation; d. multiple shooting; e. rooting; f. nursery hardening.

The plantlets from culture vessels/bottles are moved from the laboratory to a room at ambient temperature and kept open for 4-6 days. Later they are shifted to green house for primary hardening where they are first gently washed free of agar medium. This is important as sucrose in agar encourages microorganisms. 8 cm shoots with 3-4 ramified roots are planted in individual micropots in a protray. In places where weather is conducive (24-26 °C temperature and more than 80 % humidity), the plantlets are hardened for 4-6 weeks in mini-sand beds. During this period, 90-95

% humidity is maintained for the initial 6-8 days under diffused light. The humidity is slowly reduced to 70 %, light intensity raised to normal and temperatures brought to 26 °C by the end of 6 weeks.

Structures used for primary hardening vary with the climatic conditions. These can be highly sophisticated with UV-stabilized polysheet covering, multiple misting options, thermal shade net and auto-monitoring of light intensity, temperature and humidity. On the other hand, the structures can be simple with polycarbonate roofing, shade net on all sides with fogger facilities. Temperature, RH and light intensities are monitored manually using thermometer, hygrometer and lux meter, respectively.

Planting media for primary hardening range from sieved sand augmented with nutritions to mixtures of cocopeat and Soilrite with fine sand in equal proportions. NPK is provided in liquid form on weekly basis.

3.6 Secondary hardening

After primary hardening for 5-6 weeks, the plantlets are transferred from micropots to polybags. Base substrate is generally soil and sand along with low cost materials like coir pith, sawdust or rice husk. Organic manure is either in the form of farm yard manure or poultry manure. In Maharashtra, India, Press mud, a byproduct of sugar factories, has been found to provide best substrate for secondary hardening along with soil (Vasane *et al.*, 2006).

Plantlets from micropots are, dipped in fungicide solution (0.1% bavistin) and planted in polybags containing suitable substrate. Initially, these are maintained in low light intensity shade nets and 70 % RH. The plants are hardened by gradually increasing the light intensity and reducing RH (40 %). After 5-6 weeks, the plants become ready for field planting having 3-5 well developed leaves and a good mass of fibrous roots.

During both primary and secondary hardening, the stocks should be rouged for variants at weekly intervals. These could include vegetative deformities like dwarfism, leaf variegation, rosette foliage and leaf crinkiness. Other precautions to be followed are:

- The rooting media should be completely free from pathogens.
- Water used for irrigating the plants should be free from pests and pathogens.
- Sample plants from each batch should be randomly virus indexed (at least 10 plants from each batch/explant)
- While shifting primary hardened plantlets, two longitudinal cuts should be given to the micropots to facilitate further corm growth.

3.7 Manuring and plant protection in nursery

Plantlets should be 2-3 weeks old before any fertilizer is applied. 100 ml water containing 0.5 g urea, 2 g superphosphate and 1 g muriate of potash can be applied per plant. The manuring is repeated by doubling the dosage after three weeks. Spraying of commercially available micronutrient mixtures during sixth week helps in better establishment both in nursery and field. Strict sanitary measures are adopted in the nursery to avoid the risk of damage by pests and diseases either through substrate or irrigation water.

3.8 Field planting and initial management

20-30 cm tall plants with 3-5 broad leaves are ready for field planting (Fig. 9). At the time of planting, 10 g of Carbofuron is applied per plant. Watering is done soon after field planting as young micropropagated plants are sensitive to dry weather and heat. Since these are also highly susceptible to bacterial rot (*Erwinia* rot), within 3 days of planting the soil around the plants is drenched with 500 ml of 0.1 % Emisson (methyl ethoxy mercuric chloride). Recommended package of practices is strictly followed to achieve successful field establishment and subsequent vigorous growth (Figs. 9 and 10).

Ideal tissue culture raised plant

An ideal tissue culture raised plant should:

- be 30 cm in height and have a pseudostem circumference of 5.0-6.0 cm after 60 days of total hardening;
- have 4-5 photosynthetically active leaves and inter-foliar space must be not less than 5.0 cm;
- have approximately 25-30 more than 15 cm active roots at the end of secondary hardening;
- be free from any visual symptoms of leaf spot, pseudostem rot and physical deformations;
- be free from root pathogens like *Erwinia,* nematode lesions and root knots. Random checking of roots is essential to ensure health of plantation.

3.9 Testing for genetic fidelity and virus infection

Virus indexing and genetic fidelity testing are important to produce good quality disease free planting material using tissue culture technology. Details of virus detection and elimination protocols are given in Chapter 4.

Fig. 9. Young tissue culture plantation.

Fig. 10. Field planted tissue culture banana at fruiting.

Table 2. List of ISSR primers used for genetic fidelity testing

ISSR Primer	Nucleotide sequence
UBC 807	5'-AGA GAG AGA GAG AGA GT-3'
UBC 808	5'-AGA GAG AGA GAG AGA GC-3'
UBC 811	5'-GAG AGA GAG AGA GAG AC-3'
UBC 812	5'-GAG AGA GAG AGA GAG AA-3'
UBC 818	5'-CAC ACA CAC ACA CAC AG-3'
UBC 830	5'-TGT GTG TGT GTG TGT GG-3'
UBC 834	5'-AGA GAG AGA GAG AGA GYT-3'
UBC 836	5'-AGA GAG AGA GAG AGA GYA-3'
UBC 840	5'-GAG AGA GAG AGA GAG AYT-3'
UBC 841	5'-GAG AGA GAG AGA GAG AYC-3'
UBC 842	5'-GAG AGA GAG AGA GAG AYG-3'
UBC 850	5'-GTG TGT GTG TGT GTG TYC-3'
UBC 868	5'-GAA GAA GAA GAA GAA GAA-3'

Shoot tip cultures preserve genetic stability much better than callus or cell suspension cultures, yet somaclonal variation seems to be widespread among plants regenerated from banana shoot tip cultures. Commercial varieties like Robusta, Grand Naine, Dwarf Cavendish, Shrimanthi and Madhukar are highly susceptible to these variations and the off-types number up to 74 %. High level of variation is not desirable as it defeats the purpose of clonal reproduction and majority of the off-types are agronomically inferior to the parental clone. Banana and plantains have a flexible genetic make and its genetic stability under cultive is strongly influenced by external factors like growth regulators, duration of culture etc. Mild stress under *in vitro* results in reverting of the clone to its

parental type. For example, Robusta, a Cavendish clone frequently reverts to its original type Dwarf Cavendish which is not acceptable for its low stature and poor yield. Hence, genetic fidelity testing using preferably molecular marker techniques is essential to ensure the supply of true to type quality planting material. At NRCB, besides phenotype PCR technique with ISSR markers is being successfully used for screening off-types in banana (Table 2).

Figure 11 summarizes the various stage in micropropagation of banana detailed in this chapter. Using the protocol, more than 10,000 plants are expected from a single explant at the end of 320 days (Table 3).

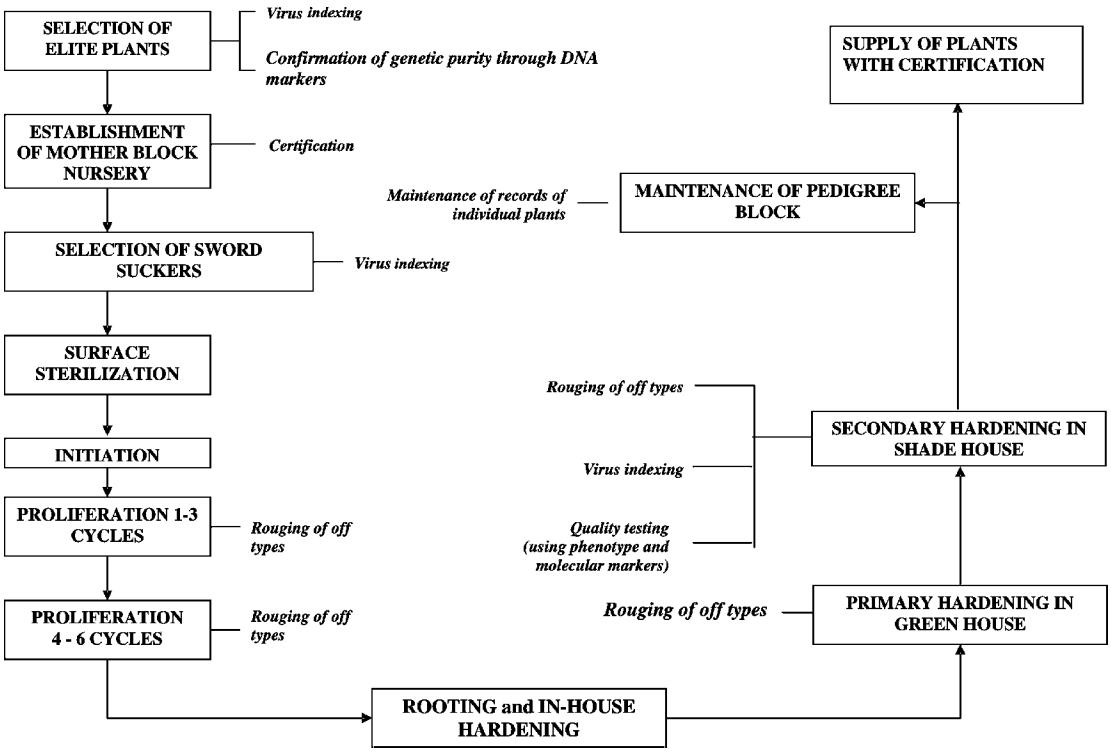


Fig. 11. Flow chart for production of quality planting material of banana through tissue culture.

Table 3. Projected multiplication rate of banana under of tissue culture from a single explant

Particulars Stage		Duration (days)	No. of plants
Initiation		25	1
Subculture stages	1st	50	3
	2nd	75-80	12
	3rd	100-110	48
	4th	125-130	192
	5th	175-180	760
	6th	200-210	3,040
	7th	225-230	12,160
Rooting		255-260	11-12,000
Primary hardening		270-280	11,500-11,000*
Secondary hardening		310-320	10,000-10,500**

*Success depends on the sophistication of the hardening structure
**Somaclones and off-types constitute the major discards

4. HEALTH MANAGEMENT IN THE PRODUCTION OF DISEASE FREE PLANTING MATERIAL THROUGH MICROPROPAGATION

The following specific interventions are required for producing virus, pathogen and pest free banana plants through tissue culture:

a) Virus diagnosis using visual and molecular detection techniques. Testing is done at mother plant selection, 2nd and 3rd subcultures and primary and secondary hardening stages.

b) Elimination of viruses.

c) Managing *Erwinia* and nematode infections during secondary hardening.

4.1 Visual diagnosis of banana viral diseases

4.1.1 Banana Bunchy Top Disease

Banana Bunchy Top disease is caused by *Banana bunchy top virus* (BBTV) belonging to the genus *Babuvirus* (family *Nanoviridae*). BBTV is an isometric virus (Fig. 12a), 18-20 nm in diameter, with a genome comprising at least six circular, single-stranded DNA components each with a size of approximately 1 Kb. Intermittent dark green dots and streaks of variable lengths are visible in leaf sheath, midrib, leaf veins and petioles of infected plants. Sometimes, the typical streaks may not appear. The leaves produced are progressively shorter, brittle, narrow and give the appearance of bunchiness (Fig. 12b). The affected plant fails to produce fruiting bunch or produces a bunch with fingers that do not develop to maturity. In case of Grand Naine, the affected plants produce bunches with extremely long or very short peduncle. Marginal chlorosis in leaf lamina can also be noticed. The emerging leaves are yellowish green and with mock iron deficiency symptoms. Vein flecking symptoms in the lamina are noticed in some varieties. When infection occurs very late in the season, the plant shows dark green streaks on the tip of the bract. Sometimes, the bract tip of male bud becomes green and leafy. Mild vein clearing and dark green blisters on mid rib and leaf sheaths have also been recorded. Symptomless infection is reported to occur for two seasons. In some clusters, plants may exhibit symptoms while others are bereft of typical symptoms.

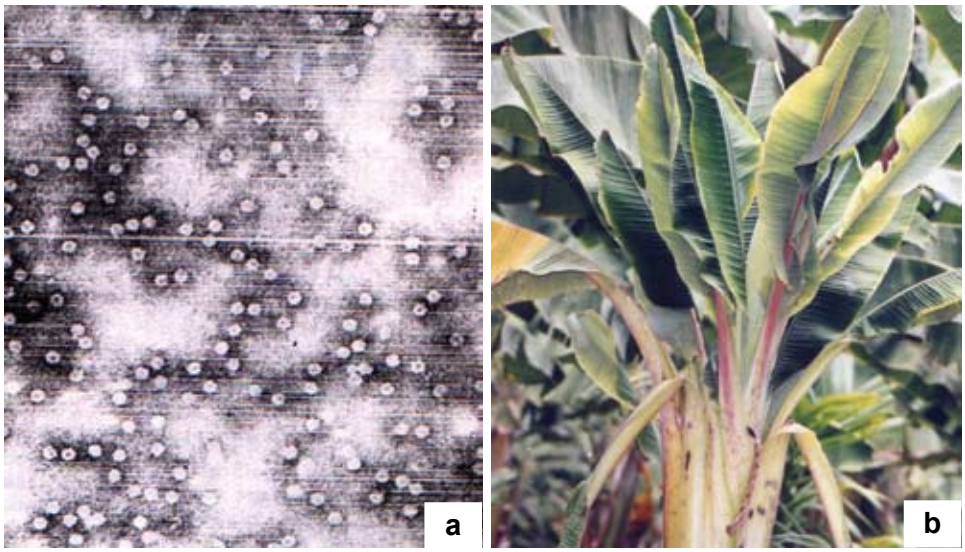


Fig. 12. Banana bunchy top virus (a); infected plant (b).

4.1.2 Banana Streak Disease

Banana streak virus (BSV), a plant pararetrovirus, causes banana streak disease. Non-enveloped bacilliform particles (Fig. 13a), 130 × 30nm in size with double stranded DNA approximately 7.5 Kbp in size are the characteristics of the virus.

