CLONING AND CHARACTERIZATION OF *FUSARIUM* WILT RESISTANCE GENE ANALOGS IN BANANA (*Musa* Spp.)

By

RAMESH

(2013-11-108)

THESIS

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KERALA, INDIA

2016

DECLARATION

I hereby declare that the thesis entitled **"Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled**"Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.)"**is a record of research work done independently by**(2013-11-108)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree,diploma,associateship,fellowship to him.

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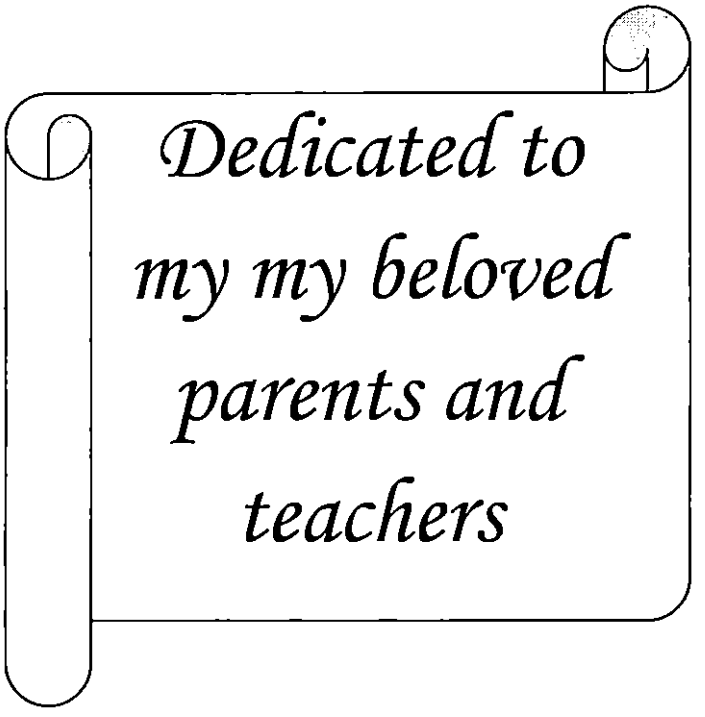
Dedicated to
my my beloved
parents and
teachers

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Abbreviations

%	Percentage
µg	Microgram
bp	Base pair
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
G	Gram
Kb	Kilo basepairs
L	Litre
M	Molar
M	Meter
Mg	Milligram
Ml	Millilitre
mM	Millimole
MT	Metric Tonnes
Ng	Nanogram
°C	Degree Celsius

OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra Violet
V	Volts
α	Alpha
B	Beta
µl	Microlitre
CET	Constitutive Expressor of Thionin
CEV1	Constitutive Expression of VSP1
CHIB	Chitinase B
COI1	Coronatine Insensitive1
EDS1	Enhanced Disease Susceptibility1
EIN2	Ethylene Insensitive2
ET	Ethylene
EAD	Fatty Acid Desaturase
HEL	Hevien-Like Protien

JA	Jasmonic Acid
JAR1	Jasmonic Acid Resistant1
JOE	Jasmonate Over Expressing
MPK4	Mitogen-activated Protein Kinase4
PAD4	PhytoAlexin Deficient4
PDF1.2	Plant Defense In 1.2
PR	Pathogenesis-Related
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SID2	SA Induction Deficient2
SSI2	Suppressor of SA Insensitivity2
THI 2.1	Thionin 2.1

Introduction

I. INTRODUCTION

Banana and plantain (Musa spp.) are among the most important crops in the world, serving as a staple food and source of income in many developing countries. Banana is also the world's leading fruit crop and consequently an important export commodity for several agricultural-based economies in Latin America, Africa and Asia, and represents the fifth most important agricultural crop in world trade (Aurore*et al.*, 2009).India is the largest producer of banana in the world and also in Asia. India alone produces 30.38 million tons from an area of 0.765 million ha (2012-2013) (http://nationmaster.com). Although Brazil has the second largest area under banana (0.49 million ha), the country ranks only fourth in terms of production. The major banana growing states are Tamil Nadu, Maharashtra, Gujarat, Karnataka, Kerala, Andhra Pradesh, Orissa, Bihar, Madhya Pradesh, West Bengal, Assam, Tripura and Manipur. Tamil Nadu has the largest area (1.25 lakh hectare) followed by Maharashtra (0.82 lakh hectare) and Gujarat (0.65 lakh hectare). Tamil Nadu ranks first in production (8.02 million tonnes), followed by Maharashtra (4.1 million tonnes) and Gujarat (4.04 million tonnes) (http://nhb.gov.in).But still there is a long way to go to achieve target yield potential, which is being threatened from time to time by various biotic and abiotic stresses associated with banana production. Among biotic stresses, *Fusarium* wilt is regarded as one of the most devastating diseases of banana, affecting plantations in almost all banana-growing countries of the world (Ploetz*et al.*, 1990). This disease is caused by the soil-borne fungus *Fusariumoxysporumformaespecialis* (f. sp.) *cubense*(FOC) (Stover, 1962b).

The first record of *Fusarium* wilt was in Australia in 1874 (Bancroft, 1876) and the disease has since been found in all banana-growing regions of the world, including Australia, Asia, Africa and Central and south America (Ploetz, 2006) except for some of the countries bordering the Mediterranean sea. It first became epidemic in Panama in 1890 and proceeded to devastate the Central America and Caribbean banana industries that were based on the 'Gros Michel' (AAA) variety in

the 1950s and 1960s, because of race 1.*Fusarium*wilt of banana can be readily diagnosed by the appearance of a number of typical symptoms. These include typical vascular disease causing disruption of translocation and systemic foliage symptoms in bananas, which eventually lead to collapse of the crown and pseudostem (Jeger*et al.*, 1995). *Fusarium*f.sp.*cubense* is also capable of infecting and surviving on a wide range of alternative hosts, including coffee and maize, both of which may be intercropped with bananas. As a result, disease diagnosis and development of control strategies, involving the use of plant varietal resistance is hampered by difficulties encountered in identification of alternative hosts and relevant pathogenic forms present.Four races of *F. oxysporum* f. sp. *cubense* have been described, only three of which affect banana (race 3 is a pathogen of heliconia). Race 1 affects the cultivars 'Maqueño', Gros Michel 'Silk', 'Pome', 'PisangAwak' and the hybrid 'I.C.2'. Race 2 affects cooking bananas, such as 'Bluggoe' and some breed tetraploids. Race 4 is most destructive since it affects race 1 and race 2 susceptible clones as well as the Cavendish cultivars (Randy and Ploetz, 2005). *Fusarium* wilt continues to pose threat among commercial Indian cultivars. Twenty-two percent of the total banana production remains at the mercy of this pathogen which includes popular cultivars like Silk (AAB), Ney Poovan (AB), Red Banana (AAA), Pome (AAB), PisangAwak (ABB) and all cooking bananas. All these years, race 1 and 2 were reported to attack the above cultivars while recently Poovan (Mysore, AAB) has lost its immunity and sporadic reports of *Fusarium* wilt on immune Mysore group is also being received.

Recent molecular research on R proteins and downstream signal transduction networks has provided exciting insights, which will enhance the use of R genes for disease control. Plants have evolved an efficient defense transduction network against pathogenic attack. Different resistance (R) gene mediated gene-for-gene resistance pathways, are well recognized branches of this network (Glazebrook, 2001; Hammond-Kosack and Parker, 2003). Definition of conserved structural motif in R proteins has facilitated the cloning of useful R genes, including several that are

functional in multiple crop species and/or provide resistance to a relatively wide range of pathogens. Numerous signal transduction components in the defense network have been defined, and several are being exploited as switches by which resistance can be activated against diverse pathogens (McDowell and Woffenden, 2003).

Resistance genes (R-Genes) are genes in plant genomes that convey plant disease resistance against pathogens by producing R proteins. The main class of R-genes consists of a nucleotide binding domain (NB) and a leucine rich repeat (LRR) domain(s) and is often referred to as (NB-LRR) R-genes.

Most of the functionally known resistance genes have been cloned and identified using transposon tagging and map-based cloning technologies, but the use of these approaches for the identification of new resistance genes is still laborious and time-consuming. On the other hand, there are eight major conserved amino acid motifs in the NBS (Meyers *et al.*, 1999) that can be used to design degenerate primers to identify RGAs by means of PCR amplification and sequencing. This approach to identify RGAs has been used in a variety of plant species, such as soybean (Kanazin*et al.* 1996; Yu *et al.*, 1996), common bean (Ferrier-Cana *et al.*, 2003; Rivkin*et al.*, 1999), alfalfa (Cordero and Skinner, 2002; Zhu *et al.*, 2002), lettuce (Shen*et al.*, 1998), coffee (Noir *et al.*, 2001), and grape (Di Gaspero and Cipriani, 2002).

Options for the control of *Fusarium* wilt are limited by ineffectual chemicalcontrol and the lack of commercially suitable resistant cultivars (Smith *et al.*, 2006).

Unfortunately, cultivated banana varieties are mostly triploid and can only be propagated asexually, making it difficult to improve this crop genetically using conventional plant breeding methods that rely heavily on cross-pollination between plants. As an alternative, the introduction of resistance genes into banana plants via

biotechnological means offers a valuable way of developing resistant banana cultivars (Sagi, 2000).

Recent studies has shown (Jusna, 2013) that the *Foc* resistant cultivar 'Palayankodan' has 'R' genes of class NBS type and can serve as source for this type of genes for molecular breeding.When cultivation is with resistant varieties no input is required from the farmer and there are no adverse environmental effects.Study will be useful for development of molecular markers linked with *Fusarium* wilt resistance for marker assisted selection. In this background the research was undertaken to 'clone and characterize the *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.)'.

Review of Literature

2. REVIEW OF LITERATURE

In the present study, an attempt has been made to clone and characterize the *Fusarium* wilt resistance gene analogs in banana variety 'Palayankodan'. The contents of this chapter are placed under the following heads:

2.1 Origin and history of banana

2.2 Introduction to *Fusarium*wilt

2.3 Crop loss due to *Fusarium* wilt

2.4 Response of plants to disease conditions

2.5 Gene network involved in disease resistance

2.6 Isolation of resistant gene

2.7 Future perspectives

2.1 Origin and history of banana

Centre of origin of banana is South East Asia, a region considered as the primary centre of diversification of the crop and where the earliest domestication has occurred (Simmonds *et al.*, 1955). This is an area bordered on the west by India and on the east by Samoa, Fiji and other South Pacific islands (Simmonds, 1996). *Musa acuminata* is said to have originated from Malaysia, while the hardy *Musa balbsiana* originated from Indochina. The low land areas of West Africa contain the world's largest range of genetic diversity in plantains (Musa AAB) (Ortiz and Vuylsteke, 1994). Conversely in East Africa, bananas are highly evolved into an important zone of secondary genetic diversity for the East African highland bananas (Musa AAA) (Smale, 2006).

The generic name Musa is derived from the Arabic word 'mouz'. Bananas were known to the early Arabs and appear in the Koran as the 'tree of paradise' (Robinson, 1996). The genus *Musa* was created by Carl Linnaeus in 1753.The name may be derived from Antonius *Musa*, physician to the Emperor Augustus, or Linnaeus may have adapted the Arabic word for banana, mauz.*Musa* is in the family Musaceae. Ancient Greek records of Alexander the Greats campaign in India describe bananas, and the Arabs have long been familiar with the banana palm which they called by its Indian name, Pala. The Romans also used this name. This led to the belief that the edible banana originated in continental South and Southeast Asia. However, later research has shown that the center of origin of the wild banana stretches from India to Papua New Guinea including Malaysia and Indonesia. In India banana is known for its antiquity from its mention in Ramayan (2020 BC), Kautilya'sarthasastra (300-400 BC). Plantain fruits resemble bananas but are longer, have a thicker skin, and contain more starch. They are a major staple in Africa, Latin America, and Asia. They are usually eaten cooked unless they are very ripe. Plantains are especially important in the humid lowlands of West and Central Africa. Banana fruits are consumed and processed in many ways and at all stages of ripening and development, leading to products with increased shelf life, such as flour, chips, and beverages. In India and other Asian countries, banana is grown for its leaves (for plates or firewood) or fiber extracted from the pseudo-stem (for ropes and fishing nets). In Southeast Asia, the flowers and terminal bud are eaten cooked or raw and the pseudo-stem is also sometimes eaten.

2.1.1 Morphology and Reproduction of Banana

Banana is a monocotyledonous, herbaceous plant. When a banana plant is mature, the corm stops producing new leaves and begins to form a flower spike or inflorescence. A stem develops which grows up inside the pseudostem, carrying the immature inflorescence until eventually it emerges at the top. Each pseudostemnormally

produces a single inflorescence, also known as the 'banana heart'. The tree stems remains underground which is known as the rhizome. Botanically rhizome is a modified stem.

Bananas are large perennial herbs with an underground stem called a corm, which is the true stem of the banana plant. The corm produces aerial shoots which arise from the lateral buds which develop into eyes and later suckers. The continuous vegetative growth of suckers perpetuates the corm's life and hence the perennial status of bananas. The aerial shoot is called a pseudostem and grows to height of 2 to 8 m depending on the variety and the conditions. The pseudostem consists of large overlapping leaf bases which are tightly rolled round each other forming a cylindrical structure almost 48 cm in diameter. The roots are initiated from the corm and they range from 50 to 100 cm in length; occasionally sub-horizontal roots reach 3 m (Blomme and Ortiz, 2000). The corm also consists of the apical meristem from which the leaves and ultimately the flowers are initiated. On average, each plant produces 35 to 50 leaves in its growth cycle.

When the banana plant has formed an average of 40 leaves (within 8 to 18 months), the terminal bud of the corm develops directly into the inflorescence which is carried up on a long smooth unbranched stem through the centre of the pseudostem emerging at the top in the centre of the leaf cluster. The inflorescence is a compound spike of female and male flowers arranged in groups. Each group consists of 2 rows of flowers, one above the other, closely appressed to each other, and the whole collection is covered by a large subtending bract. The bracts and their axillary groups of flowers are arranged spirally round the axis and the bracts become closely overlapping each other forming a tight conical inflorescence at the tip. The lower bracts of the axis enclose female flowers; the middle few bracts enclose neuter flowers (absent in some cultivars) whilst at the tip of the inflorescence male flowers occur (Purseglove, 1972). In a few cases, (*M. schizocarpa*, *M. acuminatassp*,sspand *M. acuminata*.) hermaphrodite flowers are produced (Sharrock*et al.*, 2001). The

female inflorescences develop into fingers that constitute the bunch. Banana bunches possess 4 to 12 hands (clusters), each with at least 10 fingers. In wild bananas both male and female flowers produce abundant nectar and pollen whereas in cultivated bananas, many clones lack pollen. Banana pollen is tiny and sticky, being coated with waxes and proteins held in place by sculpture elements. The quantity of pollen is an important factor to enhance the germination potential of pollen grains (Dumpe and Ortiz, 1996). The female flowers have ovaries that develop first by parthenocarpy (without fertilization) to form pulp which is the edible part of the crop. However, wild bananas exhibit cross pollination and ultimately fertilization to form seeds instead of pulp (non-parthenocarpic).

2.1.2 Basis of Modern classification

The modern method of classifying edible banana was devised by Simmonds and Shepherd (1955) and Ortiz, (1997). It originally came from two wild, seeded species, *Musa acuminataColla* (A genome) and *Musa balbisiana* (B genome) which are native to Southeast Asia. However a few cultvars may have originated from hybridization with *Musa schizocarpa* (S genome).

The major genomic groups were AA, AAA, AAAA, AB, AAB, BBB, ABBB, AAAB and AABB.

2.1.3 AB Group

This group comprises a small number of diploid hybrids of south Indian origin. The main cultivar is 'Ney Poovan' (India) which is widely distributed but unimportant commercially. Being a diploid the plants are slender and lacking in vigour although fruits are white-fleshed with pleasant, sweet acid flavour. It is highly resistant to *Fusarium* wilt disease and leaf spot.

2.1.3.1 AAB group

According to Robinson (1996) this group of triploid hybrids originated in India. Therefore a wide range of clones and somatic mutations occured here. The AAB plantains generally have starchy flesh and at maturity they are usually unpalatable unless boiled. Other AAB cultivars have sweet fruit and are used as dessert bananas which include 'Mysore', 'Silk', 'Pisang Raja'. 'Palayankodan', 'Poovan' and 'Maia Maoli'.

