MINING OF RESISTANCE GENES ASSOCIATED WITH ANTHRACNOSE INFECTION IN GREATER YAM (*Dioscorea alata* Linn.)

by

SARANYA G.

(2011-09-106)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

B.Sc.-M.Sc. (INTEGRATED) BIOTECHNOLOGY

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2016

DECLARATION

I hereby declare that this thesis entitled **"MINING OF RESISTANCE GENES ASSOCIATED WITH ANTHRACNOSE INFECTION IN GREATER YAM (*Dioscorea alata* Linn.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Place: Vellayani,
Date: 18 /11/2016

SARANYA G.
(2011-09-106)

भा कृ अनु प-केन्द्रीय कंद फसल अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद, कृषि और किसान कल्याण मंत्रालय, भारत सरकार)
श्रीकार्यम, तिरुवनन्तपुरम - 695 017, केरल, भारत

ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
(Indian Council of Agricultural Research, Ministry of Agriculture and Farmers Welfare, Govt. of India)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India

ISO 9001:2008

CERTIFICATE

Certified that this thesis entitled **"MINING OF RESISTANCE GENES ASSOCIATED WITH ANTHRACNOSE INFECTION IN GREATER YAM (*Dioscorea alata* Linn.)"** is a record of research work done by **Ms. Saranya G. (2011-09-106)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Trivandrum

Date: 18.11.2016

Dr. M. L. Jeeva
(Major advisor, Advisory Committee)
Principal Scientist
Division of Crop Protection
ICAR-Central Tuber Crops Research Institute
Sreekariyam, Thiruvananthapuram- 695 017

Phone : +91-471-2598551 to 2598554
Director (Per.) : +91-471-2598431
(Res.) : +91-471-2597211
Sr. Admn. Officer : +91-471-2598193
Fax : +91-471-2590063
E-Mail : director.ctcri@icar.gov.in
ctcritvm@gmail.com
Web : http://www.ctcri.org

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Saranya G. (2011-09-106)**, a candidate for the degree of **Master of Science (Integrated) in Biotechnology**, agree that the thesis entitled "**MINING OF RESISTANCE GENES ASSOCIATED WITH ANTHRACNOSE INFECTION IN GREATER YAM (*Dioscorea alata* Linn.)**" may be submitted by Ms. Saranya G., in partial fulfillment of the requirement for the degree.

Dr. M. L. Jeeva
(Major advisor, Advisory Committee)
Principal Scientist
Division of Crop Protection
ICAR-Central Tuber Crops Research Institute, Sreekariyam,
Thiruvananthapuram-695 017

Dr. B. R. Reghunath
Dean, Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695 522

Dr. T. Makeshkumar
Principal Scientist
Division of Crop Improvement
ICAR-Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram-695 017

Dr. K. Umamaheswaran
Associate Director (RARS (SR)) and Professor
Department of Plant Pathology
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

ACKNOWLEDGEMENT

I humbly bow my head before God Almighty for being caring and graceful to me over the hurdles, and enabling me to complete this endeavour successfully.

It is my great pleasure to acknowledge the following people who lend their helping hands and offered me all support for the successful completion of my research work. Without their support, patience and guidance, this study would not have been completed.

First and foremost, I express my deep sense of gratitude and sincere thanks to my major advisor Dr. (Mrs.) M. L. Jeeva, Principal Scientist, Division of Crop Protection, ICAR-CTCRI, for her excellent guidance, sustained interest, mentoring, encouragement, motivation, love, help and valuable support throughout the period of my research work. I really can't express my gratitude in actual sense. She gave me all time available help, advices, encouragement and sincere corporation during my research work. She gave me the confidence to describe things in the best and easiest way. Her constructive criticisms and suggestions were invaluable in the successful completion of this work.

I also express my deepest heartfelt gratitude and my sincere thanks to my committee members, Dr. B. R. Reghunath (Dean, Professor and Head, Dept. of Plant Biotechnology, CoA), Dr. T. Makeshkumar (Principal Scientist, Division of Crop Protection, ICAR-CTCRI), and Dr. K. Umamaheswaran (Associate Director (RARS (SR)) and Professor, Department of Plant Pathology, CoA), for their able guidance, sustained interest, affectionate encouragement and valuable suggestions. Their guidance helped me throughout my research work and writing of thesis.

My heartfelt appreciation to Dr. B. R. Reghunath (Dean, Professor and Head, CoA), Dr. James George (Director, ICAR-CTCRI), and Dr. C. A. Jayaprakash (Head, Division of Crop Protection, ICAR-CTCRI) for providing me all the facilities required of my work.

I also wish to express my sincere thanks to Dr. Lekha Sreekantan (former Course Director, B.Sc. – M.Sc. Integrated Biotechnology course, CoA), Dr. Swapna Alex (Course Director, B.Sc. – M.Sc. Integrated Biotechnology course, CoA) and my teachers Dr. K. B. Soni, Dr. Deepa S. Nair, and Mrs. Geetha Ramesh for their meaningful suggestions and encouragement.

A word of appreciation to all staffs of the Division of Crop Protection for their support and assistance offered during my work. My heartfelt appreciation to Dr. Vishnu S. Nath, who despite his enormous work pressure gave whole hearted assistance and encouragement throughout my project. I would like to thank Mrs. Shyni B., Mrs Smitha S., Mrs. Nijamol, Mr. Shinil T., and Ms. Surya, for their assistance during this project. I am also thankful to my seniors, juniors and friends for their support and affection during my study.

I am indebted to my parents, who sacrificed all the things for me and provided me the opportunity and desire to succeed. I also thank them for their love, support, constant encouragement and prayers, which kept me going at difficult times.

I thank all those who helped me directly and indirectly in completing my M.Sc. thesis. I apologize for being unable to name each and everyone, but, I sincerely thank each one of them.

Dedicated to my dear parents

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
A230	Absorbance at 230 nm wavelength
A260	Absorbance at 260 nm wavelength
A280	Absorbance at 280 nm wavelength
BLAST	Basic local alignment search tool
bp	Base pair
C	Cytosine
CDD	Conserved Domain Database
cDNA	Complementary deoxyribonucleic acid
cm	centimetre
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
F	Foreward primer
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare

IAA	Indole -3-acetic acid
Kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mm	Millileter
mM	Millimolar
MS	Murashige and Skoog
Mt	Metric tonnes
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NBS-LRR	Nucleotide binding site leucine rich repeats
NCBI	National centre for biotechnology information
Ng	Nanogram
Nm	Nanometer
OD	Optical density
ORF	Open reading Frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
ppm	Parts per million
PVP	Polyvinylpyrrolidine

R	Reverse primer
RGAs	Resistance gene analogues
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
S	second
sp.	Species
spp.	Species (plural)
t	Tonnes
T	Thymine
Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
U	Enzyme units
UV	Ultra violet
V	Volt
v/v	volume/volume
w/w	weight/weight

INTRODUCTION

1. INTRODUCTION

Yams are important carbohydrate rich, staple tuber foods of the tropical and subtropical regions. Yams are members the family of *Dioscoreaceae*, and belong to the genus, *Dioscorea*. 'Yam' refers to all members of the genus *Dioscorea*, containing more than 600 species. Around 10 species of yams are cultivated extensively for food, whereas numerous other species are harvested from the wild during periods of food insufficiency (Bhandari and Kawabata, 2005). The two widely cultivated edible yam species are *Dioscorea alata* and *Dioscorea rotundata*. *D. alata,* well known as greater yam or water yam is the largely cultivated species globally. Greater yams were introduced to Africa from Asia in during 16^{th} century. In India, greater yam is cultivated practically in many states, the major producers being Assam, Andhra Pradesh, Bihar, Gujarat, Kerala, Maharashtra, West Bengal and Orissa. Though there is variability existing both between and within species, all yams exhibits a similar growth habit with twining vines and shallow broadly radiating root system, which dies and are renewed annually. The economically important yam species are tuberous, producing one or a few underground tubers, which are the starch storage organs. These tubers offer means of vegetative propagation from one season to the other. Many species produce aerial tubers, or bulbils, forming means of vegetative propagation through dispersal.

Dioscorea alata is the most widespread species in the world. It is believed to have originated in south Asia, but recent studies on yam genetic have recognized Melanesia as its centre of origin. This region is also identified as the centre of maximum diversity (Lebot, 1999). Yam is considered to be the highly nutritious among the widely cultivated tropical root and tuber crops (Wanasundera and Ravindran, 1994). It has four times more protein content than cassava. It is the only tuber crop harbouring more protein content than rice in proportion to digestible energy (Bradbury and Holloway, 1987). The amino acid composition of yam protein is suboptimal in sulfur-containing amino acids, but the overall content of essential amino acids is superior to sweet potato (Bhandari *et al.*, 2003;

Splittstoesser *et al.*, 1973). Yams are an excellent source of vitamin A and C, dietary fibre and minerals. There are several constraints to yam production worldwide. Among the major constrains, diseases contribute significantly to heavy yield losses before and after harvesting. Greater yams face many challenges from wide range of pathogens like fungi, bacteria, and viruses at their different growth stages and also at times of storage of tubers.

Anthracnose disease has a considerable impact on yam production throughout the world (Nwakiti and Arene, 1978; Simon, 1993), which is caused by the pathogenic fungi *Colletotrichum gloeosporioides* (Penz.) (Nwakiti and Arene, 1978). On susceptible yam varieties, symptoms develop as small dark brown to black lesions on the surface of leaves, petioles and stems, which are surrounded by a chlorotic halo, resulting in necrosis of the leaves and vine die-back (Amusa, 1991; 1997). A few lower leaves may survive following vine die-back, but in most cases, the whole vine dies off thereby resulting in no or very poor yield. New shoots may arise from the planting material and the plants develop multiple stems in comparison to healthy plants which usually bear one or two stems. Since each stem produces a tuber, the diseased plants develop several small tubers instead of the normal one or two. The withered appearance of leaves and dieback of stem gives the plant a scorched appearance, hence the name 'scorch' disease (IITA, 1993).

Once infection is established, consequent progress of disease depends on rainfall and host variety. Susceptible varieties develop severe disease out breaks following rainstorms and cyclones. Numerous spores are produced on the infected leaf and are splashed in rain to adjacent leaves and vines (Okigbo, 2005). Farmers no longer depend on the effectiveness of benzimidazole or related fungicides for the control of anthracnose disease due to the development of fungicide resistant strains of *C. gloesporioides* (McDonald and Linde, 2002). The development of durable host plant resistance in *D. alata* against yam anthracnose disease will contribute notably to a high level of field performance (Abang *et al.*, 2003).

Successful control of anthracnose disease would promote greater widespread cultivation of yams with significant increases in overall production to meet the increasing demand for yam. In yam growing areas where anthracnose is not a serious problem currently, there is a risk of future yield losses, as novel hybrids are replacing many of the local landraces and also fungus is developing resistant to common systemic fungicide (McDonald and Linde, 2002).

Plants defend against its pathogens via mechanism based on the gene-for-gene concept, which suggests that the resistance gene product recognises a pathogen avirulence gene product resulting in defence activation (Flor, 1971). The vast majority of such plant R genes cloned to date contain nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR). A striking feature of these classes of genes is that, they are involved in gene-for-gene resistance towards a wide array of plant pathogens viz. fungi, virus, bacteria or nematodes (Bent *et al.*, 1994).

The sequence conservation of different NBS–LRR genes offers the opportunity for the use of PCR-based strategies to isolate and clone other R gene family members or analogues with the use of degenerate or specific primers for these conserved motifs. Through this approach, successful cloning of putative NBS–LRR resistance gene analogues (RGA) from many crops was possible. Identification of RGAs not only serves useful markers for mapping disease resistance genes but also as reference materials for studying the evolution and organisation of the NBS–LRR gene family in different plant species (Joshi *et al.*, 2012).

Hence the objectives of this work were formulated to identify resistance genes associated with anthracnose infection in greater yam and to study the expression of resistance genes in tolerant as well as susceptible varieties.

REVIEW OF LITRATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Tropical tuber crops mainly cassava, sweet potato, yams and aroids are widely used as vegetables, and also as raw materials for different types of industries. They constitute the staple food for millions of people especially in the under developed regions of the world (Ravi *et al.*, 1996). More than 64% of the major root and tuber crops are produced in North America, Europe, and the Soviet Union. Asia is considered the largest producer of root crops after Africa (FAO, 1985). The nutritional and economic values of root and tuber crops lies in the production of starch-filled storage organs which serve as a low cost source of carbohydrates in the diets of many people. They form the critical component in the human diet during the early evolution of mankind. Thus they form the most important food crops of very ancient origin in the tropics and sub tropics. It is thought to be associated with human existence, survival and socio-economic history of the world (Asha and Nair, 2002). With the exception of sweet potato, their production is limited to the warmer regions due to the lack of tolerance to freezing temperature conditions (O'Hair, 1990). They not only provide basic food security, but also a source of income and diversity to diet. They are also rich in proteins and are a good source of minerals and essential vitamins. Root and tuber crops have established production systems and perform well under various management levels from low to high input levels. This feature makes them important for improving the productivity and richness of agro-systems. Though the agronomic characteristics of these crops are well standardized, their food and industrial excellence are yet to be studied. However, the actual potentials of these crops are identified and realized in growing regions and this would contribute to the energy and nutritional demand for the rising population.

2.2 YAMS

The commonly cultivated yams belong to Dioscoreceae family and to the genus *Dioscorea* (Coursey, 1967). Adelusi and Lawanson (1987) suggested that they are an extremely important source of carbohydrates to a large number of people, especially of the tropical and subtropical Africa, the Caribbean, parts of Asia, Central and South America, and the Pacific Islands. *Dioscorea* spp. constitutes the staple food of the tropics (Han *et al.*, 1987). Yams are annual root tuber-bearing plants with have more than 600 species. Six of them are economically and socially important as food, medicine, and cash (IITA, 2013). Some such species of yam are water yam (*Dioscorea alata*), yellow yam (*Dioscorea cayanensis*) and white yam (*Dioscorea rotundata*) (Ike and Inoni, 2006; FAO, 1998; Zaknayiba and Tanko, 2013). Yam as a staple food crop is cultivated widely in the tropics (Thouvenel and Fauquet, 1979) and major producer is the savannah region of West Africa (Etejere and Bhat, 1986). Yams are the fifth most harvested crops, after cassava, maize, guinea corn, and beans/cowpeas. Estimations by FAO revealed that yams as the most commonly harvested crop after cassava in the world (FAO, 2012).

Yam plays significant roles in the social, economical, and cultural wellbeing of millions of people around the world. Yam is one among the major cash crops and the most consumed food crops in West African countries. Yam cultivation is considered profitable despite the high production cost and price fluctuations in the markets (Izekor and Olumese, 2010; IITA, 2013). It is estimated by IITA that an average profit per seed yam, after harvest and storage, was at over US$ 13,000 per hectare and more than 60% of people in Nigeria primarily grow yams as a source of livelihood (IITA, 2013).

On considering the nutritional quality of yams, it is an essential source of carbohydrates for the consumers, basically in the tropics and subtropics (Coursey, 1967). Despite the significance of yams as a source of food security, the attention provided to yam production is still less, as more rural dwellers are still starving.

About 26 species of Dioscorea have been reported in India (Abruna *et al.*, 1981). Currently about thirteen species are accessible in Orissa, of which two are cultivated and remaining are wild (Maharana, 1993). Yam production has undergone remarkable changes in many parts of the world. The production process involved like bush clearing, cultivation, application of chemicals, harvesting and transporting to markets are still labour demanding (Ennin *et al.*, 2009). Maximum production of food and its availability is threatened where the inputs are not used wisely (Udoh and Etim, 2007). Statistical data of 2014 showed that, the area under harvest in the world had expanded from 1.15 million ha in 1961 to 5.04 million ha in the year 2012. Yield (kg/ha) also raised from 72.35 thousand metric tons in 1961 to 116.65 thousand metric tons in 2012 (FAO, 2014).

2.2.1 Greater Yam

D. alata, also known as greater yam, or ten-month yam is more significant as food in West Africa and the Caribbean than in Asia and America where they originated. It has been under competition with the important native species *D. rotundata*. Greater yams were introduced to Africa hundreds of years ago from Malaysia by agriculturists and seafarers from Portuguese and Spanish (Martin, 1976). In terms of area under production, they are next to *D. rotundata* in the degree of utilization. *D. alata* is the highest yielding species among the different yam species. They can be stored for relatively longer periods (5-6 months) than other species after harvest. *D. alata* is very well known for its high nutritional composition, with 7.4 % crude protein content, 75-84% starch content of, and vitamin C (13.0 to 24.7 mg/100g tuber) (Osagie, 1992).

D. alata tubers are of variable shapes, the most common being cylindrical shape. The number of tubers produced ranges from one to five. The tuber flesh colour ranges from white to cream and purple (FAO, 1994). The flesh texture is generally not so firm in white yam which makes it less suitable for the preparation of most popular delicacies, than the other species of yam in the West African

regions. On the other hand, *D. alata* is it is reported to be the major staple food in Côte d'Ivoire. About 65% of people here depend on greater yam as the major source of food and income. In the West Indies, New Caledonia and Papua New Guinea, *D. alata* is the major food yam species cultivated and consumed (Orkwor, 1998). Mashed yam cuisines are greatly enjoyed in Trinidad, Tobago and in regions of Barbados.

D. alata have the Far East as its centre of origin and is considered one of the representative species due to its better availability and economic worth. The characteristic features of this species are their square stem with tubers differing in shape, and with different flesh colours - white, creamy yellow and purple. The tubers generally weigh more than 500 g (Njoh *et al.*, 2015).

2.3 ORIGIN AND DISTRIBUTION OF YAMS

The Dioscoreaceae family to which yam belongs is supposed to be amongst the most primitive angiosperms and is most likely to be originated in the Southeast Asia (Coursey, 1976). The diverse Dioscorea species apparently followed a divergent evolution process during the course of time in three continents which separated them by the creation of the Atlantic Ocean and aridness of the Middle East (Hahn, 1995). As a result, the widely cultivated food species originated in three distinct centers: Africa, South America and Southeast Asia (Alexander and Coursey, 1969). These regions were also thought to be areas of independent yam domestication, and correspond to considerable diversity. The economically important species of yam comprise of *D. alata*, *D. rotundata* and *D. cayenensis*. *D. alata* have Southeast Asia as its centre of origin, more particularly in tropical Thailand and Myanmar (Orkwor, 1998). It is presently the most diversified and broadly distributed species. The reach of Asiatic yams, mainly *D. alata* and *D. esculenta*, occured more than 2000 years back, and reached Africa in 1000 AD (Coursey, 1967). Later *D. alata* was, introduced into tropics of America from West Africa in the 16th century through travellers from Portuguese and Spain (Onwueme and Charles, 1994). *D. rotundata* and *D. cayenensis* are indigenous to

West Africa (Coursey, 1976). Among the two, *D. rotundata* is presently the leading species in terms of area under cultivation worldwide. It is widely cultivated in West Africa, the West Indies and, to a lesser degree, in East Africa. The introduction of the African yam cultivars into tropical regions of America is thought to have occured as early as the 16th century (Coursey, 1967). Lamarck gave the primary explanation of *D. cayenensis* in 1792 based on samples from French Guiana, while *D. rotundata* was described in 1813 by Poiret based on specimens from Puerto Rico prior to their African origin was recognized. These species had narrowed movement towards the regions in the east and limited to East Africa. There is very little or nearly no cultivation of the African yam species in the Asia (Onwueme and Charles, 1994).

D. bulbifera, is a native to Asia and Africa, and is characterized chiefly by the bulbil formation (aerial tubers). Wild forms of *D. bulbifera* still exists (Onwueme and Charles, 1994). Considerable differences are however observed between the wild and continental forms (Alexander and Coursey, 1969). The cush-cush yam (*D. trifida*) is the yam species originated in the tropical America that have attained importance as a food crop. However its production is restricted to the West Indies (Onwueme and Charles, 1994). Other species of yams are of minor economic importance in several tropical regions which includes species like *D. japonica*, *D. dumetorum*, *D. hispida*, *D. transversa* and *D. opposita*, (Asiedu *et al.*, 1997).

In India Yams are grown practically in Kerala, Odisha, Bihar, Maharashtra, Assam, West Bengal, Andhra Pradesh, Uttar Pradesh, Gujarat and to some extent in Tamil Nadu and North eastern states in India. Purple flesh greater yam is mostly grown in Andhra Pradesh, Uttar Pradesh and Gujarat.

2.4 PRODUCTION

Food yams are cultivated chiefly in Africa particularly in West Africa with more than 90% of the world's production from the areas called "the yam zone of

Africa" (FAO, 2002). FAO statistics also reveals that, 48.7 million tonnes of yams were produced worldwide in 2005 with highest production obtained from sub-Saharan Africa. Central and West African regions account to 74% of world's production. The chief producers are Nigeria with 34 million tonnes, Côte d'Ivoire (5 million tonnes), Ghana (3.9 million), and Bénin (2.1 million tonnes) (IITA, 2009). Sub-Saharan Africa is expected to produce 98.1% of total world production of yam by the year 2020 (Scott *et al.*, 2000a). As of 1995 to 2000, the total world production raised from 32.7 million tonnes to 37.5 million. Production increase observed is because of the increase in cultivated area but no considerable increase was found in yield per hectare. West Indies ranks second in yam-production. It is reported that West Indies produces more than 250,000 tonnes of yam of which roughly 5% is exported. The exporting contributed to annual export earnings of around $15 million (Mitchell *et al.*, 1989).

The total area under cultivation in 2009 in India was 7756 ha with a yield of 115618 Mt. Consumption of yam is relatively more in Orissa, West Bengal and Kerala compared to other Indian states. Yam cultivation area during 2010-11 in Kollam district of Kerala was 1858 ha and Kollam ranks first with 34 percent area. Pathanamthitta, Idukki, Alappuzha and Kottayam are the other major yam growing districts in Kerala (Srinivas *et al.*, 2012).

2.4.1 Economic and social importance of yam

Unlike most tropical crops, yam naturally has a dormancy period which provides the advantage of a longer storage period. This helps ensuring a constant food supply even at periods of general scarcity. Yam can be stored for up to 6 months. They can be stored for even longer depending on the storage means used. Stored yam is believed as stored wealth and it can be sold when prices become higher. Yams ranks second to cassava as the most essential tropical root crop in the world. From the nutritional view point, yams have higher vitamin C and protein content than cassava (Opara, 1999). Nutritional value of yam has been highly described by several authors in their articles on yam (Bradbury and

Holloway, 1987). South Pacific regions grow yam as a significant food crop. Processed yam products have a very high market demand internationally if well produced. The international price of processed yam flour, yam chips and pellets ranges from $120,560 - $152,362 per tonne which are higher than those of cassava products, which are sold at prices between $90,000 and $105,000. Until recent times, yams were grown as main subsistence crops in Africa. But now, it is grown as cash crop for both local and export. A Nigerian survey revealed that, when purchasing yam processed power improved at all expenditure levels. There was a tremendous increase observed in household yam consumption (Nweke *et al.*, 1992). This shows that yams continue to have a high market potential in Nigeria. Yam also has medicinal, ritual and socio-cultural significance. It is included in various ceremonies and festivities (Coursey, 1967). In parts of Nigeria, the meals offered to deities consist primarily of mashed yam. Certain customary ceremonies were celebrated with yam as the major delicacy, such as the New Yam Festival in parts of West Africa.

