

*Isolation and Characterization
of an Antifungal Protein
from *Urgenia indica*
to *Fusarium oxysporum* f. sp.
*cubense**

Chapter 3

Introduction

Fungal diseases cause severe limitation in production of major crops. To prevent plants from being destroyed by fungal pathogens, several attempts have been made. One of the alternative approaches is to produce transgenic plants resistant to diseases. Several transgenic plants, which overproduce pathogenesis-related proteins (PR proteins), have been reported to enhance resistance to fungal diseases (Jach *et al.*, 1995; Lin *et al.*, 1995; Terras *et al.*, 1995).

The outbreak of fungal disease like *Fusarium* wilt, with a devastating effect on banana production and germplasm exchange, has sparked a renewed interest in banana research. The rapid evolution in molecular techniques to isolate resistance genes from the own or a foreign gene pool and to bring these into the genome of susceptible landraces brings us closer to the generation of disease resistant/tolerant plants. For this we are dependent upon genes that encode new types of antifungal proteins, which could be introduced into the genome of banana and plantain cultivars (Schoofs *et al.*, 1998).

A preliminary and important part of the strategies in obtaining transgenic plants is the discovery and characterization of antifungal proteins from different plant species and the isolation of their encoding genes.

Antifungal proteins and polypeptides have been isolated from diverse groups of organisms, including plants, fungi, bacteria, insects and animals (Selitrennikoff, 2001). The mechanisms of action of these proteins are as varied as their sources and include fungal cell wall polymer degradation, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis, and inhibition of the cell cycle. The range of fungi inhibited by antifungal proteins is extremely broad, with plant pathogens being sensitive at micromolar levels (Selitrennikoff, 2001).

The aim of the present work was to isolate and characterize antifungal protein to *Fusarium oxysporum* f. sp. *cubense* (Foc), race 1 that infects 'Nanjangud Rasabale'. Among 15 different plant species tested, the crude extracts of *Urgenia indica* showed

inhibition to the growth of *Foc*. Hence, the present work was taken up to explore the antifungal properties with special reference to wilt pathogen of 'Nanjangud Rasabale', *Foc*.

Urgenia is one of the extremely interesting polytypic genus with about 100 species occurring in Africa, Mediterranean regions and in plains of South India (Airy Shaw, 1966). It is represented in India by about nine species (Hemdri and Swahari, 1982). Squill bulbs have long been used as a source of natural products with pharmaceutical (cardio tonic) and biocidal (rodenticide) applications (Pascual *et al.*, 1999).

Materials and Methods

Fungal culture

The wilt pathogen *Foc* was isolated from infected corm pieces of the banana cultivar 'Nanjangud Rasabale' and maintained on potato dextrose agar.

Urgenia bulbs

Urgenia bulbs were chopped into small pieces and dried open in a petri dish for 40-50 days at room temperature. The dried pieces were homogenized in a blender to obtain the powder at the finest setting.

Bulb extracts

Three grams of *Urgenia* bulb powder was extracted with 0.025 M Tris buffer (1:4 w/v) pH 6.8 in a pre-chilled pestle and mortar at 4° C. The suspension was centrifuged at 8000 rpm for 15 min at 4° C (Kuboto 6930 centrifuge, Kuboto Corporation, Japan) and the supernatant was used for purification of proteins since it contained fractions inhibitory to the growth of *Foc*.

Preparation of the acid extractable basic fraction

The pH of the crude extract was lowered to pH 3 by adding 1 N HCl and incubated at 4° C overnight. This was centrifuged at 12,000 rpm for 30 min at 4° C (Kuboto 6930 centrifuge, Kuboto Corporation, Japan) to precipitate the acidic proteins in the pellet. The supernatant was extensively dialyzed against distilled water at 4° C to neutralize the pH and subsequently lyophilized.