2.1.3.1.2 Palayankodan (AAB)/ Mysore Poovan/ Chamba

A popular cultivar grown all over the country in perennial cropping system, it is a leading commercial cultivar of southern and north-eastern states. It is cultivated for both fruit and vegetable purpose. The variety has been adjudged as the most suitable cultivar of banana for intercropping in coconut gardens. The fruit is medium to small, yellow skinned, firm fleshed with a sub-acid taste. It has a good keeping quality. The average bunch weight is 20-24 kg. Crop duration is 16-17 months and the variety is also known as Mysore Poovan, and highly resistant to *Fusarium* wilt (Uma and Sathimoorty, 2002)

2.1.3.1.3 Poovan(AB)

This is the choicest table banana for its tasty, crisp, good sour-sweet blended and pleasant flavoured fruit. Crop duration 13-15 months with bunches weighing15-18 kg each, stout fruits, turning golden-yellow on ripening, and it is highly susceptibility to *Fusarium* wilt.

2.1.3.1.3.1 Rasthali (AAB)/ Poovan

Its unique fruit quality has made Rasthali popular and a highly prized cultivar for table purpose. Crop duration 13-15 months with bunches weighing 15-18 kg each. Fruits are yellowish green throughout their development, but turn pale yellow to

golden yellow after ripening. Fruit is very tasty with a good aroma. Longer crop duration, severe susceptibility to *Fusarium* wilt, requirement of bunch cover to protect fruits from sun cracking and formation of hard lumps in fruits make crop production more expensive.

2.2 *Fusarium* wilt disease in banana

Symptoms

Fusarium wilt of banana can be readily diagnosed by the appearance of a number of typical symptoms. These include typical vascular disease causing disruption of translocation and systemic foliage symptoms in bananas, which eventually lead to collapse of the crown and pseudostem (Jeger*et al.*, 1995). Symptoms generally commence as premature yellowing of the oldest leaves which develops as a band along the margin and spreads towards the midrib. The leaves hang between the pseudostem and the middle of the lamina while still green. The leaves wilt, the petiole buckles and spreads towards the midrib. All the leaves eventually collapse where the petiole joins the pseudostem and die (Rawal, 1996). As the disease advances, more of the leaves become yellow and die. A skirt of dead leaves often surrounds the pseudo-stem. In advanced stages of disease, affected plants may have a spiky appearance due to prominent upright apical leaves in contrast to the skirt of dead lower leaves (Stover, 1962b).

Internally, symptoms first become obvious in the xylem (water conducting) vessels of the roots and the rhizome. These turn a reddish-brown to maroon colour as the fungus grows through the tissues. Occasionally, the discolouration first appears yellow in plants showing early stages of infection. When a cross-section is cut, the discolouration appears in a circular pattern around the centre of the rhizome where the infection concentrates due to the arrangement of the vessels. As symptoms progress into the pseudo-stem, continuous lines of discolouration are evident when the plant is cut longitudinally. The infection may travel all the way up to the top of

the pseudo-stem. In severe cases it may even enter the leaf petioles and the peduncle (bunch stalk) of bunched plants. However, infection has not been shown to progress into the fruit(Ploetz and Pegg, 2000).

Causal agent

Taxonomic Tree

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Subclass: Hypocreomycetidae

Order: Hypocreales

Family: Nectriaceae

Genus: *Fusarium*

Species: *Fusariumoxysporum*f.sp. *cubense*

Foc is essentially confined to the xylem elements. *Fusariumoxysporum*.f. sp. *cubense*. It is one of more than 100 formaespeciales (special forms) of *F. oxysporum* that cause vascular wilts of flowering plants. It contains pathogenic and saprophytic strains that cannot be distinguished morphologically. Colonies grow 4 - 7 mm/day on PDA at 24°C, with slight to significant aerial mycelium, and white to purple pigmentation. Sporodochia are tan to orange, and sclerotia are blue and submerged. Micro- and macroconidia are produced on branched and unbranchedmonophialides. Microconidia are 5 - 16 x 2.4 - 3.5 µm, one- or two-celled, oval- to kidney-shaped, and are borne in false heads. Macroconidia are 27 - 55 x 3.3 - 5.5 µm, four- to eight-celled and sickle-shaped with foot-shaped basal cells. Terminal and intercalary chlamydospores are 7 - 11 µm in diameter, usually globose

and are formed singly or in pairs in hyphae or conidia. Atypically for the species, chlamydosporesare not produced by isolates of *F. oxysporum* f. sp. *cubense*in vegetative compatibility group (VCG). Four races of *F. oxysporum* f. sp.*cubense* have been described, only three of which affect banana (race 3 is a pathogen of heliconia). Race 1 caused the epidemics on Gros Michel and also affects the cultivars 'Maqueño', 'Silk', 'Pome', 'PisangAwak' and the hybrid 'I.C.2'. Race 2 affects cooking bananas, such as 'Bluggoe' and some breed tetraploids. Race 4 is most destructive since it affects race 1 and race 2 susceptible clones as well as the Cavendish cultivars (Randy andPloetz, 2005).

2.2.1 Disease Cycle and Epidemiology

F. oxysporum is an abundant and active saprophyte in soil and organic matter, with some specific forms that are plant pathogenic (Smith *et al.*, 1988). Its saprophytic ability enables it to survive in the soil between crop cycles in infected plant debris. The fungus can survive either as mycelium, or as any of its three different spore types (Agrios, 1988).

Healthy plants can become infected by *F. oxysporum* if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the plant's roots. The roots can be infected directly through the root tips, through wounds in the roots, or at the formation point of lateral roots (Agrios, 1988). Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can

also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem pits (Agrios, 1988).

Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves' stomata to close, the leaves wilt, and the plant eventually dies. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly (Agrios, 1988). The resulting spores can then be used as new inoculum for further spread of the fungus.

F. oxysporum is primarily spread over short distances by irrigation water and contaminated farm equipment. The fungus can also be spread over long distances either in infected transplants or in soil. Although the fungus can sometimes infect the fruit and contaminate its seed, the spread of the fungus by way of the seed is very rare (Agrios, 1988). It is also possible that the spores are spread by wind.

2.3 Crop loss due to *Fusarium* wilt

The first description of *Fusarium* wilt of banana and plantain was by Bancroft (1876) in Australia, who was unaware that was dealing with a disease today widely recognized as one of the most destructive in the history of world agriculture. *Fusarium* wilt was responsible for the decimation of the export trades in Central and South America and the Caribbean that were based on the cultivar 'Gros Michel' (AAA genome) which is susceptible to race 1 of the fungus. It has been estimated that about 40,000 ha became unproductive over a period of 50 years (Stover, 1972). The industry was saved by changing to cultivars in the Cavendish subgroup (AAA genome), which were resistant to race 1. However, Cavendish cultivars in plantations in subtropical Australia, the Canary Islands, South Africa and Taiwan were subsequently affected by *F. oxysporum* f.sp. *cubense* subtropical race 4. More recently, Cavendish cultivars planted on a large scale in South-East Asia are succumbing to *F. oxysporum* f.sp. *cubense* 'tropical race 4' (Ploetz and Pegg, 2000).

Since its appearance, tropical race 4 has caused severe damage to Cavendish cultivars in Malaysia, Indonesia, South China, the Philippines and the Northern Territory of Australia (Ploetz, 2006; Molina *et al.*, 2008; Buddenhagen, 2009). Garcia-Bastidas*et al.*, 2014) have recently reported tropical race 4 in Cavendish bananas in Jordan, with 80% of the Jordan Valley production area affected by *Fusarium* wilt, and 20-80% of the plants affected in different farms.

Cultivars that are favoured by smallholders are also affected by *Fusarium* wilt. 'Silk' (AAB genome) is a very popular dessert banana in Brazil, India, Indonesia, Malaysia, Philippines, but it is extremely susceptible to the disease and is almost impossible to grow in some areas. Cultivars in the Pome (AAB genome) and Bluggoe (ABB genome) subgroups, and 'PisangAwak' (ABB genome) are also attacked and losses are significant. *Fusarium* wilt is a major factor limiting the production of many cultivars.

Because conventional banana planting material can carry *F. oxysporum* f.sp. *cubense* and other pathogens, the local and international movement of banana suckers and corm pieces is viewed as undesirable. This has led to the development of alternative strategies, such as micropropagation techniques for producing clean planting material.

Fusarium wilt continues to pose threat among commercial Indian cultivars. Twenty-two percent of the total banana production remains at the mercy of this pathogen which includes popular cultivars like Silk (AAB), Ney Poovan (AB), Red Banana (AAA), Pome (AAB), PisangAwak (ABB) and all cooking bananas. All these years, race 1 and 2 were reported to attack the above cultivars while recently Poovan (Mysore, AAB) has lost its immunity and sporadic reports of *Fusarium* wilt on immune Mysore group is also being received.

2.4 Response of plants to disease conditions

The plant employs several layers of defense against microbial attacks. These defense include both general and pathogen specific mechanisms, which have evolved over years. These mechanisms make plant disease the exception rather than the rule. Disease is any physiological abnormality or significant disruption in the 'normal' health of a plant. Disease can be caused by living (biotic) agents, including fungi and bacteria, or by environmental (abiotic) factors such as nutrient deficiency, drought, and lack of oxygen, excessive temperature, ultraviolet radiation, or pollution. Plants are exposed to a large number of microorganisms. In order to protect themselves from damage, plants have developed a wide variety of constitutive and inducible defenses. Constitutive (continuous) defenses include many preformed barriers such as cell walls, waxy epidermal cuticles, and bark. These substances not only protect the plant from invasion, they also give the plant strength and rigidity. In addition to preformed barriers, virtually all living plant cells have the ability to detect invading pathogens and respond with inducible defenses including the production of toxic chemicals, pathogen-degrading enzymes, and deliberate cell suicide. Plants often wait until pathogens are detected before producing toxic chemicals or defense-related proteins because of the high energy costs and nutrient requirements associated with their production and maintenance (Freeman and Beattie, 2008). Most of the microorganisms are not able to invade them because of basal resistance and induced resistance, which includes elicitor induced production of phytoalexins and pathogenesis-related (PR) proteins. Plants can activate a very effective arsenal of inducible defense responses, comprised of genetically programmed suicide of infected cells (the hypersensitive response, HR), as well as tissue reinforcement and antibiotic production at the site of infection (Hammond-Kosak and Johns, 1996). Plants have developed multiple layers of sophisticated surveillance mechanisms that recognize potentially dangerous pathogens and rapidly respond before those organisms have a chance to cause serious damage. These surveillance systems are

linked to specific pre-programmed defense responses. Basal resistance, also called innate immunity, is the first line of pre-formed and inducible defenses that protect plants against entire groups of pathogens. Basal resistance can be triggered when plant cells recognize microbe-associated molecular patterns (MAMPs) including specific proteins, lipopolysaccharides and cell wall components commonly found in microbes. The result is that living plant cells become fortified against attack. Non-pathogens as well as pathogens are capable of triggering basal resistance in plants due to the widespread presence of these molecular components in their cells(Freeman and Beattie, 2008).

Pathogens have developed countermeasures that are able to suppress basal resistance in certain plant species. If a pathogen is capable of suppressing basal defense, plants may respond with another line of defense: thehypersensitive response (HR). The HR is characterized by deliberate plant cell suicide at the site of infection. Although drastic compared to basal resistance, the HR may limit pathogen access to water and nutrients by sacrificing a few cells in order to save the rest of the plant. The HR is typically more pathogen-specific than basal resistance and is often triggered when gene products in the plant cell recognize the presence of specific disease-causing effector molecules introduced into the host by the pathogen. Bacteria, fungi, viruses, and microscopic worms called nematodes are capable of inducing the HR in plants. Once the hypersensitive response has been triggered, plant tissues may become highly resistant to a broad range of pathogens for an extended period of time. This phenomenon is called systemic acquired resistance (SAR) and represents a heightened state of readiness in which plant resources are mobilized in case of further attack. Researchers have learned to artificially trigger SAR by spraying plants with chemicals called plant activators. These substances are gaining favor in the agricultural community because they are much less toxic to humans and wildlife than fungicides or antibiotics, and their protective effects can last much longer (Freeman and Beattie, 2008). This multicomponent response requires a substantial commitment

of cellular resources, including genetic re-programming and metabolic re-allocation (Somssich and Hablbrod, 1998). Thus, defenses are kept under tight genetic control and are activated only if the plant detects a prospective invader. Plants do not have the benefit of circulating antibody system so plant cell autonomously maintain constant vigilance against pathogens by expressing large arrays of 'R genes' (Resistance genes) (Dangl and Jones, 2001; Holub, 2001; Jones, 2001).R genes encode putative receptors that respond to the products of '*Avr* genes' (*Avr*, avirulence) expressed by the pathogen during infection. The encoded R proteins usually contain a nucleotide-binding site (NB) and a leucine-rich repeat (LRR) domain. They are further classified into those that contain an N-terminal coiled coil (CC) motif or a Toll interleukin receptor (TIR) domain. Such R genes, when transferred into a susceptible plant of the same or closely related species, usually impart full resistance capability. For this reason, R genes have been used in conventional resistance breeding programs for decades (Pink, 2002). However, in many cases, pathogens eventually overcome resistance, resulting in outbreaks of large epidemics. The plant cultivar that was once 'booming' but now 'busts', forcing breeders to introduce a cultivar with a new resistance trait. Repeated boom and bust cycles in agriculture have provided material for extensive studies on various plant-pathogen interactions (Johnson *et al.*, 2003). The strong phenotypes and natural variability at R loci have also been exploited by molecular geneticists to clone the R genes and investigate their molecular modes of action.

Resistance genes (R-Genes) are genes in plant genomes that convey plant disease resistance against pathogens by producing R proteins. The main class of R-genes consists of a nucleotide binding domain (NB) and a leucine rich repeat (LRR) domain(s) and is often referred to as (NB-LRR) R-genes. Generally, the NB domain binds either ATP/ADP or GTP/GDP. The LRR domain is often involved in protein-protein interactions as well as ligand binding. NB-LRR R-genes can be further

subdivided into toll interleukin 1 receptor (TIR-NB-LRR) and coiled-coil (CC-NB-LRR). R gene-mediated resistance has several attractive features for disease control.

Resistance can be conveyed through a number of mechanisms including:

The R protein interacts directly with an Avr gene (Avirulence gene) product of a pathogen

The R protein guards another protein that detects degradation by an Avr gene

The R protein may detect a Pathogen-Associated Molecular Pattern or PAMP (alternatively called MAMP for microbe-associated molecular pattern).

The R protein encodes enzyme that degrades a toxin produced by a pathogen.

Once the R protein has detected the presence of a pathogen, the plant can mount a defense against the pathogen. Because R genes confer resistance against specific pathogens, it is possible to transfer an R gene from one plant to another and make a plant resistant to a particular pathogen (Knepper*et al.*, 2010).When induced in timely manner, the concerted response can effectively halt the pathogen growth with minimal collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, R genes are often quickly defeated by co-evolving pathogens (Pink, 2002).

Tyloseocclusions of xylem vessels are considered as a defense mechanism against attack by *Fusarium* races in resistant banana cultivars that prevent the upward spread of the fungus. Indole-acetic-acid is one positive host factor in Beckman time-space model of host-parasite interactions (Beckman, 1987; 1990; 2000).

2.4.1 Physiological, morphological and biochemical responses

A wide range of physiological changes are known to occur in response to pathogen attack. Pathogen recognition at the site of infection initiates cellular and systemic signaling processes that activatemulticomponent defense responses at local and systemic levels, and these responses result in rapid establishment of local

resistance and delayed development of systemic acquired resistance. In a generalizing view that ignores species- and interaction-specific features, theearliest components of the cellular response include directed movements of organelles and the nucleus towards the site of pathogen attack, extracellular generation of reactive oxygen species (ROS), formationof cell wall appositions mostly consisting of callose atthe site of attempted penetration, often followedby cellular collapse which is one type of programmedcell death called the hypersensitive response (HR).These processes are frequently accompanied by releaseof phenolics from disintegrating cellular compartments,which upon contact with cytosolic enzymes are chemicallymodified or polymerized. Accumulation of defensegene transcripts follows these initial events sometimesin the attacked cells, but mostly in surrounding tissue. These genes encode pathogenesis-related proteins,such as glucanases, chitinases, defensins etc., and enzymesinvolved in the biosynthesis of phytoalexins and other,often phenylpropanoid- or fatty acid-derived secondarymetabolites (Cohn *et al.*, 2001) Some of these products act directly as defensefactors, for example some pathogenesis-related proteinsand phytoalexins, whereas others apparently representsignaling elements, such as jasmonate and salicylate, someof which participate in the induction of systemic acquiredresistance. This latter type of broad resistance isaccompanied by systemic development of microlesionsand gene activation (DierkScheel, 1998). If cell wall is breached the battle will continue inside the plant, whereby an arsenal of specific and broad-spectrum antimicrobial compounds are deployed to combact the attackers. Such compounds are known as phytoalexins, the production of phytoalexins which is elicited in response to several pathogens including pathogenic fungi (Thommaet *al.*, 1999).