2.5 MORPHOLOGY

Dioscorea spp. is polyploidy and vegetatively propagated tuber crop. The shape and number of yam tubers vary largely between species. *D. rotundata* (white yam) tubers are generally large and are cylindrical in shape. They are white fleshed. *D. alata* (water yam) tubers have variable shapes. The majority of them are cylindrical in shape. Tuber ranges in size from that of a small potato sized over 7.5 feet long. Yam's flesh colour may vary according the variety. Flesh colour may be of diverse shades like off-white, purple, yellow, creamy, or pink. The skin colour of greater yam varies from off-white to pale and dark brown. Different textures exist in yams from moist and tender to coarse, dry, and mealy. Yams are greatly found in most Latin American markets, often in form of chunks, sold by weight (IITA, 2004). The varying tuber shapes and sizes are due to their difference in genetic makeup and the influence of environmental factors. Cultivated yams usually produce tubers that are roughly cylindrical in shape and weigh around 3-5 Kg. Yam tuber develops from a corm-like structure situated at

the bottom of the vine. Seldom, this corm remains attached to the tuber after crop harvest and sprouts develop from it later. When the corm is separates from the tuber, sprout emerges from the tuber near to the point of corm attachment (Huber, 1998).

The shape and number of yam tubers vary greatly between species (Huber, 1998). *D. rotundata* (white yam) tubers are basically large sized and cylindrical shaped with white coloured flesh. *D. alata* (water yam) have different shapes in tubers, the common being cylindrical, but can be extremely variable. Tuber flesh is generally white coloured and is having watery texture. It is a main food source in Africa, the Caribbean, and especially Melanesia. It has great social and cultural significance in these regions (Lebot *et al*, 2005). Tubers of *D. cayenensis* (yellow yam) are similar to that of *D. rotundata* (white yam) in many aspects. Yellow yam (*Dioscorea cayenensis*) derived its common name from its yellow coloured flesh. The presence of carotenoids gives them the yellow colour. Yellow yam is native to West Africa and is very comparable to the white yam in appearance, except for some morphological differences and a shorter dormancy compared to white yam. Bitter yam (*Dioscorea dumetorum*) popularly known as trifoliate yam, originated in Africa, with a characteristic bitter taste of its tubers.

2.6 GENETIC DIVERSITY

The poor knowledge of their genetics is one of the major limitations to maximizing the full potential of various yam species under cultivation. Among the important staple food crops, yam genetics is one of the least studied (Zoundjihekpon *et al.*, 1994). There is limited knowledge about the inheritance of main traits in yams. The understanding of the chromosome number has been reversed several times (Gedil and Sartie, 2010).

Studies regarding the morphological distinction present in *D. alata* worldwide exposed out highly plastic species. Among them the greatest diversity is found in Papua New Guinea and Philippines (Martin and Rhodes, 1977). A

variety of ploidy levels identified have indicated the existence of tetraploid, hexaploid and octoploid cultivars with basic chromosome number x = 10 (Abraham and Nair, 1991; Gamiette *et al.*, 1999). No diploid forms are identified so far.

Isozyme level variations were studied in 269 accessions of greater yam (Lebot *et al.*, 1998). The study identified 66 distinct zymotypes using four polymorphic enzyme systems. But the low polymorphism of these markers could not reveal the correlation between the zymotype groups, ploidy levels, and the phenotypic characters.

Asemota *et al.* (1996) assessed the genetic relationship among different *D. alata* cultivars using RAPD. AFLP was used to study the genetic relationships in guinea yams (Terauchi and Kahl, 1999; Mignouna *et al.*, 2002a; 2002b).

In Guinea yams, Amplified fragment length polymorphism (AFLP) had been used to study the genetic relationships (Mignouna *et al.*, 1998) and to create genetic maps of three distinct Dioscorea species (Mignouna *et al.*, 2002a; 2002b). AFLP markers, had also been been used to study the genetic variability in *D. alata* and between species of the Enantiophyllum (Terauchi and Kahl, 1999).

2.7 AGRONOMIC CHARACTERISTICS

Yams prefer tropical climates and do not perform well under frosty environment (Coursey, 1967). Temperatures beneath 20°C impede the plant growth, whereas they grow well between 25 - 30°C. Short days (10 to 11 hr) promote tuber development, whereas longer days (more than 12 hr) promote vine development (Coursey, 1982; Okezie, 1987). Rainfall of around 1000 mm for 5 - 6 months, friable, fertile, and well-drained soils are best suited for cultivating yams (IITA, 2009). Yams generally show maximum yields in areas experiencing long rainy seasons. Yams are capable of surviving long dry periods, even though the yield reduces to some amount.

Conventionally, yams are propagated through vegetative means. Whole tubers, tuber pieces or minisetts are usually used for planting (Otoo *et al.*, 1985). Growth of yam begins with a sprout from the post-dormant tuber (Passam and Noon, 1977; Onwueme, 1984). Craufurd *et al.* (2001) suggests that yams show a sigmoidal growth pattern which is common to most annual crops. A slow growth is observed during development which is followed by exponential growth during development. At this stage, maximum area of canopy is formed, which undergoes senescence after growth rates decline. Maturity of yams is not well defined though it is generally measured by the vine dryness (Okoli, 1980). Osagie and Opute (1981) reported that the physiological condition of yam tuber at the time of harvest influences its food quality and storage period.

2.8 NUTRITIONAL VALUE

Yam alone is not a balanced food and nutrient deficiency diseases still prevail among the poorer yam-growing population. However, yam is very much advanced to cassava, taro and sweet potato in protein and vitamin C content. Replacement of yams by the easily growing cassava varieties is considered to be a factor responsible for the spread of nutritional deficiency in tropics (Coursey, 1983). *D. alata* possess a high content of protein, vitamin C and lower lipids compared to *D. cayenensis*, *D. rotundata*, *D. trifida* and *D. escunlenta* (Muzac-Tucker *et al.*, 1993). *D. dometorum* is having the highest nutritional quality, with highest protein and mineral contents, but very limited studies were focused on this specie. Large post-harvest losses occur in *D. dometorum* because, if tubers are not processed or cooked within a few days after tuber harvest, becomes rigid and inconsumable (Treche and Delpeuch, 1982).Yams also have high moisture content, starch content, dry matter, minerals especially potassium, and low vitamin A content. It contains about 5-10 mg of vitamin C, and essential amino acid isoleucine. Yams also hold a steroid compound known as diosgenin. Table 1 provides a summary of the nutritional values of yams.

Table 1. Range of nutritional values of greater yam (nutrients in 100g edible portion)

Nutrient	Amount in tuber
Calories	71.00 – 135.00
Moisture (%)	65.00 - 81.00
Protein (g)	1.40 – 3.50
Fat (g)	0.20 – 0.40
Carbohydrates(g)	16.40-31.80
Fibre (g)	0.40-10.00
Ash (%)	2.3-3.9
Calcium (mg)	12.00 - 69.00
Phosphorous (mg)	17.00 - 61.00
Iron (mg)	0.70 – 5.20
Sodium (mg)	8.00 – 12.00
Potassium (mg)	294.00 - 397.00
β -Carotene (mg)	0.00 – 10.00
Thiamin (mg)	0.01 – 0.11
Riboflavin (mg)	0.01 – 0.04
Niacin (mg)	0.30 – 0.80

Source: (Opara, 1999)

2.9 CONSTRAINS TO YAM PRODUCTION AND STORAGE

A number of constrains hinder the sustainable production of yams. Factors influencing the choice of varieties which the farmers prefer include: taste preference of consumers, storability, yield, early maturity, adaptability, and the availability of planting material. Major constraints to yam production include high labor costs for most cultural practices, and other elevated cost inputs such as the cost of planting materials, pests and diseases, declining soil fertility, unreliable sources of credit and unpredictable weather conditions. In order to overcome this, adoption of new cultural practices like planting on ridges instead of on mounds to lower cost inputs. The introduction of the minisett technique for production of seed yam also helps to make seed yams available at reduced costs. Establishment of woodlots near fields helps in providing cheap staking materials for storage (Tettah and Saakwa, 1994).

The major problems associated with yam production is the high costs and lack of availability of planting materials, high labour demands (during planting, weeding, staking, and harvesting), pests and diseases, weeds and the post-harvest losses which occurs during storage (Tschannen *et al.*, 2003).

2.10 DISEASES AND PESTS OF YAM

Yams are quite free of pest problems (Hahn, 1984). The major pests of yams include: froth beetle (*Criceris libida* Dalm., *Lilioceris livida* Dalm.), yam tuber beetles (*Heteroligus* spp.), mealybugs (*Planococcus citri* Ris.), scale insects (*Aspidiella hartii* Ckll.), yam nematode causing tuber dry rot (*Scutellonema bradys*), and and yam root-knot nematode (*Meloidogyne* spp.). Resistance to nematodes had been observed in some cultivars of *D. alata*. Good field sanitation, use of pest-free propagules for planting, avoidance of time and locations where pests are most serious and crop rotation practices are the best control measures. Biological control methods, especially breeding for resistance yields greater input.

Yam anthracnose or dieback disease caused by *Colletotrichum gloeosporioides* (Penz.) is one of the most common diseases of yams, and is considered to be restricted to *D. alata* (Singh *et al.*, 1966). But, *Colletotrichum*, spp. were also reported to cause dieback disease on *D. rotundata* and *D. cayenensis* (Nwankiti and Arene, 1978). Winch *et al.* (1984) indicated that *D. trifida and D. nummularia*, a lesser valued food yam, are highly susceptible to this disease whereas, *D. pentaphylla* and *D. rotundata*, are moderately susceptible to virulent strains of *C. gloeosporioides*. The pathogen is thus considered to be not as specific as once was thought. In addition, isolates of *C. gloeosporioides* varied in virulence within *D. alata* varieties and also differ in morphology when grown on culture medium. This suggests that there is a range of virulence exists among different isolates of *C. gloeosporioides* and that control with single, highly specific fungicides must be avoided.

Leaf spot diseases caused by *Alternaria* sp., *Gloeosporium* sp., *Cercospora* spp., *Phyllosticta* sp., and *Septoria* sp. are considered less serious. Limited studies had been conducted to find resistance or disease management measures. Wilt diseases caused by *Fusarium oxysporum* and *Sclerotium rolfsii* is problem in some localized yam growing areas, but is not considered a serious problem for most yam producing regions. Since yams are propagated vegetatively, viruses are one of the major pathogens. Still, there are little records on yield losses on yams due to virus infection. Viruses are generally transmitted by *Aphis* sp. and *Myzus persicae* (Reckhaus and Nienhaus, 1981; Thouvenel and Fauquet, 1979), resulting in leaf deformation, vein-banding, shoe stringing, mottling, necrosis, severe stunting, mosaic, ring spotting and tuber flesh browning (Harrison and Roberts, 1973). Tuber rots resulting from bacterial and fungal infection are probably the economically important disease problems, because of their effect on the storage process. *Botryodiplodia theobromae, Penicillium* sp., *Drechslera rostrata, Fusarium* and *Aspergillus niger* causes majority the rotting in yams (Maheshwari *et al.*, 1983; Noon, 1978). Several others have also been associatted with postharvest losses (Coursey, 1967).

2.10.1 *Colletotrichum gloeosporioides*

Colletotrichum is one among the major plant pathogens, responsible for anthracnose disease on a wide range of hosts from grasses to trees (Dean *et al.*, 2012). The characteristics of disease involve sunken spots of different colours on leaves, tubers, stems or flowers. These spots often broaden, and lead to wilting of vines, withering, and dying (Hiremath *el al.*, 1993). The pathogen requires humid and warm conditions to establish infection. Major hosts include angiosperms, gymnosperm, ornamental plants and fruit crops, vegetables and even grasses. The primary inoculum (spores) is disseminated by rain splashes or wind, the pathogen is cosmopolitan in its distribution (Farr *et al.*, 2006). Many crops grown worldwide are susceptible to one or multiple strains of *C. gloeosporioides* (Weir *et al.*, 2012). Thus, the pathogen causing anthracnose is a major economic constrain to crop production throughout the world.

Indian subcontinent has diverse environmental conditions favouring the growth and establishment of *Colletotrichum* spp. to infect a wide array of hosts. *C. gloeosporioides*, *C. sansevieriae*, *C. falcatum*, *C. capsici*, *C. truncatum*, *C. coccodes* and *C. acutatum* are some of the species reported to cause anthracnose disease in India during the past few years in different host types (Gautam *et al.*, 2012c). A single species of *Colletorichum* is also found to infect multiple hosts using various strategies for invading host cell. This ranges from subcuticular intramural necrotrophy to intracellular hemibiotrophy. Pathogen develops an array of infection structures, like appressoria, germ tube, intracellular hyphae and secondary necrotrophic hyphae. Studies revealed that the pathogen can be parasitic (Gautam *et al.*, 2012c), saprophytic (Nicholson and Moraes, 1980), and endophytic (Lu *et al.*, 2004).

Colletorichum gloeosporioides is one among the most frequently reported plant pathogens in India, belonging to the genus *Colletotrichum*. It is identified to infect a wide range of host with characteristic disease symptoms. *Colletotrichum*, the generic name was given by Corda for *Colletotrichum lineola*, a species of the

member of the Apiaceae family, in the Czech Republic (Sturm, 1798). Penzig (1831) indicated the pathogenic nature *C. gloeosporioides* for first time. The nomenclature is based from *Vermicularia gloeosporioides*, the type specimen collected from Citrus (Latunde-Dada, 2001) in Italy. Butler (1918) reported *C. gloeosporioides* in India for the first time on coffee leaves. McRae (1924) described it as the causal organism of mango anthracnose disease. Detailed studies on *C. gloeosporioides* in India are on progress from past few decades and are still continuing.

2.10.2 Biology of *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides (Penz.) belongs to the family Phyllachoraceae and the division Ascomycota. It is an asexually reproducing facultative parasite. The fungi have two forms: *C. gloeosporioides* as an anamorph imperfect or the asexual form and *Glomerella cingulata* as the perfect teleomorph state or sexual form. *G. cingulata* is identified on a wide range of hosts. They produce acervuli inside the host tissue during mitotic (asexual) stage of life cycle. The teleomorphic stage is well known for its ability to cause serious disease conditions (Cannon *et al.*, 2012).

The pathogen prefers warm and humid conditions for spread of anthracnose disease effectively (Farr *et al.*, 2006). The fungus colonises into injured plant tissues and produces numerous acervuli and conidial structures. Conidia can extend over fairly short distances, thereby infecting other healthy host tissues. Penetration of fungi into host tissues is via specialized infection structures termed appressoria. Asexual bodies called acervli are produced during the infection within the host as small, flask shaped bodies with a cushion at the base. Short crowded conidophores arises out of them which can be observed on the surface of infected plants. Conidia break out through an opening at the tip of acervuli. Long brown coloured structures called setae emerges from the acervuli cells (Kumar *et al.*, 2001; Perfect *et al.*, 1999). The infection process, involves the formation of acervuli, conidia, appressoria and setae. The infection results in

tissue necrosis. Debris from the infected host is also a primary source of inoculums for disease spread.

In infected plant tissues, sunken, water soaked spots, expand rapidly and becomes soft. They expand and show different colours from reddish brown to tan or black, which are characteristics of anthracnose disease. But a great variability in the symptoms development by *C. gloeosporioides* is recorded from different host types. The symptoms involve sunken, water-soaked, round or oval, regular to irregular shaped and brown- red to black spots. Likewise, the fungal morphology on culture media also varies. The fungus usually produces circular, cottony or woolly colonies on culture media with pale brown or grey colour (Vidyalakshmi and Divya, 2013; Hiremath *et al.*, 1993). The mycelium of culture is hyaline, septate and branched in nature. The size of fungal conidia ranges from 15-20 µm length and 5-7 µm in width. Generally the conidia are oblong with obtuse end though other shapes are also seen (Freeman *et al.*, 2000).

The nutritional composition of culture media as well as the growth parameters such as temperature, moisture and pH also affects the growth and sporulation process of *C. gloeosporioides* (Singh *et al.*, 2006; Rani and Murthy, 2004). Studies showed that *C. gloeosporioides* exhibits maximum growth at pH of 6-7 and temperature of 25-30°C. Exposure of the fungus to alternate 12 h light cycles and 12 h dark cycles resulted in the highest mycelial growth compared to 24 h exposure to continuous light and dark cycles (Hubballia *et al.*, 2011).

2.10.3 Taxonomic description

C. gloeosporioides is a filamentous fungi and its teleomorph *Glomerella cingulata* is among the main plant pathogens world-wide. The taxonomic explanation of *C. gloeosporioides* had been described in detail by different researchers. Based on morphological and microscopic observations, a number of scientific reports had been published regarding the taxonomy of *C. gloeosporioides*. It was identified that the pathogen was studied by various

researchers and confirmed the type culture collections particular for microbial identification. The most widely accepted taxonomic descriptions of *C. gloeosporioides*, which are as follows:

Kingdom : Fungi
Division : Ascomycota
Class : Sordariomycetes
Order : Phyllachorales
Family : Phyllachoraceae
Genus : *Colletotrichum*
Species : *gloeosporioides*
Scientific Name : *Colletotrichum gloeosporioides* (Penz.) (Ajay, 2014).

2.10.4 *Colletotrichum gloeosporioides* as pathogenic fungi:

In India, *Colletotrichum gloeosporioides* is one among the important plant pathogens which causes anthracnose disease in a variety of hosts including cereals, legumes, vegetables, grasses, fruits, perennial crops and tree species (Martín and García-Figueres, 1999; Sharma *et al.*, 2005). Choudhuary in 1963 recognized *C. gloeosporioides* as a pathogen causing anthracnose of *Zea mays*. Sharma and Sharma (1969) studied the toxic metabolite production of *C. gloeosporioides* causing dieback disease in citrus.

The early symptoms of the anthracnose disease are characterized as round to oval, water sunken spots, which develops as the disease progresses. This finally leads to tissue necrosis or tissue death. Due to the variable agroclimatic situation in India, the disease symptoms show variation from region to region, host and environmental conditions. The symptoms are basically brown, round or circular spots surrounded by a yellow halo on leaves and petiole as observed in turmeric (*Curcuma longa*) in Gujarat (Patel *et al.*, 2005). In leaves and fruits of bell pepper, the symptoms of anthracnose include irregularly spreading oval spots with small or large lesions in addition to necrosis of petiole, stem and inflorescences

resulting in dieback (Gupta *et al.*, 2009; Das *et al.*, 2005). In *Aloe vera*, round or oval shaped, water-soaked greenish areas with light brown borders were observed following pathogen attack. The infection leads to loss of mucilaginous gel and death of leaves (Avasthi *et al.*, 2011).

2.11 YAM ANTHRACNOSE

A major constraint to the yam production is anthracnose disease. *D. alata* is the most widely cultivated species of yam. They are highly susceptible to this disease (Winch *et al.*, 1984; Nwankiti and Ene, 1984). Necrosis of leaf and dieback of stem leads to decline in the effective photosynthetic leaf area, and epidemics occur prior to tuber formation resulting in yield losses (Green, 1994). Anthracnose has been a serious problem in Nigeria, where the greatest production of yam takes place resulting in severe yield losses (FAO, 1994). Use of resistant cultivars forms the basis of sustainable management practices against anthracnose disease. Breeding for disease resistance is hampered by lack of knowledge regarding the variability *Colletotrichum* (Green, 1998; Green *et al.*, 2000). This pathogen is capable of infecting leaves, vines, tubers and seeds of yam, but whether the same species causes different disease symptoms are not well understood (Abang *et al.*, 2001; Green and Simons, 1994).

Infection normally starts from the lower leaves facing the soil and progress upward along the vine. This shows that the pathogen is probably soil-borne. In certain conditions, black lesions appear on the lower regions of the main vine just before the first mature leaf. In severe infections, aerial regions of certain susceptible cultivars become chlorotic and stunted, but in others the younger leaves at the early infection tends to fold and twist and become stunted, indicating toxic effects (Ebenebe, 1982). Typical anthracnose symptoms on leaves and vines of a susceptible yam variety predominate in an infected field. On the infested leaves, the cuticle is sloughed-off, leaving behind superficial fruitfications. This indicates the hypostomatic development characteristic of *C. gloeosporioides*. Young plants are highly susceptible to the disease than mature plants and early

infection results in premature death and high yield losses. The scorch like symptoms are generally seen 3 to 4 months after planting. It becomes more severe during rainy season and hence the name "lightning disease." Scorched leaves exhibits signs of burning due to toxic effects of certain chemical produced. The extent of scorching in leaves varies from one cultivar to another.

2.12 DISEASE RESISTANCE IN PLANTS

Plants have to defend themselves from attack by microbes, viruses, invertebrates, and sometimes other plants. Since plants lack a circulatory system like animals, they should possess a pre-formed or inducible defence mechanism (Walton, 1996). With the help of Mendel's studies, plant breeders understood that the resistance of plants to a disease was inherited as a sole dominant or semi dominant genes (Keen, 1990). Considerable amount of data has now accumulated regarding the genetic and biochemical basis of plant resistance (Hammond-Kosack and Jones, 1996) and the use of resistant genotypes had emerged a good strategy to control many plant diseases (Crute and Pink, 1996). R gene products function in recognizing noval pathogens inducing defence pathways.

Pathogens usually use three main strategies to attack their hosts: necrotrophy, biotrophy, or hemibiotrophy. The necrotrophs first kills its host and then metabolize their components. Some necrotrophs have a wide host range, and tissue death is generally induced by toxins or enzyme production targeted to particular substrates (Walton, 1996). *Botrytis* and *Pythium* species are some of the widely studied fungal necrotrophs. Other types of necrotrophs produce selective toxins against host that are functional over a narrow range of hosts. Host resistance against this class of pathogens can be achieved through target alteration of toxin or via toxin detoxification. The virulence of pathogen is controlled by dominant genes due to the need of producing functional enzymes or toxins. Similarly, avirulence is controlled by recessive alleles, where there is inability to produce functional toxins. Hm1 from maize is the first R gene to be isolated,

conferring resistance against the leaf spot fungal pathogen *Cochliobolus carbonum* (Johal and Briggs, 1992).

Biotrophic as well as hemibiotrophic pathogens invade live host cells and divert host metabolism to favour its growth, development and reproduction (Agrios, 1988). Such pathogens tend to infect only one or a few related host species. On the contrary, hemibiotrophic pathogens such as *Colletotrichum* and *Phytophthora*, kills surrounding host tissue during the later infection stages. Because of the specialized nature of plant- pathogen interactions, minor differences occuring in either organism can upset the balance resulting in positive or negative impacts. Incompatibility results in triggering of plant defence responses which include host cell death (localized) and hypersensitive response (Hammond-Kosack and Jones, 1996).

The inheritance of plant resistance and pathogen virulence was studied using flax (*Linum usitatissimum*) and its fungal rust pathogen *Melampsora lini*. This study revealed the concept of "gene-for-gene" model, for resistance (Flor, 1971). Complementary pairs of genes, one in host and one in the pathogen, are required for this interaction. An alteration to any of these genes results in disease development. This model is true for most biotrophic pathogens and some nematodes (Keen, 1990; Crute and Pink, 1996). R genes have been isolated from many plant species including *Arabidopsis thaliana*, which serves as an excellent model for studying plant-pathogen interactions (Kunkel, 1996).

2.12.1 Plant disease resistance genes

R-genes form an important element for imparting disease resistance to plants (Dangl and Jones, 2001). Disease resistance genes have been successfully identified from a wide variety of plant species through transposon tagging (Anderson *et al.*, 1997) and also by map based cloning (Johal and Briggs, 1992). Hammond-Kosack and Jones (1997) reported that the comparative study of R-gene sequences from large number of crop species showed the presence of

conserved amino acid regions within them such as the P-Loop, Kinase, GLPL, RNBS motifs. The mechanism of defence response in plants against various pathogens including fungi, viruses, bacteria and nematodes is explained by the gene-for-gene concept (Keen, 1990). Plant R gene products recognize, directly or indirectly, the Avr gene products in pathogen, leading to defence activation (Flor, 1971).