The disease appears initially as small golden yellow dots which later extend to form long streaks. The chlorotic streaks become necrotic giving a blackish appearance on the lamina. Necrotic streaks are also observed on pseudostem midrib and petiole (Fig. 13b). The streaks are erratically distributed over individual leaves and across the leaves of same plant. Bunch choking, abortion of bunch and seediness in fingers are seen in infected plants. Fruits get distorted and plant vigour gets reduced. Dieback and

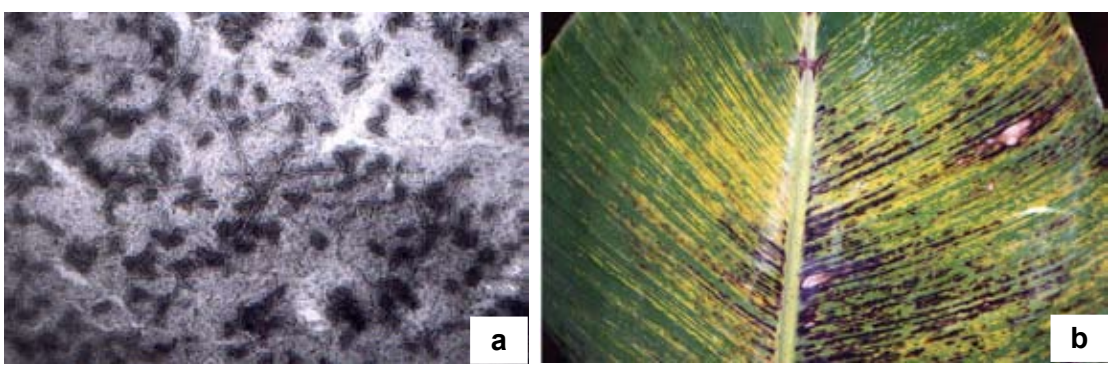


Fig. 13. Banana streak virus (a); infected leaf showing necrotic streaks (b).

internal pseudostem necrosis have also been associated with the disease. Necrosis of cigar leaf and death of entire plant has been recorded in plantain hybrids in Nigeria. Similar necrosis has been noticed in cultivars Poovan and Ney Poovan in India.

Variations in symptoms have been noticed in different banana varieties. Leaf stripping is commonly noticed in cultivars Poovan, Grand Naine and Robusta. Typical chlorotic and necrotic streak symptoms have been noticed in Poovan, Cavendish clones, Nendran and Red Banana. Extended female phase after a short male phase is observed in BSV infected Poovan. Blackish spindle shaped streaks on petiole and leaf sheaths are also common symptoms seen in Poovan. Fruit malformation and seediness in Poovan are major symptoms induced by BSV. In Rasthali, the affected plant bears small bunches with reduced finger size while pseudostem splitting and peel splitting have also been recorded. Mild to severe mosaic pattern also has been observed.

4.1.3 Banana Bract Mosaic Disease

This disease is caused by *Banana bract mosaic virus* (BBrMV). The virus belongs to the family *Potyviridae* and the genus *Potyvirus*. The genome is positive ssRNA and approximately 10 Kb in length. The particles are flexuous rod shaped and measuring 750 × 15 nm in size (Fig. 14a).

Banana bract mosaic disease is characterized by the presence of spindle shaped pinkish to reddish streaks on pseudostem, midrib, peduncle, bracts and fingers (Fig. 14b). In cultivar Nendran, the leaf orientation changes in such a way giving the appearance of traveler's palm plant. Spindle shaped waxy coating appears on the lower side of the leaf while on the upper surface spindle shaped mosaic patterns are seen. In Nendran, unusual long and very short peduncle, abortion of bunches and raised corky growth on peduncle are the symptoms of BBrMV. Necrotic streaks on fingers, leaf, pseudostem and midrib are seen in some varieties. Extended female phase after a short male phase has been observed in Nendran and Ney Poovan cultivars. In Red Banana and Robusta, leaf stripping symptoms are common in some seasons. In Robusta, fingers of infected plants stop to develop and give the appearance of pencil, explaining the Tamil local name 'pencil kai' (pencil-sized fruit). Mild mosaic symptoms on green fruits of cultivars Ney Poovan, Robusta, Karpuravalli and Poovan are recorded.

4.1.4 Banana Mosaic or Infectious Chlorosis

The disease is caused by *Cucumber mosaic virus* (CMV). The virus particles are about 29 nm in diameter and composed of 180 subunits (Fig. 15a). CMV induces mild mosaic symptoms which appear on entire leaf or part of leaf or on all the leaves

Fig. 14. Banana bract mosaic virus (a); infected plant (b).

of the infected plant (Fig. 15b). CMV is also related to heart leaf rotting symptoms in banana. Rotting of cigar leaf, internal necrosis of pseudostem and death of the whole plant are associated with CMV. Chlorotic banding, line patterns, deformation of leaf, rosette appearance of leaf arrangement are also the symptoms of CMV.

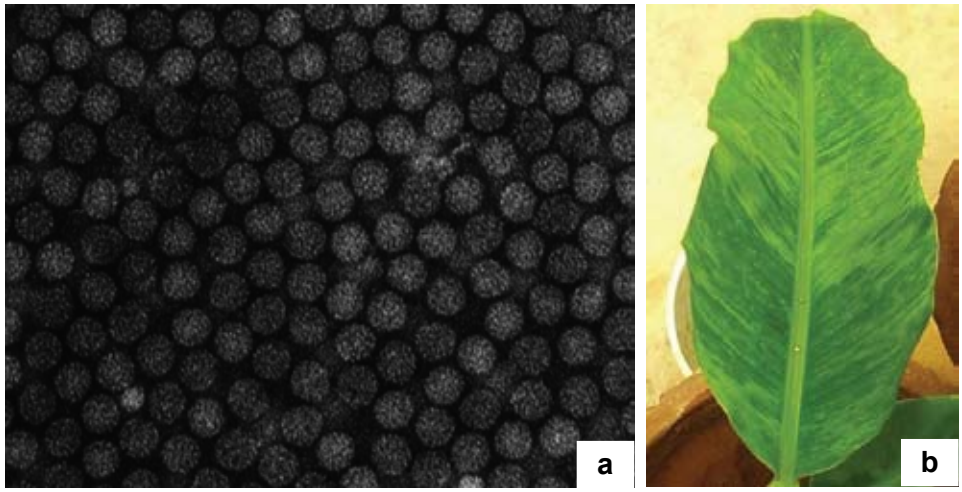


Fig. 15. Cucumber mosaic virus (a); infected leaf (b).

4.2 Virus indexing using molecular techniques

There are many published protocols for detection of banana viral pathogens. Serological tests (ELISA) are based on antigen-antibody reaction in which the viral coat protein acts as antigen and specific antibody raised against coat protein serves as antibody for immunological tests. In case of nucleic acid or viral genome based techniques, PCR, RT-PCR and NASH have been standardized and are widely used in virus detection. Principles and prototcols of these are detailed in Annexure III. At NRCB, ELISA, PCR and NASH have been standardized and are being used to detect viruses in banana mother plants and tissue culture derived plants (Table 4, Annexure III)

Table 4. Indexing techniques being used for banana virus diagnosis at NRCB

List of Viruses	Technique			
	PCR/RT-PCR/IC-PCR/DB-PCR/ IC-RT-PCR	ELISA /DIBA	(NASH) using non-radioactive DNA/RNA probes	Southern and northern blots using non-radioactive probes
Banana bunchy top virus (BBTV)	√	√	√	√
Banana bract mosaic virus (BBrMV)	√	–	√	√
Banana streak virus (BSV)	√	–	√	√
Cucumber mosaic virus (CMV)	√	√	√	√

4.2.1 Protein based methods

Detection of plant viruses by serological assay is being widely practiced since the discovery of enzyme-linked immunosorbent assay (ELISA) technique by Clark and Adams (1977). Various forms of serological assays are currently available for all seven known banana viruses. ELISA is a sensitive and efficient practical assay for banana virus indexing, when suitable antisera are available. Specific polyclonal and monoclonal antibodies are available for BBTV, BSV, CMV and BBrMV (Diekmann and Putter, 1996). ELISA tests with monoclonal antibodies (Mabs) have been commonly used for the accurate detection of BBTV (Wu and Su, 1990; Thomas and Dietzgen, 1991; Geering and Thomas, 1996; Espino *et al*., 1989). However, ELISA is of limited use when very low concentrations of BBTV occur in infected plants. Further, viruses belonging to geminiviridae are less immunogenic and yield low titre

polyclonal antisera. Geering and Thomas (1996) developed triple antibody sandwich ELISA for virus indexing of BBTV which is preferred method when compared to dot immunobinding assays and amplified ELISA. Other variants have been introduced for better detection of BBrMV, BSV, BBTV and CMV (Wanitchakorn *et al.,* 1997; Sadik *et al.,* 1997; Kiranmai *et al.,* 2005; Agindotan *et al.,* 2006; Ex-Dougdoug *et al.,* 2006).

4.2.2 Nucleic acid or viral genome based methods

4.2.2.1 Nucleic acid hybridization

New techniques based on viral nucleic acids are becoming popular and wide spread as molecular diagnostic tools. Nucleic acid hybridization of DNA or RNA probes has the advantage of being able to detect the nucleic acid of the virus in both single-stranded and double-stranded replicate forms. Radioactive isotopes like ^{32}P are used for labeling nucleic acid probes and the signal is detected by autoradiography. In recent years, non-radioactive labeling and detection using biotin/streptavidin or Dig-oxigenin (DIG) systems have been widely used for many viruses (Dietzgen *et al.*, 1994; Mas and Pallas, 1995; Mas *et al.*, 1993). When large numbers of samples are to be tested in a short time, dot blot hybridization or nucleic acid spot hybridization (NASH) is the appropriate technique. In this technique, the viral genomes are detected from crude or purified samples after hybridizing with labeled specific probes. It is easy to send the samples after spotting than sending live tissues to testing centres as they get spoilt in transit. NRCB has developed NASH technique for BBTV which is equally sensitive as PCR and the technique has been applied for detection of BBrMV, CMV and BSV.

4.2.2.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) based detection systems are now available for all banana viruses (Dietzgen *et al.*, 1999; Harper *et al*., 1999). Xie and Hu (1995) used PCR for detecting Hawaii isolates of BBTV and it was 1,000 times more sensitive than ELISA or dot blots with DNA probe. A simple, single-step plant tissue preparation protocol to reduce plant inhibitory factors interfering with PCR and suitable for the detection of BBTV in corm, leaf and root tissues has been developed (Thomson and Dietzgen, 1995). Galal (2007) used PCR for detection of BBTV in banana samples exhibiting characteristic symptoms of BBTV, and also from the viruliferous banana aphid. Selvarajan *et al.* (2007b) has also developed PCR based method to detect Indian isolates of BBTV. BBrMV was detected by RT-PCR in total nucleic acid extracts from infected plants, using specific or degenerate potyvirus group primers (Bateson and Dale, 1995; Thomas *et al*., 1997). PCR based diagnostic assay for BSV is difficult as its genome integrates with banana host genome. Complete sequences

for many species of BSV have become available for designing primers for detection (Harper and Hull, 1998: Geering *et al*., 2005). Specific PCR are now available for some BSV strains, but there are additional uncharacterized isolates not detected by PCR assay, which show great genomic and serological heterogeneity (Lockhart and Olsezewnski, 1993).

Cucumber mosaic virus (CMV) causing mosaic and chlorosis of banana has been detected by RT-PCR amplification of coat protein gene (Aglave *et al.*, 2007). Polyvalent degenerate oligonucleotide RT-PCR was used to detect *Banana mild mosaic virus* (BaMMV) and *Banana virus* X, two flexiviridae infecting *Musa* spp. BVX could be detected using species specific primers whereas PDO-inosine containing primers were found well suited for detection of BaMMV (Teycheney *et al*., 2007).

4.2.2.3 Multiplex PCR and Immuno-capture PCR

Multiple species or strains of viruses are detected simultaneously in a single PCR reaction by combining oligonucleotide primers specific for different viruses, a technique termed as Multiplex-PCR. In this technique, DNA fragments to be amplified are of different lengths and there should not be any cross reactivity between different viral species targeted. Immuno-capture PCR (IC-PCR) method combines both serology and PCR techniques. In IC-PCR, the virus particles are first trapped onto a specific antibody bound to a surface. The trapped virus particles are disrupted and the viral nucleic acid released and amplified by PCR or RT-PCR. This method is especially useful in concentrating virus particles from plant species where virus titre is low and compounds that inhibit PCR are present. Sharman *et al.* (2000) standardized multiplex-immuno-capture PCR (M-IC-PCR) for simultaneous detection of BBTV, BBrMV and CMV in banana. Simultaneous detection of Indian isolate of BBTV and BSV with duplex PCR and BBrMV by multiplex-PCR has been reported (Selvarajan *et al.*, 2004a). Salvarajan *et al.* (2004b) developed IC-PCR technique of BBTV. Immunocapture RT-PCR has also been used to detect BaMMV (Teycheney *et al.,* 2007).

4.2.2.4 Real Time PCR

Of late, a novel real-time quantitative PCR technique, Real Time PCR, has been developed for the detection and quantification of plant viruses (Dietzgen *et al*., 1999; Roberts *et al*., 2000). It is more sensitive, reliable and specific than PCR. It reduces the risk of cross-contamination, obviates post PCR manipulations, provides higher throughput, and enables quantification of virus load in a given sample. However, this technology requires more expensive equipment and reagents compared with conventional PCR.

Rapid, reproducible and specific detection of episomal BSV from crude extracts of infected plants by real time assay has been developed for large scale screening (Delanoy *et al.,* 2003). Primers have been designed to specifically amplify the episomal BSVOLV sequence of 1,336 bp size to detect in real-time by a short fluorogenic 3' minor groove binder DNA probe for 14 bp conserved BSV sequence. Due to cost factor, this technology may not be feasible for small scale banana tissue culture set up where small number of samples are handled.

4.2.2.5 Direct-binding PCR (DB-PCR)

This technique is based on the adsorption of viral particles to the surface of a polypropylene microcentrifuge tube. Direct binding PCR has successfully been used at NRCB to detect BSV, BBrMV and CMV (Selvarajan *et al.,* 2007a). This technique has also been used to detect the episomal form of BSV. This assay is cost effective and less time consuming.

4.3 Elimination of viruses

Virus affected planting material poses a major problem in propagation and exchange of germplasm, and eventually in breeding and distribution of superior genotypes. For many banana viruses, the nature of spread still remains unclear and most of the field gene banks are severely affected. Meristem-tip culture, chemotherapy, electrotherapy and cryotherapy are some of the methods applied for elimination of viruses in banana and other vegetatively propagated crops. They are used either alone or, in most cases, in combination. Kassanis (1950) first used high temperature treatment to eradicate virus from potato tubers. Later, techniques involving thermotherapy or tissue culture and frequently a combination of both have been successfully used to eradicate viruses from infected plants (Walkey, 1980).

