INDUCTION AND EVALUATION OF BANANA CULTIVAR 'NANJANGUD RASABALE' CLONES FOR *FUSARIUM* WILT RESISTANCE

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DECLARATION

I, Sandhya R. Shenoy, declare that this investigation entitled " **Induction and Evaluation of banana cultivar 'Nanjangud Rasabale' clones for *Fusarium* wilt resistance"**, submitted by me to the university of Mysore, for the award of the Degree of **Doctor of Philosophy in Biotechnology**, is the result of bonafide research work carried out by me under the guidance of Prof. H.S. Prakash, Department of Studies in Applied Botany and Biotechnology, University of Mysore, Mysore during 1997-2002.

I further declare that the results presented here have not been previously submitted for any degree or diploma, either in this or any other university.

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I certify that the thesis entitled " **Induction and Evaluation of banana cultivar 'Nanjangud Rasabale' clones for *Fusarium* wilt resistance"** submitted for the award of **"Doctor of Philosophy"** in **"Biotechnology"** to the university of Mysore by Ms Sandhya R Shenoy, is the result of bonafide research work carried out by her, in the Department of Studies in Applied Botany and Biotechnology, Manasagangothri, Mysore, under my guidance during the years 1997-2002 and it has not formed the basis for the award of any degree or diploma of this university or elsewhere before.

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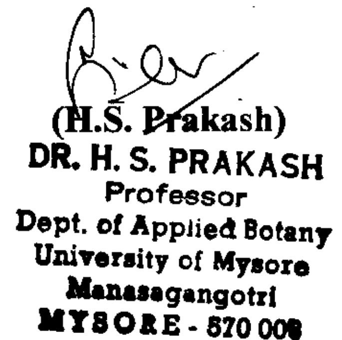
Place: MYSORE



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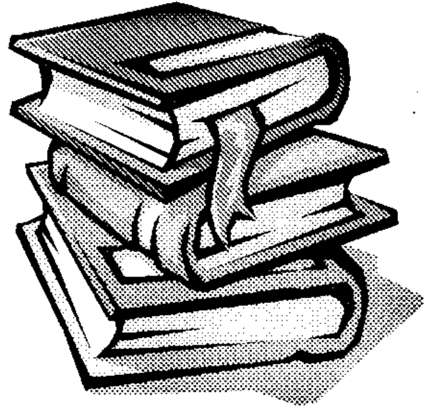

To

My parents, who encouraged me,
My teachers, who enabled me, and
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ABBREVIATIONS

2,4-D	=	2,4-dichlorophenoxyacetic acid
^{60}Co	=	Radioactive isotope of Cobalt
A	=	Absorbtion
AS	=	Adenine Sulphate
BAP	=	6-benzylaminopurine
BSA	=	Bovine Serum Albumin
Cm	=	centimeter
CM	=	Coconut milk
CTAB	=	Cetyltrimethylammoniumbromide
cv.	=	Cultivar
DC	=	'Dwarf Cavendish'
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxy-nucleotide
EDTA	=	Ethylenediaminetetraacetic acid
et al.,	=	et alii (and others)
eg.,	=	example gratia (for example)
EtBr	=	Ethidium bromide
Fig.	=	Figures
g	=	gram
h	=	hour
Gy	=	Gray
IAA	=	Indole-3-acetic acid
IAA	=	Isoamylalcohol
Kb	=	Kilobasepairs of DNA
l	=	litre
M	=	Molar
mg	=	milligram
min	=	minute
ml	=	milliliter
MS	=	Murashige and Skoog's basal medium
NAA	=	α-naphthaleneacetic acid
NaOAc	=	Sodium acetate
NRB	=	'Nanjangud Rasabale'
%	=	Percentage
PO	=	Peroxidase
RAPD	=	Random Amplified Polymorphic DNA
PCR	=	Polymerase Chain Reaction
RFLP	=	Restriction Fragment Length Polymorphism
RNA	=	Ribonucleic acid
SDS_PAGE	=	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
$T_{10} E_1$	=	Tris 10 mM EDTA 1 mM
TBE	=	Tris-borate-EDTA buffer
Tris-HCl	=	Tris [hydroxymethyl] aminomethane. HCl
u	=	Units
UPGMA	=	Unweighted Pair-Group Method with Airthmetic Means
viz.,	=	videlicet(namely)

General Introduction

Bananas and plantains are perennial giant herbs belonging to the genus *Musa*; their fruits are the fourth most important food in the developing world (May *et al.*, 1995). Approximately 10% of the world's production of bananas (85.5 million tons; FAO 1998) enters the export market to generate an important source of income for tropical and sub-tropical regions (Crouch *et al.*, 1998). More than 100 countries throughout the tropical and sub tropical regions cultivate bananas (Sharrock and Frison, 1999) and India is the world's largest banana producer (Singhal, 1999).

In light of the fact that *Musa* spp. make such an enormous contribution to food security and also provide export revenue in developing countries, it is a remarkable paradox that these crops have never benefited from traditional crop breeding. In terms of important crops for which biotechnology offers the possibility for dramatic genetic improvement, there are few opportunities as open for novel approaches as *Musa* spp. (Persley *et al.*, 1987; Artzen *et al.*, 1992).

There is a general agreement that edible bananas originated from two species, *M. acuminata* and *M. balbisiana* (Simmonds *et al.*, 1955). Edible bananas have three different ploidy levels: $2n = 2x = 22$, $3n = 3x = 33$, $4n = 4x = 44$. They have been propagated vegetatively for hundreds of years with somatic mutations providing variability. Triploids are the most numerous and widely utilized cultivars (including desert bananas used in world export trade). Efforts to breed *Musa* using conventional methods are fraught with obstacles, including low fertility, various levels of ploidy, and lack of genetic variability (Vuylsteke *et al.*, 1992). Since almost all accepted cultivars are seedless, sterile, clonally propagated, conventional breeding efforts must begin with unimproved material that has been poorly characterized for genetic traits.