The role of R gene products is to suppress the pathogen load, via processes like apoptosis at the site of pathogen attack or hypersensitive response (HR). The induction of pathogenesis related gene expression involves various physiological events such as a burst of reactive oxygen, calcium ion flux, disabling pathogen's ability to replicate and move, cell wall biosynthesis inhibition, callose deposition and systemic acquired resistance (SAR) against future attack by pathogen (Hammond-Kosack and Jones, 1996).

2.12.2 Components of gene-for-gene interaction

The resistant (R gene) response in host plant occurs only if the pathogens have an *Avr* gene for which a contrasting resistant (R) gene exists in the host (Staskawicz *et al.*, 1995). So any alterations made to either R gene or *Avr* gene, results in a compatible reaction (susceptibility).

2.12.2.1 The avirulence (Avr) genes

Avr genes are of pathogenic origin, regardless of their function for the pathogen survaival (Long and Staskawicz, 1993). *Avr* gene products must be identified by R gene products and it leads to activated defence response. Capsid protein (CP), an effector molecule and type III secretion (T3SS) are products of pathogen *Avr* genes common in fungi, viruses and bacteria. Example: Turnip crinkle virus (TCV), causing turnip crinkle disease, has CP as elicitor. In contrast, resistant host genotypes, *Arabidopsis* ecotype Di-17, encode TCV-interacting protein (TIP) that have role in binding with CP (Oh *et al.*, 1995). Site directed mutagenesis in the TIP binding region causes the loss of ability to bind with CP.

Subsequently, the mutant resistant genotype fails to induce HR. These data indicates that, both the components, TIP and CP are crucial for resistance response (Ren *et al.*, 2000; Gabriel and Rolfe, 1990).

Grennan (2006) indicated that fungi forms haustoria and delivers elicitors which the secreted effector proteins such as, '*Avr 4*' and '*Avr a*' of *Cladosporium fulvum* and *NIP1* effector molecules of *Rhynchosporium secalis* into plant apoplast which activates signaling pathways. Abramovitch *et al.* (2006) reported in his study, that *Pseudomonas pathovas* form pilus to transport the elicitor protein into plant cells. The gene expression of *hrp*-gfp indicated that *hrp A* and *hrp Y* and T3SS of *Ralstonia solonacearum* and *Pseudomonas syringae*, have important role in activation of host defensive response (Van-Gijsegem *et al.*, 1995).

2.12.2.2 The resistance (R) genes

Resistance genes also known as R-Genes, impart disease resistance against pathogens via production of R proteins inside the host cells. The major class of R-genes reported consist of a nucleotide binding site (NBS) domain and leucine rich repeats (LRR). Such domains and are referred as NBS-LRR genes. NBS-LRR class of genes were reported in many crop species including tuber crops like cassava (Roberto *et al.*, 2015) and taro (Nath *et al.*, 2013).

The NBS domain binds to either ATP or GTP. The LRR domain is involved in protein-protein interactions as well as ligand binding. Two groups of NB-LRR R-genes have been reported: toll interleukin 1 receptors (TIR-NBS-LRR) and coiled-coil (CC-NB-LRR) R-genes (Knepper and Day, 2010). The majority of R-genes studied so far, suggest their role in pathogen recognition, degrading Avr gene product and recognizing pathogen associated molecular patterns (Hammond-Kosack and Jones, 1997). Once the resistance protein detects a pathogen, the plant activates defence mechanism to eliminate the pathogen.

The knowledge about the dominant character of R genes and Avr genes had proved that R genes encode proteins which recognize Avr-gene products. Once the presence of a pathogen is recognized, the R protein activates signalling cascades that coordinate plant defence reactions to reduce pathogen establishment. R genes are expressed in healthy, unchallenged plants also in readiness for the early detection of pathogen attack. A third requirement is the capacity for swift evolution of specificity. Often new virulent strains of pathogens evolve regularly that escape specific defence responses (Dangl *et al.*, 1996; Crute and Pink, 1996). Therefore, an effective mechanism is necessary for plants for fast evolution of noval defence genes against more virulent strains of pathogen.

2.12.3 NBS-LRR genes

The NBS-LRR class of R gene is the major class of R genes that forms approximately 75% of plant R genes identified so far. The key domains of this type of gene are NBS, LRR and TIR or non-TIR domains. These domains play crucial role in defence response of resistant plant cultivars. QTL map of NBS-LRR genes were constructed in soyabean and their expression was studied in resistance and susceptible near isogenic lines following challenge with the pathogen *Xanthomonas axonopodis* and identified differentially expressed NBS LRR genes (Yang *et al.*, 2012). Moffett *et al.* (2002) proved that CC-NBS and LRR domains are necessary for *Rx* activity, conferring resistance against potato virus X (PVX) in the *Solanaceae* spp. The gene constructs in which either LRR or CC-NBS regions had been removed failed to induce HR, whereas, co-expression of both exhibited HR. One hundred and forty nine genes encoding NBS-LRR proteins were reported in *Arabidopsis* (Meyers *et al.*, 2003). Wan *et al.* (2012) identified 51 NBS-LRR genes from pepper using PCR amplification with degenerate primers. The expression study revealed that, these genes show increased transcript levels in presence of salicylic acid and abscisic acid, suggesting their role in defence signalling.

2.12. 4 Role in signal transduction pathway and specific recognition of NBS domain

On the N-terminus, NBS domain is thought to play an important role in signal transduction pathway in resistant crops. Conserved motifs among family members were identified in NBS domain. The backbone of the NBS domain consists majorly of: P-loop/Kinase-2/RNBS-C/RNBS-B/GLPL motifs (Traut, 1994). These conserved motifs are required for binding with *Avr* gene product which leads to signal transduction (Pan *et al.*, 2000). Zhang *et al.* (2004) revealed that P-loop plays important role in the NBS domain, because of the lack of HR induction identified in tobacco plants with point mutations at the P-loop region. The kinase-2 domain is associated with co-ordination of metal ion binding, mainly Mg^{2+} (Traut, 1994). P-loop and kinase-2 are important motifs in the NBS domain and act as ATP- and GTP binding sites, which allows NBS membrane fusion proteins to disassemble with other proteins during downstream signalling pathway (Traut, 1994; May *et al.*, 2001).

NBS domain also plays significant role in pathogen recognition. Though highly conserved motifs were identified in NBS domain, the sequences of conserved motifs differ among plants. This diversity in sequences is presumed to influence elicitor-specific recognition between R gene and *Avr* gene with downstream signalling pathways (Noir *et al.*, 2001; Aarts *et al.*, 1998).

2.12.5 The TIR and non-TIR NBS-LRR genes

The TIR NBS-LRR gene contains a domain similar to *Drosophila Toll* and mammalian *Interleukin-1* receptor whereas, the non-TIR that contains CC or LZ. The non-TIR subclass, such as *RPM1* and *RPS2* are found within both monocots and dicots (Pan *et al.*, 2000; Meyers *et al.*, 1999). Both CC and LZ motifs were identified in non-TIR subclass. The CC motif is a collection of 2 to 5 helices with two hydrophobic amino acids at the helix-helix interface and functions in downstream signalling pathway (Parker *et al.*, 1997). In contrast, the LZs present in *RPM1*, *RPS2* and *Prf* exist as monomers in uninfected plants. But they exist as dimers or multimers following pathogen attack. This suggests that LZ motif have

the ability to form dimers with themselves through specialized interaction with other related proteins (Landschulz *et al.*, 1998).

The mechanism of TIR protein activation of the signal transduction is still unidentified. Sequence homology is present between TIR of plants and animals. The similarity of TIR domain in these systems is predicted to have similar function in activating a defence response (Hammond-Kosack and Jones, 1997). Other than the potential of TIR to initiate signal transduction cascade, this domain also plays a role in pathogen recognition. As revealed in the studies of 13 alleles of flax *L6* and *L7*, the TIR domain showed altered response in pathogen recognition (Falk *et al.*, 1999; Ellis *et al.*, 1999).

The TIR and non-TIR genes show differences in their response to different pathogens. In *Arabidopsis*, TIR sequences functions via an *eds-1* dependent pathway, whereas some of the non-TIR sequences functions via the *ard-1* pathway (Aarts *et al.*, 1998). This indicates that TIR or CC motifs play important role in the induction of the signal transduction (Aarts *et al.*, 1998).

2.12.6 R gene evolution

The defence responces in plant are activated by interaction between R gene in plant and pathogen *Avr* genes. The domains that are assumed to play a role in specific recognition with *Avr* gene are LRR and TIR/non-TIR domains. So, the increased ability of these domains in the recognition of *Avr* gene product facilitates defence response to several pathogens (Zhou *et al.*, 2004).

Diversifying selection, unequal-cross over, chromosome breakage, mobile genetic elements, inter-locus recombination and chromosome rearrangements are reported as basic mechanisms for R gene evolution for generating diversity among domains (Ellis *et al.*, 2000; Michelmore and Meyers, 1998). Furthermore, pathogen virulence, pathogen and plant populations are also involved in evolution of R genes (Zhou *et al.*, 2004). Unequal crossing over occurs, which generates divergent forms of *T2* gene in *Arabidopsis thaliana* (Vision *et al.*, 2000). Ellis *et*

al. (1999) demonstrated that high rates of allelism have been identified in flax as a result of recombination and chromosome rearrangements. Similarly, ten of th eleven alleles identified in flax *L* locus are able induce defensive response to different races of flax rust pathogen (Ellis *et al.*, 1999).

LRR domain is thought to play major role in cell specificity recognition, thereby making its sequence more divergent (Parniske *et al.*, 1997). As a result, evolution of R gene by diversifying selection occurs greatly at the LRR domain (Mondragon-Palomino *et al.*, 2002). TIR/non-TIR motifs that plays a role in cell recognition has been evolutionary as well. Amusingly, these diversifying selections were found in non-TIR more often than TIR (Zhou *et al.*, 2004).

It was estimated that number of non-TIR subclasses was larger than TIR subclasses approximately 10 times (480 non-TIR from total 535 NBSLRR genes) in many species including rice (Bai *et al.*, 2002). Phylogenetic studies on NBS-LRR genes from different taxa including *Arabidopsis*, legume species like *Glycine max* and *Medicago truncatula* indicates that the non-TIR subfamily has greater sequence diversity than the TIR subfamily (Cannon *et al.*, 2002).

2.13 RESISTANCE GENE ANALOGUES (RGAs)

Resistance gene analogues (RGAs) are groups of genes whose sequences are partially homologous to resistance genes. They function in resistance response against similar or dissimilar pathogens. Therefore RGAs have led to the discovery of resistance genes against a wide array of diseases caused by fungi, viruses, bacteria and nematodes (Hammond-Kosack and Jones, 1996). RGAs had been studied extensively on numerous crops and have high potential in improve disease resistance in several plants.

RGAs have been successfully cloned from many crops by polymerase chain reaction (PCR) based approach using degenerate oligonucleotide primers designed from conserved domains. By using degenerate primers for conserved

motifs, RGAs were identified in many plant species such as, 243 RGA sequences in peanut (Yuksel *et al.*, 2005), 51 sequences in mango (Saboki *et al.*, 2013) etc.

2.14 PLANT TISSUE CULTURE AND STUDY OF HOST-PATHOGN INTERACTION

Yam propagation by means of seeds or tubers using conventional methods is slow and not sufficient for rapid multiplication. Tuber yield is severely reduced by many pathogenic infections spread through infected planting material and is transmitted to the subsequent generations (Ng, 1992) and deterioration of tuber quality (Mitchell and Ahmed, 1999). In vitro propagation of yams helps in overcome constraints related to availability of high quality disease free materials for planting (Wheatley *et al.*, 2005).

Tissue culture plants are currently well recognized as helpful experimental systems for the use in studying of host-pathogen relationships. Study of plant pathogen interactions using tissue culture plants can help in the production of disease free plants. These techniques have advantages for the examination of obligatory biotrophic fungi and also those with a necrotrophic lifestyle. It is in this area where many researches have been focused. Disease induction by artificial inoculation of pathogen is essential for studies of various aspects of plant pathology, such as etiology, epidemiology, disease resistance, host-parasite interaction and disease control (Xiang-Li and Wen-Hsiung, 1997). Tissue culture plantlets grown under controlled conditions of nutritional, hormonal, and environmental factors offer the use of simplified methods which allows the assessment of disease resistance and susceptibility (Saurabh *et al.*, 2015).

Testing of yam plants under environmental conditions favouring both disease development and plant growth is required to assess the reactions of different yam cultivars to anthracnose. Due to lack of standard screening methods for yam anthracnose resistance, limited advancement is done in resistance breeding until recently. Abang *et al.* (2001b) proposed a tissue culture-derived *in*

vitro technique for the rapid assessment of yam genotypes under controlled conditions. This method involves inoculation of pathogen on tissue culture-derived whole plants, for the optimization of evaluation in level of anthracnose resistance in yam accessions (Onyeka *et al.*, 2005a; 2006).

2.15 DEGENERATE PRIMERS

A primer nucleotide sequence is called degenerate, if one or more of its positions are occupied by one several likely nucleotides (Kwok *et al.*, 1994). The degeneracy of a primer sequence represents the number of different nucleotides that it contains. A possible way of amplifying a specific target sequence in genome is to design primer pairs flanking the ends of the target sequence using Polymerase Chain Reaction (PCR) technique. If highly conserved regions are identified in several genomes, then primers can be designed based on the conserved regions to amplify the region of interest. But, when the primer sequences are less conserved in several species, degenerate primers are needed. Degenerate primers are highly useful in the amplification of homologous genes from diverse organisms (Shen *et al.*, 1998). In the process known as "the candidate gene approach", already identified genes possessing similar function in one organism is considered to have their homologues amplified in related organisms by the use of degenerate primers (Deng and Davis, 2001). The degenerate primers can be of great use to isolate genes encoding proteins belonging to known families of proteins (Shen *et al.*, 1998).

The basic requirement to identify primer pairs for a set of homologous genes is to perform a multiple sequence alignment using suitable software. At least two conserved regions in the alignment have to be identified so that the region of interest is contained in between them. The next step is to check whether the designed primer satisfy the several conditions such as amount of GC-content, annealing temperature, non-formation of secondary structures etc between the conserved regions (Raddatz *et al.*, 2001; Kampke *et al.*, 2001). The final step is to find of a primer pair showing all the requirements of a good primer sequence.

2.16 PRIMER DESIGNING

A primer is a short oligonucleotide which is used in most molecular biology techniques from PCR to DNA sequencing. The primers are designed in such a way that they have sequences which are the reverse complement of a region on template DNA to which we wish the primer to get annealed. The success of a PCR is highly dependent on the selection of primers that hybridize to the complementary DNA sequence. These short nucleotide sequences function as a pair, known as the forward and reverse primers, which amplify a specific DNA sequence (Lexa *et al.*, 2001). In PCR, primers are used to determine which region in the DNA is to be amplified. The primer length is usually 18-24 nucleotides, not more than 30, and they should match the beginning and the end of the DNA fragment which we wish to amplify (Patrica *et al.*, 2009). The product of one amplification process serves as the template for the other, leading to an exponential increase of the target region. Typically primers of 20-24 bases and GC content of 45-60% with Tm of 52-58°C works best in most PCR amplification. The annealing temperature of the primer is normally set at 5°C lower than the estimated Tm (Dieffenbach *et al.*, 1993).

Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mis-hybridization to a similar sequence nearby. Most common method used is BLAST search. Here, all the possible regions to which a primer may bind can be predicted. The nucleotide sequence as well as the primer sequence can be BLAST searched. The free online NCBI Primer-BLAST tool integrates primer design and BLAST search into a single application. Many other online tools are also freely available for primer design, including some tools which focus on specific applications of PCR. The most popular tools used widely for primer designing are Primer3Plus and PrimerQuest.

2.17 POLYMERASE CHAIN REACTION (PCR)

This technique is extensively used in molecular biology to generate thousands to millions of copies of a particular DNA segment (Bartlett and Stirling, 2003). PCR is developed in 1983 by Kary Mullis. It is now the most common and often vital technique involved in medical and biological research for a variety of applications (Saiki *et al.*, 1985). In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR.

The PCR technique relies on thermal cycling, which comprises of repeated heating and cooling cycles followed by enzymatic replication of the DNA template. In PCR, the DNA segments generated is itself used as a template for next cycle of replication, resulting in the exponential amplification of DNA sequences (Sharkey *et al.*, 1994). Earlier, R-genes were identified from plants using laborious and costly techniques like map based cloning or transposon tagging (Mago *et al.*, 1999). But presently, the knowledge about the consensus in already reported R-genes makes it possible to design primers and mine out similar genes using PCR technique. By using this methodology, NBS-LRR genes have been studied from many crops including *Solanum tuberosum* (Leister *et al.* 1996), *Glycine max* (Kanazin *et al.* 1996), *Saccharum officinarum* (Rossi *et al.* 2003) etc.

2.18 RT PCR

Reverse transcription PCR is a variant of PCR used in molecular biology to study RNA expressions (Freeman *et al.*, 1999). RT-PCR qualitatively detects gene expression through creation of complementary sequences of DNA (cDNA) from RNA samples. Though RT-PCR as well as traditional PCR produces multiple copies of the target through amplification, the applications of both techniques are primarily different. Traditional PCR is used to exponentially amplify target DNA sequences, whereas RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement. The newly synthesized cDNA is thus amplified using traditional PCR technique. In addition

to studying the quality of gene expression, it can also be utilized for quantification of RNA, by incorporating qPCR into the technique (Livak and Schmittgen, 2001).

2.19 NUCLEOTIDE SEQUENCING

Knowledge about nucleotide arrangement is requisite for the basic biological research mainly in fields of biotechnology. The speed and accuracy of sequencing is achieved through modern sequencing technologies which are now mechanized (Olsvik *et al.*, 1993). The first sequencing was done by academic researchers by means of 2D-chromatography in 1970. With the emergence of fluorescence-based sequencing methods using DNA sequencer, the sequencing process has become easier and faster (Pettersson *et al.*, 2009).

R genes play a key role in pathogen recognition and in the downstream signalling cascades during disease resistance (Zipfel, 2008; Jones and Dangl, 2006). So far, more than 112 *R* genes and 104,310 putative *R*-genes from wide variety of plants species conferring resistance to about 122 pathogens had been successfully identified through sequencing (Sanseverino *et al.*, 2012). The known R genes can be grouped into several families based on the presence of certain structural motifs, which includes the nucleotide-binding sites (NBSs), leucine-rich repeat (LRR) domains, Toll/Interleukin-1 receptor (TIR) domains, coiled-coil (CC) domains and transmembrane (TM) domains which were revealed via sequencing (Martin *et al.*, 2003). Through sequencing, most prevalent *R* genes in plants of the NBS-LRR type, with the presence of an N-terminal CC or TIR domain were also identified (Dangl and Jones, 2001; Mayers *et al.*, 2003). Around 480 NBS-LRR genes encoded resistance proteins were identified in rice genome using cloning and sequencing (Zhou *et al.*, 2004).

Studies revealed that many *R* gene sequences are clustered within plant genomes (Hulbert *et al.*, 2001). To date, a large number of *R*-genes have been cloned and sequenced from several plant species, including *Arabidopsis* (Meyers *et al.*, 2003), *Medicago truncatula* (Ameline-Torregrosa *et al.*, 2008), soybean

(Innes *et al.*, 2008), rice (Bai *et al.*, 2002), *Lotus japonicus* (Sato *et al.*, 2008) and *Phaseolus vulgaris* (David *et al.*, 2009). In the *Arabidopsis* genome, 159 NBS-LRR genes were identified, and 113 of these genes occurred in 38 clusters (Guo *et al.*, 2011).

2.20 SEMI QUANTITATIVE RT (REVERSE TRANSCRIPTASE) PCR

RT-PCR is a highly sensitive method used for the detection of rare transcripts and also for analysis of samples available in limited quantities (Erlich, 1989). Quantitative measurement of specific gene expression is a critically important in understanding of the basic cellular mechanisms and the effects of various agents on cell metabolism. The use of reverse transcription along with traditional PCR is a very potent technique for the quantification of the mRNA transcripts (Bustin, 2000).

Generally, two main problems exist in the quantification of gene expression via RT-PCR. The first problem is the fact that PCR is an exponential amplification process where the product accumulates in an exponential manner. On reaching a critical point, known as the plateau phase, the rate of product formation decreases and product will be produced at an unknown rate. At this point the initial concentration of template cannot be calculated (Raeymaekers, 1993). In simple terms, even the very fine differences at the start of reaction will have a huge effect on the amount of product produced after a restricted number of cycles (Siebert, 1999). The second problem is that it is very difficult to ensure equal concentration of RNA in each reaction samples. In order to compensate for variation that may arise out of the variation in RNA input, both target gene and a reference gene that is not expected to vary in response to experimental condition has to be quantified (Thellin *et al.*, 1999). Different approaches were developed to circumvent these problems of PCR-based quantification of gene expression. One approach is the measurement of PCR products by semi-quantitative PCR technique. Here, the amount of amplification products is measured during the exponential phase of the PCR reaction only, which occurs before reaching

saturation (Reichl and Kochanowksi, 1999). This is performed for both, the housekeeping (reference) genes as well as the target genes. Gene expression of NBS-LRR genes were successfully studied in citrus plants infected by *Leiberibacter Asiaticus* (Maryam *et al.*, 2007). The result of the study showed increased gene expression in infected plants.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The study was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2015 – 2016. Details regarding the materials used and the methodology adopted for various experiments are presented in this chapter.

3.2 SAMPLE COLLECTION

Leaves of greater yam varieties Orissa Elite (susceptible to anthracnose disease) and Sree Keerthi (tolerant to anthracnose disease) were used for RGA amplification. Leaf samples for the study were collected from greater yam fields of ICAR-CTCRI as well as from tissue culture raised plants of the above varieties.

3.3 TISSUE CULTURE OF *D. alata*

Explants of susceptible (Orissa Elite) and tolerant (Sree Keerthi) varieties of greater yam were collected from the fields of ICAR-CTCRI. Nodes were used as explants to raise plantlets needed for the study. First seven nodes from the shoot tip were used as explants.

3.3.1 Media preparation and sterilization for tissue culture

Murashige and Skoog (MS) media (HIMEDIA PT021X1L) were used to establish nodal culture of plants. Each pack of MS powder was dissolved in distilled water and added sucrose (30g) and PVP (0.1%). The pH was adjusted to 5.7 with 1N NaOH/1N HCl and the volume was made to 1 L. The medium was poured into test tubes (15 ml media in 150 X 25 mm tubes) and sterilized at 121 ^{o}C and 15 psi pressure for 20 minutes (Appendix I). After sterilization the culture tubes were stored in culture room until use.

Initial inoculation of explants from field source was done in MS media supplemented with growth hormones – IAA (1.5 mg L^{-1}) and kinetin (2 mg L^{-1}) (Appendix I) (Supriya *et al.*, 2013). Subculturing of plants was done in half MS media without hormones.

All metal /glass instruments and other accessories used in the inoculation cabinet were wrapped in aluminium foil and sterilized. Scalpel blades, scissors, forceps etc were again dipped in alcohol and flamed at the time of use.

3.3.2 Explant preparation and surface sterilization

Excised nodes were coarsely trimmed to a size of 3 cm and washed under running tap water for 15 minutes. The nodes were then placed in 1 per cent laboline solution for 12 minutes followed by tap water wash and sterile distilled water. Further sterilization procedures were done inside the laminar airflow chamber. The explants were given an ethanol (70 %) wash for 2 minutes, 0.01 per cent bavistin wash for 3 minutes, 0.08 per cent mercuric chloride wash for 2 minutes, 200 ppm streptomycin wash for 3 minutes and finally 4 repeated washes with sterile distilled water. After the final washing step, the explants were spread on pre sterilized petri dishes lined with sterile blotting paper. They were then trimmed finely to an appropriate size of 1-1.5 cm.