Gel filtration

The basic fraction of the bulb was fed on to a Sephadex G-100 (Pharmacia LKB, Sweden) column (55 x 1.5 cm) equilibrated with Tris buffer (0.02 M, pH 6.8) and eluted with the same buffer. Fractions (1.5 ml) at a flow rate of 1.5 ml /6 min were collected and the protein was detected at 280 nm routinely. Every 5th fraction was tested for antifungal activity. Fractions corresponding to different peaks were pooled, reverse dialyzed against sucrose. The protein in pooled fractions was estimated by the dye binding method (Bradford 1976) using bovine serum albumin (Hi-Media, India) as a standard. The pooled fractions were used for electrophoresis.

Gel electrophoresis

SDS-PAGE was performed on 12% separating and 6% stacking slab gel according to the method of Laemmli (1970). The standard proteins for molecular mass determination obtained from Bangalore Genei, Bangalore, India, were used. Proteins were stained with silver nitrate.

Native PAGE was done cathodically by the method of Reisfeld *et al.* (1962) in 14% separating and 5% stacking gel.

Electroelution

Following, SDS-PAGE, appropriate bands were cut out of the gel by using the sidestrip method (Harlow and Lane 1988). Protein was eluted from the gel with an

Elutrap electroseparation devise (Schleicher and Scheull, Keene, NH), following the manufacturer's instructions and this purified preparation was used to test for the antifungal property of the two separated proteins in active Peak 2.

Assay for antifungal activity

Antifungal activity of the purified protein was tested on wilt pathogen *Foc* as described by Roberts and Selitrennikoff (1986). Fungal spores were harvested from well sporulating colonies and suspended in sterile distilled water. The concentration of the spore suspensions were determined in a haemocytometer and adjusted to 5×10^5 spores ml^{-1} . The freshly prepared suspensions (0.1 ml) were plated out on petri dishes containing potato dextrose agar medium for maintenance of the test fungus. To allow for spore germination and initial vegetative growth, the plates were incubated for 20 to 24 h at room temperature. At this time, circular wells were punched on the agar surface with the help of sterile tips and aliquots of the purified protein (18 μg) were pumped into the wells. Tris-HCl buffer (0.025 M pH 6.8) was also loaded, that served as control. The plates were further incubated at room temperature and observed for inhibition zones.

Immunological methods

Polyclonal antiserum

Antiserum was raised against the purified protein (from active Sephadex G-100 first peak - Peak 1) from *U. indica* bulbs in New Zealand white rabbits. For immunization, 125 μg s of protein was used. Protein mixed with 500 μl of Freund's complete adjuvant was used for the first rabbit injection; incomplete adjuvant was administered for subsequent boosts through intramuscular routes on days 0, 14, 28 and 42. The antiserum was separated from the blood 2 days after the last injection and was stored at -40°C

Protein gel blot analysis

Crude extract, basic fraction, pooled fractions P1 and P2 (active Peak 1 and active Peak 2) were subjected to SDS-PAGE on 12% separating and 6% stacking gel according to the method of Laemmli (1970). 12 µg of protein was loaded per well. Immediately after electrophoresis, gels were blotted onto nitrocellulose membrane (Millipore) using Bangalore Genei, Bangalore, electrophoretic transfer apparatus. The membrane was blocked in a blocking buffer (containing 20 mM Tris, 150 mM NaCl and 3% BSA). The blot was washed and incubated with polyclonal antibodies raised against purified protein (1:500 dilution) for 3 h. After washing the membrane, the blot probed with primary antibody was incubated with alkaline phosphatase conjugated secondary antibody (1:1000 dilution) for 3 h. Immunoreactive bands were detected by immersing the membrane in substrate solution BCIP (5-bromo-4-chloro-3-indolyl phosphate, Bangalore Genei) and the color reactions were developed using NBT (nitro blue tetrazolium, Bangalore Genei).

Partial amino acid analysis

Purified protein from the active Sephadex G-100 first peak was separated by SDS-PAGE and blotted on to PVDF (Polyvinylede Difluoride) membrane (Sigma, USA) using Bangalore Genei (Bangalore, India) electrophoretic transfer apparatus. The transfer buffer was 25 mM Tris, 192 mM glycine with 15% (v/v) methanol. The purified protein was detected on the blot by Coomassie blue staining. The band was excised from the blot and the protein was sequenced on an Applied Biosystems Sequencer (model 477 A) with online HPLC.