Although traditional breeding has been slow for *Musa* spp., application of the tools of plant tissue culture have been of significant value to crop improvement and germplasm distribution (Novak, 1992; Artzen *et al.*, 1992 ; Vuylseteke *et al.*, 1992, Arias, 1993). The uses of shoot-tip culture, cell suspension cultures and related cell culture techniques have been used in research activities to overcome limitations in crop breeding. Banana shoot-tip culture protocols are now widely in use in developed and developing countries for *Musa* multiplication (Swennen *et al.*, 1994).

The sustainable production level of bananas and plantains is threatened by pest pressure, which is increasing. Worldwide, *Mycosphaerella fijiensis* and *Fusarium oxysporum* f.sp. *cubense* (Foc) are among the most devastating fungal pathogens affecting these crops. Disease control is so far based on chemical spraying and or conventional breeding for resistant cultivars. But these methods have serious drawbacks, including high expense, ineffectiveness (e.g., towards specific races of Foc), a negative environmental impact and time consuming processes. Superficial applications of fungicides cannot effectively control soil-borne pathogens like Foc, which infects the plants through the roots and spreads *via* the vascular system.

Breeding for banana resistance is particularly difficult due to the sterile and polyploid nature of the plant and to the saprophytic-pathogenic nature of the fungus (Novak, 1992). The relationship of the pathogen with the plant host and the complex interplay occurring between them and environmental parameters results in an extremely complex situation (Beckman *et al.*, 1962; Marois, 1990). Selecting plant genotypes resistant to pathogens has become one of the major tools of combating agricultural losses and increasing productivity. However, traditional plant breeding is not able to maintain pace with pathogen evolution. Field screening is time consuming and requires a large amount of space and intensive manpower inputs. Furthermore, disease symptoms develop only after a long period of incubation and results can be misleading due to non-uniform distribution of the pathogen in soil. Plant tissue culture techniques have been used for investigating mechanism of host-pathogen relationship (Buiatti and Scala, 1984) and have been proposed as an aid in banana breeding programmes (Novak, 1992).

Cell and tissue culture technology provides the necessary tools for the development of new clones resistant or tolerant to diseases. Tissue culture facilitates mass propagation, conservation and easy movement of germplasm. Somaclonal variation has been heralded as a source of new variation in plants dreaded by biotechnologists who seek to alter only a single trait of cultivars without the uncontrollable changes that accompany the tissue culture process they rely upon (Veilleux *et al.*, 1998). Somaclonal variants with enhanced resistance to various diseases are known for many plants including banana (Hwang, 1990). Techniques

such as somatic embryogenesis and induced mutations offer new opportunities for inducing genetic variability (Novak, 1992).

Biochemical markers, especially phenolic compounds and peroxidases, have been used largely as a measure of resistance in numerous plant-pathogen interactions (Quiroga *et al.*, 2000). Plant peroxidases are localized mainly in cell walls and in vacuoles. Peroxidase is a multi-purpose enzyme that has been correlated with active plant defense mechanisms by condensing phenolic compounds to form lignin, and has been used as a parameter to discriminate between susceptibility and tolerance to Foc race 1 and 4 on diploid banana *Musa acuminata* (Morpurgo *et al.*, 1994).

Various types of DNA markers, developed in the 1980's have found a number of applications in the field of agriculture (Ranade *et al.*, 2000). The Polymerase Chain Reaction (PCR) based molecular markers have been used for analysis of genetic variation in natural populations. One such technique, using arbitrary primers, namely Random Amplified Polymorphic DNA (RAPD) provides a convenient and rapid assessment of the differences in the genetic composition of the related individuals (Welsh and McClelland 1990; Williams *et al.*, 1990). The applicability of this technique for bananas and plantains has already been demonstrated (Kaemmer *et al.*, 1992; Howell *et al.*, 1994; Bhat and Jarret 1995; Damasco *et al.*, 1996).

A molecular strategy for the creation of *Musa* cultivars resistant to fungal diseases has also been proposed (Cammue *et al.*, 1993; Sagi *et al.*, 1995; May *et al.*, 1995) which includes introduction of genes that encode new type of antifungal proteins into the genome of banana and plantain cultivars. These antifungal proteins are stable cysteine-rich peptides isolated from seeds of different species (Cammue *et al.*, 1993; Broekaert, 1992; Terras *et al.*, 1992). They share some unique properties with respect to the proposed function as antifungal tools for banana such as high *in vitro* fungistatic activity against field isolates of both *Mycosphaerella fijiensis* and *Fusarium oxysporum* f.sp. *cubense*, and the lack of toxicity for human or banana cells (Cammue *et al.*, 1993). Genes coding for antifungal proteins that show broad antifungal activity *in vitro* have been introduced into a plantain landrace and the resulting transgenic plants await field testing (Remy *et al.*, 1998).

The cultivar 'Nanjangud Rasabale' belongs to the silk subgroup Rasthali (AAB) and is a native of Nanjangud area in Chamarajnagar district of the state of Karnataka in India. It is one of the best genotypes of banana in India. However the sustainable production level of 'Nanjangud Rasabale' is threatened by dreaded disease *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *cubense*, because of which the area under cultivation is shrinking and has threatened the very existence of this valuable germplasm. The area under NRB cultivation has declined from 500 ha in 1950 to 20 ha in 1998. This decline is mainly attributed to the onslaught of *Fusarium* wilt.