3.3.3 Inoculation and incubation

Single nodes were dissected out and all inoculation operations were carried out under strict aseptic conditions inside a laminar air flow chamber, which was made sterile by alcohol wipe and exposure to UV rays for 20 minutes before use. All operations were carried out using pre sterilized instruments and glass-wares. Explants were then carefully inoculated into culture tubes using sterile forceps.

The surface sterilized single nodes were blotted on sterile filter paper and are placed in liquid MS media supplemented with IAA (1.5 mg L^{-1}) and kinetin (2

mg L^{-1}) and 0.1% PVP. Nodal cultures were done in test tubes and the nodes were held inside using sterile filter paper wicks. Once the plants are ready for subculturing, the nodes are excised and subcultured to half MS media.

The inoculated tubes were maintained in culture room at 25±1°C at under 16/8 h photoperiod of around 2000 lux light intensity provided by cool white fluorescent tubes with 60 % relative humidity inside culture room.

3.3.4 Hardening of plants

After 3 months of subculturing on half MS media, the plants were taken out of the culture tube and hardened before transferring to soil. The plantlets were taken out of the culture tubes with the help of forceps avoiding any mechanical damages. A thorough washing in tap water was done to remove the adhering medium to avoid infections in the future and the plantlets were then dipped in bavistin solution (1g L^{-1}) solution for 3 min.

Following bavistin treatment, the plants are carefully placed in plastic cups containing sterile coirpith. The hardened plants are thoroughly watered and kept in shade under a humidity range of 60-70%. Liquid MS without sucrose is sprayed every three days after transplantation. Watering is done as per requirement. When the roots are well developed and the plants are acclimatized, they were transferred to small pots (6 inch pots) with potting mixture and treated like normal plants.

3.4 CULTURING OF *Colletotrichum gloeosporioides*

Culture of *C. gloeosporioides* maintained at ICAR- CTCRI was used for the study. Culturing is done on potato dextrose agar media plates (Appendix II) with subculturing after 12 days. The PDA plates seized with paraffin film were transferred in incubator at temperature (27±2°C). After 12 days, margin of mycelial growth was transferred to new PDA plates under aseptic condition.

3.4.1 Purification and maintenance of culture

Spore masses were picked up with a sterilized wire loop and were serially diluted. Aliquots (10 µl) of spore suspension from different dilutions were placed in an improved neubar counting chamber (Marienfeld, Germany), and spores were observed at 40 X magnification using a light microscope (Nikon Eclipse E200). The smallest squares in the counting chamber are 0·05 mm x 0·05 mm, of which 80 were analysed to obtain the average spore count per square. The dilution which resulted in at least $5x10^2$ spores ml^{-1} is used for single spore isolation.

From that corresponding dilution, 10 µl (around 50 spores) was taken and plated on to the surface of agar plates and incubated over night. A single germinating spore observed under microscope was circle marked, picked up with a sterilized needle and transferred onto potato dextrose agar (PDA) plates under sterile condition and incubated. Pure culture was stored on 4°C on PDA slants (Yang *et al.*, 2008).

3.4.2 Preparation of spore suspension for artificial inoculation

Seven days old cultures were used for preparing spore suspension. The culture plates were flooded with 10 ml sterile water and the conidia were carefully scraped off with sterile needles into the solution. This is filtered through a clean cheese cloth and the filtrate is used as the spore suspension. The spore suspension is mixed well and 10 µl of spore suspension is charged to each side of an improved neubar counting chamber (Marienfeld, Germany). The number of spores in zones A to E on both sides of counting chamber is counted and average is estimated. A spore count of 2×10^6 spores ml^{-1} is used to inoculate the plants.

3.4.3 Pathogenecity testing of *C. gloeosporioides* on detached leaves

In order to test the pathogenecity of the maintained culture and also to ensure the susceptible and tolerant properties of selected plant species, a detached leaf assay was performed. Healthy young leaves from susceptible (Orissa Elite)

and tolerant variety (Sree Keerthi) were taken, and washed under tap water followed by repeated washes with sterile distilled water inside laminar air-flow chamber. Leaf samples were placed on sterile petridish containing sterile filter paper, moistened with sterile distilled water.

Each leaf was artificially inoculated with 25 µl of spore suspension containing approximately 2 x 10^6 spores ml^{-1} from a 7 days old culture. Control leaves were also kept in the same assay plate by replacing spore suspension with sterile distilled water. After 2 days of inoculation, leaves were evaluated for symptoms of anthracnose disease (Yang *et al.*, 1991).

3.4.4 Artificial inoculation of disease on tissue culture raised plants

Disease inoculation using the fungal spore suspensions of *Colletotrichum gloeosporioides* were carried out in tissue culture raised plants of Orissa Elite (susceptible) and Sree Keerthi (tolerant) for studying the host resistance genes expression during anthracnose disease development. The spore suspension (25 µl) was applied on both surfaces of young leaves using a clean paint brush and incubated at room temperature. After inoculation plants are covered with glass jars in order to maintain high humidity. Control plants were also kept by applying sterile water instead of spore suspension and maintained under similar conditions.

Leaves were collected from infected and control plants at alternate days till 5^{th} day. The RNA was isolated to study the expression of RGAs.

3.5 NUCLEIC ACID BASED STUDY OF RESISTANCE GENES

3.5.1 Isolation of DNA

Young leaves of *Dioscorea alata* plants, Orissa Elite (susceptible), and Sree Keerthi (tolerant) collected from ICAR-CTCRI fields were used to isolate DNA following CTAB method

3.5.1.1 CTAB Method of DNA Extraction (Lodhi *et al.*, 1994)

100 mg of young fresh samples of leaves were chilled and pulverized to a fine powder with liquid nitrogen using a sterile mortar and pestle and transferred into a sterile 2 ml centrifuge tube. Followed that 1ml of extraction buffer (pre-warmed at 65 °C) (Appendix III) was added to the tissue powder. Crude extract (750 µl) was transferred into a fresh 2 ml tube and incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for 30 minutes for cell lysis. The tubes were centrifuged at 10,000 rpm (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 10 minutes at 27 °C and the supernatant was transferred to a fresh 2 ml tube. After transferring the supernatant into fresh tube, 10 µl of RNase was added and incubated at 37 °C for 1 hour. Equal volume of chloroform: isoamyl alcohol (24:1) was added to supernatant and centrifuged at 15,000 rpm for 10 minutes at 4 °C. The aqueous layer after centrifugation was transferred into fresh tube and added 0.8 volume of ice-cold isopropanol and incubated at -20 °C for at least 1 hour or overnight. Following centrifugation at 12,000 *g* or 15,000 rpm for 10 minutes at 4 °C, the pellet was washed with 0.5 ml of 70 percent ethanol by centrifugation at 12,000 *g* for 5 minutes at 4 °C. The DNA pellet obtained was air dried at 37 °C for 30 minutes and then dissolved in 50 µl sterile 1X TE buffer and incubated at 37 °C for 1 hour and stored at -20 °C.

3.5.2 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard lab procedure for evaluating the integrity of DNA. A small aliquot of the isolated DNA was run on an agarose gel to visualize the quality of DNA. 0.8 percent agarose gel was prepared in 1X TAE buffer (Appendix III) and ethidium bromide was added to a final concentration of 0.5 µg L^{-1}. An aliquot of DNA sample (3 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5V cm^{-1} (BIO RAD Power Pac HV, USA) for 30 minutes. The gel was then visualized under UV light and the image was documented using Alpha Imager (Alpha Innotech, USA).

3.5.3 Nanodrop spectrophotometer analysis

The absorbance of DNA samples were recorded to determine the quantity and quality of DNA isolated. The optical density (OD) of DNA samples were measured using NanoDrop spectrophotometer (DeNovix DS-11). The spectrophotometer was calibrated to blank (zero absorbance) with 1.5 µl of sterile 1X TE buffer. Then the concentration of each of the DNA samples (1.5 µl) was recorded. The quality of DNA preparation was determined by analyzing the A_{260}/A_{280} and A_{260}/A_{230} readings.

3.5.4 PCR amplification of RGAs using degenerate primers

The Resistant gene analogues (RGA) in *Dioscorea alata* were targeted using degenerate primers already reported by Aswati and Thomas, (2007).

3.5.4.1 Gradient PCR for standardizing annealing temperature

Degenerate primers (Sigma Aldrich, USA) were used to amplify RGAs in *Dioscorea alata.*

RGA 1F (LM 638) and RGA 1R (P2 GLPL) primers (degenerate primers)

RGA 1F (forward primer) sequence: 5' GGIGGIGTIGGIAAIACIAC 3'

RGA 1R (reverse primer) sequence: 5' ARIGCTARIGGIARICC 3'

In order to standardize the annealing temperature of the primer pair used, gradient PCR was performed for the primer set. The isolated DNA sample was used for PCR amplification. The reaction mix was optimized as listed below:

2X PCR Master Mix	: 12.5 µl
Forward Primer (10 µM)	: 0.5µl
Reverse primer (10 µM)	: 0.5 µl
DNA	: 1.5 µl

Nuclease free water	: 10µl
Total volume	: 25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at temperature gradient from 45 °C to 55 °C for one min and extension at 72 °C for 1.30 min. The final extension was carried out at 72 °C for 10 min. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.5.4.2 PCR amplification of RGAs

Once the annealing temperatures were standardized for the primer pair, the DNA samples were subjected to PCR using same reaction mixture as described in section 3.4.4.1. PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the same cycling conditions were set as given in section 3.4.4.1 with an annealing temperature of 45 °C for Orissa Elite and 49 °C for Sree Keerthi. The PCR products were analyzed on agarose gel (1.5%).

3.5.4.3 Purification of PCR product

The purification of PCR amplified product was carried out with Gene JET PCR Purification kit (Fermentas life sciences, UAS). The PCR product was added to equal volume of binding buffer and mixed thoroughly until the PCR product was completely dissolved. Then 800 µl of sample was transferred to the Gene JET PCR Purification column. Following centrifugation at 12,000 *g* for 30 seconds at room temperature, the flow through was discarded and the column was placed back into the same collection tube. To the column, 700 µl of wash buffer was added and was centrifuged at 12,000 *g* for 30 seconds at room temperature. The

flow through was discarded and the column was placed back into the collection tube. The empty column was centrifuged for an additional one minute to completely remove any residual wash buffer present in the column. Then the column was transferred to fresh collection tube and the column was air dried for 20 minutes. The purified PCR product was eluted by adding 30 µl of elution buffer to the centre of the purification column, followed by centrifugation for 1 minute. After elution, the column was discarded and the purified PCR product was stored at -20 °C.

3.6 CLONING AND TRANSFORMATION

The eluted PCR product was cloned and transformed into *E. Coli* DH5α using InsTA Clone PCR Cloning Kit (Fermentas, USA). The recombinant clones obtained were analyzed by colony PCR method. Plasmid DNA was isolated from the recombinant clones and checked for PCR amplification. Recombinant plasmid was sequenced to confirm the presence of RGAs.

The cloning and transformation protocol was performed in three consecutive days.

Day1:

E. coli DH5α cells revived in Luria agar medium (Appendix IV) were used for the transformation procedure.

The ligation mix was prepared with the components as listed below.

Vector pTZ57R/T	:	3 µl
5X ligation buffer	:	6 µl
PCR product	:	4 µl
Nuclease free water	:	16 µl
T4 DNA ligase	:	1 µl

Total volume : 30 µl

After vortexing, the ligation mix was centrifuged for a few seconds and incubated overnight at 4 °C. A control reaction mix was also prepared with 4 µl of control PCR fragment (instead of sample PCR product) and incubated under similar conditions.

Day 2:

To 1.5 ml C-medium (pre-warmed at 37 °C for at least 20 min), a portion of freshly streaked bacterial culture was transferred using inoculation loop and gently mixed to re-suspend the cells. After incubating the medium at 37 °C with shaking for 2 h, the bacterial cells were pelleted by one min centrifugation. The pellet was re-suspended in 300 µl of T solution (Appendix IV) and incubated on ice for 5 min. The cells were again pelleted by centrifugation, re-suspended in 120 µl of T solution and incubated on ice for 5 min.

2.5 µl of ligation mix or control reaction mix was added to new microfuge tubes and chilled on ice for 2 min. 50 µl of prepared cells was added to each tube containing DNA, mixed, incubated on ice for 5 min and plated immediately on pre-warmed LB Ampicillin X gal/IPTG agar plates (Appendix IV). The plates were incubated overnight at 37 °C. Untransformed DH5α cells were plated on an LB Ampicillin X gal/IPTG agar plates to serve as negative control.

Day 3:

The plates were observed for the presence of recombinants. The number of transformants obtained in the sample as well as positive control plates was determined.

3.6.1 Analysis of recombinant clones – colony PCR

The recombinant clones were analyzed for the presence and orientation of the DNA insert by colony PCR using M13 primers (IDT, USA). The PCR reaction mix was formulated as:

M13 forward primer (10 μM)	: 0.6 μl
M13 reverse primer (10 μM)	: 0.6 μl
2X PCR Master Mix	: 10 μl
Nuclease free water	: 8.8 μl
Total volume	: 20 μl

A single white colony was selected and resuspended in the PCR reaction mix. A short strike was made over the culture plate (containing selective medium) in order to save the clone for repropagation. The PCR was performed with an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 30 sec, and extension at 72 °C for one min. A final extension step for 5 min was also given. The colony PCR products were resolved on agarose gel (1.5%).

3.6.2 Plasmid DNA isolation

Recombinant plasmid DNA was isolated using GeneJET Plasmid Miniprep kit (Thermo Scientific, USA). From a freshly streaked selective plate, a single bacterial colony was selected and inoculated in 1.5 ml of LB medium (Appendix IV) supplemented with ampicillin. The tubes were incubated at 37 °C for 12-16 h with shaking. After incubation, the bacterial cells were harvested by centrifugation at 8000 rpm for 2 min at room temperature. The pelleted cells were resuspended in 250 μl of the resuspension solution and vortexed until no cell clumps remains. 250 μl of the lysis solution was then added and mixed thoroughly until solution becomes viscous and slightly clear. 350 μl of the

neutralization solution was added next and mixed immediately. The cell debris was pelleted by centrifugation for 5 min. The supernatant obtained was transferred to a GeneJET spin column and centrifuged for 1 min. The flow-through was discarded and 500 µl of wash solution was added. After centrifuging the column for one min, the flow-through was discarded and the wash procedure was repeated once. An additional centrifugation for one min was done to remove any residual wash solution present in the column. The spin column was then transferred into a new 1.5 ml microfuge tube and air dried. 30 µl of the elution buffer was added to the centre of the column to elute out the plasmid DNA. The column was incubated at room temperature for 2 min, followed by centrifugation for 2 min. A second elution step was carried out with 20 µl of the elution buffer. The purified plasmid obtained was stored at -20°C.

The isolated plasmid DNA was checked on agarose gel (1%). In order to check the presence of insert DNA in the plasmid, a PCR was carried out using RGA primers.

The reaction mix used was:

Plasmid DNA	: 1 µl
Forward primer (10 µM)	: 0.6 µl
Reverse primer (10 µM)	: 0.6 µl
2X PCR Master Mix	: 10 µl
Nuclease free water	: 7.8 µl
Total volume	: 20 µl

The PCR was performed with the same cycling conditions as described in section 3.5.1. The products of PCR amplification were analyzed on agarose gel (1.5%). After confirming the presence of DNA insert by PCR method, the plasmid was send for sequencing.

3.7 DNA SEQUENCING

The isolated plasmids containing the inserts were sequenced (Genetic Analyzer ABI 3500) at the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB).

3.7.1 DNA sequence analysis

The sequence obtained was first edited with BioEdit Sequence Alignment Editor Programme version 7.2.5. The edited sequence was compared to knowm RGA sequences using NCBI BLAST program. Amino acid sequences were deduced using ExPASY translate tool. Phylogenetic tree was constructed from BioEdit aligned sequences using MEGA version 6.06 (Tamura *et al.*, 2013) using Neighbor-Joining method. Robustness of clustering was checked by bootstrapping 1000 replicates. Multiple alignment of amino acid sequences with already available database sequences were performed using CLUSTALX program of BioEdit software (Hall, 1999). A conserved domain database (CDD) search was performed at the at NCBI server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with the obtained sequence for annotations of domains and conserved functional sites on protein sequences.

The sequence information was used to design primers specific to resistant gene analogues of *Dioscorea alata*.

3.8 PRIMER DESIGNING FOR GENE EXPRESSION ANALYSIS USING RT-PCR

Primers were designed based on the obtained DNA sequence information. Primer 3 version 0.4.0 was used to design specific primers.

The designed primers were subjected to *in-silico* analysis for determining specificity and other characteristics. FastPCR programme was used to study the length of primers as well as the PCR product, self complementarity and

possibilities for primer dimer formation, GC content, melting temperatures and feasible annealing temperatures. The primer sequences thus validated by in-silico methods were sent to Sigma Aldrich (USA) for synthesis.

3.9 TOTAL RNA ISOLATION

Leaf samples of pathogen inoculated and control plants were used to isolate RNA. From the leaf samples RNA was isolated using Ambion Purelink RNA Mini Kit. The isolated RNA was stored at -20°C.

3.9.1 Ambion Purelink RNA Mini Kit (Life Technologies, USA)

Leaf sample weighed 100 mg was taken and ground into fine powder with liquid nitrogen using mortar and pestle. The powdered tissue was transferred to an RNase free micro centrifuge tube and 1000 µl of lysis buffer with 10 µl of β-mercaptoethanol was added and vortexed (Labnet Vortex Mixer, USA) briefly. After centrifugation at 20,000 *g* (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 7 min at room temperature, the supernatant was transferred to fresh tube. To the supernatant, added equal volume of 70% ethanol and mixed thoroughly to dissolve the precipitate. Then, 700 µl of sample was transferred to the RNA spin cartridge inserted in the collection tube. Centrifuged at 10,000 *g* for 30 seconds at room temperature and discarded the flow through and reinserted the cartridge into the collection tube. The above step was repeated until the whole sample had been processed. Then 700 µl of wash buffer I was added to the spin catridge and centrifuged at 12,000 *g* for 30 s. The flow through was discarded and the spin cartridge was placed into a clean collection tube provided in the kit. 500 µl of wash buffer II was added to the spin cartridge and centrifuged at 12,000 *g* for 30s at room temperature. The flow through was discarded and cartridge was reinserted and repeated the above step once again. The empty spin column was centrifuged at 12,000 *g* for 1 min at room temperature. The collection tube was discarded the cartridge was inserted into a recovery tube and air dried for 15 mins. Then 30 µl of the elution buffer (RNase free water) was added to the center of

spin cartridge and was incubated at room temperature for 1 min followed by centrifugation at 12,000 *g* for 1 min at room temperature to obtain the first elute of RNA. Again 20 µl of elution buffer was added to the spin cartridge and centrifuged at 12,000 *g* for 1 min to obtain the second elute. The isolated RNA was checked on 1.5 % agarose gel and stored at -20 °C.

3.10 EXPRESSION STUDY OF RGAs IN TOLERANT AND SUSCEPTIBLE VARIETIES

3.10.1 First strand cDNA synthesis

The RNA isolated from control as well as pathogen inoculated leaf samples of susceptible and tolerant varieties were subjected to cDNA conversion using Revert Aid FIRST strand cDNA synthesis kit (Thermo Scientific, USA).

The components of the reaction mix were as follows:

5 X Reaction buffer	: 4 µl
10mM dNTP mix	: 2 µl
Oligo $(dT)_{18}$ primers (100 µM)	: 1 µl
Revert Aid M-MuL V RT (200 U μl^{-1})	: 1 µl
Ribolock RNase inhibitor (200 U μl^{-1})	: 1 µl
Template RNA	: 2 µl
Nuclease free water	: 9 µl
Total volume	: 20 µl

All the reagents were added, mixed by vortexing (Labnet vortex mixer, USA) and flashed down. The cDNA conversion reaction was carried out in Agilent Technologies sure Cycler 8800 (USA). The reaction conditions comprised of a reverse transcription step at 42 °C for 1 hour, followed by an extension step at 72 °C for 5 mins.

3.10.2 Gradient PCR for standardizing annealing temperature

The newly designed primers were used to amplify the RGAs present in susceptible and tolerant varieties of *Dioscorea.*

D. alata DA F1 and DA R1 (20 bases)

DA F1 (Forward primer) sequence : 5'TCCATGCATCTTCTTTCCAA 3'

DA R1 (Reverse primer) sequence : 5'GGGGAAGACGACACTTGCTA 3'

The synthesized primers were diluted to a final concentration of 10 µM with sterile water to obtain the working solution. In order to standardize the annealing temperature of primers, gradient PCR was performed. The cDNA synthesized from RNA of susceptible and resistant varieties were used for PCR amplification.

The reaction mix was optimized as follows:

2X PCR Master Mix (Thermo Scientific, USA)	: 12.5µl
Forward primer (10µM)	: 1µl
Reverse primer (10µM)	: 1µl
cDNA (100 ng μl^{-1})	: 2µl
Nuclease free water	: 8.5 µl
Total volume	: 25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at temperature gradient from 50 - 60 °C for 45 seconds and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo

Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.11 GENE EXPRESSION ANALYSIS BY SEMI-QUANTITATIVE RT-PCR

The cDNA from resistant and susceptible varieties were diluted to a final concentration of 500 ng μl^{-1} with sterile water for both control and test samples of different days. PCR amplification was carried out in Agilent Technologies sure Cycler 8800 (USA) under the same cycling conditions mentioned in section 3.10.2 with annealing temperature of 55 °C. The PCR products were analyzed on agarose gel (1.5%).

Control reactions with reference gene were also kept which included a positive RT-PCR with Actin (ACT 1F and ACT 1R) primers reported in *Dioscorea* which yields a product size of 130 bp (Xiting *et al.*, 2016) with the same cycling conditions mentioned in 3.10.2 with annealing temperature of 55 °C.

The reference gene primers (Actin) used in the present study is as follows:

ACT 1F and ACT 1R (20 bases)

ACT1F (Forward primer) : 5'GGCTGATTGTGCTGTGCTTA 3'

ACT1R (Reverse primer) : 5'CTCACTCCAAGGGTGAAAGC 3'

A negative control reaction was also kept with elongation factor specific primers but with water instead of cDNA as template. Amplicons were electrophoresed on 1.5 % agarose gel. The amplicons showing expected band size (240 bp) were purified by gel elution method and sequenced.

3.11.1 Purification of PCR amplification product by gel elution method

Gel extraction of PCR fragments was achieved with GeneJET Gel Extraction kit (Thermo Scientific, USA). The PCR products were resolved on

agarose gel (1.5 %) and the amplicons were excised from the gel using a clean sharp scalpel. The gel slice was placed into pre-weighted 1.5 ml tubes and its weight was recorded. Then twice the volume of binding buffer was added to the gel slice and it was incubated at 50 - 60 °C for 10 min or till the gel slice was completely dissolved. In order to facilitate the melting process, the contents of the tube were mixed by inversion for every three min. Up to 700 µl of the solubilised gel solution was transferred to GeneJET purification column followed by centrifugation at 14,000 rpm for one min. The flow-through was discarded and the column was placed back into the same collection tube. After addition of 100 µl of binding buffer to the column, it was centrifuged at 14,000 rpm for one min. Again, the flow-through was discarded and the column was placed back into the collection tube. Then 700 µl of wash buffer was added to the column, followed by centrifugation for one min. The flow through was discarded and the empty column was centrifuged for an additional one min to completely remove any residual wash buffer present in the column. After the column was transferred into new collection tube, it was air dried for 10 min. The purified DNA was eluted by adding 50 µl of elution buffer to the centre of the purification column membrane followed by centrifugation for one min at 14,000 rpm. After elution, the column was discarded and the purified DNA was stored at -20°C.