Blast Search

Blast search analysis of the sequenced protein was carried out using the prosite database and blocks database.

Results

Using bioassay procedures coupled with biochemical methods, a protein, which is a potent inhibitor of the wilt pathogen *Foc*, was identified and isolated. The extract of *Urgenia* bulbs is inhibitory to the growth of *Foc* on potato dextrose agar (Fig. 3.1). The recovery of the enzyme after various steps of purification is presented in Table 3.1. When subjected to gel filtration chromatography on Sephadex G-100 (Fig.3.2), the extract separated into two major peaks. Both the peaks inhibited the growth of *Foc* as compared with that of control. When electrophoresed by SDS-PAGE, a single major protein was detected in Peak 1 of the Sephadex G-100 column (Fig. 3.3). The apparent molecular mass of this protein was found to be 27 kDa. Native PAGE also showed a single protein in that fraction (Fig. 3.4). However two proteins were detected in Peak 2. The apparent molecular mass of these two proteins was 27 kDa and 54.6 kDa respectively.

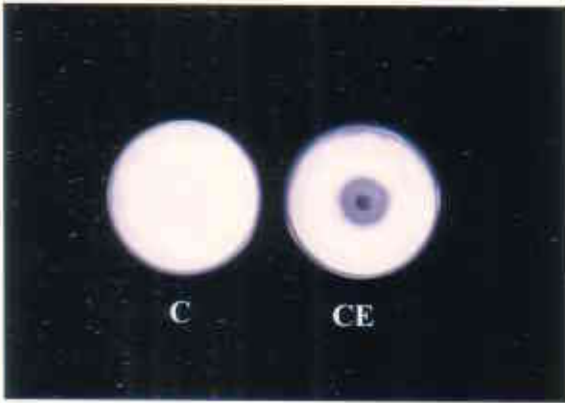
Antifungal Activity

The purified protein from active Peak 1 pooled fraction was tested for antifungal activity. Tris-HCl buffer (pH 6.8) was used as control. Circular inhibition zones were seen in *Foc* plates treated with purified protein (Fig. 3.7). No inhibition in growth was observed in the plate containing Tris-HCl buffer (Fig. 3.6).

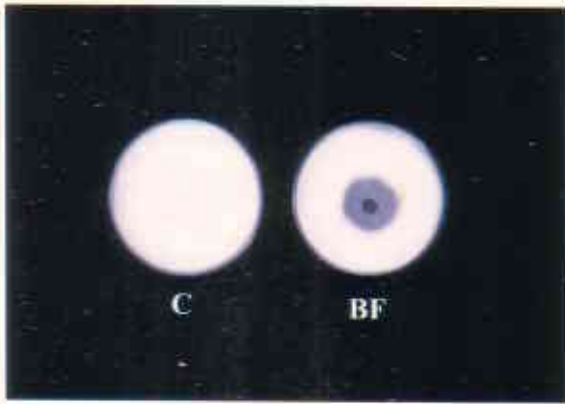
In order to ascertain which of the two proteins of active Peak 2 was antifungal, electroeluted purified preparations of these proteins were further employed to carry out antifungal assay. The inhibitory crescents due to the growth retardation of mycelia was seen around the wells loaded with purified preparation of 27 kda protein. No such inhibition of growth was seen in the plate containing the purified preparation of 54.6 kDa protein. This confirmed that the antifungal activity was due to 27 kDa protein (Fig. 3.8).