2.4.1.1 Hypersensitive response

Fungal infection of a host resistant to a fungal plant pathogen leads to the 'hypersensitivereaction', a term coined by Stakman (1915). The hypersensitive response (HR) is a mechanism, used by plants, to prevent the spread

of infection by microbial pathogens. The HR is characterized by the rapid death of cells in the local region surrounding an infection. The HR serves to restrict the growth and spread of pathogens to other parts of the plant. The HR is analogous to the innate immune system found in animals, and commonly precedes a slower systemic (whole plant) response, which ultimately leads to systemic acquired resistance (SAR) (Matthews and Ben, 2007). The HR is triggered by the plant when it recognizes a pathogen. The identification of a pathogen typically occurs when avirulence geneproducts, secreted by a pathogen, bind to, or indirectly interact with the product of a plant resistance (R) gene (gene for gene model). R genes are highly polymorphic, and many plants produce several types of R gene products, enabling them to recognize virulence products produced by many different pathogens.

In phase one of the HR, the activation of R genes triggers an ion flux, involving an efflux of hydroxide and potassium outside the cells, and an influx of calcium and hydrogen ionsinto the cell.In phase two, the cells involved in the HR generate an oxidative burst by producing reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals andnitrous oxide. These compounds affect cellular membrane function, in part by inducing lipid peroxidation and causing lipid damage.

The alteration of ion components in the cell, and the breakdown of cellular components in the presence of ROS, results in the death of affected cells and the formation of locallesions. Reactive oxygen species also trigger the deposition of lignin and callose, as well as the cross-linking of pre-formed hydroxyproline-rich glycoproteins such as P33 to the wall matrix via the tyrosine in the PPPPY motif. These compounds serve to reinforce the walls of cells surrounding the infection, creating a barrier and inhibiting the spread of the infection (Heath,2000a). There is visible necrosis of the host tissue, and the necrotic region, which arises as a result of very complex events in the host, constitutes a defensive barrier. While

thisbarrier prevents the spread of biotrophic fungi (Lamb and Dixon, 1997; Lu and Higgins, 1999), it does not prevent the subsequent spread of necrotrophic fungi. However, reactive oxygen species alone may not always be sufficient to cause programmed cell death in the HR response, and additional factors such as iron availability or production of nitric oxide may be required (Mayer *et al.*, 2001). Hypersensitive response is one of the most powerful weapons in a plant's arsenal against pathogen attack (Cohn *et al.*, 2001).

2.4.1.2 Salicylic acid and Systemic Acquired Resistance

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. After the formation of a necrotic lesion, either as a part of the hypersensitive response (HR) or as a symptom of disease, the SAR pathway is activated. SAR activation results in the development of a broad-spectrum, systemic resistance (Hunt and Ryals, 1996; Neuenschwander*et al.*, 1996). Although SAR is interesting as a paradigm for signal transduction, it may have practical value as well. An understanding of the biochemical changes leading to the resistance state could enable the development of either genetically engineered plants with enhanced disease resistance or novel mode-of-action plant protection chemicals that act by stimulating the plant's inherent disease resistance mechanisms. SAR can be distinguished from other disease resistance responses by both the spectrum of pathogen protection and the associated changes in gene expression.

Activation of the HR triggers a systemic resistance response known as systemic acquired resistance (SAR). SAR is one component of plants' integrated disease-resistance repertoire. SAR appears to be distinct from preexisting resistance mechanisms such as physical barriers or protein cross-linking and also from other inducible resistance mechanisms such as phytoalexinbiosynthesis, the hypersensitive response, and ethylene-induced physiological changes. Furthermore, SAR is not

related toresponses induced by wounding or osmotic stress(John Ryals, Scott Uknes and Eric Ward, 1994).

This response includes the accumulation of the signal molecule salicylic acid (SA) throughout the plant and the consequent expression of characteristic set of defense genes called pathogenesis related (PR) proteins (Glazebrook, 2001). A lot of evidence suggests that SA plays a key role in both SAR signaling and disease resistance. Initially, the level of SA was found to increase by several hundred-fold in tobacco or cucumber after pathogen infection, and this increase was shown to correlate with SAR (Malamy*et al.*, 1990; Métraux*et al.*, 1990; Rasmussen *et al.*, 1991). Since these reports, a considerable amount of data has established a correlation between the concentration of SA and the establishment of enhanced disease resistance not only in tobacco and cucumber but in other plants as well (Malamy*et al.*, 1990; Métraux*et al.*, 1990; Rasmussen *et al.*, 1991; Dempsey *et al.*, 1993; Uknes*et al.*, 1993; Yalpani*et al.*, 1993; Cameron *et al.*, 1994). These data, coupled with the finding that exogenous SA can induce SAR (White, 1979; Ward *et al.*, 1991; Vernooij*et al.*, 1995) and SAR gene expression (Ward *et al.*, 1991; Uknes*et al.*, 1992), led to the suggestion that SA was involved in SAR signaling.

The SAR signal transduction pathway appears to function as a potentiator or modulator of other disease resistance mechanisms. When SAR is activated, a normally compatible plant-pathogen interaction (i.e., one in which disease is the normal outcome) can be converted into an incompatible one (Uknes*et al.*, 1992; Mauch-Mani and Slusarenko, 1996). Conversely, when the SAR pathway is incapacitated, a normally incompatible interaction becomes compatible (Delaney *et al.*, 1994; Mauch-Mani and Slusarenko, 1996). The mechanism by which this modulation occurs is not understood; however, at least part of the resistance response could be due to expression of the SAR genes. Compelling evidence supporting this idea comes from the analysis of transgenic plants expressing the bacterial nahG gene encoding salicylate hydroxylase, an enzyme that catalyzes the conversion of SA to

catechol. These plants are not only unable to accumulate free SA, but they are incapable of mounting a SAR response to viral, fungal, or bacterial pathogens (Gaffney *et al.*, 1993; Bi *et al.*, 1995; Friedrich *et al.*, 1995; Lawton *et al.*, 1995), indicating that SA accumulation is required for SAR induction. SAR inducible defense responses found in most plant species and it can be activated by exogenous application of salicylic acid or its analogues like 2, 6-dichloroisonicotinic acid (INA) and benzo thiodiazole-7-carbothioc acid S-methyl ester (BTH) (Gorlach*et al.*, 1996 Qui *et al.*, 2004). Salicylic acid deficient transgenic plants expressing salicylate dehydrogenase (nahG gene) prevents the establishment of SAR (Gaffney *et al.*, 1993).

2.4.1.3 Defense signaling molecules

Plant defense in response to microbial attack is regulated through a complex network of signaling pathways that involve three signaling molecules: salicylic acid (SA), jasmonic acid (JA) and ethylene. The SA and JA signaling pathways are mutually antagonistic. This regulatory cross talk may have evolved to allow plants to fine-tune the induction of their defenses in response to different plant pathogens (Barbara and Dangl, 2002).

These signaling molecules are involved in what appear to be two major pathogen defense signaling pathways: an SA-dependent pathway and an SA-independent pathway that involves JA and ET. These pathways do not function independently, but rather influence each other through a complex network of regulatory interactions. A greater understanding of the SA, JA and ET signaling pathways and of how they modulate each other should provide insight into the mechanisms underlying the activation and regulation of defense responses. This may also provide insight into strategies that are used by plant pathogens to alter (e.g. evade or suppress) host defense responses, and thus to promote pathogen virulence and disease production (Barbara and Dangl, 2002).

2.4.1.3.1 Salicylic-acid-mediated defenses

SA has long been known to play a central role in plant defense against pathogens. SA levels increase in plant tissue following pathogen infection, and exogenous application of SA results in enhanced resistance to a broad range of pathogens. Genetic studies have shown that SA is required for the rapid activation of defense responses that are mediated by several resistance genes, for the induction of local defenses that contain the growth of virulent pathogens, and for the establishment of systemic acquired resistance (SAR). SAR is a state of heightened defense that is activated throughout the plant following primary infection by pathogens that elicit tissue damage at the site of infection. Several pathogenesis-related (PR) genes whose expression is dependent on SA are commonly used as reporters of SA-dependent defenses (Barbara and Dangl, 2002).The role of SA in plant immunity is supported by the fact that exogenous SA, or high-level endogenous SA accumulation by expression of bacterial SA synthases, induce SAR-like resistance and PR gene expression (Verberne*et al.*, 2000).Salicylic acid activates PR genes through WRKY and TGA transcription factors which bind to *cis* elements in their promoters (Dong, 2004).Effective against biotrophic and hemi-biotrophic pathogens; Existence of a dichotomous branching at an early step specified by either EDS1 or NDR1; A role of SA-dependent amplification feedback in R-mediated and basal resistance; NPR1, a central regulator of defense signaling downstream SA; Hallmark of SA-signaling activation: PR1/2/5 (Shunyuan Xiao, 2007).

2.4.1.3.1.1 Jasmonic-acid-dependent defenses

JA, a fatty-acid-derived signaling molecule, is involved in several aspects of plant biology including pollen and seed development, and defense against wounding, ozone, insect pests and microbial pathogens. JA-dependent genes that encode pathogenesis-related proteins, including Plant defensin1.2 (PDF1.2), Thionin2.1

(THI2.1), Hevein-like protein (HEL) and Chitinaseb (CHIB), are commonly used to monitor JA-dependent defense responses.

2.4.1.3.1.2 JA / ET dependent responses

The role of ET in plant defense is somewhat controversial as it contributes to resistance in some interactions but promotes disease production in others. For example, the ethylene insensitive2 (ein2) mutant of *A. thaliana* exhibits increased susceptibility to *B. cinerea*and *E. carotovora*, but decreased symptoms when infected with virulent isolates of *P. syringae* or *Xanthomonascampestrispv. campestris*. Similar divergent effects of ethylene insensitivity on disease development have also been observed in soybeans.

It is reported that salicylic acid signaling is important for resistance against biotrophic pathogens, whereas JA/ET signaling is important against necrotrophic pathogens and these signaling pathways interact with each other in complicated manner (Glazebrook, 2005; Kliebenstein and Rowe, 2008).

2.4.1.3.1.3 Cross-talk between SA and other pathways

SA vs JA/ET: Overall antagonistic relationship could be synergistic at lower concentrations or early stages of activation. Likely modulators: MPK4, BIK1 (↓SA-pathway, JA-pathway↑), WRKY70 (↑ SA-pathway, JA-pathway↓) (Shunyuan Xiao, 2007).

2.4.1.3.1.3.1 Pathogenesis-related proteins

Pathogenesis-related proteins were first observed as new protein components induced in hypersensitively reacting tobacco (Van Loon and Van Kammen, 1970). The defense strategy of plants against stress factors involves a multitude of tools, including various types of stress proteins with putative protective functions. A groupof plant-coded proteins induced by different stress stimuli, named

'pathogenesisrelated proteins' (PRs) is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment (AglikaEdreva, 2005). Since their discovery in tobacco leaves hypersensitively reacting to TMV by two independently working groups (Van Loon and Van Kammen, 1970; Gianinazzi*et al.*, 1970), pathogenesis-related proteins (initially named 'b' proteins) have used an increasing research interest in view of their possible involvement in plant resistance to pathogens. Pathogenesis-related (PR) proteins are proteins produced in plants in the event of a pathogen attack. They are induced as part of systemic acquired resistance. Infections activate genes that produce PR proteins. Some of these proteins are antimicrobial, attacking molecules in the cell wall of a bacterium or fungus. Others may function as signals that spread 'news' of the infection to nearby cells. Infections also stimulate the cross-linking of molecules in the cell wall and the deposition of lignin, responses that set up a local barricade that slows spread of the pathogen to other parts of the plant (Loon, 1985).

An important common function of most PRs is their antifungal effects – as recently shown – antiviral action. Toxicity of PRs can be generally accounted for by their hydrolytic, proteinase- inhibitory and membrane-permeabilizing ability. Thus, hydrolytic enzymes (β-1, 3-glucanases, chitinases and proteinases) can be a tool in weakening and decomposing of fungal cell walls, containing glucans, chitin and proteins, while PR-8 can disrupt gram-positive bacteria due to lysozyme activity (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001), some PRs also exhibit antibacterial, insecticidal or antiviral action, function as signals that spread 'news' of the infection to nearby cells, Infections also stimulate the cross-linking of molecules in the cell wall and the deposition of lignin, responses that set up a local barricade that slows spread of the pathogen to other parts of the plant.Chitinase activity, Peroxidase, ribonuclease and lysozyme activities, Their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability, they

inactivate the proteins secreted by the parasites in the invaded plant tissues, (Loon, 1985).

Originally, five main groups of PRs (PR-1 to PR-5) were characterized by both molecular and molecular-genetic techniques in tobacco, numbered in order of decreasing electrophoretic mobility. Each group consists of several members with similar properties (Bol*et al.*, 1990). Group PR-1 is the most abundant, reaching up to 1-2 % of total leaf proteins. PRs of group 5 share significant amino acid sequence homology with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcusdaniellii*, and have been named thaumatin-like (TL) proteins (Cornelissen*et al.*, 1986). PRs are distinguished by specific biochemical properties. They are low-molecularproteins (6-43 kDa), extractable and stable at low pH (< 3), thermostable, and highlyresistant to proteases (Van Loon, 1999). PRs in plants are coded by a small multigene family. Since their discovery, regulation of PRs has been a highly active research area. Putative plasma membranelocalized receptors of PRs inducers are suggested, and secondary signals of PRs induction, such as salicylic acid (SA), jasmonic acid and ethylene, are established. Many of these secondary signals are well-known inducers of PRs expression (Durner*et al.*, 1997; Surplus *et al.*, 1998; Anderson *et al.*, 1998; Zhou, 1999; Cameron *et al.*, 2000; Poupard*et al.*, 2003). Cross-talks are common between signaling pathways mediated by these secondary messengers. Thus, SA-independent/jasmonate dependent, and vice-versa pathways of PRs induction have been demonstrated (Mitsuhara*et al.*, 1998; Fidantsef*et al.*, 1999). It has been proven that PRs synthesis is regulated at transcriptional level; the exact mechanisms of transcriptional regulation have beenones of the most active fields of PR gene studies. Several *cis*- regulatory elements in PR-promotors mediating PR gene expression have been identified. These include Wbox, GCC box, G box, MRE-like sequence, SA-responsive element (SARE) (Zhou,1999). PR proteins are classified into 17 families (Sels*et al.*, 2008, Lio and Ekramoddoullah, 2006).

2.4.1.3.1.3.1.1 Gene for gene hypothesis

The gene-for-gene relationship was discovered by the late Harold Henry Flor who was working with rust (*Melampsoralini*) of flax (*Linumusitatissimum*). Flor was the first scientist to study the genetics of both the host and parasite and to integrate them into one genetic system. Gene-for-gene relationships are a widespread and very important aspect of plant disease resistance.Flor showed that the inheritance of both resistances in the host and parasite ability to cause disease is controlled by pairs of matching genes. One is a plant gene called the resistance (R) gene. The other is a parasite gene called the avirulence (*Avr*) gene. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding Avr gene product (Flor, 1942).

The gene for gene relationship between a host and its pathogen was postulated by Flor in 1951 on his work on linseed (*Linumusitatissimum*) rust caused by *Melampsoralini*. Subsequent studies have shown that the gene-for-gene relationship holds true in most of the cases studied extensively, and is now widely accepted. It has been found that for every resistant gene present in the host, the pathogen has a gene for virulence. Susceptible reaction would result only when the pathogen is able to match each of the resistance gene present in the host with appropriate virulence gene. If one or more resistance genes are no matched by the pathogen with the appropriate virulence gene, resistant reaction will result. In most of the pathogens virulence is recessive to avirulence (Sing, 2001).

2.4.1.3.1.3.1.2 Trench warfare hypothesis

According to Stahl *et al.*, 1999 'trench warfare' hypothesis states that the frequency of susceptible and resistant alleles cycles according to the population status of the pathogen and cost of organizing resistant alleles. Ancient resistance alleles are supposed to evolve slowly and go through cycles of advance and retreats, sometimes described as a 'birth-and death' process (Kamoun, 2001).

Trench warfare has been invoked, in which there are advances and retreats of resistance and avirulence allele frequencies to maintain dynamic and stable polymorphism. Furthermore co-evolutionary dynamics are influenced by the nature of the plant (whether it is annual or perennial) and the nature of the pathogen (for example how it is transmitted).