3.11.2 DNA Sequencing

Gel elutes of PCR products were sequenced (Genetic Analyzer ABI 3500) at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB).

3.11.3 DNA sequence analysis

The sequence obtained was first edited with BioEdit Sequence Alignment Editor Programme version 7.2.5. The edited sequence was compared to known RGA sequences using NCBI BLAST programme. BLAST analysis was performed in order to confirm the sequence is that of RGAs.

RESULTS

4. RESULTS

The results of the study entitled "Mining of resistance genes associated with anthracnose infection in greater yam (*Dioscorea alata* Linn.)" was carried out at the division of crop protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016 are presented in this chapter.

4.1 SAMPLE COLLECTION

Explants for nodal culture of *D. alata* as well as leaf samples of Orissa Elite (susceptible variety) and Sree Keerthi (tolerant variety) for DNA isolation were collected from yam growing fields of ICAR-CTCRI. The yam field as well as the disease symptoms observed in field are shown in Plate 1. The susceptible and tolerant varieties used for the study is shown in Plate 2. Only young leaves were used for DNA isolation. First 7 nodes from the tip of *D. alata* vines were used as explants for tissue culture. Tissue culture raised plantlets were used for disease inoculation and resistance gene expression study.

4.2 TISSUE CULTURE OF DIOSCOREA

Nodal culture of *D. alata* varieties Orissa Elite and Sree Keerthi were established on MS media supplemented with growth hormones - IAA (1.5 mg L^{-1}) and kinetin (2 mg L^{-1}). The inoculated tubes were maintained in culture room at 25±1 °C at under 16/8 h photoperiod of 2000 lux light intensity and 60 % relative humidity inside culture room. Fifteen nodes from each variety were inoculated, out of which 12 plants from Orissa Elite and 9 plants of Sree Keerthi were produced respectively.

Shoot induction was observed within 2 weeks of inoculation and root development was observed within 4 weeks. The plants reached a height of 8-10 cm in 3 months with about 8-10 nodes.

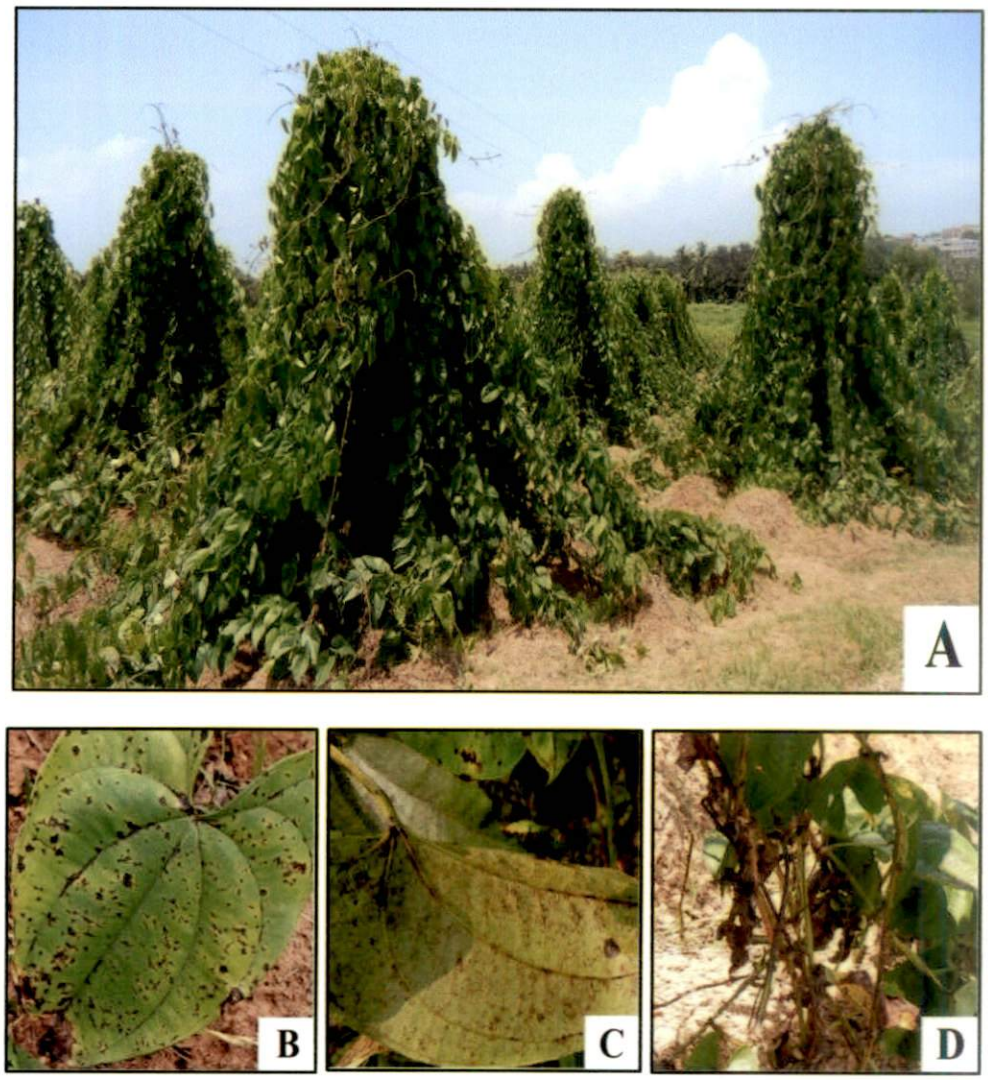

Plate1. Symptoms of anthracnose infection in greater yam. (A) Field view of greater yam. (B) Brown spots on leaf. (C) Necrosis of leaf on ventral side. (D) Die-back symptom of yam vine.

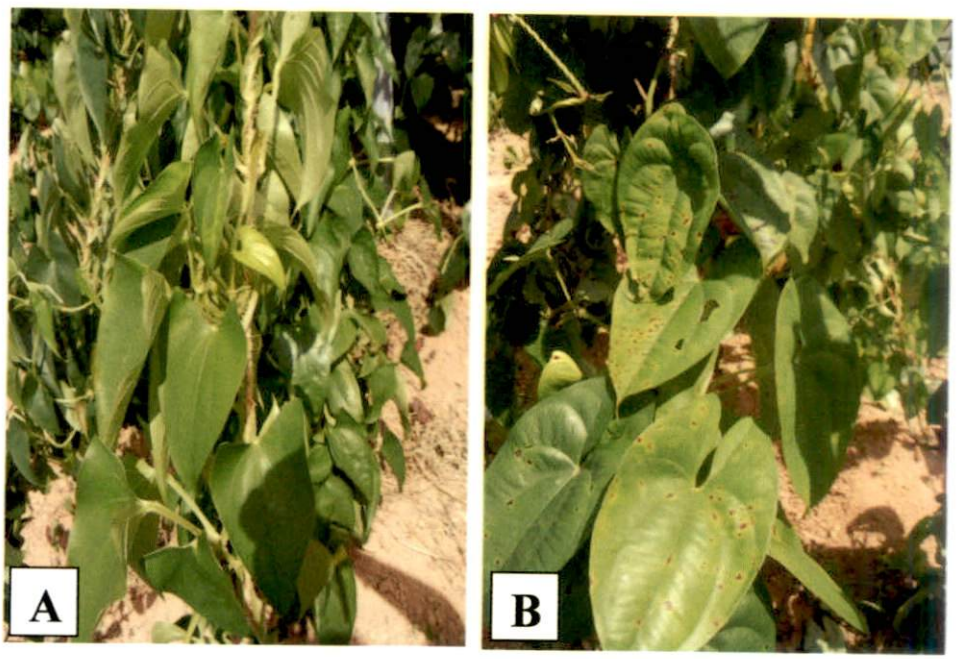

Plate 2. Susceptible and tolerant varieties used for the study. A: Sree Keerthi (tolerant variety) without disease symptoms. B: Orissa Elite (susceptible variety) showing disease symptoms.

4.2.1 Sub culturing of plants

When the plants reached a height of about 10 cm, they are sub- cultured in half-MS media without any hormones. The nodes were excised aseptically and placed in the media. Eight plants of Orissa Elite and six plants from Sree Keerthi were subcultured from which 21 plants of Orissa Elite and 10 plants of Sree Keerthi were produced.

Subcultured plants also showed shoot initiation within 2 weeks and root initiation within 4 weeks of inoculation. Some plants were also found to produce micro-tuber inside the culture tubes.

4.2.2 Hardening of tissue culture raised plants

After five months of subculturing the plants were ready for hardening. The sufficiently rooted plants were removed from culture tube and washed thoroughly to remove adhering media. They were then transplanted to plastic cups containing sterile coir pith and kept inside the growth chamber. During this period, the plants were sprayed with liquid MS medium without agar and sugar. Out of 16 and 7 plants hardened, 13 and 7 survived in the varieties Orissa Elite and Sree Keerthi respectively. Plate 3 shows the different developmental stages of tissue culture plants – Orissa Elite and Sree Keerthi. After one month they were transplanted to bigger pots containing sterile potting mixture (Plate 4). Shoot length increase and increase in leaf area were observed at this stage. Figure 1 shows the graphical representation of number of nodes inoculated, number of plants raised, number of plants subcultured and hardened.

4.3 CULTURING OF *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides, the causal agent of greater yam anthracnose disease was subcultured on potato dextrose agar media. Mycelial growth was observed within 24 hours of inoculation and spore development was found within 7-10 days of culturing. Plate 5 shows culture of *Colletotrichum*

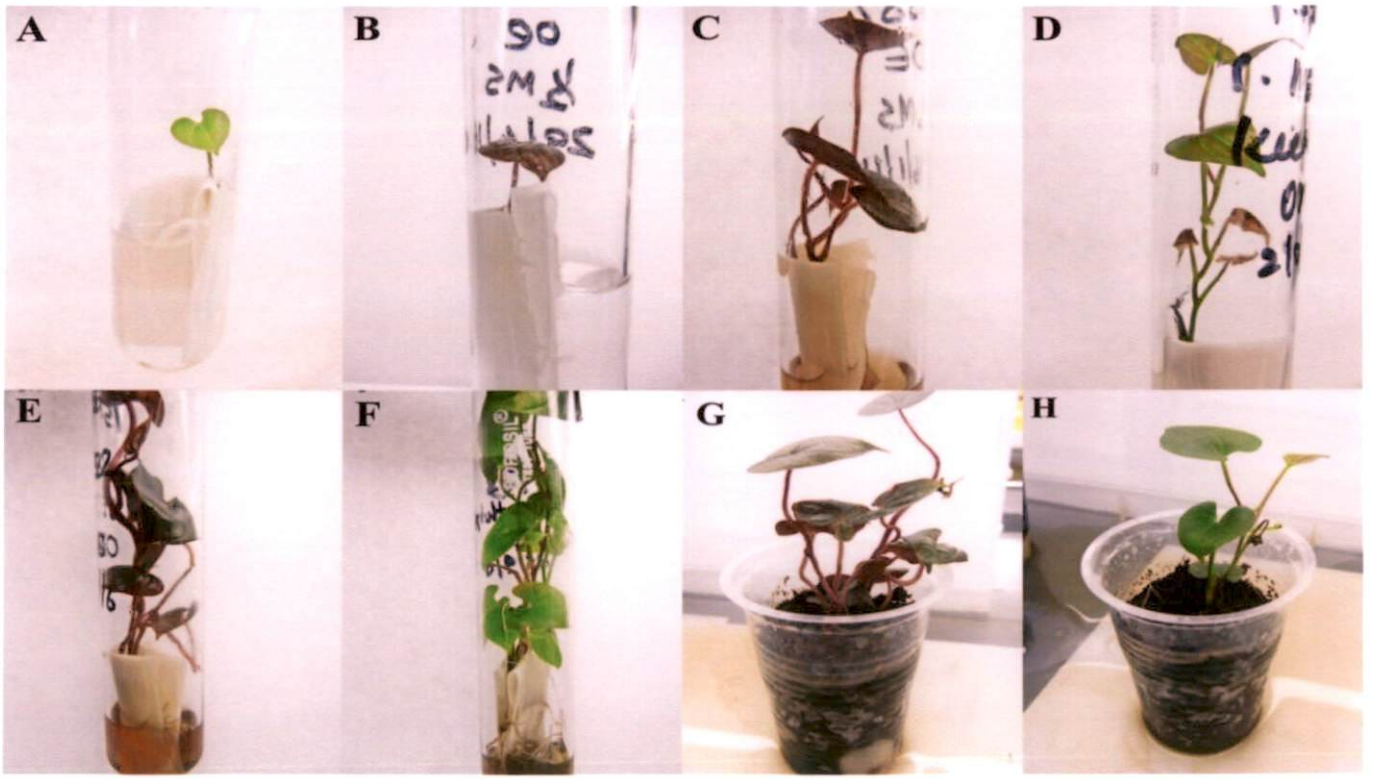

Plate 3. Nodal culture of *D. alata* varieties Sree Keerthi and Orissa Elite: Different growth stages. (A and B) shoot initiation after 1 week of inoculation. (C and D) Shoot and root development after 2 months (E and F). Adequately rooted plants which are ready for hardening. (G and H) Hardened plants in cups containing sterile coirpith.

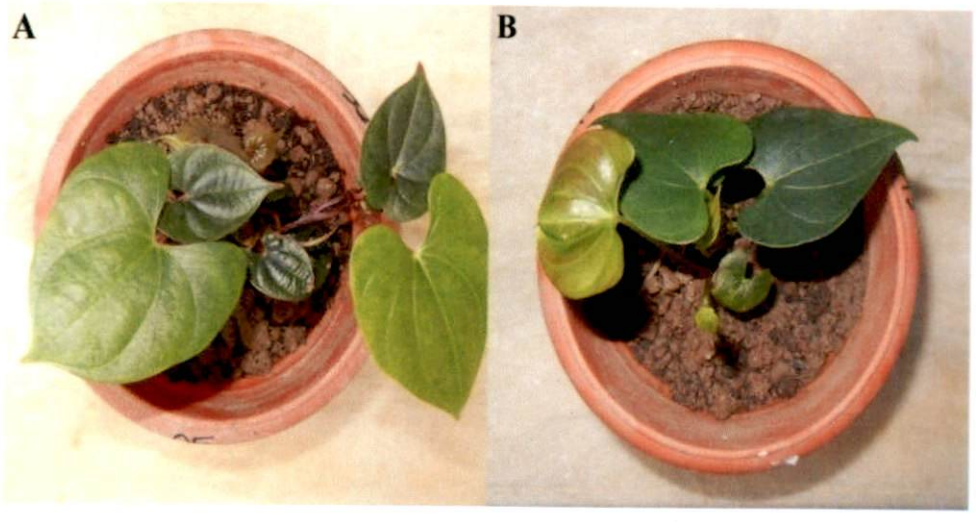

Plate 4. Hardened plants of Orissa Elite (A) and Sree Keerthi (B) in pots containing sterile potting mixture.

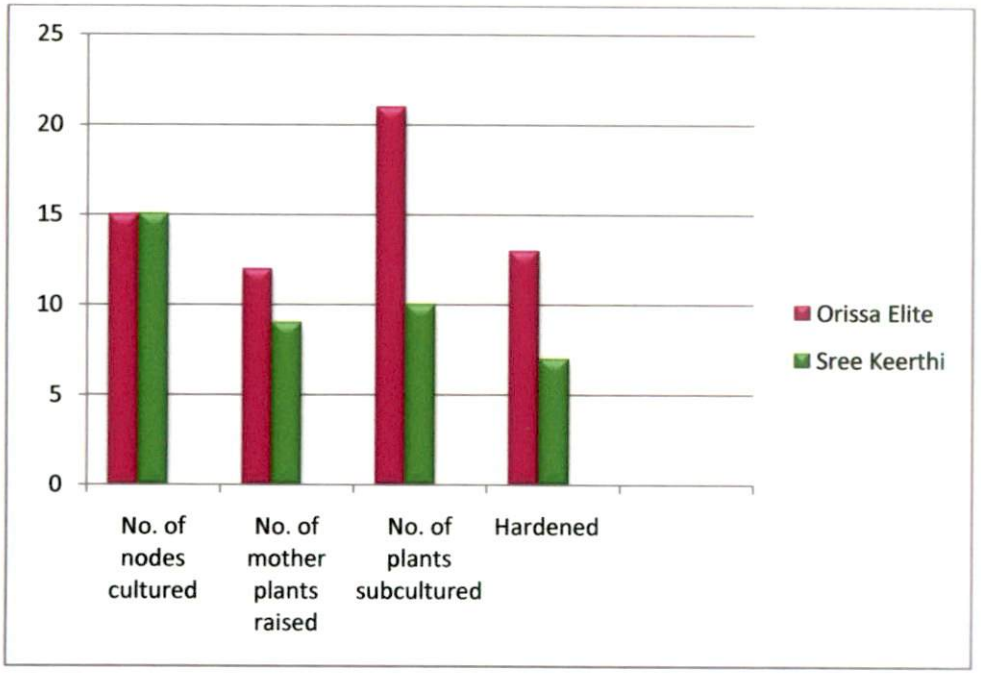

Fig. 1 Details of number of nodes inoculated, tissue culture plants raised, number of plants subcultured and hardened.

gloeosporioides and the spores produced. Subculturing of fungi was done at twelve days interval.

Virulence of culture was maintained by inoculating the culture on fresh young leaves once in every two months and re-isolation of the fungus from infected leaves (Plate 6).

4.3.1 Pathogenecity testing of *C. gloeosporioides* on detached leaves

Pathogenicity of *C. gloeosporioides* to cause infection was tested on detached young leaves of Orissa Elite (susceptible) and Sree Keerthi (tolerant). 25 µl of spore suspension was placed on leaf surface as a drop and incubated at room temperature for 5 days. Symptoms of anthracnose were developed on 3rd day after inoculation (Plate 7). Dark brown spots and necrosis were found in test leaves but the intensity was found to be less in tolerant variety (10-20%) than in the susceptible variety (40-90 %).

4.3.2 Artificial inoculation of tissue culture plants

Disease inoculation using the fungal spore suspensions were carried out in tissue culture raised plants of Orissa Elite (susceptible variety) and Sree Keerthi (tolerant variety). The spore suspension (25µl) was applied on upper and lower surfaces of leaves. Control plants were also kept by applying sterile water instead of spore suspension. These plants were used to isolate RNA and gene expression study. Leaf samples were collected on 1st, 3rd and 5th days after inoculation.

Appearence of first symptom was observed within 24 hours of infection in both susceptible and tolerant varieties. Brown spots appeared in the early infection stage which later developed into tissue necrosis. The symptoms of disease development in the susceptible variety (Plate 8) were more compared to the tolerant variety (Plate 9). On the fifth day of inoculation, damage to the susceptible plant was about 75% and tolerant plant was about 20%. The susceptible variety was completely damaged on the 6th day. Hypersensitive

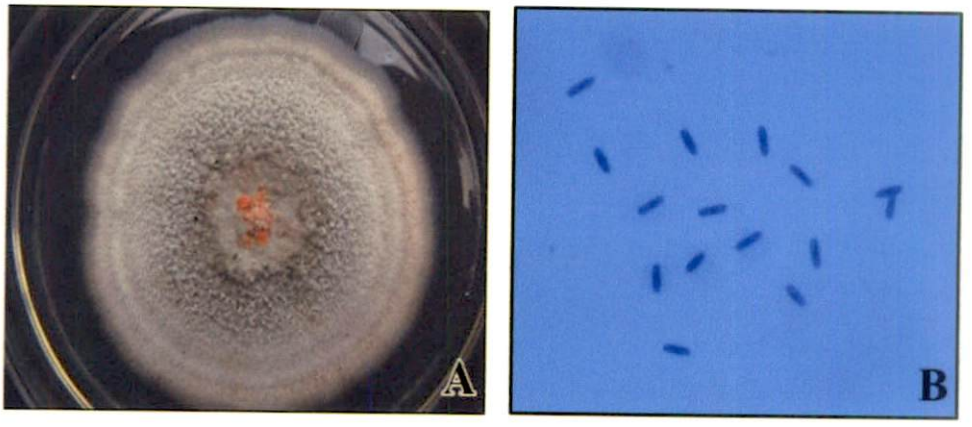

Plate 5. (A) Seven days old culture of *Colletotrichum gloeosporioides* on PDA media. (B) Spores of *C. gloeosporioides* observed under microscope (40X)

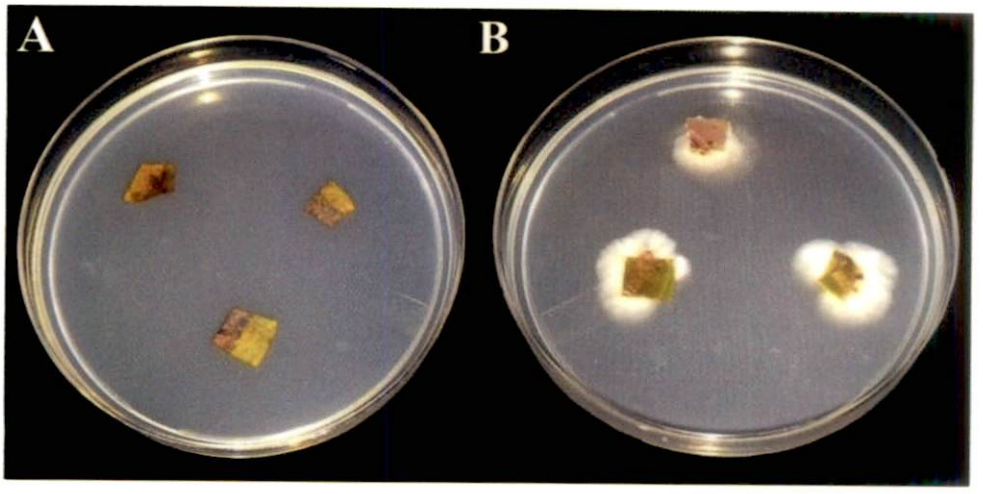

Plate 6. Maintenance of culture virulence by inoculation and re-isolation of fungi from leaf. (A) Disease inoculated leaf placed on PDA. (B) Growth of fungi from infected leaf.