Symptoms of **Fusarium** *wilt*

The external symptoms of the disease consist of successive wilting and drying up of the leaves. The first symptom of the disease is the yellowing of the inner leaf blade, which extends rapidly from the margin towards the midrib. These leaves hang withered around the pseudostem and gradually the infection spreads and all the leaves except the top leaves hang down in course of time. The top leaves, which remain green for sometime, also wither and the plants exhibit longitudinal splitting of the pseudostem.

The new suckers, which are produced, are also affected. Infected plants do not produce bunches. Even if they produce any bunches, the fruits will be small and develop very few fingers. The fruits ripen irregularly and flesh becomes pithy and acidic. When the rhizome is cut open the discoloration of the vascular bundles can be seen. The vessels are plugged and the translocation of food materials are interfered which results in wilting of the plants. The cut stem smells of rotten fish.

Characteristic symptoms of the Fusarium wilt disease are shown in **(Fig. A.).**

The Pathogen

Fusarium oxysporum f. sp. *cubense* (Foc) is an imperfect fungus. Numerous micro-conidia and macro-conidia are produced on small conidiophores. Thick walled chlamydospores are also found. They are intercalary or borne on short lateral

Fig. A. *FUSARIUM* WILT SYMPTOMS

1. Wilt Infected Plot

2. Splitting of the pseudostem

3. Colonization of the vascular bundles by Foc

4. Pure culture of Foc

5. Microscopic field showing

a) macro conidia

b) micro conidia

c) chlamydospore

branches. The micro-conidia are ovate, non-septate or single septate, hyaline thin walled and measure 5-7 to 2.5-3 μm. The macro-conidia are comparatively larger, falcate, thin-walled, hyaline and measures 22-36 by 4-5 μm. The chlamydospores are globose, thin-walled often paired and measure 7-13 by 7-8 mμ. The mycelium is both inter-cellular and intra-cellular within host tissue. Mostly the mycelium is seen in vascular tissues particularly in xylem vessels.

Foc affects species of *Musa* and *Heliconia* and strains have been classified into four physiological races based on pathogenecity to host cultivars in the field. Race 1 infects Gros Michel (AAA) and (AAB) desert cultivars like Silk and Pome; race 2 infects Bluggoe and other closely related ABB cultivars; race 3 infects *Heliconia* species and race 4 infects Cavendish group (AAA) cultivars and all cultivars susceptible to race 1 and 2 (Bentley *et al.*, 1998). Out of the four races of Foc, race 1 and 2 are reported in India (Banana compendium, 1996).

Fusarium wilt is soil-borne and the fungus enters through the roots. Entry is facilitated by root damage such as that caused by the nematode, *Radopholus similis*. The chlamydospores survive in the soil for long period of time and infect plants. Sometimes the fungus survives saprophytically in the infected rhizomes. It has also been noticed that the disease has been more prevalent in the crop, which is retained in the field for more than one season. Several factors such as soil conditions including texture, structure, temperature and fertility level of soil are responsible for disease incidence. The cultural operations and plant vigor also influence the disease incidence and spread.

Scope of the Investigation

Basic scientific research in recent years, particularly in molecular and cell biology, has provided a better understanding of plant regeneration, genetics, growth and development. This has led to the development of a wide range of techniques, collectively termed *Biotechnology* for genetic manipulation and faster regeneration of plants through cell and tissue culture. These techniques permit a more rapid and deeper insight into the prevailing genetic diversity, the mechanism by which that variation is generated, and significance of that variation in adaptation and performance of the plants.

Bananas and plantains are excellent candidates for virtually all tissue culture strategies (Krikorian *et al.*, 1984a, Krikorian, 1987; Krikorian, 1990; Vuylsteke *et al.*, 1992). However, breeding of the seed-sterile clones to improve bananas is fraught with many difficulties (Stover *et al.*, 1986, Rowe *et al.*, 1996). A number of diseases seriously affect banana and plantain plantings through out the world. The amount of time and money that must be invested in control measures using chemicals are beyond the level of subsistence farmers and pose financial problems even for large commercial operations. Breeding strategies to generate tolerance or resistance to important diseases such as the wilt caused by *Fusarium oxysporum* f. sp. *cubense* (various races) has thus far been the main breeding objective but this has yet to lead to commercially successful clones. The improvement programs are rendered all the more difficult because the potential to breed plantains and bananas is seriously handicapped by the high sterility of the clones of primary interest (Simmonds, 1955; Stover *et al.*, 1987; Novak, 1990; Crouch *et al.*, 1998).

The reliable application of cell and tissue culture, somatic embryogenesis and irradiation procedures are necessary and key components in any comprehensive biotechnology plan aimed at improvement of seed-sterile clones of *Musa*. Perusal of the literature suggests a biotechnological approach to plant protection. Studies described in this thesis analyses investigations of biotechnological techniques, micropropagation, cell/tissue culture and induced mutations to overcome problems with banana production of a local variety 'Nanjangud Rasabale'.

Large-scale cultivation of banana cultivar 'Nanjangud Rasabale' is being threatened by the emergence of *Fusarium* wilt caused by the soil inhabiting fungus

Foc race 1. Whilst efforts are being made towards developing resistant materials for commercial planting, however, there is a need for developing quick, reliable and reproducible screening techniques for mass evaluation of somaclones obtained through micropropagation, irradiation and somatic embryogenesis. Though the setting of '*Fusarium* hot spots' would facilitate the testing of field resistance, the scale of screening exercises is often limited by time and space.