Protein gel blot analysis

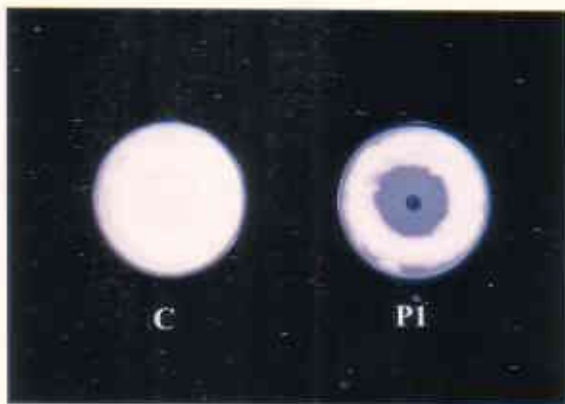
The 27 kDa protein corresponding to the purified antifungal protein was observed in the crude extract, basic fraction, active Peak 1 and Peak 2 (pooled fractions 1 and 2)



C - Control (Tris-HCl buffer pH 6.8)
CE - Crude Extract (50 μ l), protein - 154.5 μ g



C - Control (Tris-HCl buffer pH 6.8)
BF - Basic Fraction (50 μ l), protein - 63 μ g



C - Control (Tris-HCl buffer pH 6.8)
P1 - Pooled Fraction 1 (50 μ l), protein - 18 μ g

Fig. 3.1 The extract of *Urgenia* bulbs showing inhibition to the growth of *Foc*

Table 3.1 Summary of antifungal protein purification steps from *Urgenia* bulb

Step	Total Protein (mg)	Purification (fold)	Yield (%)
Crude Extract	82.4	1	100
Basic Fraction	12.6	2.45	15.29
Sephadex G-100	0.6	8.58	0.728

Elution Profile

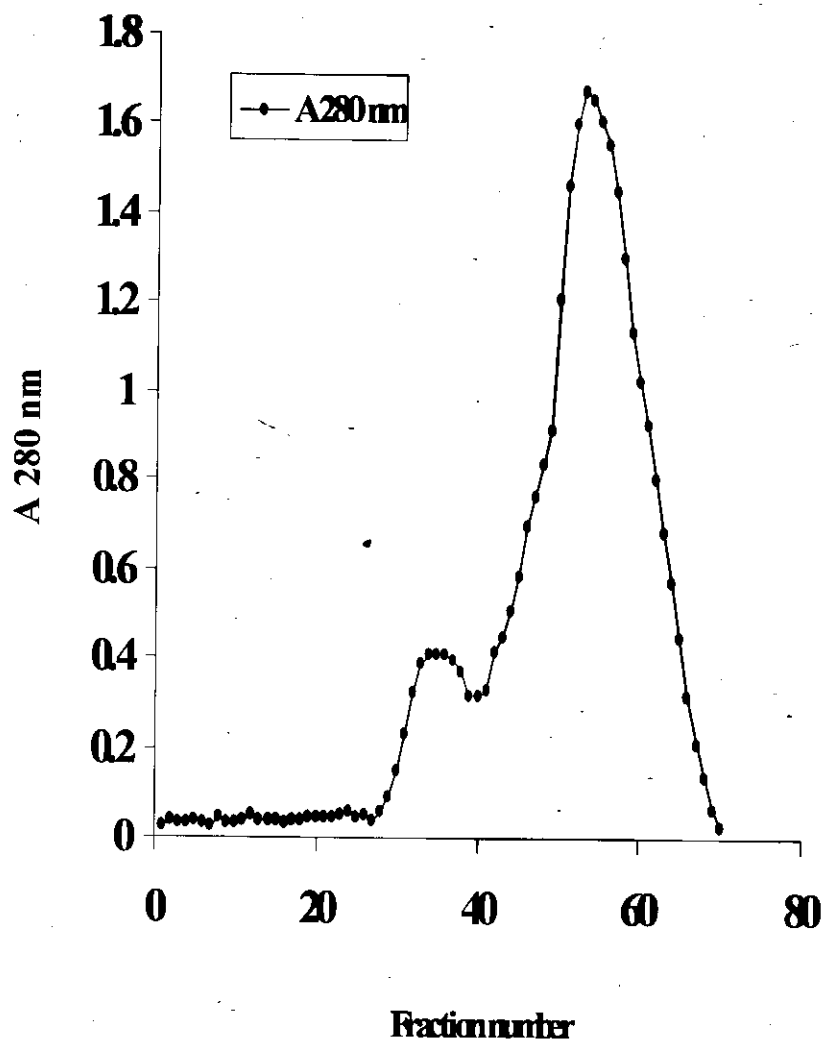


Fig. 3.1 Sephadex G-100 Column Chromatography of antifungal protein from *Urgenia indica* bulb extracts.