4.3.1 Meristem-tip culture

This technique involves the use of apical dome or shoot tip with a few leaf primordia of the size less than 1 mm in length as the explant. In banana and plantain, meristem-tip culture is considered to be the reference tool for virus eradication. The application of meristem-tip culture to eradicate viruses was initially based on the concept of meristem "immunity" towards viruses (Morel, 1948). Different researchers have shown that the probability of obtaining virus-free plants is inversely related to the size of the meristem (Faccioli and Marani, 1998).

4.3.2 Thermotherapy

Heat therapy has been used to successfully eliminate many viruses from a variety of plant species (George, 1993). The heat treatment may be done either *in vitro*

or *in vivo* and is usually combined with meristem culture for better results. CMV infected *in vitro* and *in vivo* plants of banana cv. Williams BSJ (ITC 0570) were kept for one day in a growth cabinet under artificial light with diurnal alternating periods (16 h light/8 h dark). Day temperature in the growth cabinet was initially 28 ± 1 °C which was increased at 2 ± 1 °C per day until 40 ± 1 °C was obtained. Plant material was kept at this temperature for 4 weeks, with night temperature at 25 ± 1 °C. Meristems (domes with four-leaf primordia) were then excised and transferred to MS medium. The percentage of healthy plants regenerated after a combination of meristem culture and thermotherapy was 38 % and 70 % when meristems were excised from *in vivo* or *in vitro* plants, respectively. Berg and Bustamante (1974) observed that heat treatment (35-43 °C for 100 days) performed on rhizomes (2.5 cm square and 5-7.5 cm long) in conjunction with meristem culture was inefficient for cleaning commercial banana cultivars. However, 56 plantlets out of 73 regenerated by culturing meristems of heat-treated rhizomes did not produce symptoms when extracts were used to inoculate indicator plants. CMV eradication was also achieved by Gupta (1986) in approximately 100 % of the regenerated plants when using meristem culture in combination with a two-week heat therapy (38 °C-40 °C).

4.3.3. Chemotherapy

Chemotherapy, either alone or in combination with other techniques, is becoming increasingly available as a virus elimination tool (George, 1993). Anti-viral chemicals may be either sprayed on plant or incorporated into tissue culture media. Often, a chemical therapy is followed by meristem culture. Antiviral substances such as acyclic adenosine analogue [(RS)-9-(2, 3-dihydroxypropyl) adenine [(RS)-DHPA] and ribavirin (1-b-D-ribofuranosyl-1,2,4-triazole carboxamide; Virazole®) have been used.

Meristems excised from CMV-infected banana were grown in a culture room (standard conditions) for 3 months on MS medium to which 50 mg/l of Virazole or (RS)-DHPA was added (Helliot *et al*., 2004). Concentrations of chemical compounds used for chemotherapy assays were determined after the phytotoxicity tests. The percentage of healthy plantlets regenerated from Virazole treated highly proliferating meristems reached 29 % while only 2% of plantlets were found virus-free when regenerated from (RS)-DHPA treated highly proliferating meristems.

4.3.4 Electrotherapy

Electrotherapy assays were carried out either on infected *in vivo* or *in vitro* plants (Hernandz *et al.,* 1997). Pulses of 15 V were applied for 5 min to 2-3 cm long explants containing apical meristem. The meristems were then excised and placed on an MS culture medium. The efficiency of electrotherapy in producing virus-free regenerants from BSV-infected banana plants (cv. W. Bungulan) was 40-80 %. In case

of CMV, the preliminary percentage of healthy plantlets regenerated from electrically treated explants obtained from *in vivo* plants reached 11% while no plantlet out of 27 tested was found to be virus-free when explants were obtained from *in vitro* plants (Helliot *et al*., 2004).

4.3.5. Cryotherapy

Cryotherapy of shoot tips based on cryopreservation techniques is a new method for pathogen eradication. Cryopreservation refers to the storage of biological samples at ultra-low temperature of liquid nitrogen (−196°C), and is considered an ideal means for long-term storage of plant germplasm.

Helliot *et al.* (2002) reported the utilization of cryopreservation for eradication of CMV or BSV from *Banana.* Plants of cv. Williams (AAA, Cavendish subgroup) were mechanically infected with CMV or naturally infected with BSV, and proliferating meristems were produced from the infected plants. Excised meristematic clumps were cryopreserved through vitrification using PVS-2 solution. The health status of regenerated *in vitro* plants was first checked by means of ELISA. The putative virus-free material was subsequently tested a second time following greenhouse acclimatization. The frequency of virus eradication for CMV and BSV following cryopreservation was 30 % and 90 %, respectively. In comparison, the frequency of virus-free plants regenerated directly from highly proliferating meristems, corresponding to a spontaneous eradication rate, was 0 % and 52 % for CMV and BSV, respectively. The conventional meristem culture resulted in 0 % CMV-free plants and 76 % BSV-free plants, while the cryoprotective treatment resulted in 2 % CMV-free plants and 87 % BSV-free plants.

4.4. Managing Erwinia and nematode infections during secondary hardening

4.4.1 Erwinia head rot or Tip over disease

The disease caused by *Erwinia carotovora* is widespread in banana growing areas of the world. Its incidence is high in 3-4 months old plants under high temperature conditions. The disease is also noticed at the secondary hardening stage of tissue culture raised banana. Cultivars like Dwarf Cavendish, Grand Naine, Robusta, Nendran and Thella Chakkarakeli are most susceptible while other varieties also succumb to the disease.

Rotting of collar region is the commonest symptom of this disease (Fig. 16). The leaves of affected plants show epinasty and suddenly dry out. If the affected plants are pulled out, they topple from the collar region leaving the corms with their roots. Splitting of pseudostem is observed at late stage of infection in cultivars Robusta

and Thella Chakkarakeli. If the affected plants are cut open at collar region, yellowish to reddish ooze can be seen. In early stage of infection, dark brown or yellow, water soaked areas are visible in the cortex area. In advanced stage the interior lesions may decay to such an extent as to form cavities surrounded by dark spongy tissues. The rot spreads radially towards the growing point through cortical tissues.

Drenching of tissue culture plants kept in polybags with 0.1 % Emisson or 2 % bleaching powder can control the disease. Planting tissue culture plants in soils lacking good drainage should be avoided.

Fig. 16. Erwinia head root infected banana.

4.4.2 Nematodes

Banana tissue culture plants are highly susceptible to parasitic nematodes such as root knot nematode (*Meloidogyne incognita*), lesion nematode (*Pratylenchus coffeae*), burrowing nematode (*Radopholus similes*) and spiral nematode (*Helicotylenchus multicinctus*). These enter the plant roots through contaminated water or through the pot mixture used for hardening of tissue culture plants. The tender roots of affected tissue culture plants become weak and cannot absorb nutrients. In case of root-knot nematode infestation, the plants exhibit stunting and the leaves turn pale yellow. Profuse galling with egg masses can be observed in the root system. Root knot nematodes are predominantly present at the primary and secondary hardening stages of tissue culture plants. Infestation by burrowing or lesion nematode initially leads to small cuticular sunken lesions on young corm roots. At later stage of infection, the roots exhibit extensive reddish brown lesions in the cortex which can be seen when cut longitudinally. Spiral nematode which tends to feed closer to the surface of the roots causes minimal damage compared to burrowing or lesion nematodes.

Use of clean and nematode free water, soil and other pot mixture during hardening are the prerequisite. Fumigated pot mixture should be used for secondary hardening. Application of 2-3 g of carbofuran per plant at secondary hardening stage can protect against invading nematodes. Application of VAM or neem cake also would ensure vigour and health of the plants.

5. PROSPECTS OF BANANA MICROPROPAGATION FOR QUALITY PLANTING MATERIAL PRODUCTION IN ASIA-PACIFIC

Asian countries are the major contributors to world banana production. The enhanced production witnessed over the last decades is mainly due to the increased productivity and to some extent due to area expansion. Increase in productivity has been made possible by adoption of high yielding Cavendish clones like Grand Naine and Robusta, use of quality planting material, integrated nutrient and water management and high density planting. Availability of diverse germplasm, varied production systems and positive response to Good Agricultural Practices (GAP) have contributed to wide adoption of banana across the globe. Consequently, its cultivation has extended from humid tropics to subtropics and arid subtropics, and from sea level up to an elevation of 1,500 m above sea level. High productivity has transformed banana from a backyard crop to a high value crop.

However, inadvertent use of disease infected suckers as planting material led to fast and widespread distribution of diseases in banana plantations. The importance of quality planting material was realized in developing countries in early 1980's, eventually leading to the growth of tissue culture industry. Presently, tissue culture is the major biotechnology tool that has contributed to the growth and development of banana industry enabling mass and season independent production of disease free planting material. In banana growing countries like Taiwan and Philippines, where the climate is peculiarly adverse during certain growing seasons, adjustment of crop timing has been resorted to. In China, seasonal effect of low temperature due to severe winter also causes crop damage. To alleviate these problems, bananas are grown at such a time that the adverse climatic effects are minimized or avoided. This has became possible due to tissue culture technology with the use of which large quantities of planting material are supplied in a short time and independent of season. Planting date is also being timed to coincide with market demands for fruit which follows a certain seasonal trend. Tissue culture planting material also facilitates high density planting which coupled with good package of practices substantially improves yield per unit area of land (Table 5). Due to these advantages experienced by farmers, tissue culture technology has revolutionized the production and productivity of banana and created great momentum for export and for rehabilitation of disease ravaged plantations in many Asian countries (Molina, 2002). Its contribution in enhancing the nutritional, economic and social status of farmers has been well recognized.

Table 5. Comparative advantages of using tissue culture planting material

Sl. No.	Parameters	Tissue culture plants (%)	Sucker (%)
1.	Field survival	78.3 - 94.8	87 - 98.3
2.	Reduction in crop duration	18.5 - 30.3	-
3.	Increase in yield over sucker plants	12.5 - 66.3	-
4.	Increase in number of hands	7.6 - 15.4	-
5.	Increase in fruit weight	12.5 - 46.6	
6.	Contribution to production cost	20 - 25	1 - 5
7.	Increase in cost of cultivation	18.1 - 23.5	-
8.	Returns (increase over conventional sucker planting)	28.3 - 64.5	-
9.	Income from selling sucker from TC plants (compensation on cost of production)	30.5 - 45.8	70.6 - 80.0

(Data compiled across banana varieties, production systems, and countries based on: Daniells, 1990; Qaim, 1993; Ortiz and Vuylsteke, 1996; More 1999; Rao, 2000; Sheela and Nair, 2001; Yadav *et al.,* 2005; Alagumani, 2005; Hanumantharyappa *et al.,* 2009).

5.1. Success stories of tissue culture banana adoption by farmers in Asia-Pacific

5.1.1 Australia

In Australia, the importance of tissue culture bananas has been recognized since long. But the demand for quality planting material shot up after the devastation of Australian banana industry by cyclone Larry in 2005-06. This demand could be met only through tissue culture banana production in collaboration under Quality Banana Approved Nursery (QBAN) scheme through which more planting material was produced and strategically supplied to reestablish the plantations, both backyard and commercial plantations (www.abgc.org.au).

Banana certification scheme established in mid-1990's by the Queensland Department of Primary Industries is perhaps the earliest certification program established for banana in the world (DPI&F, 2006). It is used as the fundamental management tool for the control of BBTV in Australia, and has been adopted in several other countries where the disease is a major problem. According to this system, banana plants (excluding fruits) must not enter Queensland without an Inspector's Approval, unless the plant is, a) banana tissue culture plantlet, b) is in a sealed pest proof container, c) is accompanied by either a QBAN certificate for the plant or an inspector's certificate stating the plant may be introduced, and (d) is transported in a way that prevents infestation by a banana plant pest. QBAN scheme enables Australian banana

industry to reduce the risk of spreading pests and diseases and allows growers to establish disease free blocks. Ongoing support from the government is provided to assist laboratories, nurseries and growers to overcome technical problems and improve efficiencies of the QBAN scheme. This project also supports banana virus indexing activities required for QBAN and to ensure that industry has access to specialists.

5.1.2 India

India has large banana growing area under perennial cultivation system. Poor attention and inherent difficulties in management of perennial plantations led to the built up of diseases. During 2004-2006, an FAO assisted Technical Co-operation Project entitled 'Improvement of banana production for small scale growers' was implemented in Tamil Nadu, Andhra Pradesh and Maharashtra states by the Ministry of Agriculture.The objective was to address the constraints and bridge the gaps in banana production with emphasis on quality planting material. The project also aimed at human resource development and capacity building of the farmers as well as field extension staff. Model tissue culture farms were raised and farmers were trained on the selection of quality planting material, adoption of improved production technologies and efficient post-harvest handling methods so as to meet the export standards. The technological infusion has greatly improved the production and productivity of small-scale farmers in the adopted states. The production enhanced by 18 % and productivity went up from 28 to 34 tonnes per hectare across the states (Anonymous, 2005).

In Tamil Nadu, BBT had ravaged the elite variety Hill Banana (Sirumalai) of Pulney Hills and its area had come down from 8,100 ha to less than 2,025 ha in last two decades. A major public-private partnership program involving the state government, NRCB, Hill Banana Growers' Association and local farmers was launched during 2005-06. Tissue culture protocol was standardized by NRCB and certified tissue culture bananas were produced and supplied to farmers at subsidized rates. Farmers were give incentive to remove the BBTV affected clumps and replant with healthy, certified, tissue culture plants. As a result, the area under Hill Banana got expanded by 4050 ha.

In Tamil Nadu, tissue culture plantlets of NRCB released variety Udhayam (Pisang Awak – ABB) were supplied to a number of farmers for testing. Feed back received after one year revealed that while the conventionally grown local Karpuravalli (equivalent of cv. Udhayam) yielded 25-30 kg size bunches, the tissue culture based plants of Udhayam uniformly recorded yields of 45-50 kg size bunches (Fig. 17), an increase in overall productivity by 35-40 %. As a result, the demand of tissue culture varieties among growers has increased three fold in the last two years across the states of Tamil Nadu, Kerala, Karnataka and Tripura.

With nearly 46,000 hectares under banana, Jalgaon district of Maharashtra state accounts for more than half of the total banana cultivation in India. Interestingly, after a series of orientation courses in new farming techniques for banana cultivators organized by the NRCB, Jalgaon based Jain Irrigation Systems (JISL) and the State Agriculture University, farmers who used to harvest 24 tonnes of banana per hectare in 1984-85 are now producing over 60 tonnes per hectare in the same area using tissue culture planting material and adopting improved cultivation practices.