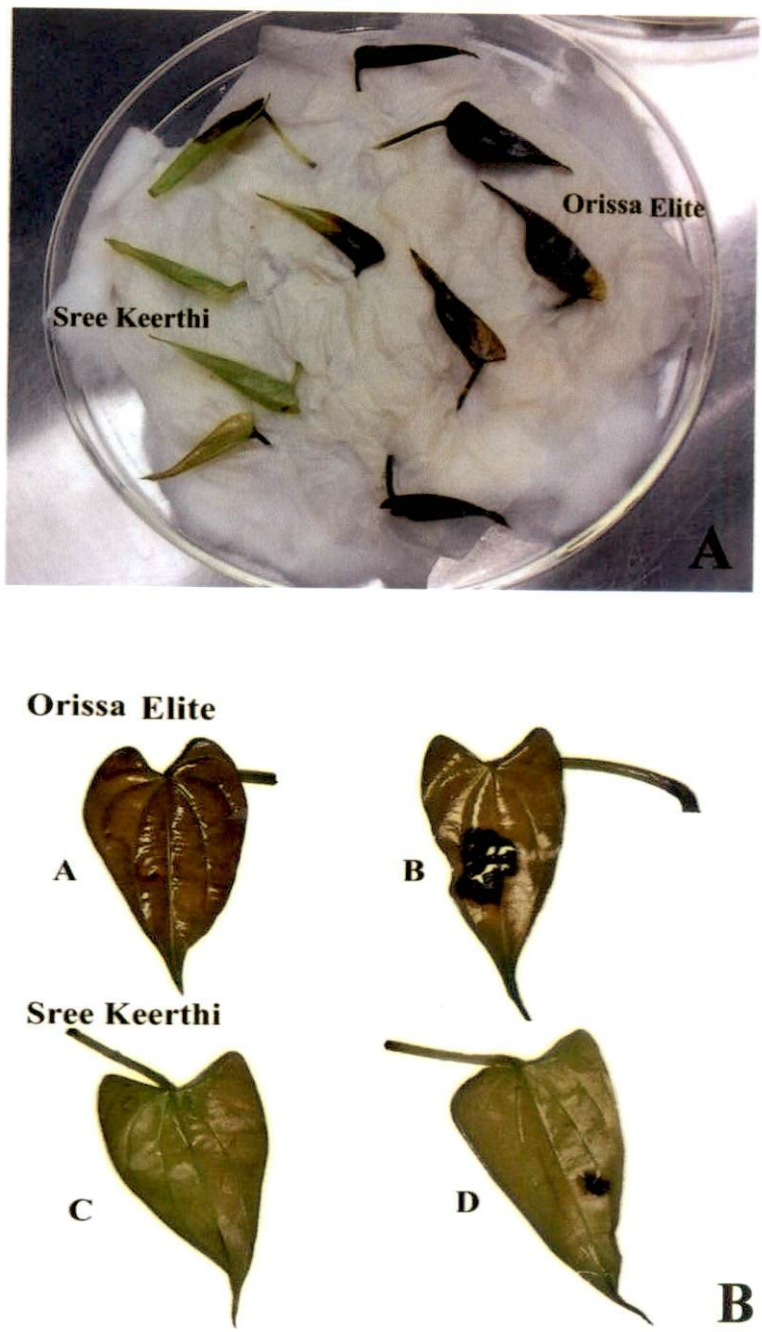

Plate 7. Pathogenecity testing of *C. gloeosporioides* on detached leaves. (A) The completely damaged leaves of Orissa Elite and less damaged leaves of Sree Keerthi on the 3rd day of inoculation. (B) Control and inoculated leaf of Orissa Elite and Sree Keerthi.

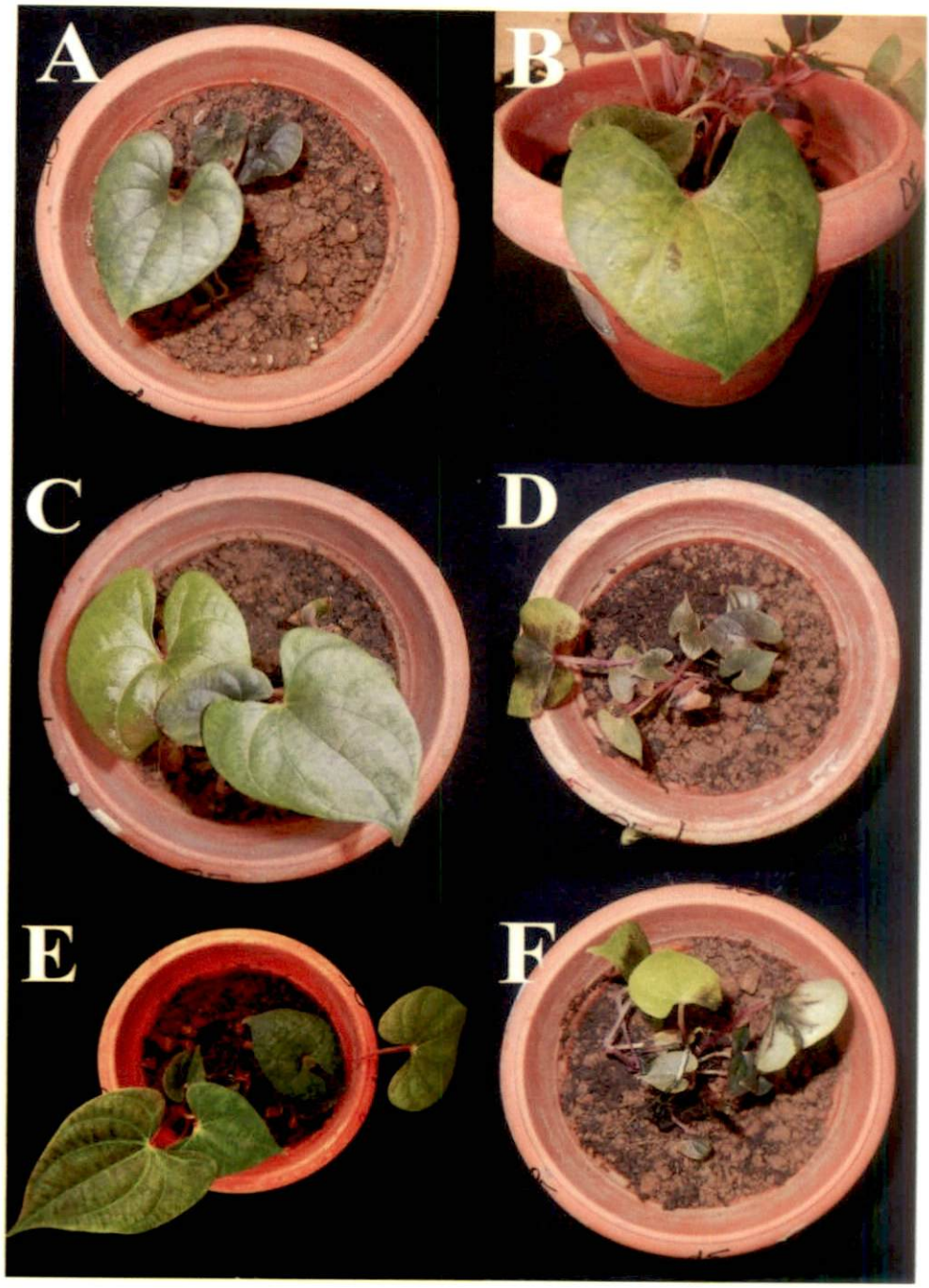

Plate 8. Artificial inoculation of the pathogen on tissue cultured plants, variety Orissa Elite. (A, C and E): Pathogen un-inoculated plants kept as control for RNA isolation on the 1st, 3rd, and 5th day. (B, D and F): Pathogen inoculated plants used as test for RNA isolation on the 1st, 3rd, and 5th day.

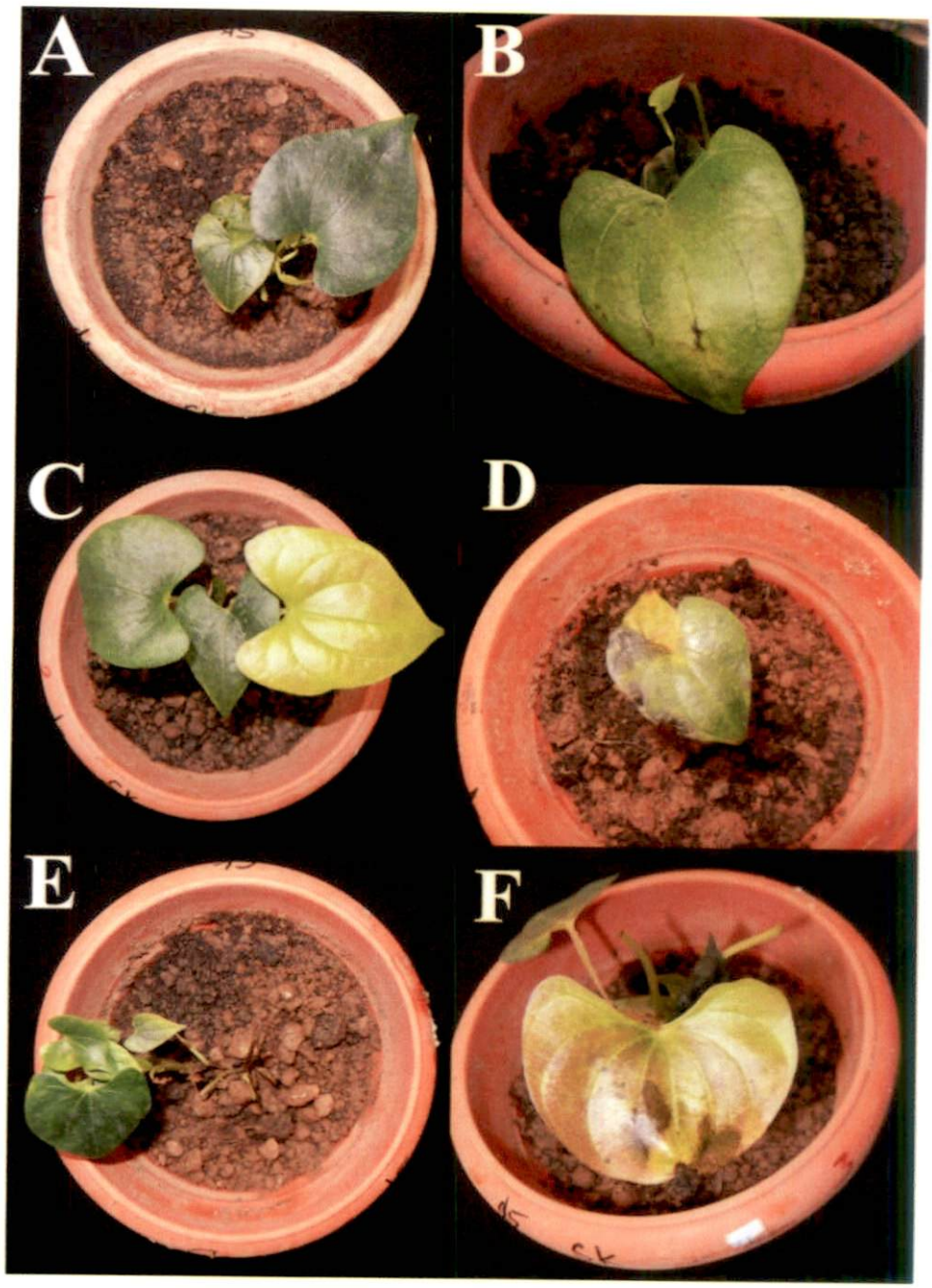

Plate 9. Artificial inoculation of pathogen on tissue culture plants, variety Sree Keerthi. (A, C and E): Pathogen un-inoculated control plants used as control for RNA isolation on the 1st, 3rd, and 5th days. (B, D and F): Pathogen inoculated plants used as test for RNA isolation on the 1st, 3rd, and 5th days.

response was observed in Sree Keerthi on the inoculated leaves, which led to shedding of some leaves.

4.4 NUCLEIC ACID BASED STUDY OF RESISTANCE GENES

4.4.1 Isolation of DNA

CTAB method of DNA isolation was carried out using leaf samples of susceptible (Orissa Elite) and tolerant (Sree Keerthi) varieties collected from yam field of ICAR- Central Tuber Crops Research Institute. The extracted genomic DNA was run on agarose gel (0.8%) and visualized under UV to observe the bands (Plate 10).

The concentration of isolated DNA was estimated using a nanodrop spectrophotometer and results are shown in Table 2.

4.4.2 Gradient PCR for Tm optimization

The annealing temperature of the degenerate primer used for amplification of RGA was standardized by using gradient PCR. Being an important step of PCR, the annealing temperature of primer pair RGA 1F and RGA 1R were optimized by conducting a gradient PCR from 45-55 ^{o}C. The optimum Tm value for better amplification was observed at 45 ^{o}C for Orissa Elite and 49 ^{o}C for Sree Keerthi (Plate 11).

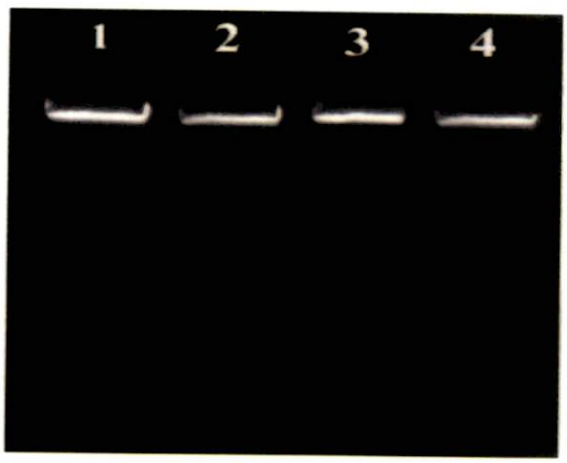

Plate 10. DNA isolated from *D. alata* varieties Orissa Elite and Sree Keerthi. Lane 1 and 2, DNA of Orissa Elite. Lane 3 and 4, DNA of Sree Keerthi.

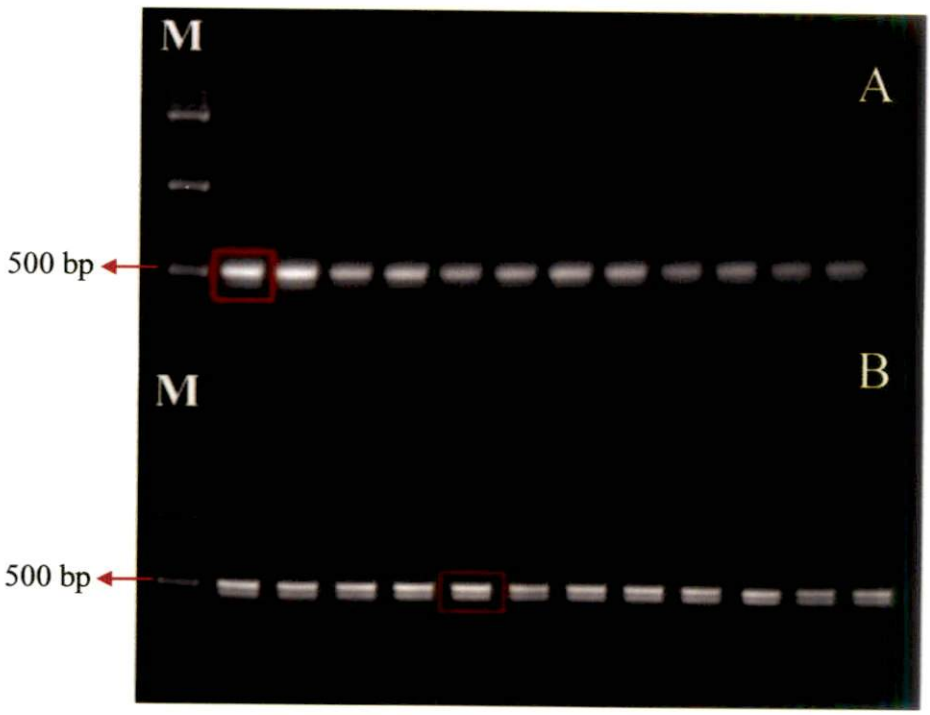

Plate 11. Gradient PCR (45 °C – 55 °C) to optimize annealing temperature. (A) Gradient PCR of Orissa Elite. Optimum Tm = 45 °C. (B) Gradient PCR of Sree Keerthi. Optimum Tm = 49 °C.

Table 2. Yield and absorbance ratio of isolated DNA estimated using nanodrop spectrophotometer.

Sample	Concentration (ng/µl)	A 260/280
DNA 1 (Orissa Elite)	801	1.74
DNA 2 (Orissa Elite)	768	1.85
DNA 3 (Sree Keerthi)	828	1.88
DNA 4 (Sree Keerthi)	740	1.79

4.4.3 PCR amplification of RGAs using degenerate primers

The annealing temperature of degenerate primers, RGA 1F and RGA 1R were standardized as 45 °C for Orissa Elite and 49 °C for Sree Keerthi. PCR amplification with the primers on DNA samples of susceptible and tolerant varieties yielded amplicons of 500 bp size when separated on 1.5 % agarose gel (Plate 12). The PCR conditions were mentioned in Sl. No 3.5.4.1. The PCR product was purified and used for cloning.

4.5 CLONING AND TRANSFORMATION

The purified PCR products were preceded with cloning and transformation for better sequencing result. The purified product was cloned using InsTA Clone PCR Cloning Kit (Fermentas, USA) and transformed into *E.coli* DH5α cells. The transformed colonies were distinguished by blue white screening in LB Ampicillin Xgal IPTG plates (Plate 13). The white recombinant colonies obtained were selected for further analysis using colony PCR with respective primers under appropriate conditions. The expected products of size 500 bp were obtained from most of the colonies analyzed by colony PCR when checked in 1.2 % agarose gel (Plate 14). The plasmid DNA was isolated from the recombinant clones and analyzed on agarose gel of 1.2 % (Plate 15).

4.6 DNA SEQUENCING

The plasmids isolated from recombinant clones were sent to the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB) with their respective primers. The sequencing results were obtained as electopherogram resulting from capillary sequencing in .aib format.

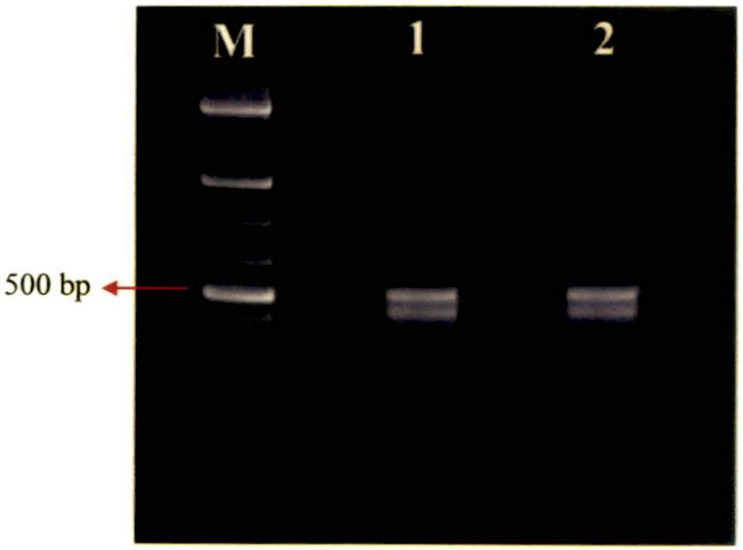

Plate 12. PCR product of Orissa Elite (Lane 1) and Sree Keerthi (Lane 2) using degenerate RGA primers separated on agarose gel. M: 1kb plus DNA marker.

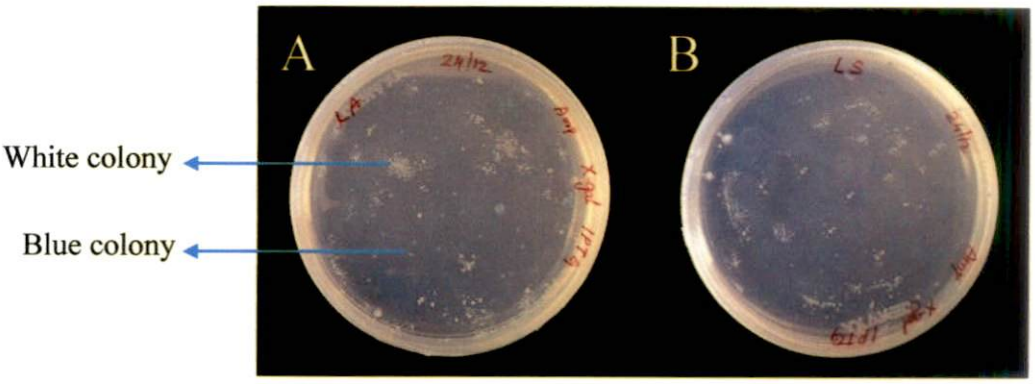

Plate 13. Luria-Bertani agar plate showing white (transformed) and blue (non-transformed) colonies of (A) Orissa Elite (B) Sree Keerthi

Plate 14. Colony PCR result of Sree Keerthi (A) and Orissa Elite (B). M: 1 kb plus DNA marker. Lane 1-9: colony PCR product. Lane 10: kit positive control. Lane 11: Kit negative control

Plate 15. Isolated plasmid DNA from the positive clones selected through colony PCR

The sequence of Orissa Elite (susceptible variety) RGA is given below:

TAGGGCTAGGGGGAGGCCCCCACACTTTTGAACAAGTTGATGAGCATA
ATCAACCAAATATGCTGGGCAACATGTTTCAGTATTTTGATTTGGGAA
GACCTTGCGAAGAAACAACTCCCGACTCTCCCTTTCATCTAAGCAACG
CAATTCATGTGGTTTGATGGTAGGATTTGCAATCTTAGCAACATTTACG
AAGCGAGTGGTGATAATAACTCTGCTTCCATTATTAACATCCGGAAAG
ACTTTTAGTAATTCAGTCCATGCATCTTCTTTCCAAACATCATCAAGAA
CGATCAAGTACTTACCTTTATGCAATTTTTCAGAAATAGCAATTGAGA
AATCTCGGATTGTATTTGCTGATGGAGTAACTGAAATTTCTGATGAGA
TTCCTTTCAAAATCTCAAAGATGGTATATTGTTGAGATATGATAACCCA
TGCAAATATATTAAAACTTCTTTTGACCTCAAGATCATTATAAACAGAT
TTAGCAAGTGTCGTCTTCCCCACCCCCCCA

The sequence of Sree Keerthi (tolerant variety) RGA is given below:

TGGGGGGGTGGGGAAGACGACGCTTGCTCAGAAGAGGTATTCCAAAA
CAATCCACAGGGAGCATCCAAGAGAAAGACTTTCACTCGCCATTACCC
GTGTGGATTTGTGTCTTCTAAGGAGGCAAACGATTTGAGAGTGTTGAA
ATCCATTGTGAAACATTTGGGTGGTAAATATAACAGTGATGACTCTGT
AGGTGAACTTCTGCATGTTCTAGGTCGAGAGGTAGATGGTAAAAAACT
GTTTTTGATTCTTGATGATGTATGGGATTCACATCTTTGGGAGAGAGTG
CTTAAAAAGCCATTGTCTAAGGTTGGTCCTGGCAGTAGAGTTTTGGTC
ACTACCAGAGATGAGGGTGTCACACTGCAAATGGGGGTTGAGTACATT
CACAATGTGCAGGAGTTGTCTGTGAAAGATGGTTGGTCATTGATGTGC
AAGCTAGTGTTCAGTGCCAACGAGGAATGTGATATGCAACAGCTGCAA
GACATTGGTATGCGAATTGTTGAGAAGTGTCATGGCCTCCCCCTAGCC
CTA

4.7 SEQUENCE ANALYSIS

Sequence analysis of the cloned fragments confirmed the presence of RGA in both the susceptible and tolerant varieties with pseudogenes identified in Sree

Keerthi. The sequence results were initially analysed and edited using BioEdit Sequence Alignment Editor Program version 7.2.5 and the obtained sequence was run through the online BLAST (BLASTx) program of NCBI. Through the BLASTx search, the input nucleotide query was translated to amino acid sequence, and the database sequences which were similar to the query were identified.

The BLAST analysis of the sequence of Orissa Elite showed similarity to disease resistance proteins of many plants, of which maximum similarity was with disease resistance protein At1g50180 of *Elaeis guineensis* (Accession no: XP_010913302.1) (Fig. 2, Table 3). BLAST analysis of the sequence of Sree Keerthi showed maximum similarity to disease resistance protein of *Phoenix dactylifera* (Accession no: XP_008779586.1) (Fig. 3, Table 4).

Domain search at NCBI server (http://www.ncbi.nlm.nih.gov /Structure/cdd/wrpsb.cgi) confirmed the presence of NB-ARC domain in both the varieties (Fig. 4 and 5).