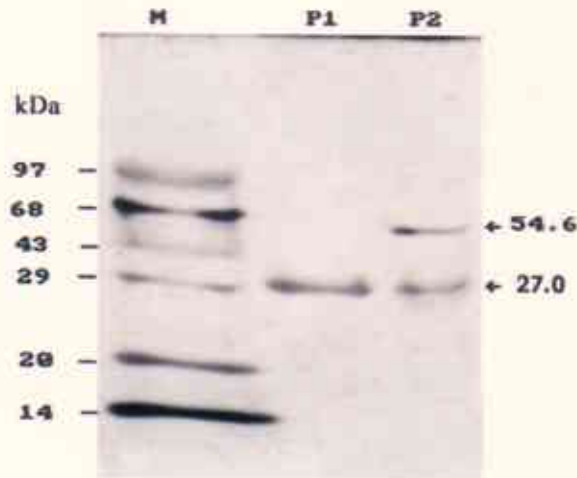


Fig. 3.3 SDS-PAGE of purified antifungal protein
 Lane 1 : Molecular Weight Markers
 Lane 2 : Active P1 (12 µg)
 Lane 3: Active P2 (12 µg)



Fig. 3.4

Native PAGE of purified antifungal protein from *Urgenia indica* bulb extracts followed by direct detection of the protein on the gel.

Lane 1 : Crude Extract
 Lane 2 : Purified Antifungal Protein



Fig. 3.5

Detection of antifungal protein by immunological reaction of CE, BF, P1 and P2 with antiserum raised against purified antifungal protein

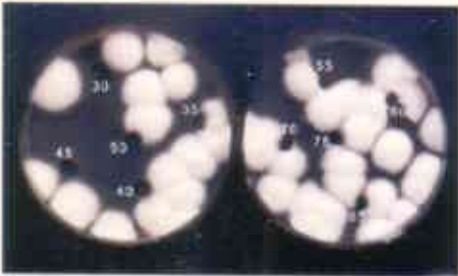
CE : Crude Extract P1 : Peak 1
 BF : Basic Fraction P2 : Peak 2

Fig- 3.6



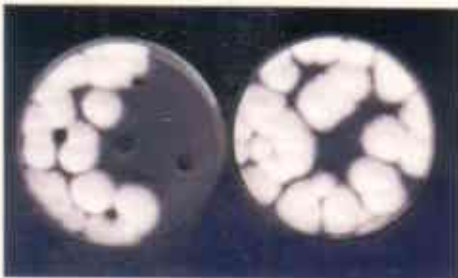
Growth of *Foc*
unaffected by Tris-HCl
buffer pH (6.8) - control

Fig- 3.7



Antifungal assay of every 5th fraction
(starting from 30th fraction)
of Sephadex G-100 gel filtration fractions
of *Urgenia indica* bulb extracts against *Foc*.
Fractions (50 μ l per well)
were placed near to *Foc* grown on PDA
for 20-24 h, followed
by further incubation at room temperature.
Numbers on the plate
indicate every 5th fraction
(starting from 30th fraction) shown in Fig. 3.2.

Fig. 3.8



1

2

- (1) Antifungal assay of the electroeluted purified preparation of 27 kDa protein from active P2 of SDS-PAGE exhibiting retardation of mycelial growth of *Foc*.
- (2) Antifungal assay of the electroeluted purified preparation of 54.6 kDa protein from active P2 of SDS-PAGE showing no inhibition of the growth of *Foc*.

using the antibodies raised against the purified protein. The clear bands of antifungal protein present at the same molecular weight in all steps of purification demonstrate that the antifungal activity from the original *Urgenia* bulbs has indeed been purified (Fig. 3.5).