Biochemical markers, especially phenolic compounds and peroxidases have been used largely as a measure of resistance in numerous plant pathogen interactions (Quiroga *et al.*, 2000). The aim of this research is to determine the levels of total phenolic compounds and peroxidases in banana cultivars 'Nanjangud Rasabale' (susceptible) and 'Dwarf Cavendish' (resistant) with differential resistance to *Fusarium* wilt and their possible role as a measure of tolerance/resistance. RAPD technique using random primers for molecular evaluation of clones obtained through micropropagation, irradiation and somatic embryogenesis for polymorphism has been studied. In addition attempts have also been made to isolate and purify an antifungal protein from *Urgenia indica* (Indian squill) to Foc race 1, which is the most potential molecular strategy for fungal disease control of banana and plantain proposed world over.

Chapter 1 deals with micropropagation, irradiation and somatic embryogenesis of banana cultivar 'Nanjangud Rasabale' and evaluation of the somaclonal variants in the sick plot, using peroxidase and phenolic compounds as early biochemical markers.

Chapter 2 summarizes the RAPD technique using random primers for differentiation in the genetic composition of the somaclonal variants obtained through micropropagation, irradiation and somatic embryogenesis. Polymorphism detected in the banding patterns of RAPD products have been described.

Chapter 3 details the isolation and characterization of an antifungal protein to Foc from *Urgenia indica.*

Review of Literature

The review of literature presented below focuses only areas that are pertinent to the present investigation.

Micropropagation

Micropropagation can be considered as an extension of the traditional methods of plant propagation. It helps in rapid clonal multiplication of superior genotypes of disease-free and pest-free plants (Smith *et al.,* 1990a). Micropropagation is especially a boon in cases where no other vegetative propagation method is available, and in cases where desired high volume of multiplication cannot be achieved by conventional means (Chadha *et al.*, 2000). Today, micropropagation of high value crops is a multi-billion industry, practiced in several small and large nurseries and commercial laboratories all over the world (Rao and Suprasanna, 1999b). Micropropagation techniques have acquired many commercial applications in horticulture, agriculture, forestry, and medicine, and in conservation of germplasm (Ortiz, 1998).

Application of micropropagation has greatly improved *Musa* germplasm handling for the purposes of clonal propagation, uniform production and breeding. Micropropagation has played a key role in plantain and banana improvement programs worldwide (Rowe and Rosales, 1996; Vuylsteke *et al.*, 1997). Planting material derived from micropropagation performs equal to or superior to conventional material (Smith and Drew, 1990a; Vuylsteke, 1998). Micropropagated plantlets establish faster, grow more vigorously, are taller, have a shorter and more uniform production cycle, and yield higher than conventional propagules (Drew and Smith, 1990; Robinson *et al.*, 1993; Vuylsteke and Ortiz, 1996). Maximum yield gains from *in vitro* derived plants range from 20% in bananas to 70% in plantains. However, this superior field performance does not appear to be consistent and requires optimal crop husbandry (Vuylsteke, 1998).

Irradiation

Mutations are defined as a sudden heritable change in the DNA of a living cell, not caused by the common phenomena of genetic segregation of genetic recombination (van Harten, 1998). Spontaneous somatic mutations of agronomic value have been utilized since the earliest phases of plant domestication in a range of vegetatively propagated crop species, including fruit trees, currants and tuber crops (van Harten, 1998). However, these occur at very low frequencies (10^{-6}) and hence not dependable for practical plant breeding.

Mutation induction and selection of desired traits in combination with *in vitro* technique offers several advantages over conventional methods, especially in the vegetatively propagated species (Maluszynski *et al.*, 1995a), such as - mutagenizing large number of samples of minute size in a small space in short duration under disease-free conditions (Ahloowalia, 1995), rapid production of large populations to separate chimeras efficiently (Kulkarni *et al.*, 1997a), uniform mutagen treatment as well as optional facility of *in vitro* selection (van Harten, 1998).

Amongst different mutagens, the gamma rays have proved to be superior, compared to other mutagens. Globally 1028 cvs. of various crops were officially released, using different types of mutagens and more than 55% of those released were credited to gamma rays alone, followed by X-rays (28.4%), whereas all the rest including chemical mutagens have contributed only about 16%. In case of rice, more than 76% cvs. (out of 217 released ones) were gamma irradiation induced derivatives (Maluszynski *et al.*, 1995b).

The frequency of mutations depends on several factors, such as appropriate selection of the plant parts to be irradiated, and also their radiosensitivity (Anon. 1977; Karmarkar *et al.*, 2001). *In vivo* plant propagules or established tissue culture explants have been mutagenized directly (Anon. 1977; van Harten, 1998). In either case,

availability of an established *in vitro* proliferation and regeneration protocol is inevitable for increased efficiency of the *in vitro* mutagenesis experiments.

Attempts have been made to bring genetic improvement in banana and plantain through *in vitro* mutation. Lopez Torres (1996) reported that the optimum dose of γ radiation from a ^{60}Co source varies from 25-60 Gy depending upon the ploidy levels of the genotype. According to Novak (1992) optimum doses are 25 Gy for diploids, 35 Gy for AAA triploids, 40 Gy for AAB and ABB triploids, and 50 Gy for AAAA tetraploids.

Smith *et al.* (1995) exposed micropropagated bananas to γ radiation from a ^{60}Co source and they established a LD_{50} at 40 Gy. Optimal dose for irradiating explants was 20 Gy and at this dose visual changes were apparent and plant survival was sufficiently high (73%) to make the technique practical on a larger scale. In addition, they also reported that irradiation of Dwarf Parfitt (AAA – Cavendish), produced an extra dwarf that was horticulturally inferior but resistant to *Fusarium oxysporum* race 4 and another taller clone Giant Parfitt, with horticultural potential that retained its resistance.

An early flowering mutant of Grand Naine has been produced by irradiation at International Atomic Energy Agency in Austria and given the identification number GN-60A (Novak *et al.*, 1990). GN-60A was micropropagated and the progenies were selected for early flowering and outstanding bunch yield (Mak *et al.*, 1996). The selected GN-60A was named Novaria (combining Novak and Austria) and this could have a significant impact in subtropical countries where long cycle time due to cold winters is a major disadvantage (Robinson, 1996).