Phylogenetic tree was constructed with similar sequences obtained through BLAST analysis. The tree constructed at 1000 bootstrap replicates. Phylogenetic analysis revealed that the amino acid sequences of Orissa Elite shows maximum similarity with the disease resistance protein of *Elaeis guineensis* and Sree Keerthi to disease resistance protein RGA3 of *Phoenix dactylifera* than with the other species used to construct the phylogenetic tree (Fig. 6).

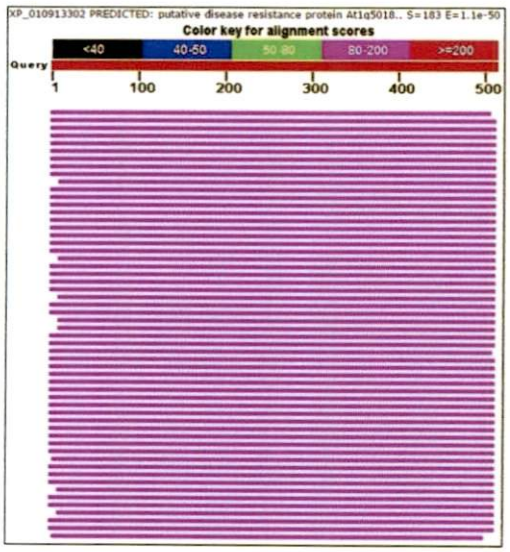

Fig 2. BLAST result of Orissa Elite RGA

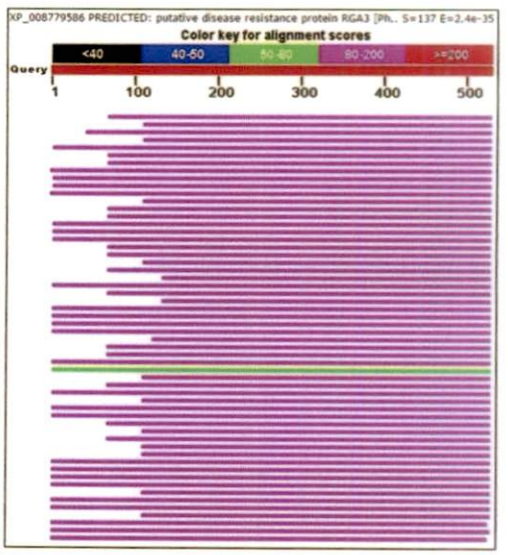

Fig 3. BLAST result of Sree Keerthi RGA

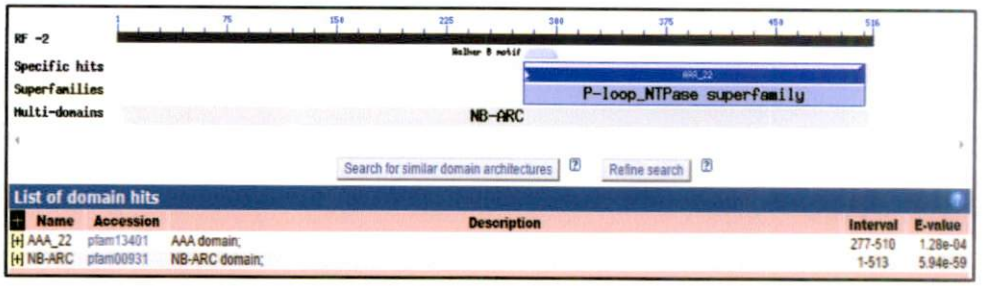

Fig. 4 Conserved domain database (CDD) search result of Orissa Elite showing the NB-ARC domain

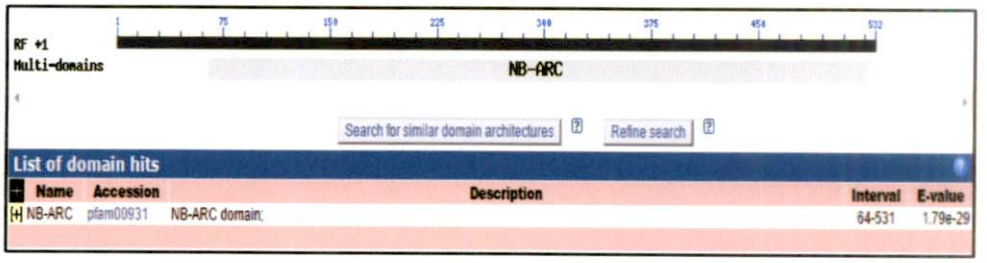

Fig. 5 Conserved domain database (CDD) search result of Sree Keerthi showing the NB-ARC domain

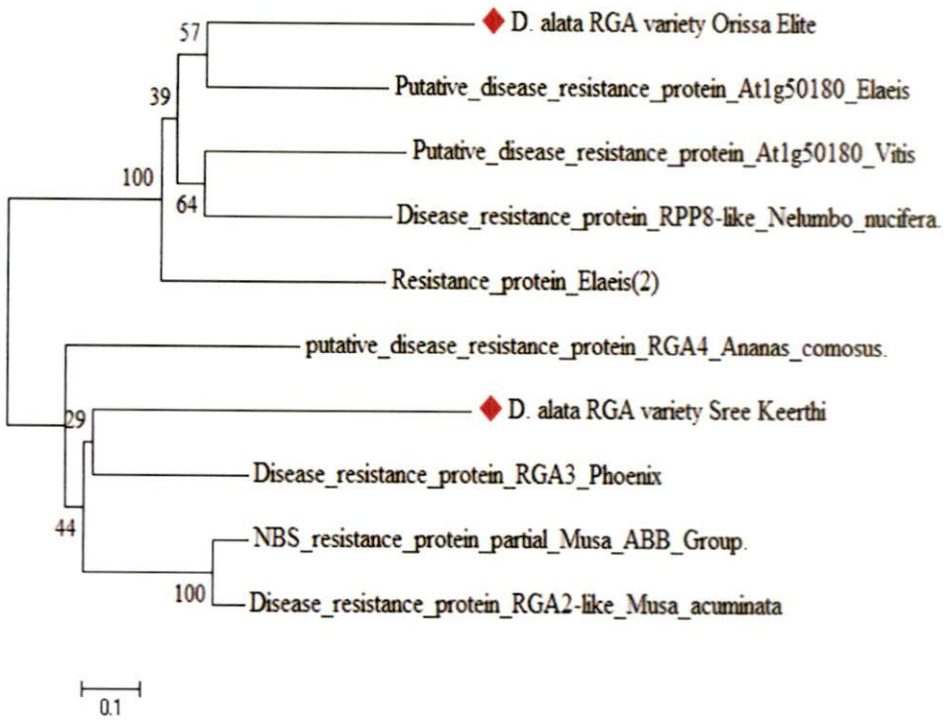

Fig. 6 Phylogenetic tree based on the amino acid sequences of the RGAs of Orissa Elite and Sree Keerthi and other similar plant species available in the NCBI database. The tree was constructed by Neighbor-Joining method using MEGA 6.06. Numbers indicate the percentage support of branching based on bootstrap analysis (1000 replications). The scale bar indicates 0.1 substitutions/site.

Table 3. Amino acid sequence identity between RGA of Orissa Elite and other plant species

Accession No.	Similar species	RGA sequence identity (%)
XP_010913302.1	*Elaeis guineensis*	50
XP_008778490.1	*Phoenix dactylifera*	50
XP_003633890.1	*Vitis vinifera*	44
XP_015578467.1	*Ricinus communis*	44
KDO65577.1	*Citrus sinensis*	41
XP_009764018.1	*Nicotiana sylvestris*	43
AEQ61802.1	*Sesamum indicum*	43
OAY40597.1	*Manihot esculenta*	45
XP_016483410.1	*Nicotiana tabacum*	42

Table 4. Amino acid sequence identity between RGA of Sree Keerthi and other plant species

Accession No.	Similar species	RGA sequence identity (%)
XP_008779586.1	*Phoenix dactylifera*	44
XP_010914651.1	*Elaeis guineensis*	48
CBW30231.1	*Musa balbisiana*	40
XP_009392750.1	*Musa acuminate*	41
OAY78760.1	*Ananas comosus*	43
ABS82598.1	*Dracaena marginata*	45
AJG44202.1	*Cocos nucifera*	46
ABS82600.1	*Sansevieria trifasciata*	42
ADC54141.1	*Cynodon dactylon*	35

Multiple alignment of amino acid sequences with already available database sequences were performed using CLUSTALX program of BioEdit software. Multiple alignment result revealed the presence of conserved resistance nucleotide binding sites (RNBS-B and RNBS-C), kinase-2, P-loop and GLPL motifs (Fig. 7). In this, the P-loop and GLPL motifs were used as the priming sites. In addition, the analysis also showed a tryptophan (W) residue at the end of kinase-2 motif, which is a characteristic feature of non-TIR subclass of NBS-LRR R-genes (Meyers *et al.*, 1999).

4.8 PRIMER DESIGNING

The primers were designed for the amplification of partial resistance gene analogue of *D. alata* varieties Orissa Elite and Sree Keerthi based on the most favourable combination of conserved regions in the multiple aligned nucleotides. *D. alata* resistance gene analogue specific primers were synthesized from Sigma Aldrich (USA).

The analysis of primers using FastPCR programme revealed good GC content and annealing temperature, and also the designed primers did not exhibit any hairpin formation and 3' complementarity.

The synthesized primer pair is as follows:

DA F1 (Foreward primer) sequence : 5'TCCATGCATCTTCTTTCCAA 3'

DA R1 (Reverse primer) sequence : 5'GGGGAAGACGACACTTGCTA 3'

4.8.1 Gradient PCR for Tm optimization

After the synthesis of primers, the annealing temperature was standardized by using gradient PCR. Being an important step of PCR, the annealing temperature of the primer pair was optimized by conducting a gradient PCR from 50-60 ^{o}C, and the optimum Tm value for better amplification was observed at 55 ^{o}C (Plate 16).

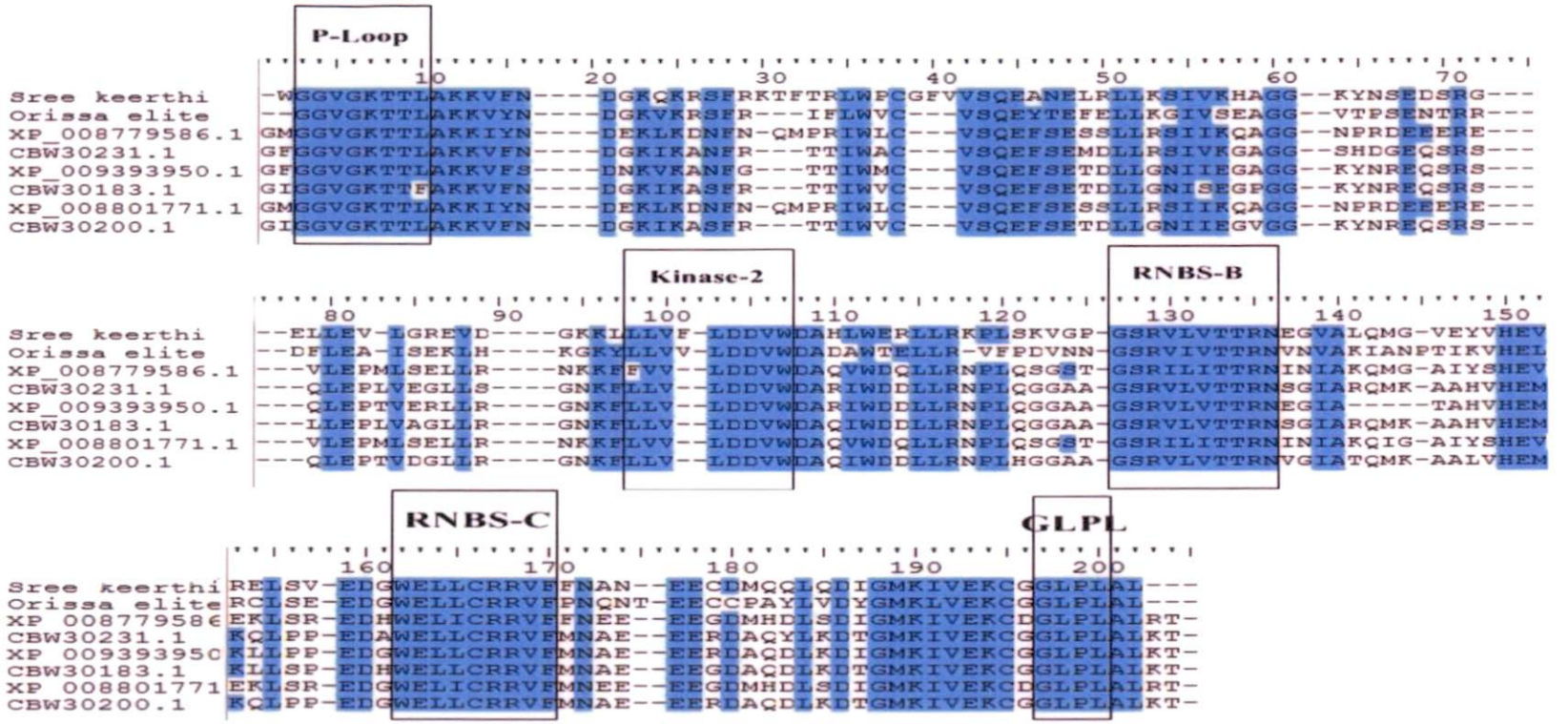

Fig 7. Alignment of amino acid consensus sequences of RGAs of Orissa Elite and Sree Keerthi with similar sequences available in the NCBI database. Conserved regions are highlighted in blue. Conserved domains of Kinase-2, R-gene specific nucleotide binding sites (RNBS-B and RNBS-C), P-Loop and GLPL are boxed. P-Loop and GLPL are the priming sites.

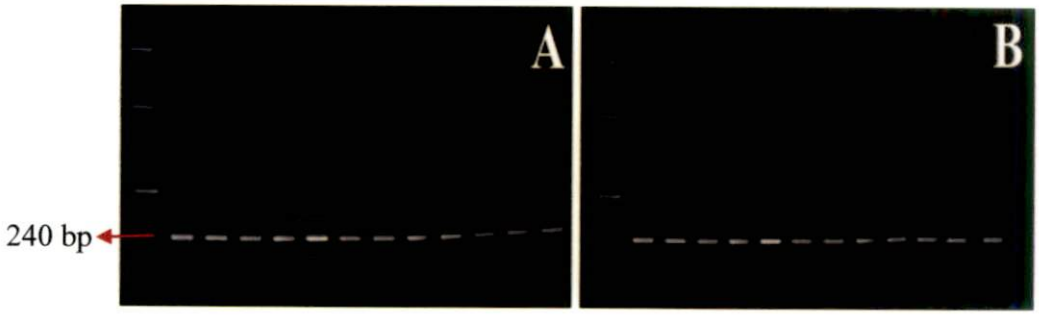

Plate 16. Gradient PCR (52 °C – 62 °C) to optimize annealing temperature. (A) Gradient PCR of Orissa Elite. (B) Gradient PCR of Sree Keerthi. Optimum Tm for both varieties = 55 °C.

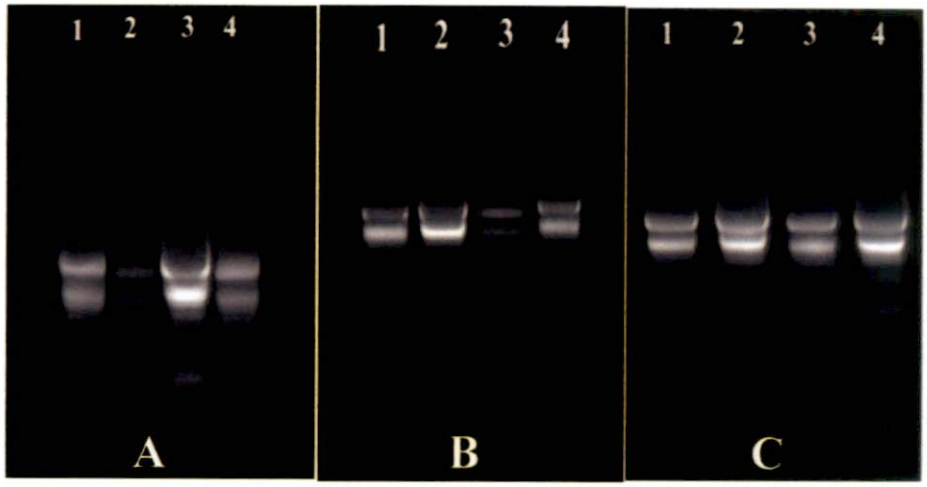

Plate 17. Total RNA isolated on 1st day (A), 3rd day (B) and 5th day (C) after inoculation. Lane 1: RNA from control plant of Orissa elite, Lane 2: RNA from control plant of Sree Keerthi, Lane 3: RNA from pathogen inoculated plant of Orissa Elite, Lane 4: RNA from pathogen inoculated plant of Sree Keerthi.

4.9 ISOLATION OF TOTAL RNA

RNA was isolated from pathogen inoculated and uninoculated plants of both the varieties were carried out using Ambion Purelink RNA Mini kit. Leaf samples of both the varieties collected on 1st, 3rd and 5th days of inoculation from pathogen inoculated and control plants were used to isolate RNA. The isolated RNA was resolved on agarose gel (1.2 %) and two distinct RNA bands were observed which showed no apparent RNA degradation (Plate 17). The concentration and purity of isolated RNA on different days was analysed using nanodrop spectrophotometer (Table 5).

4.10 EXPRESSION STUDY OF R-GENE IN TOLERANT AND SUSCEPTIBLE VARIETIES

cDNA was prepared from the isolated RNA samples of pathogen inoculated and control plants as mentioned in section 3.10.1. The cDNA samples were measured and adjusted to a final concentration of 500 ng μl^{-1} and was used for expression study through semi-quantitative reverse transcriptase PCR. The RGA present in the control and test plants of susceptible and tolerant varieties were targeted using the newly designed specific primers DA F1 and DA R1. The primer pair amplified the RGA present in both the varieties and yielded amplicons of size 240 bp in both the varieties when separated on 1.5 % agarose gel (Plate 18). Positive control reactions were also kept, which included a RT-PCR of the cDNA samples with primers ACT 1F and ACT 1R (reference gene actin), which yielded products of size 130 bp. A negative control reaction was also kept with all the reaction components but with water instead of cDNA template. Plate 19 shows the positive and negative controls with reference gene actin.

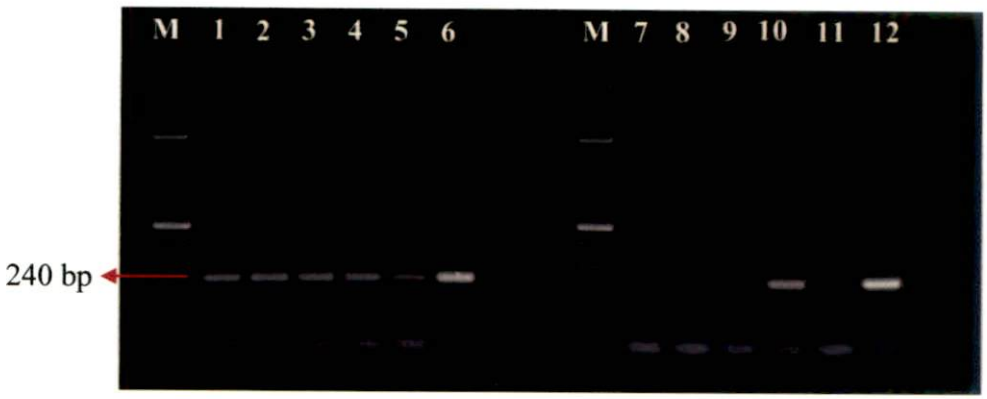

Plate 18. Semi quantitative RT-PCR of Orissa Elite and Sree Keerthi on 1st, 3rd, and 5th days after inoculation. M: 1 kb plus DNA marker. 1, 3 and 5: Control plant samples from 1st, 3rd, and 5th day from Orissa Elite. 2, 4 and 6: Pathogen inoculated test plant samples on 1st, 3rd, and 5th day from Orissa Elite. 7, 9 and 11: Control plant samples from 1st, 3rd, and 5th day from Sree Keerthi. 8, 10 and 12: Pathogen inoculated test plant samples on 1st, 3rd, and 5th day from Sree Keerthi.

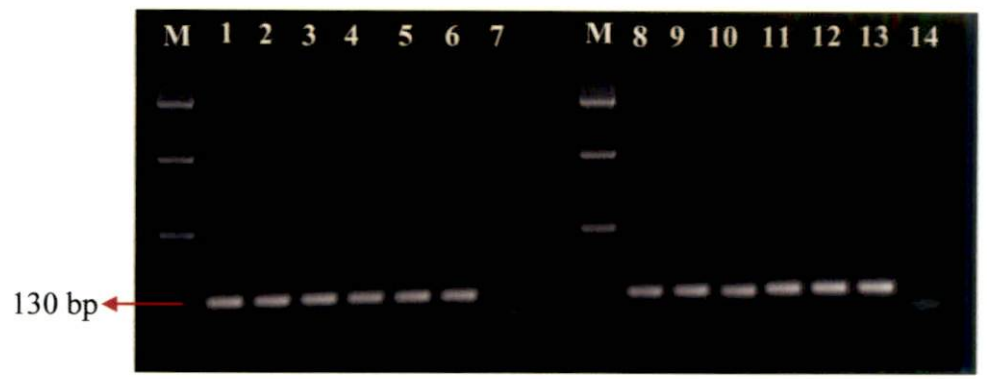

Plate 19. Reference gene (Actin) used for semi quantitative RT-PCR. M: 1kb plus DNA marker. 1, 3 and 5: Control plant samples from 1st, 3rd, and 5th day from Orissa Elite. 2, 4 and 6: Pathogen inoculated test plant samples on 1st, 3rd, and 5th day from Orissa Elite.. 8, 10 and 12: Control plant samples from 1st, 3rd, and 5th day from Sree Keerthi. 9, 11 and 13: Pathogen inoculated test plant samples on 1st, 3rd, and 5th day from Sree Keerthi. 7 and 14: Negative control

Table 5. Concentration and absorbance ratios of isolated RNA on different days

Day	Sample	RNA yield (ng/µl)	A 260/280	A 260/230
Day 1	OE C	1015	2.12	2.03
	OE T	1114	2.09	2.2
	SK C	985	1.98	2.16
	SK T	1099	2.0	1.98
Day 3	OE C	1034	2.14	2.05
	OE T	974	1.89	2.11
	SK C	986	2.0	2.08
	SK T	1091	2.13	2.13
Day 5	OE C	1138	2.0	1.97
	OE T	1256	2.0	2.0
	SK C	1062	2.1	2.06
	SK T	1055	1.96	2.16

*OE C – Control plant RNA of Orissa Elite; OE T – Inoculated test plant RNA of Orissa Elite; SK C – Control plant RNA of Sree Keerthi; SK T – Inoculated test plant RNA of Sree Keerthi

On analyzing the expression pattern of both the varieties, it was found that, the control plants also showed RGA expression. On analyzing the gene expression of pathogen inoculated test plants samples, it was observed that, the tolerant plant (Sree Keerthi) showed increased expression from the 3rd day following pathogen inoculation. Increase in gene expression was found in the susceptible variety (Orissa Elite) only on the 5th day after pathogen inoculation.

The expression profile of reference gene was analyzed in control and test samples of both the varieties. The reference gene showed stable expression pattern in control and test plants of both the varieties and is not altered by the experimental condition. The negative control reaction did not produce any bands when resolved on agarose gel.