Fig. 17. Tissue culture raised Udayam.

In view of the high frequency of off-types resulting from tissue culture, the Department of Biotechnology (DBT), Government of India in collaboration with Indian Council of Agricultural Research (ICAR) and Ministry of Agriculture developed standards for banana tissue culture and also standards for assessing tissue culture facilities (Annexure IV, V). DBT has, launched the National Certification System for Tissue Culture Raised Plants (NCS-TCP) under which a number Accredited Test Laboratories (ATLs) includes some for the banana virus indexing and genetic fidelity testing have been established (Annexure VI). These also include protocols for the preparation of buffers, PCR mix and the ISSR primers to be used exclusively for genetic fidelity testing. The ATLs certify the tissue culture raised planting materials based on standards approved by the Ministry of Agriculture. Increasing awareness among the farmers who insist upon certified plants from supplier has been noticed, a positive trend towards developing a strong tissue culture based banana industry (Annexure VII).

5.1.3 Philippines

Traditional cultivation of bananas in the Philippines through perennial system led to the build up of pests and diseases (Molina, 2002; Molina *et al*., 2009). Severe epidemics of BBTV practically eliminated the livelihoods of many such growers in the northern Philippines, who used to supply the popular variety Lakatan to Manila. Initially, tissue culture bananas were produced by the government sector which was not cost effective. Further, the small farmers were not familiar with the production practices for growing tissue culture bananas and there was low uptake of tissue culture technology. But under public-private partnership mode, the private

tissue culture laboratories succeeded in producing Lakatan at a lesser cost with the same quality (Molina *et al.,* 2006). Rooted tissue culture seedlings produced at participating private tissue culture laboratories are sent by air to Manila from where they are delivered to the provinces where they are reared to planting stage in village nurseries. Growers are also trained on improving their banana production system, and the advantages of using disease-free, TC planting material as opposed to suckers are being demonstrated. Although plants derived from TC became reinfected in the field, the symptoms appeared late and usually occurred in the ratoon crop. In heavily infested areas, the value of annual cropping was evident. This work is helping in the formulation of a sustainable Lakatan production system for small-scale farmers. Further, tissue cultured bananas were found to be free from nematodes reducing the need for nematicidal treatments. This not only reduced the input cost but also reduced the environmental hazards. These were the primary attractions for the Philippine farmers to adopt tissue culture bananas.

5.1.4 Taiwan

In Taiwan, the epidemics of Fusarium wilt race 4 on the ruling export variety, Grand Naine, almost wiped out the banana industry during 1990's (Hwang and Su, 2000). Extensive planting of millions of TC plantlets enabled Taiwan Banana Research Institute (TBRI) together with growers to select somaclonal variation derived varieties, which had desirable agronomic traits such as shorter plant height, bigger bunches, shorter maturing, and most importantly, resistance to Fusarium wilt race 4. This innovative breeding approach started in 1983 has resulted in improved commercial varieties contributing significantly to the rehabilitation of the Taiwanese banana industry.

TBRI and National University of Taiwan are involved in indexing and certification of tissue culture plants in the country (Molina, 2002). Department of Plant Pathology, National Taiwan University has developed reliable and sensitive methods for detection of viruses. Implementation of such modern virus indexing technique in the banana micropropagation system in Taiwan has greatly improved the efficiency of tissue culture process and minimized the risk of virus being distributed through TC plantlets. In Taiwan, direct use of suckers from commercial plantation for planting is prohibited and the seedlings are always procured from virus free stock foundations. The stock plants raised in insect-proof, vector-free screen house are inspected constantly for virus symptoms and subjected to virus indexing regularly. For plantlets showing symptoms suspected of viral infection, regular inspection for off-type mutants and virus indexing are made mandatory before plantlets are released to the growers (Hwang and Su, 2000).

5.2 Economics of tissue culture banana productions

Despite the superiority of tissue culture plants, high initial cost was one of the main reasons for their low adoption. Several studies have been carried out to estimate the costs and returns, identify the factors influencing the cost of production, assess the resource use efficiency in tissue cultured banana so as to identify the factors determining the adoption of tissue cultured banana and the risks involved (Daniells, 1990; Rao, 2000; Alagumani, 2005; Hanumantharaya *et al.*, 2009).

Results of economic analyses in India indicated that the cost of production per bunch is higher in tissue culture bananas (US$ 1.05) than in sucker raised plants (US$ 0.87) (Table 6). This is mainly due to the high cost of tissue culture bananas, plant protection chemicals and labour. However, the gross and net income from tissue cultured bananas is higher than those from conventional planting material by 35.4 % and 42.4 %, respectively which indicate the economic advantage of tissue cultured bananas. This has been attributed to the efficient utilisation of resources by tissue culture bananas (Alagumani, 2005). Probit analysis revealed that gross income and bunch weight have a positive and significant influence on the adoption of tissue culture bananas.

Table 6. Comparative income from tissue-culture and sucker-propagated bananas

Sl. No.	Particulars	TC propagated bananas		Sucker propagated bananas	
		INR	US$	INR	US$
1.	Mean yield (bunches/ha)	2,663		2,416	
2.	Mean price received (/bunch)	94.47	1.90	76.42	1.52
3.	Value of main product (/ha)	251,573	5,031.50	184,630	3,692.60
4.	Value of by-product (/ha)	1729	34.58	2,518	50.36
5.	Gross income (/ha)	253,302	5,066.00	187,149	3,743.00
6.	Total expenses (/ha)	141,040	2,820.80	108,294	2,165.90
7.	Net income (/ha)	112,262	2,245.20	78,855	1,577.10
8.	Cost of production per bunch	52.31	1.05	43.78	0.87
9.	Net income per bunch	42.16	0.84	32.64	0.65

Source of original data: Hanumantharya *et al.* (2009)

5.3 The way ahead

A survey conducted by FAO revealed that the traditional suckers are being used as planting material by more than 92 % of the growers across the world while

approximately only 8 % have access to tissue culture based planting material. However, with increased realization of the potential advantages of the use of tissue culture plants, a quantum increase in their demand has been witnessed in all Asian countries over last two decades. In India, a minimum of 900 million plantlets are needed in order to bring at least one third of the banana growing area under tissue culture based cultivation. With a current production of only 40-80 million plantlets, there is an enormous potential for growth of tissue culture industry in the coming years (Table 7).

Table 7. Market projections for tissue culture banana plants in India

			Year		
			2004-05 (million)	**2007-08 (million)**	**20010-11 (million)**
Anticipated	Volume		27.50	64.00	80.00
	Value	INR	330.00 @₹ 12/plant	768.00 @₹ 12/plant	960.00 @₹ 12/plant
		US$	6.60	15.36	19.20
Actuals	Volume		30.00	51.00	-
	Value	INR	2750 @₹ 15/plant	765.00 @₹ 15/plant	-
		US$	7.20		

- Survey report of DBT, India and compiled information from various other sources

Considering the high rate of consumption of conventionally propagated plants in the domestic market and the potential of replacement of at least a part of this requirement by tissue culture plants for improving overall productivity and for strengthening the industry, the Department of Biotechnology (DBT) and the Small Farmers' Agri-Business Consortium (SFAC), India, commissioned a market survey in 2005 aimed at selecting commercially important TCPs, current domestic demand and projections, major consumer segments and providing recommendations for improving the demand and strengthening the tissue culture industry. The results indicated that the annual need for tissue culture plants was 44 million of which banana constituted 41%. Based on this survey, the growth in demand for tissue cultured bananas was expected to increase at a high rate of 25.3% by 2009-2010 (DBT, 2005). Similar trends have been noticed in many Asia-Pacific countries where adoption of tissue culture based planting material has enhanced banana production.

Despite the fact that tissue culture plants have several advantages over conventional suckers, its application is hindered by high initial cost of planting material. This calls for adoption of innovative low cost tissue culture technologies to cut down the cost without compromising the quality. Mechanization in media dispensing, dish washing, cheaper alternatives for media components like agar and sucrose and harnessing natural light over fluorescent light have been tried with success. But up-scaling of these technologies and their commercialization is yet to be achieved.

Macropropagation has a high potential as a cost effective rapid propagation method for banana planting material multiplication. With the required low technology inputs, the technology can easily be adopted by farmers. With a capacity of producing 50-60 plantlets/sucker in a span of 4-5 months, a farmer needs only about 50 good quality mother suckers to cater to planting material requirement for one hectare (2,500 plants). The average cost of production, which works out to US$ 0.02 per plantlet, is far below the cost of even conventionally produced suckers. Small nurseries can be encouraged take up commercial macropropagation with little capital investment.

The production cost of tissue culture plants can also be reduced through the adoption of improved technologies like drip irrigation, high density planting and integrated nutrient and pest management strategies. Of late, the annual planting system of Cavendish clones has been extended to 1+2 crop cycles (1 main crop and 2 ratoons) wherever there is no threat of Fusarium wilt, tropical race 4. This 1+2 cropping system has been commercially adopted in most of the traditional Cavendish (cvs. Grand Naine, Dwarf Cavendish and Robusta) ruling areas in India. After harvest, 4 suckers per plant are extracted and sold in the market @US $1/10 suckers. This allows the farmer to recover the initial investment in tissue culture planting material within two years and by the third year make some profits. Thus, the concept of high initial investment can be strategically converted into a gainful business with proper management.

Somatic embryogenesis with the adoption of bioreactor for up-scaling can also be standardized as an alternative to shoot tip culture technology. Following validation for genetic fidelity and stability across well known commercial varieties, somatic embryogenesis technology can be exploited commercially. With a potential to produce very large number of plantlets, the cost of somatic embryogenesis based TC plants is expected to come down drastically to the level affordable even by small farmers.

Somatic embryogenesis

Somatic embryogenesis is the formation of an embryo from a cell other than a gamete or the direct product of gametic fusion. Somatic embryogenesis technique in banana is aimed to (a) develop a high throughput micropropagation system, and (b) as a regeneration system useful for genetic improvement.

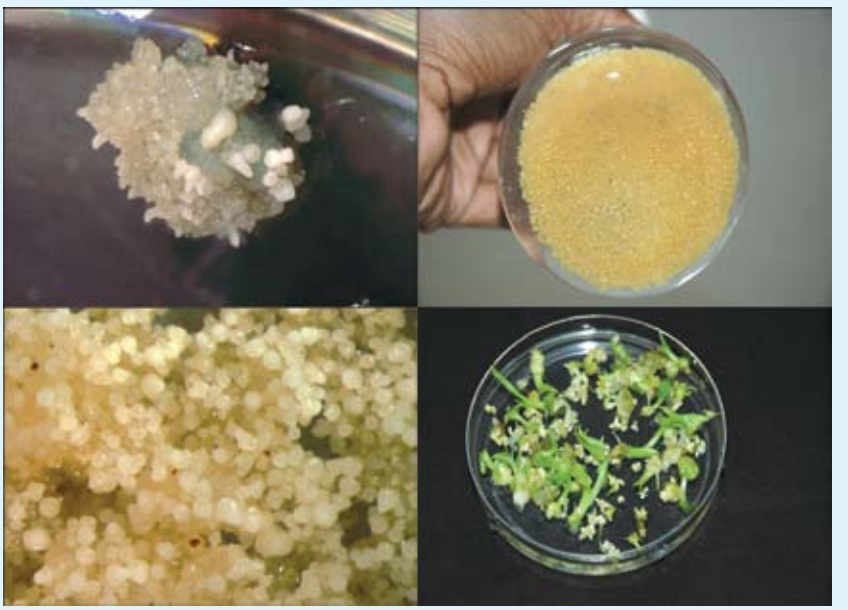
The choice of explant for somatic embryogenesis depends on the regeneration capacity of the cells. Flower buds, proliferating meristem, zygotic embryos, rhizome and leaf sheath have been used as explants to produce callus and eventually the embryogenic cell suspensions. Among them, proliferating meristem and immature male flower buds are commonly used. Both explants have their own advantages and disadvantages. Though using proliferating meristem as explant is time consuming and labour intensive, yet it shows better response in terms of high success rate over immature male flower buds. On the other hand, immature male flower buds require less time and labour input but availability of explant is season dependent.

The explants is initiated in a suitable medium and maintained in dark conditions without subculture for 5-9 months depending on the explant and cultivar. Embryogenic callus is initiated in liquid medium to develop embryogenic cell suspension with repeated media replenishments. A good suspension is obtained within 3-6 months with high regeneration capacity. Plants can be regenerated which are eventually hardened and field planted. Detailed published protocols are available for various explants and commercial cultivars.

Propagation through embryogenic cell suspension (ECS) enables production of large numbers of 'reproductive units' (60,000 to 1.35 million somatic embryos per liter of medium) with the presence of both root and shoot meristems in the same element. High regeneration capacity of embryogenic cell suspensions and faster manipulation of liquid cultures make mass clonal propagation through somatic embryogenesis quite attractive. Indeed, 1 ml settled cells of a highly regenerable cell suspension can yield upto more than 100,000 plants.

In banana, somatic embryogenesis is a powerful tool for mass production of quality planting material. Although, initial establishment of cell suspension is time consuming, once developed it is a continuous source of explants for regeneration (Table 8). This protocol has been well established in many commercial cultivars like Grand Naine, Silk, Plantains and East African Hill Bananas. But its commercial exploitation as a mass production tool is still

elusive because of problems of sustainability of suspension quality over a period of time, variable per cent regeneration and field confirmed results of genetic stability. Though initial field trials of ECS derived plants have confirmed stability and genetic purity in varieties like Rasthali (AAB) and Nendran (AAB), large scale testing of explants, varieties and suspension ages needs to be carried out. Once proven stable under field conditions, somatic embryogenesis can open up new vistas for large scale production of quality planting material in banana.



Most of the countries have national seed policies for promoting quality seed production. However, these often do not include vegetatively propagated crops, which thus get marginalized in terms of support for quality production, farmer awareness and education. Creation of awareness of quality plants through vegetative propagation especially using suckers, macropropagated plantlets and tissue culture plants is important for farmers as well as industry. Although many private and public sector nurseries are involved in large scale production of banana planting material, desirable level of awareness of quality standards is still lacking.