2.4.1.3.1.3.1.3 Ligand receptor model

The receptor-ligand hypothesis predicts that resistance proteins detect pathogen infection by directly interacting with avirulence proteins, triggering defense signaling (Jennifer, 2005). This enables recognition of the pathogen and subsequent elicitation of an array of plant defense responses that eventually lead to resistance (Keen, 1990). But a direct physical interaction between Avr and R proteins has only been shown for the *AvrPto-Pto* and *AvrPita-Pi-ta* pairs (Tang *et al.*, 1996; Scofield *et al.*, 1996 and Jia*et al.*, 2000).

2.4.1.3.1.3.1.4Guard model

Lack of evidence for direct *Avr*-R interactions stimulated scientists to propose new models for *Avr* perception by resistant plants. This led to the formulation of 'guard hypothesesby Van der Biezen and Jones (1998) which predicts that R protein confers recognition of *Avr* factors only when these Avr factors are complexed with their host virulence targets. R proteins activate resistance when they interact with another protein (a guardee) that is targeted and modified by the pathogen in its quest to create a favourable environment.

Guard: typical R proteins (NBS-LRR, RLP, RLK). Guardee: host targets of pathogen effector proteins R proteins (guard) monitor the change of activity or status of host (guardee) targeted by the pathogen effector (i.e.*Avr* in the presence of R). Examples of likely Guard-Guardee; *prf-pto*; RPM1-RIN4; RPS2-RIN4; RPS5-PBS1; *cf2-avr2*, (Shunyuan Xiao, 2007).

It involves recognition of distinct microbial structures- pathogen-associated molecular patterns (PAMPs) - with pattern recognition receptors (PRRs). Some of these stereotypical consequences of virulence factors and pathogens may include altered endosomal trafficking and changes in the cytoskeleton. These recognition mechanisms would work to complement classical pattern recognition mechanisms(Dodds *et al.*, 2014). Resistance is triggered when the R protein detects an attempt to attack its guardee, which might not necessarily involve direct interaction between R and Avr proteins (McDowell and Woffenden, 2003). R proteins can function either by directly detecting the corresponding Avr protein (the receptor-ligand model) or by perceiving alterations in plant machine that are targets of Avr protein action in the promotion of pathogen virulence (the guardee hypothesis) (Dangl and McDowell, 2006).

2.4.1.3.1.3.1.4.1 Non-host resistance/Basal resistance

Non-host resistance is the resistance shown by an entire plant species to all genetic variants of a pathogen (Heath, 1997; Mysore and Ryu, 2004; Nürnberger andLipka, 2005). However,presuming that pathogenic microbes co-evolve with radiatingplant species during evolution (Heath, 1997; Inuma*et al.*, 2007),we may expect that some plant species are currently in theprocess of losing or acquiring the host status toa certain microbial species, taking some intermediate positionbetween host and non-host status, as reported for barley inresponse to*Puccinia* rust fungi (Atienza*et al.*, 2004). Basal resistance is the complement of the term 'basic compatibility', which results from the capacity of the microbe to effectively overcome the defense mechanisms that plant species mount against unadapted microbial intruders (Heath, 1997).

Plant exhibit non-specific broad range resistance triggered by certain conserved Microbe/pathogen-assisted Molecular Patterns (MAMPs/PAMPs) called non host resistance or basal resistance. Major identified MAMPs are flagellin, (a

highly conserved 22 amino acid peptide of bacterial flagella), elongation factor-Tu, fungal chitin, β-glucanases etc. (Schwessinger and Zipfel, 2008). Perception of pathogen associated molecular patterns (PAMPs) by pattern recognition receptor (PRRs) constitutes the first layer of plant innate immunity and is referred to as PAMP-triggered immunity (PTI) (Zipfel, 2008). PAMP detection is an important component of non-host resistance in plants and serves as an early warning system for the presence of potential pathogens. Binding of PAMP to the appropriate PRR leads to downstream signaling events and ultimately, to the induction of basal systems (Ingle *et al.*, 2006). NHR acts at special level; A major contribution to safety of plants; More evolutionary ancient; Believed to be multi-layered; Activated upon recognition of PAMPs (pathogen-associated molecular patterns) such as LPS (lipopolyscharrides) and Flagellin of bacteria, chitin and glucan of fungi etc (Shunyuan Xiao, 2007).Plants have acquired the ability to recognize the presence of some of these effector proteins, which leads to a quick and hypersensitive response (HR) to arrest and terminate pathogen growth (Postel and Kimmerling, 2009).

2.5 Gene network involved in disease resistance

Recent molecular research on R proteins and downstream signal transduction networks has provided exciting insights, which will enhance the use of R genes for disease control. Plants have evolved an efficient defense transduction network against pathogenic attack. Different resistance (R) gene mediated gene-for-gene resistance pathways, are well recognized branches of this network (Glazebrook, 2001; Hammond-Kosack and Parker, 2003). Some of the components functioning in the pathogen-induced defense network also play roles in other physiologic or developmental pathways. A few genes, which positively regulate both disease resistance and development, have been identified. For example, the Arabidopsis erecta, encoding a leucine-rich repeat receptor-like kinase, quantitatively enhances plant resistance to bacterial wilt and regulates development of all aerial organs (Godiard*et al.*, 2003). Definition of conserved structural motif in R proteins has

facilitated the cloning of useful R genes, including several that are functional in multiple crop species and/or provide resistance to a relatively wide range of pathogens. Numerous signal transduction components in the defense network have been defined, and several are being exploited as switches by which resistance can be activated against diverse pathogens (McDowell and Woffenden, 2003).

Plant disease resistance pathway consist of two major classes of genes: those involved in events associated with the pathogen effector recognition of, and interaction with, elicitor effector molecules from pathogens, and those evolved in defense response (Godiard*et al.*, 2003). Sequence analysis of the predicted proteins reveals that resistance genes of diverse origin and with different pathogen specificity share similar structural motifs, indicating that a common surveillance strategy has been adopted by plant species to detect invading pathogens.

Based on structures, cloned resistance genes can be grouped into five classes: The majority of R proteins that are activated upon effector recognition fall into five classes based primarily upon their combination of a limited number of structural motifs. Class 1 consists of just one member, *Pto* from tomato, which has a serine/threonine kinase catalytic region and a myristylation motif at its N terminus. Class 2 comprises a large number of proteins having a region of leucinerich repeats (LRRs), a putative nucleotide binding site (NBS), and an N-terminal putative leucine-zipper (LZ) or other coiled-coil (CC) sequence. Class 3 is similar to class 2 but instead of the CCsequence these proteins have a region with similarity to the N terminus of the Toll and Interleukin 1 receptor (IL-1R) proteins that is therefore referred to as the TIR region (Gregory *et al.*, 2003). The successful cloning of plant disease resistance genes provides the starting point for dissecting the resistance pathways in plants. Both genetic and biochemical approaches have been used to identify and clone genes involved in the signal transduction pathways and defense responses. Genetic dissection of resistance pathways using mutant analysis has led to the identification of several important genes (Innes, 1998). For instance two novel

genes, NDR1 and EDS1 have been identified in *Arabidopsis*, which encode signal transduction components that are activated by the products of multiple resistance genes (Century *et al.*, 1997; Falk *et al.*, 1999).

In the defense system of plants, direct and indirect interactions are two alternative mechanisms to explain the gene-for-gene model. In the direct interaction, pathogen Avr effectors associate directly with plant R-genes to trigger signaling. For example, rice R-gene *Pi-ta* was shown to directly interact with *Avr-Pita* from *Magnaporthegrisea*. Likewise, a direct interaction was observed between L genes (a group of resistant genes to flax rust) and their corresponding rust *Avr* genes in flax (Manoj, 2015).

2.5.1 Resistance genes against *Fusariumoxysporum f. sp. Cubense* in banana

Natural sources of resistance againstPanama disease have been reported in wild bananas (Ploetz and Pegg, 2000), the introgression of this resistance into edible cultivars by conventional breeding has been hampered by problems associated with triploidy and low fertility (Roux *et al.*, 2004). The development of a reliable and efficient genetic transformation system for banana (Becker *et al.*, 2000; Khanna*et al.*, 2004) provides the opportunity for the generation of disease-resistant bananas using a molecular breeding approach. To date, however, no R gene/s capable of conferring resistance to race4 has been reported. One potential source of *Fusarium* R genes is the wild diploid banana, *Musa acuminata* ssp. *malaccensis*, which shows resistance to race 4 (Ploetz and Pegg, 2000). The majority of plant R genes encode proteins with cytoplasmic nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains that confer resistance to a wide variety of pathogens and pests including viruses, bacteria, fungi, nematodes and insects (Dangl and Jones, 2001). The NBS-LRR proteins are thought to recognize pathogens and respond by activating signal transduction pathways leading to disease resistance (Belkhadir*et al.*, 2004). The C-terminal LRR region has been considered the candidate pathogen recognition domain while the N-

terminal region, including the NBS, is thought to be involved in signaling (Belkhadir*et al.*, 2004). The LRR domain is the most variable region in closely relatedNBS-LRR proteins and is under diversifying selection (Michelmore and Meyers, 1998; Richter and Ronald, 2000). NBS-LRR genes are abundant in plant genomes with 149 and 480 isolated from *Arabidopsis* and rice, respectively (Meyers *et al.*, 2003; Zhou *et al.*, 2004) mostly organized in clusters (Hulbert *et al.*, 2001). The NBS-LRR class of R genes is divided into two distinct subclasses based on the presence or absence of an N-terminal domain that shows similarity to the *Drosophila* Toll and the human Interleukin-1 receptor (TIR) (Meyers *et al.*, 1999; Pan *et al.*, 2000). Widely distributed in both monocotyledonous (monocot) and dicotyledonous (dicot) species, the non-TIR subclass commonly has an N-terminal region comprising a predicted coiled-coil structure, sometimes in the form of a leucine zipper (Meyers *et al.*, 1999; Pan *et al.*, 2000; Hulbert*et al.*, 2001). The TIR subclass, however, appears restricted to dicot species. Genes that confer resistance to *Fusarium* have been isolated from tomato (Simons *et al.*, 1998) and melon (Joobeur*et al.*, 2004) by mapping approaches. Both the tomato I2 and melon Fom-2 genes were shown to encode non-TIR-NBS-LRR type R genes suggesting that targeting this class of genes may facilitate the isolation of genes from wild banana species that confer resistance to *Fusarium*. The highly conserved motifs in the NBS domain have been targeted to isolate NBS-type sequences by PCRbased strategies using degenerate primers. Using this approach, resistance gene candidates (RGCs) have been isolated from soybean (Kanazin*et al.*, 1996; Yu *et al.*, 1996; Graham *et al.*, 2000), potato (Leister *et al.*, 1996), lettuce (Shen*et al.*, 1998), rice and barley (Leister *et al.*, 1998), wheat (Seah*et al.*, 2000), sunflower (Ayele-Gedil*et al.*, 2001), common bean (Rivkin*et al.*, 1999; Lopez *et al.*, 2003), strawberry (Martınez-Zamora *et al.*, 2004), apple (Calenge*et al.*, 2005) and recently from banana (Pei *et al.*, 2007). The PCR approach has also been useful in isolating resistance gene candidates of the *Pto*-type from banana (Peraza-Echeverria *et al.*, 2007).

2.6 Isolation of resistance gene

Over the past few years, many plant disease-resistance genes (R genes) and related genes have been sequenced. Most of the functionally defined R genes cloned to date encode products with similar amino acid sequences and structural domains irrespective of whether they confer resistance to viruses, bacteria, fungi, nematodes, or insects (Baker *et al.*, 1997; Bent, 1996; Ellis *et al.*, 2000; Hammond-Kosack and Jones, 1997). These sequences include nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. These domains seem to be components of signal transduction systems involved in plant resistance against pathogens (Michelmore and Meyers, 1998); so far, the only role demonstrated for NBS–LRR-encoding plant genes is either in disease or pest resistance (Michelmore, 2000). Therefore, the majority of plant resistance genes are members of large gene families that encode NBS and LRR motifs. Many are present in complex gene clusters (Ferrier-Cana *et al.*, 2003; Meyers *et al.*, 1998; Noël *et al.*, 1999). It is estimated that the*Arabidopsis* genome contains approximately 200 NBSencodinggenes (Meyers *et al.*, 1999). The list of known resistancegenes with NBS and LRR includes, among others,N from tobacco; RPS2, RPP5, and RPM1 from *Arabidopsis*;L6 and M from flax; Mi from tomato; and Xa1 from rice(Pan *et al.*, 2000). In several higher plant taxa, such as legumespecies, functional resistance genes of this type havenot yet been identified; although many sequences coding forinternal domains conserved in known resistance genes havebeen isolated from numerous plant species. These sequenceshave been named resistance gene analogues (RGAs) or resistancegene homologues (RGHs) (Cannon *et al.*, 2002). RGAshave been isolated from several legumes such as soybean, common bean, cowpea, pea, chickpea, and alfalfa and relatedspecies (Cannon *et al.*, 2002; Chida*et al.*, 2000;Cordero and Skinner, 2002; Creusot*et al.*, 1999; Ferrier-Cana*et al.*, 2003; Geffroy*et al.*, 1998; Graham *et al.*, 2000;Kanazin*et al.*, 1996; Rivkin*et al.*, 1999; Yu *et al.*, 1996; Zhu*et al.*, 2002), as well as from many non-leguminous species.

NBS plant resistance genes are subdivided into two families based on the presence or absence of an N-terminal region with homology to *Drosophila* Toll and the human interleukin-1 receptor (TIR region) (Meyers *et al.*, 1999; Pan *et al.*, 2000; Young, 2000), namely TIR and non-TIR genes. The non-TIR type commonly has a predicted leucine-zipper (LZ) domain or a putative coiled-coil (CC) domain at the Nterminal region (Pan *et al.*, 2000). The non-TIR family is widely distributed in both monocotyledonous and dicotyledonous species, whereas the TIR family appears to be restricted to dicotyledonous species, since TIR-class genes have not been detected in genomic or expressed sequences from any monocot species (Meyers *et al.*, 1999; Pan *et al.*, 2000; Cannon *et al.*, 2002). In Arabidopsis, approximately 150 sequences fall into the TIR group and 50 into the non-TIR group (Meyers *et al.*, 1999).

Most of the functionally known resistance genes have been cloned and identified using transposon tagging and map-based cloning technologies, but the use of these approaches for the identification of new resistance genes is still laborious and time-consuming. On the other hand, there are eight major conserved amino acid motifs in the NBS (Meyers *et al.*, 1999) that can be used to design degenerate primers to identify RGAs by means of PCR amplification and sequencing. This approach to identify RGAs has been used in a variety of plant species, such as soybean (Kanazin et al. 1996; Yu *et al.*, 1996), common bean (Ferrier-Cana *et al.*, 2003; Rivkin*et al.*, 1999), alfalfa (Cordero and Skinner, 2002; Zhu *et al.*, 2002), lettuce (Shen*et al.*, 1998), coffee (Noir *et al.*, 2001), and grape (Di Gaspero and Cipriani, 2002).

2.7 Future perspective

Banana (*Musa* spp.) is one of the most important fruit crops in the world in terms of production and consumption. *Fusarium* wilt is caused by soil-borne fungus *Fusariumoxysporum* (f. sp.) *cubense* (*Foc*) is regarded as one of the most devastating diseases of banana, affecting plantations in almost all banana growing countries of the world (Ploetz*et al.*, 1990). Options for the control of *Fusarium* wilt are limited by

ineffectual chemicalcontrol and the lack of commercially suitable resistant cultivars (Smith *et al.*, 2006).

Unfortunately, cultivated banana varieties are mostly triploid and can only be propagated asexually, making it difficult to improve this crop genetically using conventional plant breeding methods that rely heavily on cross-pollination between plants. As an alternative, the introduction of resistance genes into banana plants via biotechnological means offers a valuable way of developing resistant banana cultivars (Sagi, 2000).

Recent studies has shown (Jusna, 2013) that the *Foc* resistant cultivar 'Palayankodan' has 'R' genes of class NBS type and can serve as source for this type of genes for molecular breeding. Study will be useful for development of molecular markers for marker assisted selection and cloning of full length 'R' genes for *Fusarium* wilt resistance.

Development of gene construct for *Fusarium* wilt resistant gene. Development of disease resistant/tolerant transgenic banana cultivars through RGA mediated resistance. Development of molecular markers linked with *Fusarium* wilt resistance for marker assisted selection.

Materials and Methods

3. MATERIALS AND METHODS

The details regarding the experimental materials and methodology adopted for conducting various aspects of the present study 'Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.), are presented in this chapter. This study was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT-BT Complex, College of Horticulture, Vellanikkara during the period from 2013-2015. Materials used and methodologies adopted for the study are described in this chapter.