Significant contributions by various workers in the field of ionizing radiation utilized for irradiation using several plant parts has been tabulated.

Crop	Explant	Reference
Dianthus	Nodal Segments from *in vitro* shoots	Casselles *et al.* (1993)
Cherry	Leaves from *in vitro* shoots	Yang and Schmidt (1994)
Cassava	Axillary buds and shoot tips	Ahiabu *et al.* (1997)
Pineapple	*In vitro* multiple shoot tips	Osei-Kofi *et al.* (1997)
Cassava	Somatic Embryos	Lee *et al.* (1997)
Sugarcane	Embryogenic callus	Asad *et al.* (1997)
Citrus	Protoplasts	Li *et al.* (1998)
Rose	Leaves from *in vitro* shoots	Ibrahim *et al.* (1998)
Grape vine	*In vitro* shoot tips	Charbaji and Nabulsi (1999)
Chrysanthemum	*In vitro* multiple shoot tips	Goo *et al.*, (1999)
Banana	Embryogenic callus	Kulkarni *et al.* (2000)
Potato	Nodal segments from *in vitro* shoot tips	Al-Safadi *et al.* (2000)
Chrysanthemum	Rooted cuttings	Mandal *et al.* (2000)
Pear	*In vitro* shoot tips	Predieri and Zimmerman (2001)

Somaclonal Variations

The term somaclonal variation refers to all variability observed among tissue culture regenerated plants (Larkin and Scowcroft, 1981). Somaclonal variations can be advantageous in several ways viz., *in vitro* selection using phytotoxins, improved resistance to disease, virus and nematodes, tolerance to herbicide, salt, nutritional improvement, agronomic traits and male sterility (Karp, 1994). Somaclonal variation could arise either from pre-existing genetic variation within the explants or due to the stresses induced during *in vitro* passage (van Harten, 1998). In both the cases, the

variation can either be genetic (heritable) or epigenetic (developmental) (Skirvin *et al.*, 1994). In recent times, this exciting field has been extensively reviewed (Duncan, 1997; Brar and Jain, 1998). Until the mid-1970s, somaclonal variations were considered undesirable and were therefore discarded because, then, the main emphasis was on clonal propagation and genetic stability of the cell cultures (Bajaj, 1990).

When the plants are regenerated from *in vitro* cultivated somatic cells via a callus phase, increased genetic variation is noticed in the generated plants (somaclones). Thus, somaclonal variation has been observed in plants regenerated from shoot tips, protoplasts, other cultured explants (immature embryo, flower, leaves, rhizome etc), microspores, anthers and ovaries, and also in plants regenerated from cultured tumorous tissues. This phenomenon has been studied extensively in bananas and plantains (Israeli *et al.*, 1991; Vuylsteke *et al.*, 1991, 1996; Novak, 1992; Cote *et al.*, 1993; Ortiz *et al.*, 1995) where variations in useful traits like plant height, growth habit, fruit shape and size, pseudostem colour, fertility status, disease and pest resistance have been described. The extent of somaclonal variation depends on a number of factors *viz.* the genotype, ploidy level, tissue culture procedure employed and time in culture, the source of explant cells and the culture media used (Withers, 1993; Israeli *et al.*, 1995). Somaclonal variation is important for increasing genetic variability in the genus *Musa* particularly with respect to pest or disease resistance (Trujillo and Garcia, 1996).

Many somaclonal variants have been screened for enhanced fruit quality and resistance to diseases and pests and are being used in conventional breeding for developing superior banana or plantain hybrids (Withers, 1993; Ortiz *et al.*, 1995; Vuylsteke *et al.*, 1997). Yet there is hardly any variant, which has been released on its own merit for commercial cultivation. Vuylsteke *et al.* (1991) have observed that cultivars vary widely in terms of *in vitro* stability. Many of the variants are stable through several cycles, others revert to the source plant. Despite the associated deleterious effects (van Harten, 1998), somaclonal variations have potential applications in crop improvement for obtaining superior types.

The potential usefulness of somaclonal variation in crops first became apparent in sugarcane (Brar and Jain, 1998). Somaclonal variations have also been observed in case of tree species such as poplars (Antonetti and Pinon, 1993; Saieed *et al.*, 1994). Martinez *et al.* (1997) have reported on the evaluation of the somaclones obtained via anther callus for leaf parameters in grape. Similarly, tissue culture induced morphological and cytological variations in grape have been reported by (Kuksova *et al.*, 1997).

Somaclonal variations in case of woody ornamental species have been observed by Rout *et al.* (1998). In case of apple, somaclonal variants resistant to fire blight were isolated by Chevreau *et al.* (1998). Selection of stable somaclonal variant from *Citrus sinensis* from cell suspension cultures has been reported by Lin *et al.* (1999).

Somatic Embryogenesis

Somatic embryogenesis is the production of embryo-like structures (embryoid) from somatic cells. It has several distinct stages, beginning with induction of embryogenic state, followed by differentiation into an embryo, and subsequent maturation, germination and conversion into plants. Somatic embryos can develop and germinate to form plants in a manner analogous to germination to zygotic embryos. It offers advantages over regeneration *via* organogenesis. First among these advantages is that complete propagules are formed. As somatic embryos have both shoot and root meristems, separate shoot induction and root induction steps are not required (Parrott, 1993). Another advantage is that somatic embryogenesis tends to be less prone to somaclonal variation than organogenesis. Percentage of off-types does not exceed 2% (Scoofs, 1998). Furthermore, this can be particularly advantageous during genetic transformation of a given genotype, in which case changes other than the engineered trait are undesirable. Thus this technology provides not only exciting alternative for a large-scale clonal propagation and somatic hybridization but also a fast cell regeneration system for genetic engineering.