The partial amino acid sequence of the purified antifungal protein has been shown below.

NH₃-SVSSIVSRAQAQPPKPSSHA FDRMLLHRNDGACQA
 KGFYTYDAFVAAAAAFSGFGTTGSADVQKRELAQTSHE
 TTGGWATAPDGAFWGYCFKQERGASSDYCTPSAQWP
 CAPGKR YYGRGPIQLSHNYNYGPAGRAIGVDLLANPD
 LVATDATVSADRAAGRVPGFVITNIINGGIECGHGQD
 S-COOH

Blast analysis

The protein is found to share ~ 70% homology to 26 kDa endochitinase 2 precursor from *Hordeum vulgare* (barley) and significantly homologous (50-70%) to endochitinase a/b/1/2/3 precursors of other eudicots and monocots. It shows conserved domain Glyco_hydro_19, of chitinase class I.

Pattern identification

The sequence signatures found in the sequence:

1. From 32 –54th residue Chitinases family 19 signature 1 (prosite database)

Chitinase. of family 19 (also known as classes IA or I and IB or II) are enzymes from plants that function in the defense against fungal and insect pathogens by destroying their

chitin-containing cell wall. Class IA/I and IB/II enzymes differ in the presence (IA/I) or absence (IB/II) of a N-terminal chitin-binding domain.

2. From 27-186 Glycoside hydrolase family 19 (blocks database)

Glycoside hydrolase family 19 comprises enzymes with only one known activity; chitinase (EC 3.2.1.14).

3. From 38-183 Chitin binding domain (blocks database)

A number of plant and fungal proteins that bind N-acetylglucosamine contain this domain. The domain may occur singly or multiply and is thought to be involved in recognition or binding of chitin subunits.

Discussion

In the present study, an antifungal protein was isolated and purified from *Urgenia indica* and its partial amino acid sequence deduced. The antifungal activity of this purified protein on wilt pathogen Foc was tested. The results indicated that the acid extractable basic protein was capable of inhibiting the growth of Foc, (*Fusarium* wilt), thus indicating that the activity is due to basic protein species. This apparently supports the reports that to date most of the known antifungal peptides are highly basic (Tailor *et al.*, 1997; Koo *et al.*, 1998).

Partial amino acid sequence analysis showed that the purified antifungal protein is found to share a high homology to 26 kDa endochitinase from *Hordeum vulgare* with a conserved domain Glyco_hydro_19, characteristic of chitinase class I proteins. In addition, the sequence signatures from 38-183 indicated the presence of chitin binding domain; possessed by chitinase class I proteins. However, this protein lacked the N-terminal cysteine-rich domain of ~ 40 amino acids present in all known class I chitinases (Selitrennikoff, 2001). These data suggest that the purified antifungal protein is a

putative endochitinase and could be a bonafide member of class II chitinases. The molecular weight of this protein was found to be 27 kDa on SDS-PAGE. This result is in agreement with the known reports that molecular mass of class II chitinases is 27-28 kDa (Selitrennikoff, 2001).

Virtually all plant chitinases that have been isolated to date are endochitinases (Roberts and Selitrennikoff 1988; Majeau *et al.*, 1990). Chitinases have been isolated from fungi (Mathivanan *et al.*, 1998; Kang *et al.*, 1999), tobacco (Melchers *et al.*, 1994), cucumber, beans (Ye *et al.*, 2000), peas, grains (Huynh *et al.*, 1992) and bacteria (Chernin *et al.*, 1997) and have known to have potent antifungal activity against a wide variety of human and plant pathogens, including *Trichoderma reesei*, *Alternaria solani*, *Alternaria radicina*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Guignardia bidwelli*, *Botrytis cinerea*, and *Coprinus comatus*.

In summary, the results in this study show that antifungal protein from *Urgenia* bulbs is capable of inhibiting the growth of the wilt pathogen *Fusarium oxysporum* *in vitro*. The protein could be attributed to glycoside hydrolase family 19 proteins, which are chitinases class II involved in defense against fungal pathogens by destroying their chitin-containing cell wall. Its putative mechanism of action may be that it binds and recognizes chitin subunits and subsequently degrades them.