3.1 Materials

3.1.1 Plant materials

Banana (*Musa acuminata*) varietiesPalayankodan (Mysore Poovan AAB) reported as resistant to *Fusarium* wilt and Poovan(Rasthali AAB)assusceptible were used in the study and suckers were obtained from the Banana Research Station of Kerala Agricultural University located at Kannara.

3.1.2 Laboratory chemicals, glass wares and equipment items

All the chemicals used in the study were of good quality (AR/GR grade) obtained from various firms such as Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq*DNA polymerase, dNTPs, Taq buffer and primers used in this study were supplied by Bangalore Genei Ltd. or Sigma. All the plasticwares were obtained from Axygen and Tarson India Ltd. ISSR and RAPD primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd. pGEM-T Easy Vector System II from Promega. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). NanoDrop® ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Veriti (Applied Biosystem) and Agilent thermal cycler.Agarose gel electrophoresis was performed in horizontal gel electrophoresis unit (BIO-RAD, USA).

3.1.3 Bioinformatic software

Bioinformatics software used in this study was accessed from Distributed Information Centre (DIC), of KAU, attached to CPBMB, College of Horticulture.

3.2 Methods

3.2.1 Screening of resistant variety for *Fusarium* wilt incidence

The suckers were raised in polythene bag of 12' diameter holding 8 kg potting mixture which were then maintained in open condition at CPBMB. The suckers of Palayankodan and Poovan varieties were raised in polythene bag in the month of June- July. In each variety 4 plants were raised and were used for screening studies. Plants of 2 months old were artificially inoculated with the inoculum of *Fusariumoxysporum.*

3.2.2 Inoculation of plant genotypes with pathogen

Pure culture of *Fusariumoxysporum*(f. sp.)*cubense*, (*Foc*) has obtained from the Banana Research Station Kannara. It was inoculated on potato dextrose agar medium in sterilized Petri plates and incubated at room temperature, 26±2^{0}C, at the Department of Plant Pathology, College of Horticulture.

Inoculum was prepared by inoculating the sterilized potato dextrose broth with mycelia disc (size 1 cm) of 7 day old culture of the pathogen @ 1disc/50 ml broth. The inoculated flasks were placed on a rotary shake incubator with rotation speed of 160 rpm at 25^{0}C for 7 days. The concentration of inoculum was adjusted 10^6spores ml^{-1} by serial dilution. Plants (Palayankodan and Poovan varieties) were inoculated with this spore suspension by root feeding method. Roots were surface sterilized with 50 percent alcohol and washed with three changes of sterile water and slanting cut was made on the root tip portion and root was inserted to a small polythene cover containing 10 ml inoculums and kept for 24 hrs.

The inoculated plants were kept for 2 months under open condition for the disease development. Plants were observed for *Fusarium* wilt infection symptom development and scored as resistant or susceptible based on symptoms.

3.2.3 Molecular marker studies

DNA was isolated from both resistant and susceptible genotypes and same amplified with reported degenerate primers for *Fusarium* wilt resistant gene and were observed for polymorphism.

3.2.4 Genomic DNA extraction

Young tender, pale green leaves were collected in the morning hours on ice from each of the populations. The surface was cleaned by washing with sterile water and wiping with 70 per cent alcohol. The fresh leaves were ground into a fine powder in liquid nitrogen along with β -mercaptoethanol and PVP using sterile ice-cold mortar and pestle, in order to prevent browning due to phenol oxidase activity.

Among the most commonly used protocols, CTAB method developed by Doyle and Doyle (1990) was used for the extraction of genomic DNA. Details are as follows.

Reagents

I. CTAB buffer (2X):

- 2 per cent CTAB (w/v)
- 100mM Tris (pH 8.0)
- 20mM EDTA (pH 8.0)
- 1.4M NaCl
- 1 per cent PVP

II. 10 per cent CTAB solution:

- 10 per cent CTAB (w/v)
- 0.7M NaCl

III. TE buffer:

- 10mM Tris (pH8.0)
- 1mM EDTA

IV. Chloroform: Isoamyl alcohol (24:1 v/v)

V. Isopropanol (100%)

VI. Ethanol 70 per cent and 100 per cent

VII. Sterile distilled water

Procedure:

0.2 gram of clean leaf tissues was ground in presence of liquid nitrogen in pre-chilled mortar and pestle in 10µl of β-Mercaptoethanol(100%) and a pinch of Poly Vinyl Pyrrolidone (PVP).

The homogenized sample was transferred into an autoclaved 2ml centrifuge tube and 1ml of pre-warmed extraction buffer was added (total 1ml).

- The contents were mixed well and incubated at 65°C for 20 to 30 minutes with occasional mixing by gentle inversion.

- After the completion of incubation the sample was taken out and equal volume of (1ml) of chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversions to emulsify. Spun at 12,000 rpm for 15 minutes at 4°C

- After centrifugation, the contents got separated into three distinct phases.

- Aqueous topmost layer - DNA and RNA
- Interphase - Fine particles and proteins

- Lower layer - Chloroform, pigments

- The tubes were carefully taken out from the centrifuge without disturbing the different layers. Transferred the top aqueous layer to a clean centrifuge tube and added 1/10th volume of 10 per cent CTAB solution and equal volume of chloroform:Isoamyl alcohol (24:1) and gently mixed by inversions.
- The contents were centrifuged at 12,000 rpm for 15 minutes at 4°C.
- The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by quick gentle inversions till the DNA got precipitated. The tubes were kept at -20°C for half an hour for complete precipitation of DNA.
- After the expiry of time the tubes were taken out and centrifuged at 10,000 rpm for 05 minutes at 4°C. The pellet at the bottom of the centrifuged tube was observed and the supernatant was gently poured off.
- The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol. Spun for 5 min at 10,000 rpm and decanted the ethanol.
- The pellet was left for air drying for 10 to 15 minutes under room conditions with the open tube kept upside down.
- The pellet was dissolved in70µl of sterilized water and stored at -20°C.

3.2.4.1 Quality and quantity checking of extracted DNA

(I) Agarose gel electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis and spectrophotometer.

Reagents and equipments

1. Agarose - 0.8 per cent (for genomic DNA)
2. 50X TAE buffer (pH 8.0)

    - 10 mlTris buffer (1M)

    - 45 ml glacial Acetic acid

    - 2 ml M EDTA (0.5M)

3. Tracking/loading dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml): 5 µl/ 100 ml of agarose gel composition of reagents is provided in Annexure II

Procedure

- The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

- Prepared 0.8 per cent agarose (0.8 g in 100ml) was prepared in a glass beaker or conical flask with 100 ml 1X TAE buffer. Micro waved for 45 to 60 seconds until agarose was dissolved and solution was clear.

- Solution was allowed to cool to about 42 to 45°C and 4µl Ethidium bromide was added at this point and mixed well.

- This warm gel solution was poured into the gel tray to a depth of about 5 mm. and allowed to solidify for about 30 minutes at room temperature.

- The comb was subsequently removed and the tape used for sealing removed, the tray was placed in electrophoresis chamber, and covered (just until wells are submerged) with TAE buffer (the same buffer used to prepare the agarose).

- To prepare samples for electrophoresis, 1 µl of 6x gel Tracking/loading dye was added for every 5µl of DNA solution. Mixed well and loaded 6µl DNA sample per well.

- Electrophoresis was carried out at 70 volts until the dye has migrated two third the length of the gel. On completion of the electrophoresis, the gel was documented in a gel doc machine.

- Intact DNA appeared as orange fluorescent bands. The degraded was appeared as smear. The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.2.4.2 Gel documentation

Gel documentation was done with BioRad gel documentation system using PDQuest™ software. PDQuest is a software package for imaging, analyzing, and data basing the gels. An image of a gel was captured using the controls in the imaging device window and displayed on computer screen.

3.2.4.3Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was further checked using NanoDrop ND-1000 spectrophometer. Nucleic acids show absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance was recorded at both wavelengths and purity was indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicated that the DNA is pure and free from proteins. The quantity of DNA in the

pure sample was calculated using the relation OD_{260} equivalent to 50 ng double stranded DNA/ml sample.

OD at 260 nm = 50 ng DNA/ml

Therefore OD260 X 50 gives the quantity of DNA in ng/ml.

Procedure for quantity detection using Nanodrop

- NanoDrop spectrophotometer connected to the System and the software ND-1000 was opened.
- The option Nucleic acid was selected.
- With the sampling arm open, 1µl distilled water we pipette onto the lower measurement pedestal.
- The sampling arm was closed and a spectral measurement was started using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.
- The reading was set to zero with blank sample.
- 1µl of the sample was pipetted onto measurement pedestal and measure was selected.
- When the measurement was complete, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping was sufficient to sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.2.4..4Purification of DNA

The DNA which had RNA contamination was purified by RNase treatment, further precipitated, washed and air dried.

Reagents

- Phenol: chloroform mixture (24:1, v/v)
- Chilled Isopropanol (100 %)

- 70 per cent ethanol
- TE buffer
- Chloroform: Isoamyl alcohol (24:1, v/v)
- 1 per cent RNase

One per cent solution was prepared by dissolving RNase (Sigma, USA) in TE buffer at 100°C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20°C.

Procedure

- To 100 µl DNA sample, 1% RNase solution (2 µl) was added and incubated at 37°C in dry bath for 1 hour.
- The volume was made up to 250 µl with distilled water.
- Equal volume of chloroform: isoamyl alcohol (24: 1) mixture was added and mixed gently.
- Centrifuged at 12,000 rpm for 15 minutes at 4°C.
- The aqueous phase was transferred into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24: 1) was added
- Centrifuged at 12,000 rpm for 15 minutes at 4°C.
- The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by quick gentle inversions till the DNA gets precipitated. The tubes were Kept at -20°C for half an hour for complete precipitation.
- The mixture at -20°C for 30 minutes was centrifuged at 10,000 rpm for 15 minutes at 4°C.
- The DNA pellet was washed with 70 per cent ethanol.
- The pellet was, air dried and dissolved in 50 to 100 µl sterilized water.

- The samples was loaded on 0.8 per cent agarose gel at constant voltage of 100 V and the quality of DNA tested The shearing during RNase treatment was also checked.

3.2.5 Molecular markers for identification of *Fusarium* wilt resistant genes

Degenerate primers (markers) were used in this study to identify genes governing the resistance to *Fusarium* wilt of banana in cultivar Palayankodan.

Table 1:Degenerate primers used in DNA amplification

NBS name	Peptide	Primer sequences encoded (5' —3')	References
F1(F)	P-loop	GGDGTDGGNAARACWAC	Deng *et al.*, 2000
F2(R)	GLPL	AANGCHAGNGGYAANCC	Deng *et al.*, 2000
F3(F)	P-loop	GGWATGGGWGGWRTHGGWAARACHAC	Lee *et al.*, 2003
F4(R)	GLPL	ARNWYYTTVARDGCVARWGGVARWCC	Lee *et al.*, 2003
F5(F)	P-loop	GGIGGIGTIGGIAAIACIAC	Peraza-Echeverria *et al.*, 2008
F6(R)	GLPL	AAGIGCTAAGIGGIAAGICC	Peraza-Echeverria *et al.*, 2008
F7(F)	Kinase	GTNYTNGAYGAYGTNTGG	Dequan Sun *et al.*, 2010
F8(R)	Kinase	TAGTTGTRAYDATDAYYYTRC	Dequan Sun *et al.*, 2010
F9(F)	P-loop	GGNGGNRTIGGIAARACIAC	Dequan Sun *et al.*, 2010
F10(R)	GLPL	GAGGGCNARNGGNAAICC	Dequan Sun *et al.*, 2010

Note: Codes for mixed bases: R=A/G, W=A/T, M=A/C, K=G/T, Y=C/T, S=C/G, H=A/T/C, D=A/T/G, V=A/C/G, B=C/G/T, N=A/T/G/C, I=hypoxanthine

There are 10 primers out of which 5 are forward and 5 are reverse primers. From these 10 primers as - forward + reverse, 25 primer combinations were made as shown below.

Table 2: Primer combination

F1(F)+F2(R)	F3(F)+F2(R)	F5(F)+F2(R)	F7(F)+F2(R)	F9(F)+F2(R)
F1(F)+F4(R)	F3(F)+F4(R)	F5(F)+F4(R)	F7(F)+F4(R)	F9(F)+F4(R)
F1(F)+F6(R)	F3(F)+F6(R)	F5(F)+F6(R)	F7(F)+F6(R)	**F9(F)+F6(R)**
F1(F)+F8(R)	F3(F)+F8(R)	F5(F)+F8(R)	F7(F)+F8(R)	F9(F)+F8(R)
F1(F)+F10(R)	F3(F)+F10(R)	F5(F)+F10(R)	F7(F)+F10(R)	F9(F)+F10(R)

3.2.6 PCR analysis

The PCR conditions required for effective amplification with degenerate primers require appropriate proportions of the components of the reaction mixture and appropriate annealing temperature. The reaction mixture comprised of template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2ml PCR tubes. The PCR was carried out in Thermal Cycler (proflea from Applied Biosystems, or sure cycler from Agilent).

The thermocycler was programmed for desired number of cycles and temperatures for denaturation, annealing and polymerization, based on the number of nucleotides in the primer and method of assay.

3.2.6.1. Conditions

Genomic DNA at the concentration of 30-40ng was amplified using selected degenerate primers as per the protocol given by Peraza-Echeverria *et al.*, 2007 and Dequan Sun et al 2010.

PCR reaction mix

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture is given below

a) Genomic DNA (30 ng) - 1.0 µl

b) 10X Taq assay buffer A - 2.0 µl
c) dNTP mix (10 mM each) - 1.6µl

d) Taq DNA polymerase (3U) - 0.6µl

e) Primer (10 pM) (each F+R) - 2µl

f) Autoclaved distilled water - 12.8µl

Total volume - 20.0 µl

PCR conditions

The amplification was carried out with the following programme

95°C for 3 minutes - Initial denaturation

94°C for 45 sec - Denaturation
47°C for 30 sec - Primer annealing 35 cycles
72°C for 1minute - Primer extension

72°C for 10 minutes - Final extension

4°C for infinity to hold the sample

3.2.6.2. Agarose gel electrophoresis

140 µl reaction mixture was dispensed into 0.2 ml PCR tube. PCR was carried out under PCR conditions as mentioned above. Amplified products electrophorased run on 1.2 % agarose gel and observed for polymorphism

Reagents and equipments

1. Agarose - 1.2 per cent (for genomic DNA)
2. 50X TAE buffer (pH 8.0)

- 10 mlTris buffer (1M)

- 45 ml glacial Acetic acid

- 2 ml M EDTA (0.5M)

3. Tracking/loading dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml): 5 µl/120 ml of agarose gel composition of reagents is provided in Annexure II.

Procedure

- The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

- Prepared 1.2 per cent agarose (1.2 g in 120ml) was prepared in a glass beaker or conical flask with 100 ml 1X TAE buffer. Micro waved for 45 to 60 seconds until agarose was dissolved and solution was clear.

- Solution was allowed to cool to about 42 to 45°C and 4µl Ethidium bromide was added at this point and mixed well.

- This warm gel solution was poured into the gel tray to a depth of about 5 mm. and allowed to solidify for about 30 minutes at room temperature.

- The comb was subsequently removed and the tape used for sealing removed, the tray was placed in electrophoresis chamber, and covered (just until wells are submerged) with TAE buffer (the same buffer used to prepare the agarose).

- To prepare samples for electrophoresis, 1 µl of 6x gel loading dye was added for every 5µl of DNA solution. Mixed well and loaded 6µl DNA sample per well.

- Electrophoresis was carried out at 70 volts until the dye has migrated two third the length of the gel. On completion of the electrophoresis, the gel was documented in a gel doc machine.

3.2.7Gel documentation

Gel documentation was done with BioRad gel documentation system using PDQuest™ software. Documented gels were observed for polymorphism between resistant and susceptible genotypes.

3.2.8 Cloning of PCR amplicon

3.2.8.1 Elution of polymorphic amplicon

PCR product was eluted using AxyPrep PCR Clean up Kit (Axygen. Biosciences). Procedure was followed as per the manufacturer's guidelines

1. Excised the agarose gel slice containing the DNA fragment of interest with a clean, sharp scalpel under ultraviolet illumination. Briefly placed the excised gel slice on absorbent toweling to remove residual buffer. Transferred the gel slice to a piece or plastic wrap or weighing boat. Minced the gel into small pieces and weigh. In this application, the weight of the gel is regarded as equivalent to the volume. For example, 100mg of gel is equivalent to a 100 µl volume. Transfer the gel slice into a 1.5 ml microfuge tube.