The development of somatic embryos can be achieved by employing the appropriate explant, culture media and environmental conditions. Mohan Ram and Steward (1964) were first to obtain slow growing cell suspensions from immature fruit-derived callus in *Musa*. Novak *et al.* (1989) reported recovery of plants from somatic embryos obtained in cell suspensions from rhizome tissue in diploid and triploid banana cultivars. Novak (1992) reported that embryogenic callus was induced from basal leaf sheaths and rhizome tissue from AA, AAA and ABB clones, when placed in liquid medium with zeatin. Success has also been obtained with meristematic "scalps" of cooking banana cv. Bluggoe as explants (Dhed'a *et al.*, 1991). According to Escalant and Teisson (1989, 1993) calli from immature zygotic embryos and triploid somatic embryos from male flowers were excellent materials (explants) for somatic embryogenesis in *Musa*. Meristematic tissues from shoot tips can also be induced to undergo somatic embryogenesis (Cronauer and Krikorian, 1983, 1986). It is thus evident that several plant tissues can serve as explant, with the caveat that stage of development or maturity of the tissue can be very important. However, the recent reports (Escalant *et al.*, 1994a; Grapin *et al.*, 1998; Ganapathi *et al.*, 1999) indicate that immature female and male flowers are the best explants. With their use, the technique can be extended to all types of *Musa*.

Production of somatic embryos from the selected explant may proceed either directly or indirectly after some form of callus culture. The procedure adopted earlier consisted in cultivating immature zygotic embryos of wild species on a semi-solid medium with picloram (4-amino 3,5,6-trichloropicolinic acid) to obtain an embryogenic callus with somatic embryos. The somatic embryos were germinated and developed into plantlets (Cronauer and Krikorian, 1988; Escalant and Teisson, 1989). Later, Escalant *et al.* (1994b) described a method for deriving primary somatic embryos (or pro-embryos) from the explants and their subsequent development into somatic embryos by direct secondary embryogenesis or cell suspension cultures.

To derive primary embryos, the explants (male flowers) are cultured in a semi-solid medium consisting of mixture of auxins. The first callus appears after one to two

months and the first embryos after three to four months. Callogenesis consists of the production of a compact nodular callus with high starch content. Certain external parts of the callus is transformed succeedingly into translucent, flaky areas on which embryogenic cells with large protein reserves appear. Somatic embryos follow. Escalant *et al.* (1994b) succeeded in obtaining somatic embryos from a variety of banana genotypes (commercial AAA and AAB cultivars) by this method. The embryos thus produced are perfect and can regenerate whole plants after transfer to a germination medium.

Scalps, somatic explants derived from *in vitro* proliferating meristem cultures, can be generated from any given banana or plantain landrace (*Musa* spp). A pre-culture of meristems on a medium with 100 μM BA enhanced significantly the competence for somatic embryogenesis. Out of 21 landraces covering all diploid and triploid genome groups and the most important banana types, 18 cultivars showed an embryogenic response. From 15 accessions an embryogenic cell suspension could be established (Schoofs *et al.*, 1998).

Defense-related enzyme

Plants in nature are subject to constant attack by plant pathogenic microbes. Nevertheless, only a very limited number of these succeed in infecting the plant. This lack of success is caused by in part, plant defense, which can be of either structural or chemical in nature. The natural protection of plants, against pathogens is based on a variety of constitutive barriers already present in the plant before the actual attack. The combined effect of all these barriers is referred to as "constitutive resistance". In addition, plants can activate protective mechanisms upon contact with invaders; this is termed as "induced resistance".

Although many thousands of pathogens are present in nature, individual plant species are susceptible to only a very small number of these pathogens. In many cases, when a pathogen comes in contact with a plant, the potential pathogen attempts to infect

the plant. However, only a few specific cases the pathogen has the ability to somehow suppress or nullify the constitutive or inducible defenses of the plant to stop further invasion by the pathogen. Based on this information, it has been concluded that susceptibility to disease is at least partly due to overcoming performed defenses or to a failure of the plant to induce its defenses rapidly enough to stop a pathogen rather than being solely based on heritable "genes for resistance".

Peroxidase

Peroxidases (H_2O_2 oxidoreductase; EC 1.11.1.17) are hemoglycoproteins that occur widely in plants and have been associated with resistance to plant diseases. Plant peroxidases are monomeric, heme–containing proteins that are usually glycosylated isoperoxidases, arising from the transcription of different genes or from post-translational modification and are widely distributed within both intra and extracellular environment (Bestwick *et al.*, 1998). Peroxidases have been implicated in the hypersensitive response and play an active role in the H_2O_2-dependent polymerization of hydroxycinnamyl alcohols (monolignols) during the final stages of lignin biosynthesis (Miyazawa *et al.*, 1998). Peroxidases also catalyze rapid H_2O_2-dependent cross-linking of cell wall proteins such as the hydroxyproline-rich glycoproteins and proline-rich glycoproteins (Everdeen *et al.*, 1998), as well as cross-links between other wall components (Fry, 1986; Iiyama *et al.*, 1994) in plants. As a result of oxidative cross-linking reactions, cell walls may be strengthened and may function as physical barriers against invading pathogens. The reinforcement of the wall reduces susceptibility to wall–degrading enzymes, possibly restricts diffusion of pathogen-derived toxins to the host, and in the case of some fungal pathogens acts as a mechanical barrier to physical penetration towards the protoplast (Bestwick *et al.*, 1998).