Tissue culture for quality planting material in banana has become a success story in Australia because of the well defined certification system implemented by QBAN or other official certification agencies. In several other countries on

Table 8. Different phases and durations in regeneration of plantlets through somatic embryogenesis in banana

Phase	Stage of plant material	Duration (months)
Preparation of embryogenic competent explants	Homogeneous proliferation of callus	5-14
Embryogenic induction	Induction of embryogenic complex	4-7
Initiation of suspension and maintenance	Embryogenic cell suspensions	3-6
Formation of somatic embryo	Regeneration of somatic embryos	2-3
Germination of somatic embryos	Rooted plantlets in test tubes	2-4
Primary hardening	5 to 7 cm height	1
Secondary hardening	10-15 cm height with 3-5 leaves	1-2
Total		18-36

the other hand, technical standards required for certifying live material have not been adopted very well which has been the major reason for slow success of tissue culture industry. Although improved quality can be imposed through legislation, self-imposition of standards by industry can give better dividends. Creation of an apex regulatory body at national level to monitor the tissue culture production and supply system can improve the growth and success of banana tissue culture industry. Along with, there is a need for improving the available quality testing tools, especially virus indexing and fidelity testing, and periodic training of technical staff on the new technologies. Development of affordable advanced techniques like "dip stick method" along with multiplex PCR for virus diagnostics will help in adoption of tissue culture technology to reach all strata of banana growers.

Regional Cooperation has a great role to play in making this technology available all across the Asia-Pacific region. Countries like Australia, India, Taiwan and China have well developed virus diagnostic and fidelity testing systems. BAPNET (Banana and Plantain Network for Asia and Pacific) and BIOVERSITY – France (INIBAP) have been working on rationalization of Banana Virus Indexing protocols at global and regional levels in collaboration with Australia, India and Philippines. This network can now offer training on fidelity testing and virus indexing to other Asian countries. In fact, training of production personnel as well as farmers in technical, regulatory and business aspects of micropropagation based planting material production is important to upscale the back yard banana industry in the region to a commercial enterprise.

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ANNEXURE I

Important Banana Commercial Varieties Grown in Asia-Pacific

Variety / Country	Cavendish		Silk	Poovan	Pisang Awak	Monthan		Plantain
Australia	Grand Naine	Ladies Finger	Silk	Mysore	P. Awak	Monthan	Red	Plantain
Bangladesh	Grand Naine	Jahai, Governor	Malbhog	Champa	Kanthali	Kachkol	-	-
India	Grand Naine	Robusta	Malbhog	Poovan, Champa	Karpuravalli, Kanthali	Monthan, Kachkel	Red banana	French Plantain
Indonesia	P. Badak, P. Ambon Jepang	P. Badak, P. Ambon Putin	P. Rajah Sereh	P. Keling	P. Awak	P. Keling	P. Raja Udang	P. Candi, P. Byar
Malaysia	P. Serendah	P. Buai	P. Rasthali	P. Keling	P. Siem, P. Awak	P. Abu Bhujal	Morado	P. Tanduk
Philippines	Grand Naine	Bungulan, Tudok	Latundan	Inangel	Katali	Madurang	Agniswar, Sevvazhai	Bhngoaism
Sri Lanka	Grand Naine	Kolikkudu	Red banana	Embul, Kadali, Honderawala	Alukesel, Neer, Kaddu	Hamban Puwalu, Sura Mondan	Rathambala	Angaviaru, Nendrapalam
Taiwan	Grand Naine	GCTCV	Novaria	-	-	-	-	-
Thailand	Klui Kom Khieo Khom	Grand Naine	Klui Nam	Klui khai Farang, Klui Lanka	Klui Namwa	Klui Nam Mi, Muk Khieo	Klui Bat	Klui Khai
Vietnam	Chuoi Tieu Lun, Chuoi Va Huong	Chuoi Tieu Vanh,	Chuoi Goong	Chui Comchua	-	Chuoi Ngop cau	Chuoi Com Lua	Chuoi Sung Bo

ANNEXURE II

Culture Media Used at Different Stages of Banana Micropropagation (for one litre)

Ingredients	Initiation medium	Shoot multiplication medium	Rooting medium
Activated charcoal	-	-	2.50 mg
Agar	-	7.5 g	7.5 g
Ammonium nitrate	1.65 g	1.65 g	1.65 g
L-ascorbic acid	10.0 mg	10.0 mg	-
6-bezyl amino purine	4.0 mg	4.0 mg	-
Boric acid	6.2 mg	6.2 mg	6.2
Calcium chloride	440.0 mg	440.0	440.0
Cobalt chloride	0.025 mg	0.025	0.025
Copper sulphate	0.025 mg	0.025	0.025
Ferric EDTA	36.7 mg	36.7	36.7
Glycine	2.0 mg	2.0 mg	2.0 mg
Indole-3acatic acid	1.0 mg	1.0 mg	-
Indole-3-butyric acid	-	-	1.0 mg
Magnese sulphate	22.3 mg	22.3	22.3
Magnesium sulphate	370.0 mg	370.0	370.0
Myo-inositol	100.0 mg	100.0 mg	100.0 mg
α-Naphaleneacetic acid	-	-	2.0 mg
Nicotinic acid	0.5 mg	0.5 mg	0.5 mg
Potassium dihydrogen ortho phosphate	170.0 mg	170.0	170.0
Phytal gel	2.0 g	-	-
Potassium iodide	0.83 mg	0.83	0.83
Potassium nitrate	1.9 g	1.9 g	1.9 g
Pyridoxine hydro chloride	0.5 mg	0.5 mg	0.5 mg
Sodium molybdate	0.25 mg	0.25 mg	0.25 mg
Sucrose	30.0 g	30.0 g	30.0 g
Thiamine hydro chloride	0.1 mg	0.1 mg	0.1 mg
Zinc sulphate	8.6 mg	8.6	8.6
ptt	5.8	-	-

ANNEXURE III

VIRUS TESTING PROTOCOLS FOR BANANA ADOPTED AT NRCB

I. Detection of CMV by Direct Antigen Coating-ELISA (DAC-ELISA)

1. Principle

In this method young leaf samples are finely ground with carbonate buffer and the extract is added with chloroform and after centrifuging, the aqueous part is collected and loaded onto the ELISA wells. Then virus specific polyclonal antibodies are added, washed three times and then antirabbit Ig conjugated with alkaline phosphatase enzyme added. Finally, add the substrate (p-nitro phenyl phosphate, pNPP in case of alkaline phosphatase conjugate) and the resulting yellow color developed can be read in ELISA reader and virus can be quantified in the test sample. At least two negative controls (healthy plant sap) besides buffer controls will be used in order to have more confident background values. It is also advised to cross-adsorb antiserum with healthy plant sap to prevent false positives.

2. Requirements for DAC-ELISA Test

2.1 Equipments

- ELISA Reader with printer
- Balance, pH meter, incubator
- Magnetic stirrer
- Microcentrifuge
- Deep freezers (-20 °C, -80 °C)
- Refrigerator
- Micropippettes (10 μl, 50 μl, 100 μl)
- Multichannel adjustable micropippette (4-well type)
- Tissue grinder/pestle and mortar
- Polystyrene microtips

2.2. Supplies

- Microplates (polysterene, 96 wells)/strips
- Antisera (polyclonal/monoclonal)
- Antigen (virus affected plant material)
- Enzyme (alkaline phosphatase) labeled goat anti-rabbit IgG
- Goat Anti-rabbit enzyme conjugate (universal conjugate)
- Substrate (p-nitrophenyl phosphate-PNPP)

2.3. Buffers/Reagents

- **Stock buffer (phosphate buffer- saline, pH 7.4)**

NaCl	8.0 g
$Na_2HPO_4.2H_2O$	1.44 g
$Na_2HPO_4.12H_2O$	2.90 g
Na_2HPO_4	1.50 g
KH_2PO_4	0.20 g
KCl	0.20 g
Distilled water	to make 1 litre

- **Wash buffer (PBS-Tween, PBS-T)**

  Add 0.5 ml Tween – 20 to 1 litre PBS.

- **Coating buffer (Carbonate buffer, pH 9.6)**

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	To make 1 litre

- **Enzyme conjugate diluent/buffer (PBS-T polyvinyl-pyrrolidone and ovalbumin, PBS-TPO)**

  Add 20.0 g polyvinyl-pyrrolidone (PVP, MW 40,000) and 2.0 g egg ovalbumin to 1 litre PBS-T.

- **Antibody diluent/buffer**

  Same as PBS-TPO

- **Substrate buffer (diethanolamine buffer, pH 9.8)**

Diethanolamine	97 ml
Distilled water	800 ml

Adjust the pH to 9.8 with 1N HCl, add about 67 ml and make up the volume to 1 litre with distilled water.

- **Blocking solution**

  Add 5.0 g bovine serum albumin (BSA/ spray dried milk (SDM) to 1 litre PBS-T.

- **Fixing solution**

NaOH	120.0 g
Distilled water	to make 1 litre

(All buffers contain 0.02 % sodium azide as a preservative)

3. DAC-ELISA step by step protocol

1. Weigh 1g of test samples/healthy tissue and grind using autoclaved mortar and pestle in coating buffer containing 2 % (w/v) PVP at a ratio of 1:1 (w/v) at room temperature and low speed centrifuge (ca. 12,000 g) for 2 min.
2. Dispense 200 µl extract from test as well as healthy samples to each well of the microtitre plate. Cover the plate and incubate at 37 °C for 1 h.
3. Decant and wash the plate by flooding the wells with PBS-T for about 3 min. Repeat wash and soak operations thrice, and shake out residual liquid draining on a paper towel.
4. Dispense 200 µl blocking solution (Bovine Serum Albumin, 1%, w/v) to each well. Incubate at 37 °C for 1 h to block polystyrene well reactive surfaces.
5. Wash the plate three times as in step 3.
6. Dispense 200 µl of antiserum (primary antibody) diluted in PBS-TPO to each well. Incubate at 37 °C for 2 h.
7. Wash the plate three times as in step 3.
8. Dispense 200 µl anti-rabbit immunoglobulin alkaline phosphatase) diluted in PBS-TPO to each well. Incubate at 37 °C for 2 h.
9. Wash the plate three times as in step 3.
10. Dispense 200 µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) (0.5 – 1.0 mg/ml) solution in substrate buffer to each well. Incubate at 37°C for 2 h.
11. Measure the intensity of color in each well at 405 nm by using ELISA reader. Compare the absorbance values of the test samples with healthy control. Take a print out for record (consider samples showing absorbance (OD_{405}) values more than two times of healthy control as positive).

Helpful Hints:

- Draw test plan in checkerboard format and include minimum of two replications for each treatment.
- Include negative (healthy) control to validate the assay.
- Determine the optimum dilutions of various reagents to be used.
- Do not wash or rinse microtitre plates prior to use. Once used plates should not be reused.
- Use separate beakers for preparing antigen, antiserum, conjugate and substrate dilutions.
- Dilute antisera according to their titre value and commercial conjugates as per the manufacturer's directions.
- Antisera used should be free from cross-reacting antibodies against host proteins to avoid false positive reactions. Otherwise cross-absorb the antiserum with sap extracted from healthy plant by incubating required quantity of antiserum in about 1:50 dilution of healthy plant sap extracted in PBS. Incubate at 37°C for 1 h and centrifuge at 10,000 rpm for 5 min. The supernatant is then directly used.
- Always use freshly prepared substrate buffer.
- PNPP is photo-degradable. Hence cover with aluminium foil to avoid direct contact with light. Use as quickly as possible, once PNPP solution is prepared.

II. Detection of BBTV or BSMysV by PCR

1. Principle

Polymerase chain reaction is a technique that amplifies DNA, enabling preparation of millions or even billions of copies of a DNA molecule in a very short time. The procedure has been developed to detect very small quantities of nucleic acid present in a sample by *in-vitro* amplification of a segment of the DNA situated between two regions of known nucleotide sequence. Such a segment is flanked by two oligonucleotides, which serve as primers for a series of reactions that are catalyzed by a DNA polymerase. The amplification consists of cycles involving template denaturation, primer annealing and extension of the annealed primers by DNA polymerase, resulting in an exponential accumulation of specific fragment. Since the primer extension products, synthesized in one cycle will serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. PCR significantly enhances the probability of detecting rare sequences in heterogeneous mixtures of DNA. PCR involves three stages:

- The DNA is denatured to convert the duplex molecule into two single strands
- Primers are annealed to the target single stranded DNA
- The primers are extended by nucleotide addition by the action of DNA Polymerase.

In banana, PCR is performed for detection of DNA viruses namely BBTV and BSV.

2. Requirements of Test

2.1 Equipments:

- Thermal cycler
- Horizontal gel electrophoresis unit with power pack
- Gel documentation unit with computer and printer
- Refrigerated microcentrifuge
- Deep freezers (-20 °C, -80 °C)
- Micropippettes (1μ, 10 μl, 50 μl, 100 μl, 1000 μl, 5000 μl)
- Tissue grinder/pestle and mortar
- Polystyrene microtips

2.2. Supplies:

- PCR tubes (500 μl/200 μl)
- Virus affected plant tissue/healthy tissue
- Specific primers
- DNeasy kit
- DNA amplification kit
- Pestle and mortar
- Liquid nitrogen

2.2.1.Chemicals and Solutions for electrophoresis:

- **Ethidium bromide (10 mg/ml):**

Dissolve 1 g ethidium bromide in 100 ml H_2O and transfer to a dark bottle and store at 4 °C.

- **0.5 M EDTA (pH 8.0):**

  EDTA: 186.1 g

  Dissolve in 800 ml H_2O. Adjust the pH to 8.0 with 10 N NaOH. Make the volume to 1 litre. Dispense into aliquots and sterilize.

- **Running buffer (50 X TAE (Tris Acetate EDTA, pH 8.0)**

Tris Base	242.0 g
Glacial acetic acid	57.1 ml
EDTA(0.5M, pH 8.0)	100 ml

Adjust pH 8.0 with 1 N NaOH. Make up volume to 1 litre. Dilute 50 X TAE buffer to 1 X before use.

3. PCR step by step protocol

3.1 Isolation of total nucleic acid:

Extract total DNA from approx. 100 g/mg of sample using DNeasy kit according to the manufacturer's instructions and store this DNA at -20 °C. Use this DNA template for polymerase chain reaction (PCR).