   Note: Alternatively, the gel slice can be placed into the 1.5 ml microfuge tube and then crushed with a pipette tip or other suitable device. Spin the tube for 30 seconds at 12,000xg to consolidate the gel at the bottom of the tube.
2. Added 3x sample volume of buffer DE-A

   Note: The color of the buffer DE-A is red. This color is used to add contrast in the next step, so that any pieces of unsolubilizedagarose can be visualized

3. Resuspended the gel in buffer DE-A by vortexing. Heated at 75^0C until the gel is completely dissolved (typically, 6-8 minutes). Intermettentvortexing (every 2-3 minutes) will accelerate gel solubilization.

   Note: Do not heat the gel for longer than 10 minutes.
4. Added 0.5x buffer DE-A volume of buffer DE-B, mix.

   Note: The color of the mixture will turn yellow after the addition of buffer DE-B.
5. Placed a miniprep column into a 2 ml microfuge tube (provided). Transfer the solubilized agarose from the step 4 into the column. Centifuged at 12,000xg for 1 minute.
6. Discarded the filtrate from the 2 ml microfuge tube. Returned the miniprep column to the 2 ml microfuge tube and added 500 µl of buffer W1. Centrifuged at 12,000xg for 30 seconds.
7. Discarded the filtrare from the 2 ml microfuge tube. Returned the miniprep column to the 2 ml microfuge tube and added 700 µl of buffer W2. Centrifuged at 12,000xg for 30 seconds.
8. Discarded the filtrate from the 2 ml microfuge tube. Placed the miniprep column back into the 2 ml microfuge tube. Added a second 700 µl aliquot of buffer W2 and centrifuged at 12,000xg for 1 minute.

   Note: Two washes with buffer W2 were used to ensure the complete removal of salt, eliminating potential problems in subsequent enzymatic reactions, such as ligation.
9. Discarded the filtrate from the 2 ml microfuge tube. Placed intominiprep column back into the 2 ml microfuge tube. Centrifuged at 12,000xg for 1 minute.
10. Transferred the mini prep column into a clean 1.5 ml microfuge tube. To elute the DNA added 25-30 µl of eluent or deionized water to the center of the membrane. Let it stand for 1 min at room temperature . Centrifuged at 12,000xg for 1 minute.

    Note: Pre-warming the eluent at 65^0 C will generally improve elution efficiency.
11. Purified PCR product was checked on nanodrop and stored at -20^0Cfor further cloning works.

Cloning of PCR amplicon

3.2.8.2 Preparation of competent cells

Competent cells required for plasmid transformation were prepared using the competent cell preparation kit of GeNei, Bengaluru in LB agar media.

Procedure

Day I

- The desired strain *E.coli* DH5α culture was inoculated into10 ml LB broth from master plate.
- The flasks were incubated in shaker at 37^0C; 120 rpm for 16 to 18 hrs.

Day II

- From the 10 ml *E.coli*suspension took 1 ml and inoculated into 50 ml LB broth. Incubated in shaker at 37^0C; 120 rpm for 3 hrs.
- After 3 hrs of incubation 50 ml *E.coli* suspension was transferred into 2 Okridge tubes (Each containing 25 ml), and kept on ice for 15 minutes. Recovered the cells after 15 minutes of centrifugation at 3500 rpm for 10 minutes at 4^0C.
- After centrifugation drain off the media then added 30 ml of $MgCl_2$ solution (80 mM $MgCl_2$:20 mM $CaCl_2$), the kept on ice for 15 minutes.
- Aftre 15 minutes again centrifuged at 3500 rpm for 10 minutes at 4^0C.
- Discarded the media and kept the okridge tubes on ice then added 2 ml 0.1 M $CaCl_2$.Again centrifuged at 3500 rpm for 10 minutes at 4^0C.
- Discarded the media then added 2 ml of 10 % Glycerol/$CaCl_2$solution.
- Dispense the suspension as 100 µl aliquots to sterile chilled 1.5 ml tubes and stored at -80^0C until use.

3.2.8.3 Screening of competent cells

The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker. The procedure is as follows:

- Prepared 50 ml LB media and 50 ml LB broth
- The competent cells kept at -80^{0}C were thawed on ice.
- pUC18 was diluted to 1:10 dilution. Added 1µl of pUC18 to thawed competent cells.
- The contents were mixed gently and kept on ice for 30 minutes.
- Meanwhile, water bath was set to 42^{0}C.
- The tube was rapidly taken from ice and a heat shock at 42^{0}C was given exactly for 90 seconds. Without shaking, the tube was placed back on ice for 5 minutes.
- Added 250 µl of LB broth to vial under sterile conditions and was inverted twice to mix the contents.
- The tube was incubated at 37^{0}C for 1 hour with shaking.
- The transformed cells (100, 100µl) were pated on LB agar/ampicillin (50mg/l) overlaid with IPTG (6µl) and X-gal (60µl).
- The plates were incubated overnight at 37^{0}C.

Ligation

Ligation procedure was followed as per the manufacturer's guidelines.

Procedure

1. The pGEM-T vector was briefly centrifuged to collect contents at the bottom of the tubes.
2. The following ligation reaction was set up as described below:

Table 3: Components of ligation mixture

Component	Volume
2X Ligation Buffer	5.0 µl
pGEM-T vector (50ng)	1.0 µl
Eluted product	3.0 µl
T4 DNA Ligase (3 units/µl)	1.0 µl
Total volume	**10.0µl**

3. The reaction was mixed by pipetting and incubated for 1 hr at room temperature. Then it was kept at 4^0C overnight

3.2.8.4 Transformation of ligated product

Reagents

1. Ampicillin – 5mg/ml in water
2. IPTG – 200mg/ml in water
3. X-gal – 10mg/ml in DMSO

The procedure followed for plasmid DNA transformation is as follows:
The ligated PCR product was added to 100 µl of thawed competent cells and kept on ice for 40 minutes. Heat shock was given at 42^0C for 90 seconds in a water bath and immediately placed back in ice for 5 minutes. LB medium (250 µl) was added to the cells and incubated at 37^0C for 1 hr on shaker at 160 rpm. The aliquots of transformed cells (100 and 100 µl) were placed on LB agar/Ampicillin plate (5 mg/ml) / IPTG (6 µl) / X-gal (60 µl) plates and incubated overnight at 37^0C.

3.2.8.5 Confirmation of presence of insert by colony PCR

Confirmation of insert presence was carried out by performing colony PCR using F9f+F6r primer. The following protocol was used for colony screening by PCR.

1. A Master Mix was prepared and the regents were in the sequential order as shown in the Table.
2. The contents were mixed well and transferred 50 µl of the master mix into the PCR tubes on ice.
3. Picked an individual colony and resuspended in 20 µl of the PCR master mix.
4. The following PCR programme was run immediately:

   Step 1: 95^{0}C for 2 min - Initial denaturation
   Step 2: 94^{0}C for 45 sec - Denaturation
   Step3: 47^{0}C for 30 sec - Annealing
   Step 4: 72^{0}C for 1 min - Extension
   Step 5: 72^{0}C for 10 min - Final extension
   Step 6: 4^{0}C for 10 min - Cooling of samples

   35 Cycles
5. The PCR products were analyzed on 1 per cent agarose gel.

Table4: Composition of PCR Master mix

Components	Volume per reaction (µl)
10X Taq buffer A	2.0
dNTP mix (10mM)	1.6
Primer F9f+F6r	2.0
Taq DNA polymerase (0.3 units)	0.6
$MgCl_2$ (25mM)	1.0
Sterile H_2O	11.8
Total volume	**20.0**

Sequencing of clone

The clone was cloned in pGEMT and sequenced using forward and reverse primer F9f+F6r. Clone was sent for sequencing to DNA sequencing facility of Scigenome Kochi.

3.2.8.6*Insilico* analysis of sequence

The DNA sequence was obtained was analyzed with various online bioinformatics tool.

Vector screening

To remove the vector regions present in the sequences, vector screening was performed using Vecscreen tool (www.ncbi.nlm.nih.gov/VecScreen) provided by NCBI. The vector sequence was removed using Bioedit- Biological sequence alignment editor tool.

Searching for Homology

The nucleotide sequences of the sequence was compared with the sequences available in nucleotide database using BLAST tool (www.bcbi.nlm.nih.gov/Blast; Altschul*et al.*, 1997) provided by NCBI. Nucleotide- Nucleotide sequence comparison was done using Blastn tool, while nucleotide- protein sequence comparison was done using Blastx. The best sequence alignment results were noted and saved.

Results

4. RESULTS

The results of this study entitled 'Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.)' undertaken during the period from 2013 to 2015 at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter.

4.1. Screening of resistant variety for *Fusarium* wilt incidence

Two months old plants of resistant Palayankodan and susceptible Poovan varieties were inoculated with the spore suspension of *F. oxysporum* f. sp. *cubense* by root feeding method with spore suspension having a concentration 10^6spores/ml for 24 hrs during the months of June- July, 2014. Plants were observed for symptom development of *Fusarium* wilt incidence. The external symptoms of *Fusarium* wilt as yellowing of the leaves appeared in the leaves of plants of Poovan variety. Leaf margin turned pale green to yellow which eventually die off and the outer leaves die completely and hang down on the pseudostem as a skirt. In the advanced stages of disease, brown-reddish discoloration of the internal vessels of the pseudostem also occured. There were no symptoms in Palayankodan plants. Palayankodan plants were resistant to *Fusariumoxysporum* f. sp. *cubense*.Plants were observed for two months.

Plate 1. Palayankodan plants (AAB)

Plate 2. Poovan plants (AAB)

Plate 3. Culture of *Fusarium oxysporum*f. spp. *Cubense*

Plate 4a. Infecting resistant variety Palayankodan with *Fusarium oxysporum*

Plate 4b. Infecting susceptible variety Poovan with *Fusarium oxysporum*

Inoculation of pathogen by root feeding method

Plate 6. Inoculated Poovan plant showing *Fusarium* wilt symptom

Plate 7. Fusarium wilt symptom in cross section of Rhizome

4.2 Molecular marker studies

4.2.1Isolation of DNA

DNA was isolated fromplants of varietiesPalayankodan and PoovanbyCTAB method developed by Doyle and Doyle (1990) as described in section 3.2.4.The samples were run on 0.8 per cent agarose gel inTAE buffer buffer (1X). Single intact bands were obtained indicatinggood quality DNA. DNA from both samples quantified specrophotometrically and is presented in Table 1. The ratio for $OD_{260}/OD_{280}$for the samplesranged between 35-40ng. Good quantity of DNA extracted by CTAB method was used for PCR analysis.

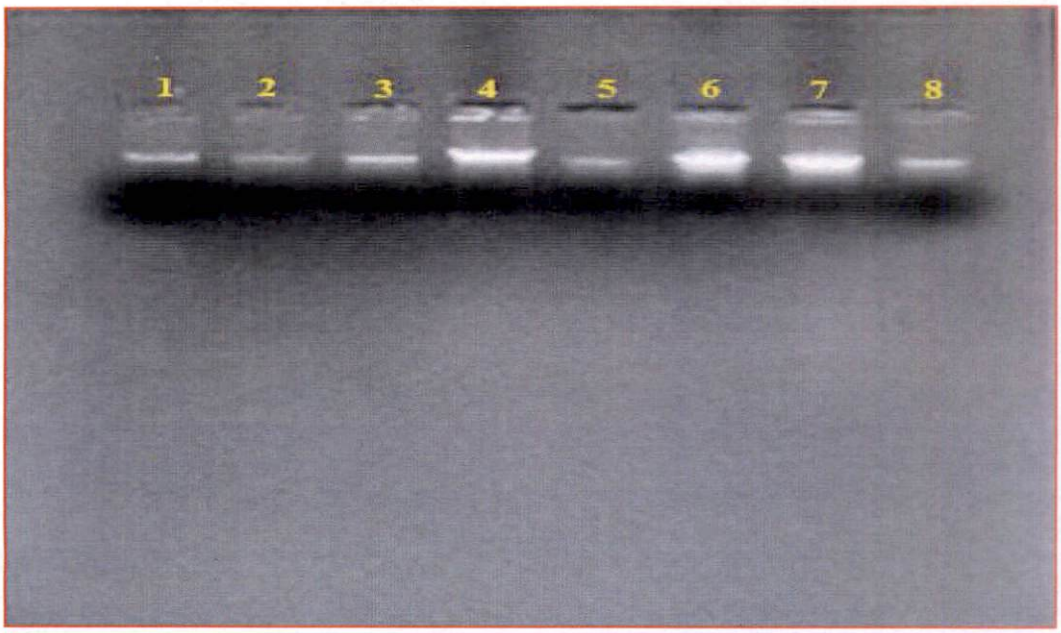


Fig 1. Isolated DNA after RNase treatment: 1 – 4 :Palayankodan , 5 – 8 : Poovan

4.2.2 Purification of DNA

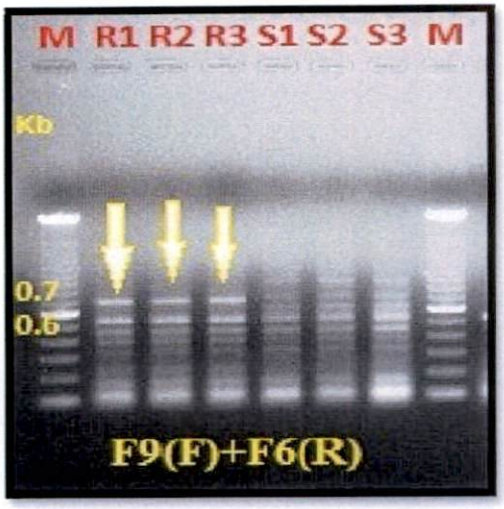
DNA was purified with 1 per cent RNase to eliminate the RNA contamination.

Table 5: Analysis of quality and quantity of DNA

	Sample	DNA yield (ng/ µl)	$A_{260/280}$
Palayankodan	1	1408.0	1.82
	2	1152.1	1.87
	3	1210.3	1.78
	4	1802.6	1.96
Poovan	1	2002.8	2.05
	2	1354.7	1.75
	3	1223.2	1.81
	4	1206.9	1.85

4.3. PCR amplification

PCR amplification of DNA samples from both resistant and susceptible varieties were done with 25 forward and reverse primer combinations. The list



M- Marker (100 bp plus)

R1, R2 & R3 palayankodan genotypes showing polymorphism

S1, S2 & S3 – Susceptible genotypes

F9(F) – Forward primer

F6(R) – Reverse primer

Fig 2. PCR using F9(F)+F6(R) primers showing polymorphism

ofprimers used is given in the section 3.2.5. PCR reaction mixture were prepared as given in the section 3.2.6.1 and was amplified under PCR conditions mentioned in the section 3.2.6.1. The amplified product was electrophoresed on 1.2 per cent agarose gel, asper the procedure described in the section 3.2.6.2. The electrophoresed DNA profile in the gel was documented in gel document machine and was observed for polymorphic bands among samples of resistant and susceptible varieties. 700bp polymorphic band was observed in resistant genotypes. The polymorphic band was eluted, cloned and sequenced. The sequence was analyzed using bioinformatics tools.

4.4. Cloning of PCR amplicon

4.4.1. Preparation and screening of competent cells

The competent cells were prepared as described in section 3.2.8.2 and were checked for competency by transforming withplasmids (pUC18) having ampicillin resistance. A large number of blue colonies were obtained (Plate8) which indicated a high degree of transformation efficiency. Thus the competent cells prepared were found ideal for cloning amplicons.