The role of peroxidases in plant defense reactions has been reported in many elegant publications (Gaspar *et al.*, 1980; Moerschbacher, 1992; Ray *et al.*, 1998; van der Westhuizen *et al.*, 1998; Wang and Liu, 1999). Peroxidases can play a role in cell wall toughening and in the production of toxic secondary metabolites. Its simultaneous

oxidant and anti-oxidant capabilities can make it an important factor in the integrated defense response of plants to a variety of stresses (Hammerschmidt *et al.*, 1982; DeJaegher *et al.*, 1985). Peroxidases are also implicated in lignin biosynthesis (Sato *et al.*, 1995; Quiroga *et al.*, 2000), ethylene production and suberization (Peyrano *et al.*, 1997; Quiroga *et al.*, 2000). Peroxidase activity has been positively correlated with plants resistance to pathogen attack. Upon inoculation with a pathogen, the peroxidase activity increased immediately in the resistant cultivar as compared to a susceptible cultivar that either did not show, or showed delayed increase in the peroxidase activity. In addition, resistant cultivars often show an earlier induction of peroxidase activity than susceptible ones (Vera-Estrella *et al.*, 1992; Scott-Craig *et al.*, 1995). Peroxidase over expressing plants showed a significantly increased resistance to pests, possibly through the production of phenolic radicals capable of cross-linking plant proteins and carbohydrates rendering them indigestible to insects (Lagnimini *et al.*, 1993).

Morpurgo *et al.* (1994) have reported the use of peroxidase as a parameter to discriminate between susceptibility and tolerance of *Musa acuminata* clones to Foc race 1 and race 4. Several workers have used polyphenol diversification and isozymes polymorphism to differentiate *Musa* clones (Bonner *et al.*, 1974; Rivera, 1983; Jarret and Litz, 1986 a, b; Jarret 1990; Espino and Pimentel, 1990; Horry, 1993; Jarret and Gawel, 1995; Ford-Lloyd *et al.*, 1997).

Phenolic Compounds

Phenolic compounds that impregnate host reaction products are thought to play an important role in the resistance process, either making physical barriers stronger or chemically impervious to the previously mentioned hydrolytic enzymes of the pathogen (Beckman 1987; MacHardy and Beckman 1981). Enzymes that are instrumental in the formation of these compounds have been previously studied in many different hosts, including banana. For example, phenol-oxidizing enzymes mainly peroxidases are associated with many different vascular diseases (Mace and Wilson 1964; Mace *et al.*, 1972; Meuller and Beckman 1978).

RAPD –based markers for characterization of genetic variability

The Random Amplified Polymorphic DNA (RAPD) technique offers a powerful tool to detect DNA polymorphism among plants, microorganisms and animals. In particular, RAPD can be used to distinguish different clones and cultivars (Galderisi *et al.*, 1999). RAPDs are strongly dependent on experimental conditions. Careful optimization of each step of the amplification reaction is needed to achieve the necessary reproducibility of the RAPD data (Micheli *et al.*, 1994; Micheli and Bova, 1997). The quality and quantity of template DNA, the source of the DNA polymerase and the amplification conditions are critical factors. RAPD has the advantage of being technically simple and rapid and has been used for plant genetics and phylogenetic studies (Yamamoto *et al.*, 1994; Demeke *et al.*, 1996).

King *et al.* (1991) and Wolfe and Gessler (1992) reported the availability of RAPD markers that aid in mapping genes coding for agronomically important characters, which increased the efficiency and reduced the time-scale of plant breeding. RAPD markers generated by the PCR were used to differentiate between morphologically indistinguishable strains and varieties (Welsh and McClelland, 1990; Welsh *et al.*, 1991; Goodwin and Annis, 1991).

The RAPD technique is central around the polymerase chain reaction (PCR), a technical innovation that has swept through molecular biology laboratories and has been applied in a wide range of DNA-handling situations. PCR is a method, which allows a particular section of the DNA of an organism to be repetitively copied (amplified) so that eventually it is present in such a high quantity that it can be simply stained by a fluorescent dye and visualized by fluorescent emission when DNA fragments are separated by electrophoresis. In order that copying can occur, short lengths of DNA (usually known as primers) are added to the PCR mix. Differences in banding pattern represent differences between DNA samples, and hence diversity (Ford-Lloyd *et al.*, 1997).

Kaemmer *et al.* (1992) and Bhat *et al.* (1995a) have observed that oligonucleotide fingerprinting and RAPD technology are more efficient in differentiating clones within each AAA, AAAA, AAB and ABB genomes. Also RAPD is a very fast way of obtaining information about genetic variation (Williams *et al.*, 1990; Welsh and McClelland, 1990). The RAPD technique has been applied in *Musa* to discriminate the genomic composition of triploid cultivars and varietal identification (Kammer *et al.*, 1992; Howell *et al.*, 1994; Bhat *et al.*, 1995b). This has also been used to study pattern of diversity amongst germplasm collections from different geographic regions such as the Indian subcontinent (Bhat and Jarret, 1995). Dwarf off-types and general levels of somaclonal variation have been identified using RAPD markers (Damasco *et al.*, 1996; Ford-Lloyd *et al.*, 1997). Along with other type of markers, RAPD has been used for the development of linkage maps of diploid banana (Faure *et al.*, 1993). Kulkarni *et al.* (1999) have reported RAPD-profile variation amongst cultivated, wild and irradiation-derived variants of banana. However, RAPD markers can suffer from problems of reliability and transferability, which may limit their application and selection programmes (Ellswoth *et al.*, 1993).