3.2 PCR amplification:

Assemble PCR reaction components on wet ice and prepare amplification mix by dispensing into ~ 200 µl microfuge tube in the order listed below

Sl. No	Reagents	Volume
1	10X PCR buffer with 15mM $MgCl_2$	5.0 µl
2	10mMdNTP mix	4.0 µl
3	Tag polymerase	0.5 µl
4	Distilled water	34.5 µl
5	Forward primer	1.0 µl
6	Reverse primer	1.0 µl
7	Template	4.0 µl
	Total volume	50 µl

Mix well. Centrifuge the tubes for 10-20 seconds to drain the walls. Place the tubes in the PCR apparatus and set the program to initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 49-53 °C for 1 min and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min.

After PCR, if necessary, keep the reaction mixtures at 4 °C overnight.

3.3. Analysis of amplicons:

1. Melt 0.5 g agarose in 50 ml 1 X TAE running buffer and add ethidium bromide (0.5 mg /ml) to the agarose after cooling to around 50 °C.
2. Pour the melted agarose into the casting tray for polymerization and fill the buffer tank with running buffer (1 X TAE) and remove the comb.
3. Load 10 µl RT-PCR products with 2 µl 6X loading dye and run the gel at 60 volts for 2 h along with marker.
4. Examine the gel under ultraviolet transilluminator and photograph.

III. Detection of BBrMV or CMV by RT-PCR

1. Principle

This technique is used for detection of viruses having RNA as their genome. In banana, two viruses namely *Banana bract mosaic* virus and *Cucumber mosaic virus* has RNA genome. This protocol describes the testing procedure of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for the detection of BBrMV or CMV using primer mediated *in vitro* reaction involving amplification of target nucleic acid sequences in which reverse transcriptase enzyme is used to convert the viral genome into cDNA before performing PCR with suitable amplification conditions. Since these are RNA viruses, either one step or two step RT-PCR is used to amplify a segment of RNA that lies between two regions of known sequences. One step RT-PCR will have four phases in the process viz., (i) cDNA synthesis using reverse transcription at 42 °C; (ii) denaturation at a high temperature (90-95 °C); (iii) annealing of target specific primers, and (iv) primer extension by a thermostable DNA polymerase. In two step RT-PCR, cDNA is synthesized first and then PCR is performed in a separate tube using cDNA as template with set of virus specific primers. The resulting amplified product is then resolved by agarose gel electrophoresis and visualized in a UV transilluminator.

2. Requirements of Test

2.1 Equipments:

- Thermal cycler
- Horizontal gel electrophoresis unit with power pack
- Gel documentation unit with computer and printer
- Refrigerated microcentrifuge
- Deep freezers (-20 °C, -80 °C)
- Micropipettes (1 µ, 10 µl, 50 µl, 100 µl, 1000 µl, 5000 µl)
- Tissue grinder/pestle and mortar
- Polystyrene microtips

2.2. Supplies:

- PCR tubes (500µl/200 µl)
- Virus affected plant tissue/healthy tissue
- RNeasy kit, specific primers
- First strand cDNA synthesis kit

- DNA amplification kit
- DEPC treated water, tips, and tubes
- Pestle and mortar
- Liquid nitrogen

2.2.1.Chemicals and Solutions for electrophoresis:

- **Ethidium bromide (10 mg/ml):**

  Dissolve 1 g ethidium bromide in 100 ml H_2O and transfer to a dark bottle and store at 4 °C.

- **0.5 M EDTA (pH 8.0):**

  EDTA: 186.1 g

  Dissolve in 800 ml H_2O. Adjust the pH to 8.0 with 10 N Na OH. Make the volume to 1 litre. Dispense into aliquots and sterilize.

- **Running buffer (50 X TAE (Tris Acetate EDTA, pH 8.0)**

Tris Base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5M, pH 8.0)	100 ml

Adjust pH 8.0 with 1 N NaOH. Make up volume to 1 litre. Dilute 50 X TAE buffer to 1 X before use.

3. RT-PCR step by step protocol

3.1. Isolation of total nucleic acid:

Weigh test/healthy sample (~100 mg) and cut into strips. Extract RNA using RNeasy kit according to the manufacturer's instructions and store in -80 °C. Use this RNA as template for reverse transcription – polymerase chain reaction (RT-PCR).

3.2. cDNA amplification:

Assemble RT-PCR reaction components on wet ice and prepare amplification mix by dispensing into ~ 200µl microfuge tube in the order listed below

- Total RNA- 10.0 µl
- Reverse primer-1.0 µl
- Mix gently and spin down for 3-5 seconds in a microfuge
- Incubate the mixture @ 70 °C for 5 min
- Chill on ice and collect by brief centrifugation
- Place the tube on ice, add the following components

S.No	Reagents	Volume
1	Rnase inhibitor	1.0 µl
2	RT buffer (5X)	4.0 µl
3	10mM dNTP mix	2.0 µl

- Mix gently and collect by centrifugation
- Incubate at 37 °C for 5 min
- Add reverse transcriptase (200 μ/μl)-1.0 μl
- Mix the solution well and incubate at 42 °C for 1 hour in PCR machine
- Stop the reaction by heating at 70 °C for 10 min
- Chill on ice
- This product can be directly used for PCR amplification

3.3. PCR amplification

Sl. No	Reagents	Volume
1	10X PCR buffer with 15mM $MgCl_2$	5.0 µl
2	10mMdNTP mix	4.0 µl
3	Tag polymerase	0.5 µl
4	Distilled water	34.5 µl
5	Forward primer	1.0 µl
6	Reverse primer	1.0 µl
7	Template	4.0 µl
	Total volume	50 µl

Initial denaturation at 95 °C for 2 min was followed by 30 cycles of denaturation at 94 °C for 20 sec, annealing at 45-53 °C for 1 min and extension at 72 °C for 20 sec with a final extension at 72 °C for 10 min.

After PCR, if necessary, keep the reaction mixtures at 4 °C overnight. Analyze the amplified product by electrophoresing 10μl from the total reaction on 1 % agarose gel in Tris acetate EDTA (TAE) containing ethidium bromide.

3.4. Analysis of amplicons:

- Melt 0.5 g agarose in 50 ml 1 X TAE running buffer and add ethidium bromide (0.5 mg/ml) to the agarose after cooling to around 50 °C.
- Pour the melted agarose into the casting tray for polymerization and fill the buffer tank with running buffer (1 X TAE) and remove the comb.
- Load 10 μl RT-PCR products with 2 μl 6X loading dye and run the gel at 60 volts for 2 h along with marker.

- Examine the gel under ultraviolet transilluminator and photograph.

Helpful Hints:

- Always perform a healthy control reaction.
- Use fresh gloves for RNA isolation and each reaction set-up.
- Wear gloves while handling agarose gels containing ethidium bromide.
- Ensure that the RNA used is inactivated at 70 °C for 5 min and then snap chill on ice.
- Thaw and vortex all the reagents before use (except DNA polymerase and reverse transcriptase).
- Optimize the annealing temperature for the primer pair before setting the reaction.
- Keep all the reagents at -20 °C after use for long storage.
- Ensure 50X TAE buffer is diluted to 1X buffer before running the gel.

General remarks on handling RNA

- Proper microbiological, aseptic technique should always be used when working with RNA.
- Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent Rnase contamination.
- Use sterile and disposable polypropylene tube.
- Non disposable plastic ware should be thoroughly rinsed with 0.1 M NaOH, 1mM EDTA followed by RNase free water.
- Glassware can be treated with 0.1 % DEPC (Diethyl pyrocarbonate) water, allow to stand overnight at 37 °C and then autoclave.
- Solutions should be treated with 0.1 % DEPC water and incubated over night. Autoclave for 15 minutes to remove any trace of DEPC. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer.

IV. Nucleic acid spot hybridization (NASH)

1. Principle

This protocol describes the testing procedure of NASH for detecting very small amount of virus in the plant material. The detection is based on mobilization of target (virus) nuclic acid (NA) on to a solid matrix followed by hybridization with NA probes under appropriate conditions. Nitrocellulose or charged nylon membranes are the commonly used solid matrix for hybridization. Both radioactive probes (^{32}P DNA probes) as well as non-radioactive probes (chemiluminscent, digoxigenenin-labelled cRNA probes) can be used for nucleic acid spot hybridization.

This technique involves denaturation of viral nucleic acid if it is double stranded; a spot of the sample extract is applied on to a nitrocellulose or positively charged nylon membrane. The membrane is baked for binding the nucleic acid on to it. Then the non-specific binding sites are blocked by incubation in a pre-hybridization solution containing salmon sperm DNA. Labeled probes are used for hybridization with nucleic acid present in the samples. The excess probes is washed off and the probes bound to the target quantified. In dot blot or slot-blot formats the samples are spotted on a membrane using a template having either round holes or slots. The template is an apparatus, which draws the sample through the membrane by suction and enables dilute samples to be concentrated on the membrane. The dot-blot does not give any information on the size or number of species of the target nucleic acid.

2. Requirements

2.1. Equipments

- Hybridization oven
- Hybridization flask
- UV Cross linker
- X-ray film developing facility
- Vacuum oven
- Shaking water bath
- Bag sealer

2.2. Supplies

- Nitrocellulose or nylon membranes
- Pestle and mortar/tissue grinder
- Virus infected and healthy plant samples
- X-ray cassettes

3. NASH protocol using non radioactive probe

3.1. Preparation of solutions and buffers for hybridization experiment

3.1.1. Solutions and buffers required for detection

Buffer 1

Maleic acid, 0.1 mol/l

NaCl, 0.15 mol/l

pH 7.5 (+20 °C) Adjust with concentrated or solid NaOH and autoclave

For chemiluminescent detection:

Buffer 1, autoclaved,

+ 0.3 % Tween 20 (w/v). Do not autoclave buffer 1 containing Tween 20.

Buffer 2

Blocking reagent stock solution, diluted 1:10 in buffer 1, autoclaved (final concentration: 1% Blocking reagent)

Antibody-AP conjugate solution for colorimetric detection NBT/X-Phosphate

Dilute anti-digoxigenin-AP conjugate to 150 mU/ml (1:5.000) in buffer 2.

Antibody-AP conjugate solution for chemiluminescent detection

Dilute anti-digoxigenin-AP conjugate to 75mU/ml (1:10.000) in buffer 2.

Buffer 3

Tris-Hcl,100 mmol/l pH 9.5 (+20 °C)

NaCl, 100 mmol/l

$MgCl_2$, 50 mmol/l

Prepare buffer 3 from corresponding stock solutions to avoid $MgCl_2$ precipitation

TE buffer

10 mmol/l Tris-Hcl, pH 8 (+25 °C)

1 mmol/l EDTA

Denaturation solution 1 (For southern transfer)

0.5 N NaOH

1.5 mol/l NaCl

Neutralization solution 1 (for southern transfer)

0.5 mol/l Tris-Hcl, pH 7.5

3 mol/l NaCl

DNA dilution buffer

50 μg/ml hering sperm DNA in 10mM Tris-Hcl

1 mmol/l EDTA, pH 8.0

Standard hybridization buffer

5x SSC

% sodium-lauroylsarcosine

0.02 % SDS

1 % Blocking reagent from Blocking reagent stock solution.

Modified hybridization buffer + 50 % formamide

(Mostly for RNA hybridization or chemiluminescence detection)

5x SSC

50 % formamide, deionized

0.1% sodium-laurylsarcosine

0.02% SDS

2% Blocking reagent.

20 x SSC

3 mol/l NaCl

300 mmol/l sodium citrate,pH 7.0.

Washing solution 2x

2x SSC

0.1 % SDS

Washing solution 0.1x

0.1x SSC

0.1 % SDS

N-lauroylsarcosine

10 % (w/v)

Filtered through a 0.2 – 0.45 μm membrane.

SDS

10 % (w/v)

Filtered through a 0.2 – 0.45 m membrane.

Probe stripping solution

0.2 N NaOH

0.1 % SDS

3.1.2. Solution and buffers required for RNA/Northern Blots

Dimethyldicarbonate treated water

Dissolve dimethyldicarbonate to 1 % (v/v) in a 50 % ethanol/water mixture, mix redist, water 1:10 with this solution (=0.1 % dimethyldicarbonate); incubate for 30 minutes at room temperature, then autoclave

RNA dilution buffer

Mix dimethyldicarbonate treated water with 20 SSC and formaldehyde in a volume ratio of 5:3:2

Standard hybridization buffer (formamide)

Recommendaton for RNA probes

50 % formamide, deionized

5x SSC

2 % Blocking reagent

0.1 % (w/v) N-lauroylsarcosine

0.02 %(w/v) SDS

RNA loading buffer

Make up a fresh solution

250 μl formamide,deionised

83 μl formaldehyde 37 % (w/v)

50 μl 10x MOPS buffer

0.01 % (w/v) bromophenol blue

50 μl glycerol

Fill up to 500 μl with DEPC- treated water.

10x MOPS

Morpholinopropansulfonic acid, 200 mmol/l

Sodium acetate, 50 mmol/l

EDTA,10 mmol/l

pH 7.0, autoclaved

After autoclaving, the solution will turn yellow.

3.2.1. DNA Dot blotting protocol

1. Isolate DNA or RNA from the samples to be tested or just homogenise the leaf samples and centrifuge the sap in a microfuge at 10,000 rpm for 5 min. Dilute the collected supernatant in 10 x SSC. A positive control and also a negative control from a virus-free plant should be included
2. Denature the target DNA for 10 minutes at 95 °C
3. Chill directly on ice
4. Mark the membrane lightly with a pencil to identify each dilution spotting. Mark the membrane for orientation
5. Wet the membrane in 20 x SSC and place it on a sheet of filter paper
6. Dispense 2 μl of each of the samples onto the membrane and dry the spots at room temperature
7. Fix the DNA to the membrane by UV cross-linking or baking in an oven at 80 °C for 1-2 hours
8. The membrane can be used immediately for prehybridization or can be stored dry at 4°C for further use

3.3 Probe preparation

1. Add 1 μg template DNA and autoclaved double distilled water to a final volume of 16 μl to a reaction vial
2. Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling in ice
3. Mix DIG-High Prime thoroughly and add 4 μl to the denaturated DNA
4. Mix and centrifuge briefly
5. Incubate for 20 hrs at 37 °C

3.4 Pre-hybridization and hybridization

1. Place the blot in hybridization bag containing 20 ml standard prehybridization solution per 100 cm^2 of membrane surface area
2. Seal the bag and prehybridize at the anticipated hybridization temperature for at least 1 hour (optimal hybridization temperature for a specific probe will depend on the length of the probe and on the extent of its sequence homology with target sequence). If roller tubes are used for hybridization, use at least 6 ml per tube
3. Several membranes can be processed in the same sealed bag as long as there is pre-hybridization solution to cover all the membranes and the membranes can move freely in the bag
4. Discard the pre-hybridization solution

5. Add the hybridization solution containing the DIG-labeled probe and allow the probe to hybridize
6. DNA probe concentration should be 5.25 ng/ml hybridize overnight at 68 °C; if using a dsDNA probe, heat in a water bath for 10 min. to denature the DNA. Chill immediately on ice
7. Dilute the probe in standard hybridization solution. The required amount of standard hybridization solution for 10 × 10 cm is at least 2.5 ml.