Plate 8. Checking competency of *E. coli* cells for transformation

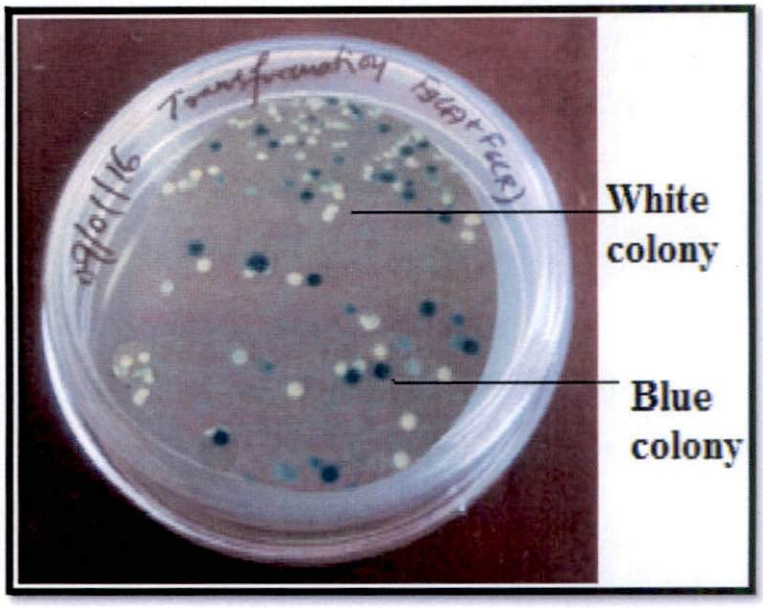


Plate 9. Blue-White screening of transformed *E. coli* cells

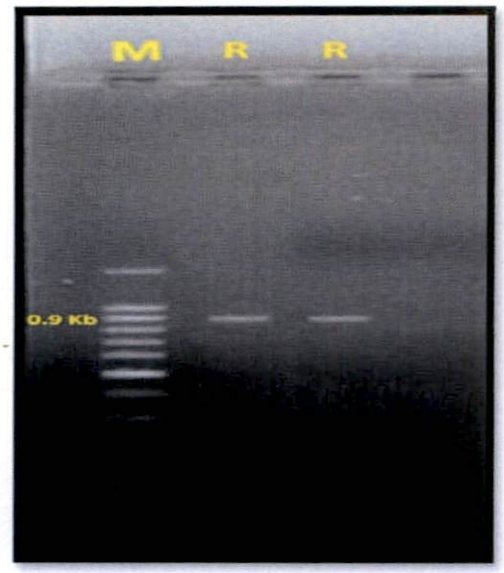
4.4.2. Transformation of ligated product

The ligated product was used to transform the prepared competent cells using the heat shock method and was incubated at 37^0C.

Large number of blue and white colonies was obtained after overnight incubation when the transformed cells were cultured in LB/ampicillin media, overlaid with X gal and IPTG.

4.4.2.1. Confirmation of recombinants by colony PCR

The white colonies were further checked for the presence of insert by colony PCR amplification using F9(F)+F6(R) primer. The PCR products when checked on 1.2 per centagarose gel showed amplicon of 900 bp sizes. This confirmed the presence of insert in the plasmid.



M- Marker (100bp plus)

R- Recombinant clone

Fig 3. Confirmation of recombinants by colony PCR

4.5Sequencing of clone

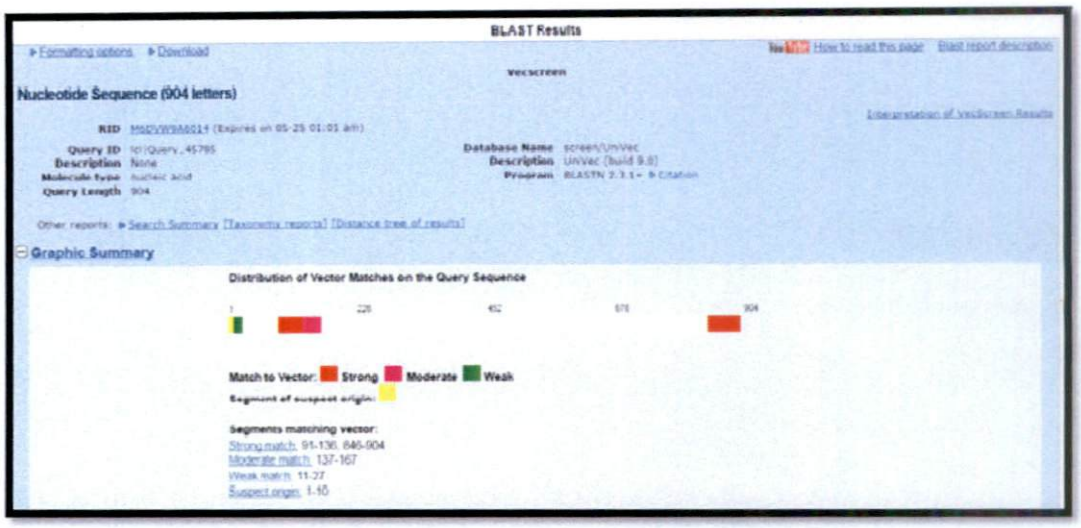
The clone in which the presence of insert was confirmed were screened out and were sequenced in automatedsequencer by outsourcing. Outsourcing was done atGenie Bangalore. The sequence data were obtained in the form of nucleotide sequence.

4.5.1Sequence data analysis by Bioinformatics tools

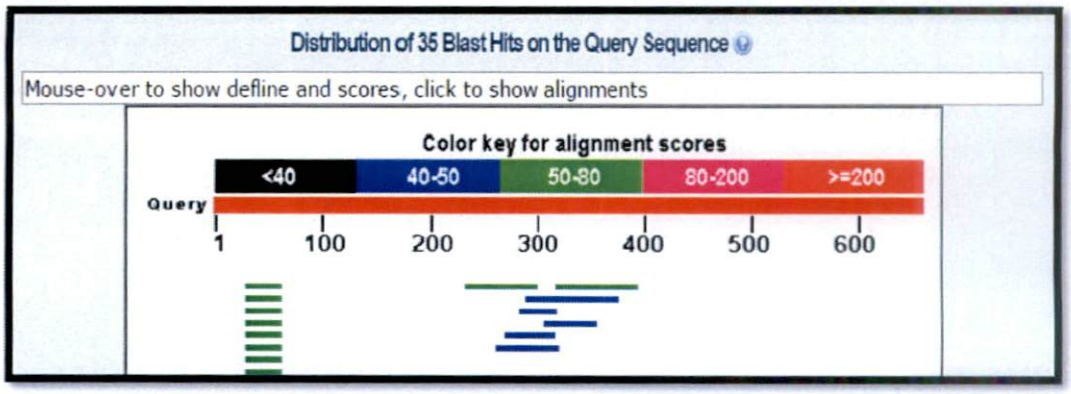
Sequence data after vector screening were subjected to Blastn, Blastx and Blastpanalysis.The sequence data obtained for the clone was of 904bp in size and vector screening indicated vector sequence from 91-136 and 846-904 bp. After vector screening, the total sequence obtained for further analysis was 678bp as detailed below,

5'GGCATGGGCGGCTGATACGACATCGCCTACGTGGGCAAGACCACCCTGGCC
CAGAAGATCTTCAACGACCCCAAGACCCAGGACATCTTCCAGGTGAGCAGCA
TCGCCTACATGAAGGGCCTGGGCGTGAGGGACCCCGAGGTGCAGAGGGACAG
GGCCGCCGAGGCCGACCACCAGAGCCCCAGGGTGACCATCAGGAGGCCCGGC
TGAACCACCGGCAGGACCTGACCCAGGCCCAGCTGCAGCCCCTGCTGAGGCA
CCCCCTGAGGCGCCAGGGCCTGCAGCTGGTGCTGGACCACCTGCGGCAGGCC
CACCAGGACCAGGTGCGGCTGGACCTGCTGAGGCACCCCCTGCTGCAGGGCC
GCCTGGCCCACCGCCGGCAGCAGCTGCAGGCTGAGGGGCGACCACCAGGGCA
GGGAGCACAGGCAGGCCGACGGCCAGAGGCAGGACCCCCAGGGCGAGAGGC
TGGTGCACACCATCCAGCTGCTGCCCGACGACAGCGGCTGGGAGCTGCTGTGC
AAGAAGGCCTTCGTGAGCGTGATGACCAGCAGGGTGAGGAGGAGGGGCGGC
CACGGCGAGCCCGAGGGCAGGGGCCTGAGGCACAGGGAGCAGGTGCACACC
AACGCCTGACACCAGTGAAGCACCAGCTGAGGCCCCGCCCTGAGGAGGCAGG
ACAGT 3'

Plate 10- Nucleotide sequence after Vecscreen

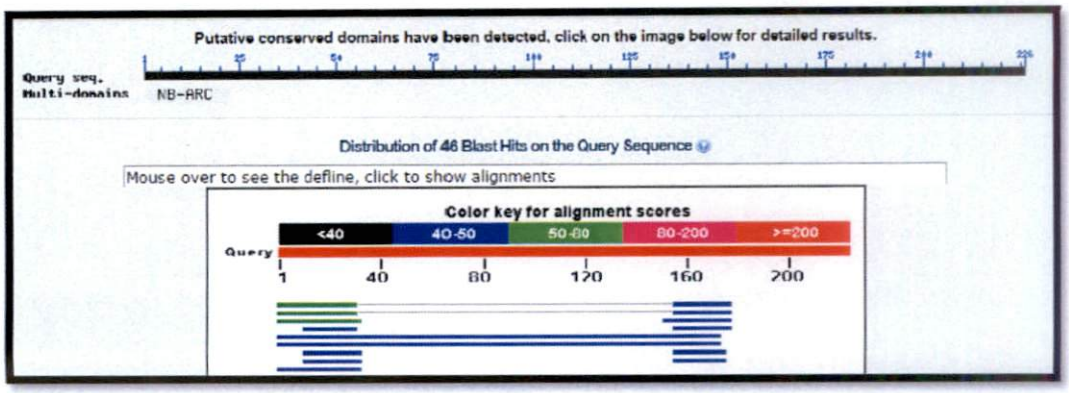


4. Vecscreen output



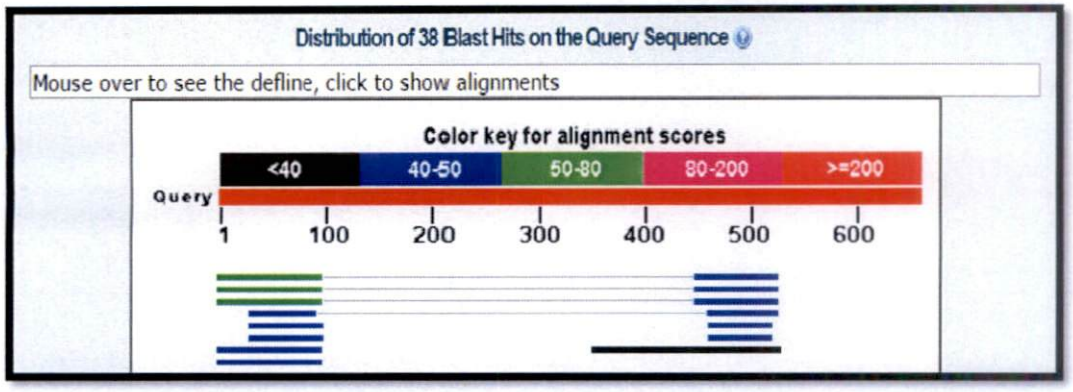
Musa AAB Group isolate YX-39 resistance protein gene, partial cds	50.0	50.0	9%	0.071	77%	HQ199834.1
Branchiostoma floridae hypothetical protein, mRNA	50.0	196	13%	0.071	72%	XM_002606051.1
Sorghum bicolor hypothetical protein, mRNA	50.0	50.0	9%	0.071	77%	XM_002451271.1
Musa ABB Group NBS resistance protein (RGA-I) gene, partial cds	50.0	50.0	9%	0.071	77%	EU855839.1

Fig 5. Blastn output



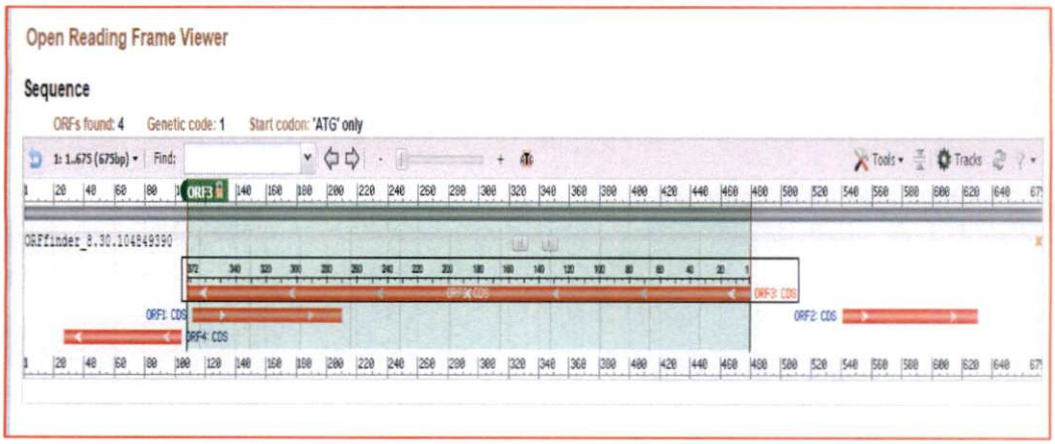
NBS-LRR disease resistance protein [Musa AAB Group]	51.2	99.7	24%	1e-04	81%	CAP66295.1
NBS resistance protein [Musa ABB Group]	51.2	99.7	24%	1e-04	81%	ACK44407.1
PREDICTED: putative disease resistance protein RGA3 [Musa acuminata subsp. malaccensis]	50.8	99.7	26%	5e-04	74%	XP_009389174.1

Fig 6. Blastp output



NBS-LRR disease resistance protein [Musa AAB Group]	51.2	100	27%	1e-04	76%	CAP66295.1
NBS resistance protein [Musa ABB Group]	51.2	100	27%	1e-04	76%	ACK44407.1
PREDICTED: putative disease resistance protein RGA3 [Musa acuminata subsp. malaccensis]	51.6	101	27%	3e-04	74%	XP_009389174.1

Fig 7. Blastx output



Label	Strand	Frame	Start	Stop	Length (bp | aa)
ORF3	-	2	479	108	372 | 123
ORF1	+	1	112	210	99 | 32
ORF2	+	1	541	630	90 | 29
ORF4	-	2	104	27	78 | 25

Fig 8. ORF analysis

4.5.1.1 BLASTn analysis

Blastn result indicated 77 per cent similarity with Musa AAB group resistance protein gene (HQ1999834.1). It also showed 77 per cent similarity with Musa AAB group NBS protein RGA of accession no-EU855839.1 (Fig. 5).

4.5.1.2 BLASTp analysis

Blastp result indicated 81 per cenr similarity to NBS-LRR disease resistance protein Musa AAB group accession no-CAP66295.1. It also showed 81 per cent similarity to NBS resistance protein of accession no-ACK44407.1 (Fig. 6).

4.5.1.BLASTx analysis

When a query was made with protein sequence to check in a database using a translated nucleotide query (Blastx), the sequence was shown to be similar to NBS-LRR disease resistance protein Musa AAB group of accession no-CAP66295.1 with 76 per cent similarity (Fig. 7).

4.5.1.4 ORF analysis.

ORF analysis had shown four ORFs in the sequence and ORF three had contributed for proteins involved in resistance mechanism (Fig. 8).

Discussion

5. Discussion

Banana is one of the important commercial fruit crops not only in India but also in the world. Susceptible to a number of fungal pathogens, as a number and bacterial pathogens, nematodes, viruses and insect pests, among their the greatest threats to global banana production is currently caused by *Fusariumoxysporum* f. sp. *cubense*. There exist no control strategy against the pathogen and control involves cultivation on disease free areas. Natural disease resistance banana breeding has been limited due to the triploid and sterility factors of banana. Although resistant sources are available in diploid banana cultivars, they were found to be either male or female-sterile, non-parthenocarpic, and produce a lower bunch weight. Hence, the development of new disease resistant high yielding varieties through the intervention of biotechnology tools is of paramount importance for the *Musa* industry. Although ranked as the fourth most important food commodity in terms of production value after rice, wheat and maize, genetic improvement of *Musa* has been limited. It is important to identify disease resistance genes in banana and investigate their role in disease resistance conditions. The information generated would be useful for disease resistance breeding in banana.

Plant disease resistance genes (R genes) encode proteins that detect pathogens. It can be grouped into several superfamilies, based on protein domains. The vast majority of genes cloned so far belong to the NB-LRR, eLRR, or LRR-Kinase superfamilies(Dangl and Jones, 2001; Holub, 2000; Jones, 2001). Thesesuperfamilies were initially identified in tomato, tobacco and *Arabidopsis*. In many cases, a single R gene can provide complete resistance to one or more strains of a particular pathogen, when transferred to a previously susceptible plant of the same species. For this reason, R genes have been used in conventional resistance breeding programs for decades (Pink, 2002). As a source of resistance to pathogens exist in germplasm, across the Musa genes, introgrcssion of R genes into susceptible cullivars offers potential for overcoming current constraints with conventional breeding.