RAPD markers are used to determine the genetic stability of long-term shoots of Japanese black pine (*Pinus thunbergii* Parl) (Goto *et al.*, 1998). RAPD technique was also used to develop PCR – based markers linked to downy mildew resistance genes in lettuce and the amplified RAPD products were cloned and sequenced (Paran and Michelmore, 1993). Galderisi *et al.* (1999) have developed a comprehensive RAPD based procedure for routine molecular typing of various plants. Identification, preservation of the genetic resources of cultivated cole *Brassica oleracera* L. and the analysis of genetic variation by RAPD was carried out by Divaret *et al.* (1999). Sun *et al.* (1997) demonstrated the efficiency of RAPD analysis for the identification of markers linked to the yellow rust resistance gene in wild emmer wheat *Triticum dicoccoides*.

Eleven apple cultivars were differentiated using RAPD markers obtained by PCR (Koeller *et al.*, 1993). A set of bands consistent in their presence or absence was chosen to create a differentiating band pattern. The applicability of RAPD technique for bananas

and plantains has been demonstrated by Kaemmer *et al.* (1992); Howell *et al.* (1994); Bhat and Jarret (1995); Damasco *et al.* (1996)

Usefulness of RAPD profiles has been reported for the diversity analysis of *Citrus*, the linkage map development and hybrid identification (Yamamoto *et al.*, 1998; Cristofani *et al.*, 1999; Elisiaro *et al.*, 1999), grape varietal classification and linkage analysis (Vidal *et al.*, 1999; Lodhi *et al.*, 1997), cultivated and wild genotypes of papaya (Jobin *et al.*, 1997; Magdalita *et al.*, 1997) and in mango (Lopez *et al.*, 1997).

Antifungal Proteins

Plants have no immune system, and yet they successfully fight infection with both constitutive and induced defense mechanisms (Vigers *et al.*, 1991). Their resistance is mainly based on a dynamic defense system composed of antimicrobial agents of different molecular weight classes (Cammue *et al.*, 1994). The best studied of these are the phytoalexins, secondary metabolites with a broad antimicrobial spectrum that are specifically synthesized upon perception of appropriate defense-related signal molecules. The production of phytoalexins depends on the transcriptional activation of a series of genes encoding enzymes of the phytoalexin biosynthetic pathway. During the last decade, however, it has become increasingly clear that some plant proteins can play a more direct role in the control of phytopathogenic fungi. Several classes of proteins with antifungal properties have now been identified including chitinases, β-1,3-glucanase, chitin binding lectins, zeamatins, and thionins and ribosome-inactivating proteins.

Chitinases are one of the first antifungal proteins reported (Molano *et al.*, 1979; Schumbaum *et al.*, 1986). Schumbaum *et al.* (1986) demonstrated that purified bean endochitinase was a potent inhibitor of hyphal extension of the deuteromycete *Trichoderma viride*.

Roberts and Selitrennikoff (1988) showed that barley, maize and wheat chitinases were more potently antagonistic to *Phycomyces blakesleanus* and *Trichoderma reesi*.

Extracts from infected pea pods had high ß-1,3-glucanase and chitinase activities and inhibited growth of 15 out of 18 tested fungi (Mauch *et al.*, 1988b). Roberts and Selitrennikoff (1990) isolated a membrane acting protein from maize belonging to thaumatin class that had profound antifungal activity. Vigers *et al.* (1991) reported the presence of zeamatin, a 22-kDa protein that had potent activity against a variety of fungi that acted by causing membrane permeabilization.

Arlorio *et al.* (1992) demonstrated that purified chitinase alone or in the presence of ß-1,3-glucanase, both from infected pea pods arrested the growth of *Trichoderma longibrachiatum* hypha. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L) seeds with broad antifungal spectrum was reported by Terras *et al.* (1992). Two 28-kDa chitinases, designated chitinase A (chit A) and chitinase B (chit B) were purified from maize seeds to homogeneity by Huynh *et al.* (1992). They reported that the antifungal activity of chit A against the growth of *Trichoderma reesi*, *Alternaria solani* and *Fusarium oxysporum* was greater than chit B.

Isolation of seven antifungal proteins from Brassicaceae species was reported by Terras *et al.* (1993). These proteins were basic, multimers of 5-kDa polypeptide specifically inhibiting the fungal growth. It was reported that only specific basic isoforms of tobacco chitinase and ß-1, 3-glucanase were antifungal against various fungi tested *in vitro* (Sela-Buurlage *et al.*, 1993). Three proteins from grain sorghum that have potent action against *Fusarium moniliforme* (Kumari and Chandrashekar, 1994) were isolated and partially characterized. They were 18, 26 and 30 kDa in size respectively. Their mode of action has been studied. The 18 kDa protein acts by releasing fungal cell wall material, while the 26 and 30 kDa proteins act on the membranes. The levels of these proteins were related to grain hardness (Kumari *et al.*, 1994).

Ji and Kuc (1996) reported antifungal activity of ß-1,3-glucanase and chitinase purified from cucumber (*Cucumis sativus*) on *Colletotrichum lagenarium*. A 22 kDa chitinase and 26 kDa ß-1,3-glucanase were isolated from cowpea (*Vigna unguiculata*) seeds and these were shown to deter development in an *in vitro* assay of the

phytopathogenic fungi *Colletotrichum lindemuthianum* and *Colletotrichum musae* (Gomes *et al.*, 1996).

Peptides with antimicrobial properties are present in most if not all plant species. The potential of antimicrobial peptides for engineering disease resistance in crops has been obtained with chimeric transgenes based on a thionin, a plant defensin and a lipid transfer proteins (Broekaert *et al.*, 1997).

The genes encoding antifungal proteins are currently being used by agribusiness to create genetically modified plants tnat have increased fungal resistance in the field (Selitrennikoff, 2001). Genes coding for antifungal proteins that show broad antifungal activity *in vitro* have been introduced into a plantain landrace and the resulting transgenic plants await field testing (Remy *et al.*, 1998).