The genes encoding many antifungal proteins are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field (Selitrennikoff, 2001). Expression of cloned chitinase genes in transgenic plants has provided evidence of their role in plant defense (Yun *et al.*, 1997). Thus it may be assumed that the antifungal protein gene from *Urgenia indica* may be a useful candidate for genetic engineering of 'Nanjangud Rasabale' banana plantlets for increased tolerance against Foc.

Summary

Fusarium wilt has a long and destructive history in the world's banana producing regions. Also known as Panama disease, it is caused by a variable soil borne fungus, *Fusarium oxysporum* f. sp. *cubense*. Less publicized, but potentially more destructive epidemic outbreak has affected non-exported, locally consumed cultivar 'Nanjangud Rasabale'. Since this preferred cultivar has been heavily damaged and eliminated by *Fusarium* wilt in many of the local areas of 'Nanjangud' town of Chamarajnar district in India, there has been an urgent need to develop or identify resistant replacements that would be accepted by local consumers. Hence the investigation entitled "Induction and evaluation of banana cultivar 'Nanjangud Rasabale' (NRB) clones for *Fusarium* wilt resistance" was undertaken with the following objectives

- (i) **Raising NRB plantlets through micropropagation and somatic embryogenesis and their evaluation for wilt resistance**
- (ii) **Evaluation of NRB somaclones using molecular markers**
- (iii) **Isolation and characterization of an antifungal protein from *Urgenia indica* to Foc**

The cultivar 'Nanjangud Rasabale' belongs to the silk subgroup Rasthali (AAB) and is a native of Nanjangud area in Chamarajnar 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.

Investigations of biotechnological techniques, micropropagation, somatic embryogenesis and induced mutations were undertaken to overcome problems with banana production of cv. 'Nanjangud Rasabale'. Protocols for micropropagation and somatic embryogenesis were established to obtain disease-free plantlets.

Although micropropagation of banana cultivars has been shown to be a simple method for the production of a high number of new plants in a relatively short period of time, in the present study only fewer shoots were produced by cultivar 'Nanjangud Rasabale'. This reflected the accumulated mutations in the different donor ramets used as explants, which in turn could be due to physiological differences between explants caused by the sucker location on the mother rhizome. However, a well-established micropropagation system allowed the preparation of hundreds of units for mutagenic irradiation.

The *in vitro* shoots were irradiated with 0, 5, 10, 20 and 30 Gy in a ^{60}Co irradiator. After irradiation, shoot tips exhibited significant differences in radiosensitivity and post-irradiation recovery. LD_{50} that reduced the growth and morphogenetic performance of the shoots was found to be 10 Gy in our experiments. No plants were recovered from shoot tips exposed to 30 Gy. In case of 20 Gy irradiated plantlets, in early stages of plant development, the irradiation affected emergence and expansion of the younger leaves indicating damage to the apical meristem.

Male flower buds cultured on MS medium supplemented with 18.01 μM 2,4-D, 5.37 μM NAA, 5.71 μM IAA and 4.09 μM *d*-biotin showed enlargement of the floral primordia and the development of whitish embryogenic callus after 2-3 months of inoculation. Six-month-old embryogenic callus showed several developed embryos on the surface and the smear preparations of the callus showed globular and heart shaped embryos. Histological sections with 10-month-old embryogenic callus revealed somatic embryos with a root apex and a shoot apex. Transfer of 10-month-old callus on half strength MS medium with 0.5 g/l malt extract, 0.1% activated charcoal supplemented with Morel's vitamins, and 0.2% gelrite, green plumule emerged from the embryos followed by the development of roots. About 30 well grown plantlets were regenerated through the process of somatic embryogenesis.

Somaclones obtained through micropropagation, irradiation and somatic embryogenesis were evaluated for *Fusarium* wilt resistance. Pre-selection of resistant

somaclones using biochemical markers such as peroxidase and phenols prior to field trials was tried.