The strong phenotypes and natural/variability at R loci have also been exploited by molecular geneticists to clone the R genes and investigate their molecular modes of action. Five principal classes of R-gcncs have been identified, based upon conserved protein domains (Martin *et al.*,2003). The most abundant class isthe cytoplasmic nucleotide-binding site-leucince-rich repeat (NBS-LRR) proteins (Rommens and Kishore, 2000). The other classes comprise proteinswith extracytoplasmic LRRs (eLRRs) anchored to a transmembrane (TM) domain (receptor-likes extracellular LRRs, cytoplasmic Ser/Thr kinases without LRRs and proteins with a membrane anchor fused to a coiled coil (CC) domain. The common NBS-LRR encoding proteins currently include over 20 functionally proven R-genes fromdiverse plant species (Van Der Biezen*etal.*, 2002).

Gene products are composed of a conserved N-terminal NBS and variable length C-terminal LRR domain of 10 to 40 short LRR motifs (Cannon *et al.*, 2002). The NBS domain isimportant for ATP binding and hydrolysis and is believed to be involved in signal transduction, triggered by the presence of the pathogen (Tamelin*et al.*, 2002). The LRR domain is likely to be involved in protein-protein interactions, recognizing pathogen elicitor molecules (Fluhr, 2001). A high mutation rate in the LRR contributes to genetic variability, necessary for specific recognition of diverse pathogens (Michelmore and Meyers, 1998). There are two major subfamilies of plant NBS-LRR proteins, defined by the presence of Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) motifs in the amino-terminal domain. Although TIR-NBS-LRR proteins (TNLs) and CCNBS-LRR proteins (CNLs) are both involved in pathogen recognition, the two subfamilies are distinct both in sequence and in signaling pathways and cluster separately in phylogenetic analyses using their NBS domains (Meyers *et al.*, 1999; Pan *et al.*, 2000).

The present study was aimed to identify and characterize *Fusarium* wilt resistant genesin banana var: Palayankodan

5.1. Screening of resistant variety for *Fusarium* wilt incidence

Inoculation of banana plants with *Fusariumoxysporum* f. sp. *cubense* was done as described in section 3.2.2. Resultsshowed that cultivar Palalyankodan plants were resistant to *Fusariumoxysporum* f. sp. *cubense*. Poovan plants were susceptible to *Fusariumoxysporum* f. sp. *cubense*. The external symptoms appeared firstly as yellowing of the lower leaves of Poovan varieties. Leaf margin turned pale green to yellow which eventually died off and the outer leaves died completely and hang down the pseudostem as a skirt.

Plants artificially inoculated with *F. oxysporum* f. sp. *cubense* developed typical symptoms of yellowing and dying of outer leaves with brownish rot of the vascular tissues. The most characteristic symptoms of the disease are the brown-reddish discoloration of the internal vessels of the pseudostem. Jeger*et al.* (1995) reported that *Fusarium* wilt is a typical vascular disease causing disruption of water translocation, systemic foliar symptoms and plant collapse. Confirmed resistant Palayankodan genetic material was used for molecular studies.

5.2. Molecular characterization of resistant loci

5.2.1. Isolation, Purification and Analysis of DNA

DNA was isolated from tender leaves of Palayankodan and Poovan, following CetylTrimethyl Ammonium Bromide (CTAB) method described by Rogers and Bandich (1994) with minor modification to yield good quality DNA (Described in section 3.2.4).

The quality of DNA was analysed by subjecting it to agarose gel electrophoresis and Nanodrop spectrophotometer analysis. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. The DNA sample revealed high quantity with respect to high molecular weight with little smearing and low amount of RNA at the bottom of the gel profile. RNA was removed from the sample by the treatment of RNase. In the present investigation, the

RNasetreated DNA sample on electrophoresis showed a high molecular weight DNA with very discrete band. This indicated that the DNA under test was of good quality.

In the spectrophotometer analysis, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samplesand ratio between 1.8 and 2.0 were considered to be of high quality and the same were used for molecular marker analysis.

5.3. Molecular markers for identification of *Fusarium* wilt resistant genes

Most of the molecular markers developed by PCR (Polymerase Chain Reaction technology)amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. In the present study, degenerate primers were utilized for molecular characterization of resistance gene analogs in banana.

Genomic DNA at the concentration of 30-40ng was amplified using selected degenerate primers as per the protocol given by Peraza-Echeverria *et al.*, 2008 and Dequan Sun *et al* 2010.

5.4. Cloning of PCR amplicon

Competency of DH5-α *E.coli* cells was confirmed by transforming the cells with plasmid (pUC18) having ampicillin resistance. *E.coli* cells alone could not grow on ampicillin containing media, as they lack the gene for ampicillin resistance. But the competent cells harbouring the plasmid could grow in media. In the present study, large number of blue colonies was observed on LB/ampicillin plate overnight incubation at 37^0C, confirming the competency of *E.coli* (DH5-α) cells for transformation.

For cloning of DNA fragments pGEMT vector of approximately 3 kb size were used. pGEMT vector contains F9(F)+F6(R) primer polymerase promoters that flank a multiple cloning region within the peptide - coding region of the enzyme p-

galactosidase. Thus, due to insertional inactivation of the peptide region, the recombinants can be directly identified by the blue-white screening of indicator plates. The vector contains multiple restriction sites within the multiple cloning regions thus facilitating easy ligation of insert and its release by digestion with restriction enzyme.

In the present study, the ligated product containing DNA fragments were used to transform the competent cells. The white colonies could be easily distinguished and picked up from the selection media containing 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal) and isopropyl thiogalactoside (IPTG).

pGEMT vector contained polycloning sites inside a gene encoding for β-galactosidase. Thus, insertion of a new sequence would disrupt the reading frame of galactosidase encoding gene. As a result of a-complementation, the bacterial cell and vector together provided the complete protein, because one part of the gene was present in bacteria while the other in vector (Ullmann*et al.*, 1967). The colonies which have not taken up the plasmid further utilized the substrate and appeared as blue colonies on chromogenic substrate, X-gal (Horwitz*et al.*, 1964). Due to the disruption of α-complementation, all the transformed colonies harbouring the recombinant plasmid appeared as white.

The presence of the insert was confirmed by direct PCR amplification of recombinant colonies with F9(F)+F6(R) primers. Amplicons for blue and white colonies differed in their size. The PCR product of blue colony plasmid had lower molecular weight since it lacked the insert and only F9(F)+F6(R) regions present in it could get amplified. While, the PCR product of white colonies gave different banding patterns each with higher molecular weight as the insert also got amplified along with F9(F)+F6(R) regions of the vector.

5.5. Sequencing of DNA clone

Since there is no automated sequencing facility at KAU and it would be highly laborious to sequence the clones manually, the outsourcing facility provided by Scigenome, Kochi was utilized. The sequence data were obtained in the form of nucleotide sequence. The sequence data obtained for the clone was of 904 bp in size.

5.5.1.*In Silico*analysis of the DNA sequence

Blastn result indicated 77 per cent similarity with Musa AAB group resistance protein gene (HQ1999834.1). It also showed 77 per cent similarity with Musa AAB group NBS protein RGA (EU855839.1). Blastp result indicated 81 per cenr similarity to NBS-LRR disease resistance protein Musa AAB group accession no-CAP66295.1. It also showed 81 per cent similarity to NBS resistance protein of accession no-ACK44407.1. Blast x result indicated 77 per cent similarity with NBS-LRR disease resistance protein Musa AAB group of accession no-CAP66295.1.Sequence was subjected to ORF finder and identified 4 ORFs. Among them ORF3 of length 372 is found to be the larger in length coding for R- gene.

5.6. Conclusion

Palayankodanresistant and Poovansusceptible varieties were used for studies. Artificially inoculation studies with the inoculum of *Fusariumoxysporum*confirmed resistance of Palayankodan and susceptibility of Poovan to the disease.DNA isolated from both resistant and susceptible genotypes, were PCR amplified with 25 degenerate primers for NBS-LRR resistance genes.and the primer F9(f)+F6(R) has given the polymorphic band of 700 bp.Polymorphic PCR amplicon was eluted and cloned using pGEMT vector system in*E-coli* DH5-alpha cells. Cloned product was sequenced and sequence information was subjected to *Insilico* analysis. Sequence showed homology to existing NBS-LRR disease resistance sequence 81 per cent similarity (*Musa* AAB group) in database.

5.7 Future line of study

1. Development of molecular markers linked with *Fusarium* wilt resistance for marker assisted selection.

2. Development of gene construct for *Fusarium* wilt resistant gene.

3. Development of disease resistant/tolerant transgenic banana cultivars through RGA mediated resistance.

Summary

6. SUMMARY

The study entitled 'Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana (*Musa* spp.)' was undertaken during the period from 2013 to 2015 at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara. The objective of the study was to clone and characterize Fusarium wilt resistance gene analogs in banana using reported degenerate primers.

The salient findings of the study are as follows:

1. Banana (*Musa acuminata*) varieties Palayankodan(AAB) reported as resistant to *Fusarium* wilt and Poovan(AAB) as susceptible were used in the study.
2. The suckers of Palayankodan and Poovan varieties were raised in polythene bag in the month of June- July. Plants of 2 months old were artificially inoculated through root-feeding with the inoculum of *Fusariumoxysporum*.Symptoms appeared within a period of one month on leaves and after two months on rhizome of Poovan and not in Palayankodan. Thus the susceptibility of Poovan and resistance of Palayankodan was confirmed.
3. DNA was isolated from both susceptible and resistant genotypes using CTAB method developed by Doyle and Doyle (1990).The RNA contamination was completely removed through RNase treatment.
4. Good quality DNA was used for the study. The quality and quantity of DNA was analyzed by NanoDrop ND-1000 spectrophotometerand the absorbance ratio ranged from 1.80-2.0, which indicated good quality DNA and the recovery was high.

5. Reported and designed degenerate primersbased on conserved regions of NBS-LRR R geneswere used in this study to identify genes governing the resistance to *Fusarium* wilt of banana in cultivar Palayankodan.
6. DNA from resistant and susceptible genotypes was amplified with 25 primer combinations. PCR products obtained were separated on 1.2 per cent agarose gel stained with ethidium bromide dye.Gel documentation was done with BioRad gel documentation system using PDQuest™ software. Documented gels were observed for polymorphism between resistant and susceptible genotypes. Only one primer, F9(F)+F6(R) showed polymorphism with an amplification of 700 bp.
7. The polymorphic PCR product was eluted using AxyPrep PCR Clean up Kit (Axygcn. Biosciences). Procedure was followed as per the manufacturer's guidelines.
8. The eluted PCR product was cloned in pGEMTvector system. The required Competent cells for plasmid transformation were prepared using the competent cell preparation kit of GeNei, Bengaluru in LB agar media.
9. The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker.
10. The ligated product was used to transform the prepared competent cells using the heat shock method and was incubated at 37^0C. The transformed cells appeared as white colonies and non transformed ones as blue colonies.
11. The transformed white colonies were further checked for the presence of insert by colony PCR amplification using F9(F)+F6(R) primer. The PCR products when checked on 1.2 per cent agarose gel showed amplicon of 900 bp sizes. Thus confirmed the presence of insert in the plasmid.
12 The clone in which the presence of insert was confirmed was screened out and was sequenced in automated sequencer by outsourcing. Outsourcing was done at Vision Scientific Bangalore.The sequence data obtained for the clone was

of 904bp and vector screening indicated vector sequence from 91-136 and 846-904 bp. After vector screening, 678bp.was available for further analysis.

13 Sequence data after vector screening were subjected to Blastn, BlastxBlastp and ORF finder analysis.

14 Blastn result indicated 77 per cent similarity with Musa AAB group resistance protein gene (HQ1999834.1). It also showed 77 per cent similarity with Musa AAB group NBS protein RGA (EU855839.1). Blastp result indicated81 per cenr similarity to NBS-LRR disease resistance protein Musa AAB group accession no-CAP66295.1. It also showed 81 per cent similarity to NBS resistance protein of accession no-ACK44407.1. Blast x result indicated 77 per cent similarity with NBS-LRR disease resistance protein Musa AAB group of accession no-CAP66295.1.

15 Sequence was subjected to ORF finder and identified 4 ORFs. Among them ORF3 of length 372 is found to be the larger in length coding for R- gene.

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Appendices

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis system	: Biorad, USA
Thermal cycler	: Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	: Biorad, USA
NanoDrop ND-1000 spectrophotometer	: NanoDrop Technologies Inc. USA

ANNEXURE II

Reagents required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

   CTAB (Cetyl trimethyl ammonium bromide) : 2 g

   Tris HCL : 1.21g

   EDTA : 0.745 g

   NaCl : 8.18 g

   PVP : 1.0 g

   Adjust the pH to 8 and made up final volume up to 100 ml.

2. **Chloroform- Isoamyl alcohol (24:1 v/v)**

   To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

3. **Chilled isopropanol**

   Isopropanol was stored in refrigerator at -20^0 C and was used for the study.

4. **Ethanol (70 %)**

   To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

5. **TE buffer (pH 8, 100 ml)**

   Tris HCl (10 mM) : 0.1576 g

   EDTA (1 mM) : 0.0372 g

   The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE III

Composition of buffers and dyes used for Gel electrophoresis

1. **TAE Buffer 50x**

   Tris base : 242 g

   Glacial acetic acid : 57.1 g

   0.5 M EDTA (pH 8.0) : 100 ml

2. **Loading dye (6x)**

   0.25 % bromophenol blue

   0.25 % xylene cyanol

   30 % glycerol in water

2. **Ethidium bromide**

   The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in dark bottles.

Abstract

CLONING AND CHARACTERIZATION OF FUSARIUM WILT RESISTANCE GENE ANALOGS IN BANANA (*Musa* spp.)

By

RAMESH

(2013-11-108)

ABSTRACT OF THE THESIS

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Kerala Agricultural University

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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Centre for Plant Biotechnology and Molecular Biology

Defense Seminar

Name: Ramesh

Admission No.: 2013-11-108

Major Advisor: Dr. P. A. Valsala

Venue:UG 4th year class room

Date: 31-08-2016

Time: 10.00 am

Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana *(Musa* spp.)

Abstract

Banana is one of the important fruit crops of India. It is susceptible to several fungal pathogens, nematodes, viruses and insect pests. The greatest threats to global banana production are *Fusarium* wilt or Panama wilt caused by *Fusariumoxysporum*f. sp. *cubense (Foc).* Control of the pathogen is difficult and mainly involves the use of disease free suckers. Although disease resistance exists in some banana cultivars, introducing resistance into commercial cultivars by conventional breeding is difficult due to its triploid nature and sterility factors of banana.

The study entitled "Cloning and characterization of *Fusarium* wilt resistance gene analogs in banana *(Musa* spp.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, Vellanikkara during the period 2013-2015 with an objective to PCR amplify the genomic DNA from *Fusarium* wilt resistant banana genotype with primers specific to 'R' genes of TIR-NBS-LRR class for cloning and characterization of resistant gene analogs.

Palayankodanresistant (Mysore Poovan AAB) and Poovan susceptible(Rasthali AAB)varieties of banana were used for the present study.*Fusarium* culture was isolated from roots of infected banana plant and cultured inPDA (Potato Dextrose Agar) media. Fungal spore was suspended in sterile water and filled in small polythene bag. Artificial inoculation – root feeding of inoculum of water suspension was done. The symptoms of *Fusarium* wilt was observed two months after infection. Results confirmed resistance of Palayankodan and susceptibility of Poovan

The DNA was isolated fromPalayankodan (resistant) andPoovan (susceptible)genotypesand isolated DNA was subjected to RNase treatment. Quality checking was done using 0.8% agarose gel electrophoresis and quantity analysis was done using nanodrop ND-1000 spectrophotometer. Thirty five ng of DNA was used as template DNA for PCR amplification using reported degenerate primers. Twenty five primer combinations were made using five pair of degenerate primers. Among those combinations only one primer combination F9(F)+F6(R) showed polymorphic band of 700bp which was eluted, purified and cloned in pGEMT easy vector system. The presence of insert was confirmed by colony PCR and the eluted product was sequenced by outsourcing. The sequence obtained was subjected Blastn, Blastx and Blastp analysis and was compared with NCBI database. The sequence showed similarity with NBS-LLR resistant gene of *Musa* spp. Open reading frames (ORFs) were also identified using ORF finder software and four ORFs were identified

For further validation, new primers were designed from 675 bp region of NBS-LLR class of resistant gene using primer3 plus software with an expected band size of 430bp. The DNA from both infected and healthy samples were amplified with designed primers and the expected bands were obtained in DNA samples of healthy

(resistant) plants where as it was absent in DNA samples of infected (susceptible) plants.

The degenerate primer F9(F)+F6(R) and designed primer F-TGAGCAGCATCGCCTA. R- GCCTGACACCAGTGAAGC can be used for *Fusarium* wilt disease diagnostics. Sequence information with respect to above primers amplicons can be used for synthesis of gene construct for genetic engineering.