3.5. Detection by using chemiluminescent system

1. Wash the membrane twice, 5 min per wash in 2X wash solution at room temperature. The washes remove unbound probe. Otherwise could lead to high background
2. Wash the membrane twice, 15 min per wash in 0.5 X wash solutions. Long probe100 bp should be washed at 68 °C
3. For shorter probe, the washing temperature corresponds to the hybridization temperature
4. Wash the membrane in wash buffer for 5 min
5. Incubate the membrane in blocking solution for 30 min
6. Incubate the membrane in antibody solution for 30 min
7. Wash the membrane in wash buffer for 15 min
8. Equilibrate the membrane in detection buffer 5 min
9. Apply CSPD for the membrane in 5 min
10. (CSPD application: Place the membrane between two sheets of acetate. Gently lift the top sheet of plastic with a sterile pipette. Add approximately 0.5 ml per 100 cm^2 of diluted CSPD to the top surface of the membrane)
11. Spread the substrate over the surface
12. Rock the membrane gently to distribute the reagent over the surface
13. Lower the top sheet of plastic and with a damp lab tissue
14. Gently wipe the top sheet to remove any bubbles present on the sheet and to create a liquid seal over the membrane
15. Seal semi-dry membrane in plastic bag
16. Incubate the membrane for 15 min at 37 °C
17. Expose to X-ray film for 20 min
18. The X-ray film can be developed by placing the film in Developer 2 min followed by water 2 min and finally place it in fixer 2 min. The film can be washed in running water

3.6. Immunological detection

1. Wash the membrane in TBS buffer (0.2 ml for each square centimetre of membrane)
2. Incubate at room temperature for 30 min in the same amount of blocking solution in a hybridization flask
3. Wash the membrane for 1 min in the same amount TBS buffer

4. Dilute the alkaline phosphatase-conjugated antibodies to DIG up to 150 U/ml (1:5000) in TBS buffer (2 μl per 10 ml buffer)
5. Incubate the membrane in approx. 10 ml diluted conjugated antibody suspension for 30 min
6. Remove unbound antibodies by washing with 20 ml of TBS buffer (twice for 15 min each)
7. Incubate the membrane for 2 min in 10 ml of AP buffer
8. Transfer the membrane to 5ml freshly prepared substrate solution and incubate in the dark. Observe the development of purple colour in dimmed light
9. As soon as the bands or spots of interest are detected (usually within 12 h), stop the reaction by washing the membrane for 5 min with 10 ml of TE buffer
10. Make a photocopy or photograph of the wet membrane. The membrane can be stored in sealed plastic bags with TE buffer at room temperature

ANNEXURE IV

BANANA TISSUE CULTURE STANDARDS

Government of India NCS-TCP

1. Department of Biotechnology, Government of India has prescribed the requirements for the tissue culture industry as per the National Certification System for Tissue Culture Plants (NCS-TCP). Following are some of the requirements pertaining to disease freedom for banana tissue culture production.
2. Laboratory and green house facilities used for production of plantlets shall be maintained free of vectors of banana pathogens. Failure to keep such pests under control may cause rejection of all lots maintained in the facility. All potting or growth media shall be sterile. Water sources used in the laboratory or green houses operation shall be treated or otherwise rendered free of all pathogens.
3. Hygienic conditions shall be strictly observed during micropropagation, potting, planting, irrigating, movement and use of equipment and other laboratory and green house practices to guard against the spread of diseases or pests in the facilities used for banana plant multiplication.
4. The green house (protected environment) must be insect proof and be equipped with a double-door entrance, provision for disinfection prior to entering the protected environment and insect proof ventilation screening on intakes and exhaust openings. The persons entering the protected environment should use Wellington boots (plastic boots) and change lab-coat in the changing area to reduce the chances of inadvertent introduction of vector insects clinging to clothes.
5. All samples of banana varieties being initiated should be tested in an accredited laboratory and be free of viruses (*Banana bunchy top virus, Cucumber mosaic virus, Banana bract mosaic virus, Banana streak virus*) and other endophytic or epiphytic bacteria and fungi.
6. On application for inspection, the mother cultures as developed above are eligible for certification. The micropropagation facility to be inspected must have been approved by the competent authority. All stocks must have a valid variety identification and disease testing report at any time during multiplication process.
7. The initiating facility must maintain following information on each variety for review and audit by the competent authority at least once in a year: variety identification, date of initiation, origin and testing results from an accredited laboratory.
8. Tests must be carried out on a minimum of 0.1 % (at least ten) plantlets for each variety by the accredited laboratory. Such tests will be valid so long as cultures of that particular batch are under production (subject to a maximum of 8 passages). No plant should contain *Banana bunchy top virus, Cucumber mosaic virus, Banana bract mosaic virus, and Banana streak virus* and other endophytic or epiphytic bacteria and fungi.
9. Valid pathogen testing results are required at the $2^{nd}/3^{rd}$ subculture stage prior to the bulking up of the cultures.
10. Effective sanitation practices including insect and disease monitoring and prevention must be adhered to.

11. No field-produced banana plants can be grown in the protected environment (greenhouse/ polyhouse) along with tissue cultured plants
12. Varieties must be separated by physical barriers (such as proper tagging), which will prevent varietals mixtures
13. Before dispatch to the farmers, the tissue cultured plants growing in the nursery should be tested for the absence of the viruses.
14. For establishing clonal fidelity, the sample size should be 0.1 % of the batch size with a minimum of 10 plants.
15. If testing performed by an accredited laboratory reveals the presence of banned viruses, fungi or bacteria, the tissue-cultured plants should not be dispatched from the premises of the production laboratory and entire material should be destroyed.
16. The concerned laboratory/agency producing the tissue culture raised material should issue a certificate to the effect that BTC have been produced as per guidelines.

Labelling Banana Tissue Culture

1. Banana tissue culture plants (BTC) shall be supplied in containers. A cloth-lined label of 12cm × 6cm containing following information shall be affixed on the container

Crop	**:**	**Banana**	**Lable No.:**
Variety	:		
Class of material	:	BTC	
Lot No.	:	Batch	
Accredited test laboratory and certification reference note	:		
Date of certification	:		
Production Agency (Name and address)	:		

1. The label shall be rubber stamped with signature, name and designation of the concerned agency. Colour of the label shall be diagonally yellow No. 356 (IS 51978) and opaline green (IS No. 275)
2. BTC producing agency shall maintain the account of labels printed and issued.

BIOVERSITY Recommendations for tissue culture propagation of Musa

In view of the problems encountered in relation to the appearance of BSV symptoms in banana plants of certain genotypes following multiplication by tissue culture, the application of tissue culture for *Musa* mass propagation needs to be reassessed. However, it should be noted that mass propagation is frequently of Cavendish-types of banana and currently there is no evidence of BSV expression in these banana cultivars following passage through tissue culture. In such genotypes, therefore, BSV is considered unlikely to be a cause for concern.

If micropropagation of other *Musa* genotypes is proposed, the risk of BSV expression exists. The recommendations for the mass propagation of non-Cavendish genotypes are as follows:

- test mother plants for the presence of the virus before initiating tissue cultures;
- test representative samples of the progeny;
- consider utilizing other mass propagation techniques if the genotypes are being proposed for commercial multiplication.

Modified BIOVERSITY germplasm health statement (to accompany tissue culture material when distributed)

The germplasm designated below was obtained from a shoot-tip cultured in vitro. Shoot-tip culturing is believed to eliminate the risk of the germplasm carrying fungal bacterial and nematode pathogens and insect pests of Musa. However, shoot-tip cultures could still carry virus pathogens.

Screening for virus pathogens

A representative sample of four plants, derived from the same shoot-tip as the germplasm designated below, has been grown under quarantine conditions for at least six months, regularly observed for disease symptoms and tested for virus pathogens as indicated below following methods recommended in the FAO/IPGRI Technical Guidelines for the Safe Movement of Musa Germplasm for the diagnosis of virus diseases.

ITC code	Accession name	Origin	Serology - ELISA				Electron microscopy		
			CMV	BBTV	BSV	BBrMV	Basilli	Isometric	Filamen

CMV = cucumber mosaic virus
BBTV = banana bunchy top virus
BSV = banana streak virus
BBrMV = banana bract mosaic virus
Bacill = bacilliform virus particles - includes BSV
Iso = isometric virus particles - includes CMV
Fil = filamentous virus particles - includes BBrMV
[+] = test positive
[-] = test negative
[o] = test inconclusive
[ ] = test not undertaken

The information provided in this germplasm health statement is based on the results of tests undertaken at INIBAP's Virus Indexing Centres by competent virologists following protocols current at the time of the test and on present knowledge of virus disease distribution. It should be noted that some virus-tested (negative) hybrids, after in vitro multiplication, have developed BSV infection and may express symptoms. Neither BIOVERSITY, nor its Virus Indexing Centre staff assume any legal responsibility in relation to this statement.

Signature: **Date:**

This statement provides additional information on the phytosanitary certificate of the plant germplasm described herein. It should not be considered as a substitute for the official "Phytosanitary Certificate" issued by the plant quarantine authorities of Belgium.

ANNEXURE V

SCORE CARD FOR ASSESSING TISSUE CULTURE AND GREENHOUSE FACILITIES IN INDIA

1. INFRASTRUCTURE

A. Laboratory Facilities

Washing room

Facilities for washing, drying and storing of glassware and storing of glassware Quality of washing Overall cleanliness	a. Depending on the volumes, washing may be done manually or through a machine but the quality of washing must be good. b. Contaminated cultures should not be stored. They should be washed as soon as possible. c. All the contaminated cultures must be autoclaved before washing with a detergent. If the contamination levels are very high then the glassware (infected cultures only) should be left overnight in chromic acid after autoclaving and washed with detergent the following day. d. The glassware must be washed under running tap water to ensure that no traces of media or detergent are left behind. e. After washing with ordinary water, the culture vessels should be rinsed with deionized water before drying. f. Drying may be done by leaving the jars in an inverted position overnight. Petriplates and other glassware may be dried in an oven. g. There should be a proper mechanism for disposal of used agar. h. Overall cleanliness must be maintained.	5 Marks

Media preparation room

Availability of equipment for media preparation and autoclaving Quality of chemicals Quality of culture vessels. Maintenance of records. Operational efficiency of media preparation (amount of	a. The media preparation room must have all the basic equipment such as weighing balance (electronic), pH meter, conductivity meter, microwave oven, deionizer/distillation unit/RO water facility, autoclave, etc. b. The chemicals should be of analytical grade from a reputed company. c. The details of the media must be recorded and the trays/racks containing media should be properly labeled.	10 Marks

media prepared everyday, proper labeling of media, etc.) Cleanliness	d. All the parameters pertaining to autoclaving such as the time when the autoclave was switched on, when the desired pressure was achieved, autoclaving time, etc. must be recorded. e. As much as possible, high operational efficiency should be maintained to save on manpower. f. After autoclaving, the medium should ideally be stored for 2-3 days so that if something has gone wrong with autoclaving, microbial contamination is detected before the medium is put to use. g. The medium must be stored in clean area where very high level of sterility (at least Class 1000) is maintained.	
Inoculation room Equipment Sterility levels Technical competence of the operators Operational efficiency (number of cultures handled by each operator, labeling of cultures, contamination losses)	a. The inoculation room should have at least sterility level of Class 1000. b. The room must be fumigated periodically with sterilant. c. The airflow of the laminar airflow cabinet should be checked periodically. d. Besides flaming, the tools (forceps, scalpels, etc.) should also be autoclaved periodically. e. Instead of rectified spirit, use of glass bead sterilizers should be favoured as the former is a potential fire hazard. f. Regular monitoring of air borne microbes in the laboratory is a must. g. Operators working in the laboratory must remove their foot wears outside the room and wear clean (preferably autoclaved) laboratory coats. h. During sub-culturing, at a time only one clone/ genotype should be handled to avoid any mixing. i. Due emphasis should be given to the efficiency of the operators (the number of jars handled, multiplication rates, contamination losses, etc.) j. Proper record of species, clone, passage number, media operator names, etc. should be maintained.	10 Marks

Growth room Availability of equipment Adequate facilities to maintain stringent conditions for temperature and RH Sterility levels	a. The growth room should be equipped with racks, AC, heat convector, temperature and humidity controller, photo period stimulator, shakers etc. b. High sterility levels (Class 10000) should be maintained with periodic check on airborne contaminants. c. The room must be fitted with UV lights. It should also be fumigated periodically especially during the rainy season to keep the contamination under control. d. Restricted entry.	10 Marks
B. Hardening Facilities **Transfer area** *Ex vitro* management Selection of proper container and potting mix a. Only one clone to be washed at a time.	b. Hardening trays should be properly labeled. c. Selection of the hardening container and potting mix to be done as per the requirement of the species. d. Drying of plants should be avoided by transferring them to the mist room/greenhouse immediately after transfer to the potting mix. e. Water used for irrigation must not be hard (rich in salts) f. Excessive watering of plants to be avoided. g. Due consideration should be given to the texture and pH of the soil used for hardening. h. All records pertaining to number of plants transferred, date of transfer, etc. should be maintained for future reference.	10 Marks
Greenhouse/polyhouse/ shade area Necessary facilities for proper hardening of plants through adequate control of temperature and RH	a. Stringent control of temperature and RH. b. There should not be any leakage for the inside air to facility for ventilation to control excess RH during rainy season. Excessive watering of plants to be avoided. It must be ensured that direct sunlight does not fall on the plants but at same time there should be sufficient natural light in the greenhouse. c. Adequate provision for artificial light for species that are high light demanders. d. Plants should be monitored regularly for their growth and presence of any disease or pest. e. Dead plants should be removed immediately to avoid any possible attack of saprophytic fungi.	10 Marks

	f. Fungal infection in greenhouse particularly during rainy season is very common. If present, the plants should be sprayed with suitable fungicides. g. Wherever possible, use of compost at the greenhouse stage should be avoided because that may invite contamination. h. Any kind of treatment given to the plant such as fertilizer, fungicides, pesticides, etc. must be recorded for reference just in case something goes wrong with the plants. i. All mortalities taking place in the greenhouse/ polyhouse should be recorded to arrive at the transplantation losses.	
Nursery Adequate space and facilities for irrigation Proper management	a. Nursery should have some shade area where the plants could be kept till they are hardened enough to be kept under direct sunlight. b. Only fully decomposed organic manure to be used. Partially decomposed manure will do more harm than any good to the plant. c. There should be adequate facilities for irrigation. d. Nursery beds should be properly leveled so as to avoid any water-logging. e. Regular weeding. f. Regular shifting of plants to prevent the roots from entering the ground.	5 Marks
	2. QUALITY CONTROL	
Selection of clones and maintenance of germplasm Selection of high yielding clones Maintaining the germplasm in proper disease-free conditions.	a. Following points must be recorded while selecting the mother plant: b. Geographical location of the mother plant or the area where mother plant is growing. c. Micro-climatic conditions prevailing in that area. d. Various growth attributes of the mother plant (height, diameter of the stem, yield, etc.). e. Origin of the mother plant (seedling raised or vegetatively raised). f. High yielding clones should only be used for micropropagation work. g. The mother plants should be maintained in disease-free environment so that the chances of initiating aseptic cultures remain high.	5 Marks