An important feature of plant resistant responses towards invading pathogens is thought to be the rapid production of defense related enzymes. Peroxidase, mentioned as defense related enzyme, showed its involvement in banana-*Fusarium* wilt host-pathogen interaction. Both susceptible 'Nanjangud Rasabale' and resistant 'Dwarf Cavendish' banana cultivars showed different pattern of peroxidase accumulation on infection with *Fusarium oxysporum* f. sp. *cubense*. In resistant banana cultivar, high levels of peroxidase activity, was observed first on 7th day and subsequently on 28th day after inoculation with the pathogen. On the other hand, susceptible banana cultivar failed to recognize the pathogen attack and early increase in peroxidase activity was not noticed. This showed that, unlike the resistant cultivar, susceptible banana cultivar fails to show early reaction against the pathogen attack and this helps the pathogen to establish itself in the host and cause disease. Also, an increased amount of phenols was observed in the inoculated resistant 'Dwarf Cavendish' cultivar suggesting that phenols are implicated in the defense mechanism.

Although, the observed peroxidase levels and phenol content were low in susceptible cv. 'Nanjangud Rasabale', employed as a measure of resistance during field screening, tissue culture studies carried out in the present work showed that somaclones derived through micropropagation, irradiation and somatic embryogenesis are a source of variability. The importance of these processes lies in the very fact that about 33 somaclones were found tolerant and continue to survive in the sick plot. This therefore suggested the importance of somaclonal variants in 'Nanjangud Rasabale' cultivar improvement.

RAPD-PCR analysis demonstrated that arbitrarily chosen commercial decamer primers can be used to generate amplified fragments of genomic DNA that can differentiate banana somaclones. This method is rapid and simple and produces repeatable results. DNA amplification was carried out in the presence of 10-mer primers. Of the 22 10-mer primers used for RAPD-PCR analysis, six primers detected enough genetic variability among the somaclonal variants of banana cultivar 'Nanjangud Rasabale' to allow for complete differentiation. By selecting only

strongly (and therefore consistent) amplified DNA segments as informational bands, variations in minor bands resulting from different amplifications were excluded. The level of polymorphism tested in this study indicated that distinction between different somaclones was possible with a small number of appropriate primers.

A molecular strategy for the creation of *Musa* cultivars resistant to *Fusarium* wilt is the introduction of genes that encode new types of antifungal proteins into the genome of banana and plantain cultivars. Antifungal proteins and peptides have been isolated from diverse groups of organisms, including plants, fungi, bacteria, insects and animals.

In the present investigation, the isolation and characterization of an antifungal protein to *Foc* from *Urgenia indica* bulb extracts following aqueous acid extraction, Sephadex G-100 gel filtration technique and bioassay procedure has been demonstrated. Partial amino acid sequence of this protein was deduced. Purification was achieved ~ 8.58 fold. The antifungal protein is acid extractable basic protein with an apparent molecular weight of 27 kDa on SDS-PAGE, capable of retarding the mycelial growth of *Foc*. Partial amino acid sequence showed that the purified antifungal protein was found to share a high homology to 26 kDa endochitinase from *Hordeum vulgare* with a conserved domain Glco_hydro_19, characteristic of chitinase class I proteins. In addition the sequence signatures from 38-183 indicated the presence of chitin binding domain, possessed by chitinase class I proteins. However, this protein lacked the N-terminal cysteine-rich domain of ~ 40 amino acids present in all known class I chitinases. From the above findings, it was therefore concluded that the purified antifungal protein is a putative endochitinase and could be a bonafide member of class II chitinases.

The results of the present investigation demonstrated that the tissue culture techniques – micropropagation, irradiation and somatic embryogenesis employed to obtain tolerant/resistant plantlets are the source of variability for the improvement of cultivar 'Nanjangud Rasabale'. In addition, cloning of the purified antifungal protein (putative endochitinase) in genetic manipulation program for 'Nanjangud Rasabale' cultivar is suggested, which will certainly serve as a valuable guideline for saving endangered 'Nanjangud Rasabale'.