Explant Apical or axillary bud.	a.	Choice of the explant is a critical factor in the success of the micropropagation protocol. Since axillary branching method is the most favoured method for *in vitro* clonal propagation, only apical or axillary bud should be used as the explant for micropropagation work. While excising the explant from the mother plant, following points must be properly recorded:	5 Marks
	b.	Location of the explant on the mother plant.	
	c.	Season (month) in which the explants have been derived.	
	d.	Any pre-treatment given to the mother plant before excising the explant.	
Virus indexing Testing the plants for known viruses and ensuring their elimination before micropropagation	a.	Before starting with the micropropagation work, the material should be tested for the presence of the known viruses.	5 Marks
	b.	If the presence of virus is established then these must be eliminated through meristem culture or a combination of techniques.	
	c.	Only virus-free tissue should be used for further micropropagation work.	
Number of multiplication cycles and clonal uniformity Number of multiplication cycles Ensuring that multiplication is only through axillary shoots and not adventitious Ensuring clonal uniformity of plants by molecular methods Carrying out field trials and confirming the yield before undertaking mass distribution of TC plants	a.	In general the multiplication cycles should not exceed 10 passages. However, this number is not fixed and would vary with the species under consideration.	10 Marks
	b.	Operators should be thoroughly trained so that they can draw a distinction between the adventitious and axillary shoots. Only axillary shoots should be used for micropropagation work.	
	c.	The plant tissue should be tested for the presence of systemic bacterial contamination by culturing the tissue after every 3-4 passages on LB medium.	
	d.	Clonal uniformity may be established morphologically through field trials.	
	e.	Proper field data must be collected and appropriately analysed.	
Overall quality of the plants	a.	At the time of dispatch it must be ensured that the plants are fully hardened and are of transplantable size. (b) A small handout giving all necessary information about after-care of the tissue cultured plants should be provided to all growers for reference	5 Marks

3. TECHNICAL SUPERVISION AND MONITORING			
Monitoring of the production process and the staff involved therein	a.	Strict monitoring of the entire production process covering all the activities that are performed in media room, inoculation room, growth room and hardening area is a must.	10 Marks
Technical competence of the production supervisory staff	a.	The managers, scientists and the supervisory staff involved in production must have very sound technical knowledge of the subject so that they could deal with any eventuality that may arise during course of production.	
	b.	There should be at least two supervisors (one in the clean area to monitor laboratory activities and one in the hardening area for after care and for monitoring field activities) in the production facility.	
Operators		The operators may or may not have very sound scientific background but they must be thoroughly trained by the supervisors and the professional staff before they undertake any skilled job such as media preparation or inoculations.	

ANNEXURE VI

Department of Biotechnology, Government of India recognized tissue culture companies under NCS-TCP.

Sl. No	States	TCPs
1.	Andhra Pradesh	A.G. Bioteck Laboratories (India) Ltd Anantha Biotechnologies Brookfields Biotech Pvt. Ltd. ACE Agro Technologies Agri Vitro Tech Laboratories S & S Agro Biotech Pvt. Ltd. Godrej Agrovet Ltd. (Plant Biotech Division)
2.	Chattisgarh	Devleela Biotechs Aditya Biotech Lab & Research Pvt Ltd.
3.	Haryana	Technico Agri Sciences Ltd. Micropropagation Technology Park, TERI. Sheel Biotech Ltd.
4.	Gujarat	Shaili Biotech (P) Ltd. Cadila Pharmaceuticals Ltd. (Agro Division) Arcadia Agro Sarjan Biotech Pvt. Ltd.
5.	Karnataka	Shri Ramco Biotech Labland Biotech Pvt. Ltd. Greenearth Biotechnologies Ltd. MSR Biotech Pvt. Ltd.
6.	Kerala	L.J. International Ltd. (AVT)
7.	Maharashtra	K.F. Bioplants Pvt. Ltd. Reliance Life Sciences Pvt. Ltd. H.U Gugle Agro Biotech Co. Pudumjee Plant Laboratories Ltd. Nirmeeeti Biotech. Jain Irrigation Systems Ltd. Ajeet Seeds Ltd. Rise N Shine Biotech Pvt. Ltd.
8.	Tamil Nadu	Growmore Bioteh Ltd. SPIC Agro Biotech Centre
9.	Uttarakhand	Aryave Biotech Pvt. Ltd.

ANNEXURE VII

PRODUCTION CAPACITY OF TISSUE CULTURE COMPANIES IN INDIA

State	Company Name	Production Capacity (millions per annum)	Crop / s
Andhra Pradesh	Anantha Biotechnologies	3.50	Banana
Andhra Pradesh	Brookfileds Biotech Pvt. Ltd.	1.50	Banana
Andhra Pradesh	Phytica Biotech Pvt. Ltd.	1.00	Banana
Andhra Pradesh	Sai Lara Biotechnologies	0.50	Banana
Andhra Pradesh	Agri Vitro Tech Laboratories	3.00	Banana and other crops
Andhra Pradesh	S & S Agro Biotech Pvt. Ltd.	1.50	Banana and other crops
Andhra Pradesh	A.G. Bioteck Laboratories (India) Ltd.	3.00	Banana and other crops
Andhra Pradesh	Godrej Agrovet Ltd.	5.00	Banana and other crops
Chattisgarh	Aditya Biotech Lab & Research Pvt Ltd.	1.00	Banana and other crops
Chattisgarh	Devleela Biotechs	1.00	Banana and other crops
Gujarat	Sarjan Biotech Pvt. Ltd.	5.00	Banana
Gujarat	Shaili Biotech (P) Ltd.	6.00	Banana and Sugarcane
Gujarat	Arcadia Agro	3.60	Banana and other crops
Gujarat	Cadila Pharmaceuticals Ltd.	10.00	Banana and other crops
Haryana	Sheel Biotech Ltd.	6.00	Banana and other crops
Karnataka	Lakshmi Biotech	3.00	Banana
Karnataka	Green Earth Biotechnologies Ltd.	3.00	Banana and other crops
Karnataka	Lab and Biotech Pvt. Ltd.	3.00	Banana and other crops
Karnataka	MSR Biotech Pvt. Ltd.	2.50	Banana and other crops
Karnataka	Shri Ramco Biotech	5.00	Banana and other crops
Karnataka	K.F. Biotech Pvt Ltd.	3.00	Banana.
Maharashtra	H.U Gugle Agro Biotech Co.	5.00	Banana
Maharashtra	Jain Irrigation Systems Ltd.	60.00	Banana
Maharashtra	Seema Biotech	2.00	Banana
Maharashtra	Rise N Shine Biotech Pvt. Ltd.	15.00	Banana and other crops
Maharashtra	Gargi Biotek Pvt. Ltd.	15.00	Banana and other crops

Maharashtra	Nirmeeti Biotech	2.40	Banana and other crops
Maharashtra	Ajeet Seeds Ltd.	1.00	Banana and other crops.
Tamil Nadu	SPIC Agro Biotech Centre	7.00	banana and other crops
Tamil Nadu	Growmore Bioteh Ltd.	10.00	Banana and other crops
Uttar Pradesh	Tissue Culture Facility, Biotech Park	0.60	Banana and other crops
Uttarakhand	Aryave Biotech Pvt. Ltd.	2.00	Banana and other crops
West Bengal	Boinchi Bioplants Pvt. Ltd.	2.00	Banana and Anthurium
West Bengal	Vedic Synergy Biotechnologies Ltd.	7.00	Banana and other crops
West Bengal	Elegant Flower Company Pvt. Ltd.	1.50	Banana and other crops

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ANNEXURE VIII

LIST OF LABORATORY ITEMS REQUIRED IN MICROPROPAGATION

Essential items

Consumables	Purpose
Glassware/plasticware like conical flasks, volumetric flasks, beakers, measuring cylinders, graduated pipettes, pasture pipettes, test tubes, Petri dishes, reagent bottles	For various laboratory activities like stock preparation, media preparation.
Erlenmeyer flasks (250 ml)/culture jars/bottles	For initiation and multiplication of shoot tips
Plastic containers (1 lit), knife, sickle	For explant preparation and transferring them to laboratory
Stainless steel forceps (pointed and broad), scissors, scalpels (No. 22 and 11), scalpel blade holders, sterile surgical blade, Petri dishes, watch glasses, stainless plates, stereo zoom microscope, hand lens	For initiation of shoot tip and meristem culture
Filter paper (Whatman No. 1), tissue paper, ball pipette, pH paper, caps, cotton plugs, cellophane tape, parafilm, aluminium foil, glass rods, blotting paper, rubber bands, autoclavable bags	For general purpose laboratory use

General consumables

Consumables	Purpose
Laboratory coats, protective hoods, goggles, slippers	For the use of the laboratory workers
Slippers, gloves (disposable and latex), washing brushes of various sizes, tub, buckets	For the use of the persons washing glass ware
Soap, absorbent and nonabsorbent cotton, gauze cloth, brown paper, sieves, measuring tape	For laboratory use
Polythene bags, earthen pots, plastic pots, tags and labels, permanent marker, twine	For net house purpose
Glass marking pens, note books, data sheets, measuring scales, eraser, tapes of different colors	For recording data, labeling
First aid box, fire extinguisher	For laboratory safety

ANNEXURE IX

SOME DO'S AND DON'TS

Stock preparation and maintenance

Do's

- Stocks for same medium or different media should be prepared separately, labeled and maintained separately.
- Separate spatula should be used for weighing different chemicals.
- The stocks should be kept either in refrigerator/room temperature or light/dark as per the temperature and light conditions required.

Dont's

- Transparent bottles should not be used to store light sensitive chemicals.

Sterilization of glassware

Do's

- Glasswares used for media preparation should be washed with detergent, rinsed with distilled water and dried in hot air oven.
- Both new and used culture vessels should be autoclaved before washing with detergent.
- Contaminated culture vessels should be autoclaved without opening the lid. Autoclaving should be done for 20 hr at 121 °C and 20 psi.
- After decontaminating, the water in autoclave should be refilled.

Dont's

- Media sterilization and decontamination should not be carried out at the same time.

Sterilization and culture initiation

Do's

- Required time should be given for surface sterilization and the explants should be washed 4-5 times with sterile distilled water before initiation.
- The shoot tips should be quickly excised and initiated in medium to avoid browning of the explant.

Don'ts

- During preparation of shoot apices for culture, do not exert too much pressure while removing the tender leaf sheaths.
- To avoid tissue blackening or death, concentration and time of surface sterilants should not be exceeded.

Shoot multiplication

Do's

- Phenolics in multiplication phase can be avoided by the addition of antioxidant L-ascorbic acid.
- BAP concentration may be increased to 5 mg/l in multiplication medium for enhancing the multiplication rate.
- Use of phytagel as solidifying agent in subculture medium enhances the multiplication rate in banana.

Don'ts

- Do not use high concentration of BAP in initiation medium.
- The combination of BAP with other plant growth regulators is genome dependant, which may not work for other cultivars.
- Avoid more than four shoots while subculturing.

Rooting

Do's

- Plants with more than four leaves should be transferred to rooting medium.
- Healthy planlets can be obtained in rooting by the addition of activated charcoal (0.5 mg/l) along with NAA (2 mg/l) and IBA (1 mg/l).
- Plantlets with thick fibrous root system should be transferred to next phase of hardening.

Don'ts

- Rooting hormones in excess may induce abnormal rooting.
- Don't transfer the shoot tips in multiplication stage for rooting. Use only healthy and mature shoot.

Acclimatization

Do's

- Plantlets in rooting medium should be kept at room temperature for 24 hrs before hardening.

Hardening

Do's

- Plantlets should be washed well in running tap water to remove all traces of the medium.
- Maintaining 80 % humidity will enhance the survival rate of hardened plants.

Planting in polybags

Do's

- Combination of sand, soil and vermicompost (1:1:1) should be used as substrate for secondary hardening.
- Thirty day old plantlets (primary hardening) should be used for secondary hardening.

Don'ts

- Excess watering should be avoided during irrigation of the polybags.

Field planting

Do's

- The field should be kept ready well before planting with adequate application of organic manure.
- After planting, the field should be inspected at regular intervals for off-types.

ASIA-PACIFIC CONSORTIUM ON AGRICULTURAL BIOTECHNOLOGY

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), was established in 2003 under the umbrella of the Asia-Pacific Association of Agricultural Research Institutions (APAARI) — an initiative of Food and Agriculture Organization that has been promoting appropriate use of emerging agri-technologies and tools in the region.

APCoAB's mission is "To harness the benefits of agricultural biotechnology for human and animal welfare through the application of latest scientific technologies while safeguarding the environment for the advancement of society in the Asia-Pacific Region".

APCoAB's main thrust is:

- To serve as a neutral forum for the key partners engaged in research, development, commercialization and education/ learning of agricultural biotechnology as well as environmental safety in the Asia-Pacific region.
- To facilitate and promote the process of greater public awareness and understanding relating to important issues of IPR's *sui generis* systems, biosafety, risk assessment, harmonization of regulatory procedures, and benefit sharing in order to address various concerns relating to adoption of agricultural biotechnology.
- To facilitate human resources development for meaningful application of agricultural biotechnologies to enhance sustainable agricultural productivity. as well as product quality, for the welfare of both farmers and consumers.