4.10.1 Sequencing

A 240 bp amplification product was excised out and gel eluted to obtain the purified product. This was sent to the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB) with their respective primers for sequencing. The sequencing results were obtained as electopherogram resulting from capillary sequencing in .aib format. The obtained sequencing results are as follows:

The RGA sequence of the 240 bp amplification product is as follows:

CAACCAGGTCTTGCCTTTCTTCATTTTTCAGAACATTTACTGAGAGGGT
TTGGATTGTATCTTCTGATGAAGTTACTGAAATTTCTGATGAGATTCCC
TTCAAAATCTCAAGGATGGTATATTGTTGAGATATGATTACCCATGCA
AATATATTAAAACTTCTCTTGACTTCAAGATCATTATAAACAGATTTAG
CAAGTGTCGTCTTCCCCCCCA

3.10.2 Sequence analysis

Analysis of the sequence information confirmed the amplification products are that of RGA, The sequencing results were initially analysed using BioEdit Sequence Alignment Editor program version 7.2.5 and the obtained sequence was run through the online BLAST x program of NCBI.

BLAST analysis of the sequence showed similarity with resistance protein of many plant varieties with maximum similarity with *Phoenix dactylifera* (Accession no: XP_008778490.1) (Fig. 8). Dioscorea RGA sequence also showed similarity with disease resistance proteins of other plants like *Vitis vinifera*, *Ricinus communis*, *Solanum demissum*, *Hevea brasillensis* etc. (Table 6).

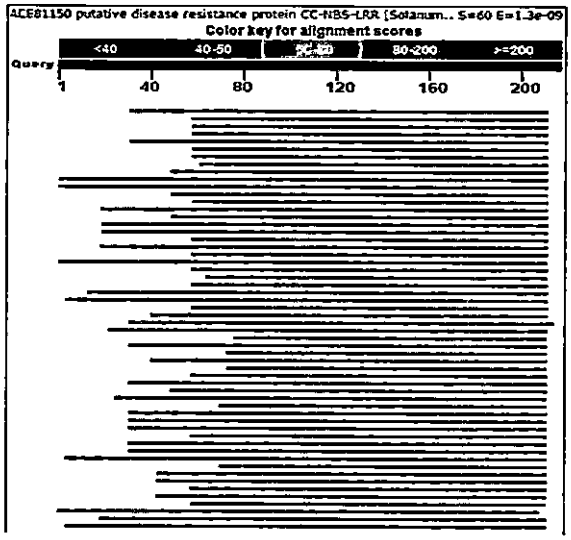

Fig. 8 BLAST analysis of *D. alata* RGA using newly designed primers

Table 6. Amino acid sequence identity between RGA of *Dioscorea* obtained through PCR with newly designed primers and other plant species

Accession No.	Similar species	RGA sequence identity (%)
XP_008778490.1	*Phoenix dactylifera*	54
CBI38087.3	*Vitis vinifera*	51
AID55054.1	*Solanum habrochaites*	45
XP_002524222.2	*Ricinus communis*	45
ACV85787.1	*Hevea brasiliensis*	44
ACE81150.1	*Solanum demissum*	43
ERN19243.1	*Amborella trichopoda*	45
AAZ07910.1	*Ipomoea batatas*	47
XP_006340276.1	*Solanum tuberosum*	49

DISCUSSION

5. DISCUSSION

Tropical root and tuber crops are among the most consumed foods after cereals. Tuber crops occupy an important place in the dietary habits of underprivileged and marginal regions especially during food scarcity. India has a rich genetic diversity of root and tuber crops particularly yams (Hahn, 1995). Anthracnose or die back disease is a serious foliar epiphytotic disease of *D. alata* worldwide resulting in severe yield losses. The causal agent of the anthracnose is an ascomycete fungus, *Colletotrichum gloeosporioides* (Ayodele, *et al.*, 2000). Different isolates of *Colletotrichum* cause considerable damage in a variety of crops such as coffee, cereals, fruits, vegetables, legumes and even grasses (Bailey and Jeger, 1992). Taking into account, the economic and medicinal value of *Dioscorea*, it is high time to develop effective anthracnose management strategies. Exploiting the natural host plant resistance is possibly the most promising and durable strategy to mitigate anthracnose disease.

Most plant-pathogen interactions involve the presence of resistance (R) genes that facilitate plants to identify pathogens and trigger defence response (Grant *et al.*, 1998). Resistance gene analogues (RGAs) are a group of potential R-genes with conserved motifs and structural similarities. Understanding of sequences of R genes as well as the genomic location and organisation, will contribute to study mechanisms underlying plant resistance to pathogens. This will be of great value in breeding crop improvement for disease resistance. Considering this point, the present study was undertaken to isolate the resistance gene analogues (RGAs) in *D. alata* and to study the difference in expression of RGAs during anthracnose infection in susceptible (Orissa Elite) and tolerant (Sree Keerthi) varieties. In this study, the RGAs were identified in greater yam utilizing two methodologies: PCR amplification with degenerate primers and database mining.

PCR approaches with degenerate primers designed from the NBS region of already reported disease resistance genes from other species has led to the cloning of many R-gene-like sequences in several other crop species (Kanazin *et*

al. 1996; Leister *et al.* 1996; Yu *et al.* 1996). This study is the first attempt for PCR-based isolation of Rgene-like sequences (RGAs) from *Dioscorea*. The primers reported by Aswati and Thomas in 2007 are used for RGA amplification. The most key factors in degenerate oligo-primed PCR are the design of the primers and the optimization of PCR conditions. The primers should be designed to regions with minimal degeneracy in codon usage (Compton, 1990). The primer pair used in this study targets the P-Loop and GLPL conserved domains of NBS-LRR gene.

The detection of conserved motifs within the NBS-LRR type resistance genes helps in the use of PCR based approach of isolating and cloning *R* genes using degenerate or specific primers. This method offers an alternative to the conventional methods of map-based cloning and transposon tagging (Seah *et al.*, 1998). In this study, the PCR amplification produced products of 500 bp size, which were cloned and sequenced. The amino acid sequences of RGAs cloned in the present investigation were compared with other known *R* genes in the database which confirmed the presence with highest similarity to *Elaeis guineensis* (Accession no: XP_010913302.1) for Orissa Elite and *Phoenix dactylifera* (Accession no: XP_008779586.1) for Sree Keerthi.

Amino acid sequence alignment using BioEdit software revealed that the *Dioscorea* RGAs are homologous to NBS regions of well-characterized R genes from other plant species. The terminal amino acid residue present at the end of kinase-2 motif of RGAs can be used to predict with 95% precision whether the R-gene belong to the TIR or non-TIR subclass of NBS–LRR genes (Meyers, *et al.*, 1999). A tryptophan residue (W) present at the terminus of kinase-2 motif is distinguishing feature of the non-TIR subclass of NBS-LRR sequences. Presence of aspartic acid (D) or asparagine (N) residue represents the TIR NBS–LRR sequences. With the help of this information, it was identified that the *Dioscorea* RGAs belongs to the non-TIR NBS–LRR subclass. No TIR-type RGA sequences were identified from any of the *Dioscorea* RGAs studied. This is in concord with the previous reports on RGAs suggesting the lack of non TIR subclass of NBS-

LRR genes in monocotyledons (Pan *et al.*, 2000; Meyers *et al.*, 1999; Cannon *et al.*, 2002). Meyers *et al.* (2003) reported that TIR proteins vary notably at the N-terminus. Conserved motifs in the NBS domains are also associated with these subclasses. TIR- and non-TIR NBS-LRR lineages are believed to have ancient evolutionary relations.

Multiple stop codons were identified in the amplified RGA sequence of Sree Keerthi variety, suggests them to be non-functional genes or pseudogenes but it was also included for further analysis regardless of the presence of stop codons or frame shift mutations observed during sequence translation. In a related study on *Pinus monticola*, 50% of the RGAs cloned were identified to be pseudogenes (Liu and Ekramoddoullah, 2003). Similar non-coding sequences were also reported in the paralogs of R genes *Cf9*, *Xa21*, *Dm3* and *Pto* (McDowell *et al.*, 1998; Song *et al.*, 1997) using PCR based approach (Aarts *et al.*, 1998; Vicente and King, 2001; Kanazin *et al.*, 1996; Leister *et al.*, 1996). Discovery of pseudogenes in plant R genes suggests their involvement in advancement of noval specificities by recombination and gene manipulations (Michelmore and Meyers, 1998). Pseudogenes are also known as *poto*genes (Brosius and Gould, 1992) because they have the capability for arising into new functional genes. As suggested by Stahl *et al.* (1999) and Michelmore and Meyers (1998), R-genes need not be under rapid evolution in order to keep in pace with changes in the pathogen. But evolution occurs fairly slowly to offer resistance against pathogen populations which are heterogeneous in space and time.

In many plants studied so far, NBS-LRR genes are proved to be exclusively devoted to defence responses against pathogens. But, the expression levels of RGAs are still under discussion owing to their low transcript levels (Graham *et al.*, 2002). Only few reports are available in the literatures regarding R-gene expression. The available data on R-gene expression demonstrates that they are expressed comparatively at low levels before pathogen attack (Hulbert *et al.*, 2001; Hammond-Kosack and Jones, 1997) but are induced or up-regulated following pathogen challenge (Wang *et al.*, 1999; Yoshimura *et al.*, 1998).

In this study, the difference in gene expression in a highly tolerant *Dioscorea* cultivar, Sree Keerthi, was compared with that present in a susceptible cultivar, Orissa Elite. Sterile *in vitro* raised whole plants were used for the assays, in contrast to similar studies where tubers, detached leaves, or non-sterile plants were used (Collinge and Boller, 2001; Beyer *et al.*, 2001; Wang *et al.*, 2005; Tian *et al.*, 2006). Sterile conditions were maintained in order to avoid interactions with other organisms (beneficial/pathogenic). The plants were also taken care, to avoid wound responses not coupled with the infection process. These are taken into consideration as many pathogenesis related or defense genes are also induced following abiotic stresses similar to wounding (Vignutelli *et al.*, 1998; Bertini *et al.*, 2003). In the present investigation *in vitro* micro-propagation of *D. alata* was carried out using nodal segment as explants. MS media supplemented with IAA and kinetin was used for *in vitro* multiplication. In this work, *D. alata* was multiplied in liquid MS medium. Similarly method of *in vitro* propagation of yam species was tried using solid MS medium by Martine and Cappadocia (1992).

To study the difference in expression of RGA in susceptible and tolerant varieties of *Dioscorea,* a Semi-Quantitative RT-PCR was performed with pathogen un-inoculated control plants and pathogen inoculated test plants raised through tissue culture. RNA was isolated on the 1st, 3rd, and 5th day following pathogen inoculation and was used for the gene expression study. The sequence information was used to design specific primers for RT-PCR using Primer3 online tool. Each reverse transcription product was normalized prior to co-amplification as the yields of the reverse transcription product may vary. For each sample studied, efficiency of reverse transcription was first evaluated by amplifying the housekeeping (reference) gene. Specifically designed RGA primers were used along with actin primers already reported which serves as a reference gene during PCR amplification. Specific primers that were designed from sequence information were used to amplify *D. alata* RGA which yielded 240 bp amplicons. The actin primers used yielded 130 bp amplicon on PCR amplification.

The expression levels of the isolated genes were studied relatively to the constitutively expressed actin gene. Results revealed that RGA was expressed in both healthy and infected plants. The RGA expression was found to be increased in the infected plants compared to healthy plants. This indicates an up-regulation of gene expression following fungal infection. The induction of RGA up-regulation was observed in the 3rd day of infection in Sree Keerthi, whereas, it was observed in Orissa Elite only on the 5th day of infection. It may be one of the reasons which make Sree Keerthi tolerant to anthracnose disease. The raise in gene expression in inoculated plants confirms that the RGA expression is induced due to the interaction of *Colletotrichum* with *Dioscorea*. A similar expression pattern was observed in the up-regulation of RGAs in chickpea in early response to *Fusarium* wilt (Gutierrez *et al.*, 2012) which is in agreement to our results. Thus the results obtained indicates that RGAs are present in *D. alata* varieties Orissa Elite and Sree Keerthi and their expression was induced and increased due to *Colletotrichum* infection. The increase in transcript level observed in the third day of pathogen inoculation on Sree Keerthi seems to indicate their role in early pathogen recognition, thereby makes it tolerant.

R-gene expression studies in other species have also confirmed their constitutive expression at low levels, prior to pathogen attack (Hulbert *et al.*, 2001) whereas, they are induced subsequent to pathogen attack as in the case of tobacco (Yoshimura *et al.*, 1998). Many RGAs were found to be upragulated in tobacco following infection by TMV and *Phytophthora* (Gao *et al.*, 2009). The current investigation showed that the RGA expression levels of *Dioscorea* RGAs were increased following challenge inoculation with *Colletotrichum*, indicating their functional role in the defence response against anthracnose. Thus, the identification of *Dioscorea* RGAs helps providing basic knowledge for further elucidating the molecular mechanism of anthracnose resistance in *Dioscorea*.

Development and Use of resistant cultivars are the most successful and economic approach against anthracnose. Hence, the discovery of noval R-genes is one among the top priorities in crop improvement for disease management. Plant-

pathogen interaction involves activation of several signal transduction events resulting in an array of defence responses against pathogens, thereby enabling the plant to overcome additional pathogen challenges (Anil *et al.*, 2014) in the future. Stimulation of defence pathways in plants involves recognition of effectors molecules produced by the pathogen Avr genes, by the products of host R-genes (Dangl and Jones, 2001). During the past 2 decades, R genes have been successfully identified and cloned from a large number of plant species, which belongs to the NBS-LRR family. Structural resemblance among R genes imparting resistance to diverse pathogens indicates the highly conserved nature of the plant resistance (Dangl *et al.*, 1996). RGAs are now widely utilized to develop molecular markers for mapping and tagging disease resistance in plants. RGAs also help in the isolation of full-length resistance genes, and to analyze the evolutionary basics of these genes in diverse species. R genes can be utilized for developing of disease resistant crop varieties, as an eco-friendly substitute to the use of chemical measures for disease management (Gururani *et al.*, 2012).

As conclusion, the RGAs studied in this work behaved differently in the response time in the tolerant and the susceptible varieties following pathogen challenge. This shows their role in the tolerance response against pathogen. Thus, the identified RGAs could be a good start point for advanced studies in *Dioscorea* such as candidate gene mapping for anthracnose resistance. In addition, the genes used for normalization could be useful for further qPCR studies in *Dioscorea*.

SUMMARY

6. SUMMARY

The study entitled "Mining of resistance genes associated with anthracnose infection in greater yam (*Dioscorea alata* Linn.)" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during 2015-2016. The objective of this study was to identify the resistance genes associated with anthracnose infection in greater yam and to study its expression in susceptible and tolerant varieties.

Young leaf samples of greater yam varieties Orissa Elite (susceptible) and Sree Keerthi (tolerant) were used for DNA isolation and PCR amplification of resistance gene analogues (RGAs). First seven nodes from the tip of the vines were used to produce tissue culture plants and were used for pathogen inoculation, RNA isolation and gene expression study. DNA isolation was carried out using CTAB method and Ambion Purelink RNA mini kit for RNA isolation. Tissue culture of greater yam was carried out in MS media. Full MS media supplemented with IAA (1.5 mg L^{-1}) and kinetin (2 mg L^{-1}) was used for shoot initiation from nodal segments. Subculturing was done in half MS media. Plants developed sufficient shoots and roots and were ready for subculturing within three months. After five months of subculturing, plants were hardened in sterile coirpith for one month and later transferred to sterile soil in pots. These plants were inoculated with spore suspension of seven days old culture of *Colletotrichum gloeosporioides* grown in potato dextrose agar media. Control plants were also kept without spore inoculation. Leaf samples collected on 1st, 3rd, and 5th day from these plants were used for RNA isolation and semi quantitative RT-PCR to study gene expression.

PCR based amplification of RGAs was carried out using degenerate primers already reported which targets the P-Loop and GLPL conserved domains of RGAs. PCR amplification of RGAs in genomic DNA of greater yam resulted in the production of amplicons of the predicted size (500 bp) based on previous reports on RGA sequences. The PCR product was purified and the amplicons

were cloned into *E. coli* DH5α cells. A total of 10 clones were selected from each variety and the presence of gene insert in the transformed colonies was confirmed by colony PCR. The recombinant plasmid DNA was isolated from the transformed colonies and was sequenced. The sequence data was analyzed through BLAST program and similar sequences were identified. Conserved domain search of the sequences showed that the sequences belong to NB–ARC (nucleotide-binding and similarity to *Apaf*-1, R genes and *Ced*-4) domain. A phylogenetic tree was constructed with the amino acid sequence of greater yam and similar sequences obtained through BLAST search. Phylogenetic tree revealed that the amino acid sequence of Orissa Elite is similar to disease resistance protein of *Elaeis guineensis* and Sree keerthi to disease resistance protein of *Phoenix dactylifera*.

Based on the nucleotide sequence information of greater yam, specific primers were designed using Primer3 online software. The analysis of primers using FastPCR programme revealed good GC content and annealing temperature, and also the designed primers did not exhibit any hairpin formation and 3' complementarity. The newly designed primers produced 240 bp products in both the varieties and it was sequenced and analysed using BLAST program to confirm that the newly designed primers amplify RGAs. This primer is used for semi-quantitative RT PCR to study the gene expression.

To study the gene expression, RNA samples were collected from pathogen inoculated and un-inoculated control plants on 1st, 3rd, and 5th day of inoculation. Test plants were inoculated with spore suspension of *C. gloeosporioides* and control plants with sterile water. These plants were maintained under humid conditions and leaf samples were collected on alternate days for isolating RNA.

cDNA was prepared out of the RNA samples and was used for semi-quantitative RT PCR. The RGAs present in the inoculated and control plants were amplified using the newly designed primers and with cDNA as template. Amplification was observed in both the varieties with products of size 240bp.

Positive control reactions kept with Actin primers, reported in *Dioscorea* spp. yielded products of 130 bp size which was same as the expected size. Negative control reactions were also kept by replacing cDNA template with sterile water.

On analyzing the expression pattern of both the varieties, it was found that, the control plants also show RGA expression under normal conditions. On analyzing the inoculated test plant sample expression, it was observed that, the tolerant plant (Sree Keerthi) showed increased expression from the 3rd day onwards which was not observed on susceptible variety (Orissa Elite). On the fifth day, the susceptible variety also showed increased expression but was comparatively less than that of the tolerant variety.

The expression profile of reference gene was analyzed in control and test samples of both the varieties. The reference gene showed stable expression pattern in control and test of both varieties and is not altered by the experimental condition. The negative control reaction did not produce any band when resolved on agarose gel.

REFERENCE

7. REFERENCE

Aarts, M. G., Hekkert, B., Holub, E. B., Beynon, J. L., Stiekema, W. J., and Pereira, A. 1998. Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 11: 251-258.

Abang, M. M., Green, K. R., Wanyera, N. W., and Iloba, C. 2001. Characterization of *Colletotrichum gloeosporioides* from yam (*Dioscorea* spp.) in Nigeria. In: Akoranda, M. O. and Ngeve, J. M. (eds.), *Root Crops in the 21st Century*. Proceedings of the 7th Triennial Symposium of the International Society of Tropical Root Crops – Africa Branch, Cotonou, Benin: ISTRC–AB/IITA Ibadan, Nigeria, pp. 613-615.

Abang, M. M., Mignouna, H. D., Green, K. R., and Asiedu, R. 2001b. Development of a tissue culture based whole plant method for assessing anthracnose disease reactions in water yam (*Dioscorea alata* L.). In: *Proceedings of the 5th Biennial Conference of the African Crop Science Society*, Lagos, Nigeria, pp. 21-26.

Abang, M. M., Winter, S., Mignouna, H. D., Green, K. R., and Asiedu, R. 2003. Molecular taxonomic, epidemiological and population genetic approaches to understanding yam anthracnose disease. *Afr. J. Biotech.* 2: 486-496.

Abraham, K. and Nair, G. P. 1991. Polyploidy and sterility in relation to sex in *Dioscorea alata* L. (*Dioscoreaceae*). *Genet.* 83: 93-97.

Abramovitch, R. B., Anderson, J. C., and Martin, G. B. 2006. Bacterial elicitation and evasion of plant innate immunity. *Natl. Rev. Mol. Cell Biol.* 7: 601-611.

Abruna, P., Diaz, N., and Vicente-Chandler, J. 1981. Studies on management of a wild yam (*D. alata*) and a wild trainer (*Xanthosoma* sp.). *J. Agric. Univ. Publs. Rec.* 65: 56-61.

Adelusi, A. A. and Lawanson, A. O. 1987. Disease-induced changes in carotenoid content of edible yams (*Dioscorea* spp.) infected by *Botryodiplodia theobromae* and *Aspergillus niger*. *Mycopathologia* 98: 49-58.

Agrios, G. N. 1988. *Plant Pathology* (3rd Ed.). Academic press, London, 334 p.

Ajay, K. G. 2014. *Colletotrichum gloeosporioides*: Biology, pathogenicity and management in India. *J. Plant Physiol. Path.* 2: 21-27.

Alexander, J. and Coursey D. G. 1969. The origins of yam cultivation. In: *The domestication and exploitation of plants and animals*. Aldine Publishing Company, Chicago, pp. 405-425.

Ameline-Torregrosa, C., Wang, B. B., O'Bleness, M. S., Deshpande, S., Zhu, H., and Roe, B. 2008. Identification and characterization of nucleotide-binding site-leucine-rich repeat genes in the model plant *Medicago truncatula*. *Plant Physiol.* 146: 5-21.

Amusa N. A. 1991. Extraction, characterisation and bioassay of metabolites of some plant pathogenic species of *Colletotrichum* sp. Ph.D. thesis, University of Ibadan, Nigeria, 187p.

Amusa, N. A. 1997. Fungi associated with anthracnose symptoms of yam (*Dioscorea* spp.) in South-west Nigeria and their roles in. *Crop Res.* 13: 177-183.

Anderson, P., Lawrence, G., Morrish, B., Aylive, M., Finnegan, E., and Ellis, J. 1997. Inactivation of the Xax rust resistance gene M associated with the loss of a repeated unit within the leucine-rich repeat coding region. *Plant Cell* 9: 641–651.

Anil, K., Das, S. N., and Podile, A. R. 2014. Induced defense in plants: A short overview. *Plant Natl. Acad. Sci.* 84: 669-679.

Asemota, H. N., Ramser, J., Lopez-Peralta, C., Weising, K., and Kahl, G. 1996. Genetic variation and cultivar identification of Jamaican yam germplasm by random amplified polymorphic DNA analysis. *Euphytica* 92: 341–351.

Asha, K. I. and Nair, M. C. 2002. Ethnic knowledge system on wild *Dioscorea* (yams) by the Kanikkars of Southern Western Ghats, Kerala. *Indian J. Plant Genet. Res.* 15: 146-149.

Asiedu, R., Wanyera, N. M., Ng, S. Y. C., and Ng, N. Q. 1997. Yams. In: Fuccillo, D., Sears, L., and Stapelton, P. (eds.). Biodiversity in trust: Conservation and use of plant genetic resources in CGIAR centers. Cambridge University Press, Cambridge, UK, pp. 57-66.

Aswati N. R. and Thomas G. 2007. Isolation, characterization and expression studies of resistance gene candidates (RGCs) from *Zingiber* spp. *Theor. Appl. Genet.* 116: 123-134.

Avasthi, S., Gautam, A. K., and Bhadauria, R. 2011. First report of anthracnose disease of *Aloe vera* caused by *Colletotrichum gloeosporioides*. *J. Res. Biol.* 6: 408-410.

Ayodele, M. A., Hughes, J. A., and Asiedu, R. 2000. Yam anthracnose disease. In: *Field symptoms and laboratory diagnostics*. International Institute of Tropical Agriculture, Ibadan, pp. 1-16.

Bai, J., Pennill, L. A., Ning, J., Lee, S. W., Ramalingam, J., Webb, C. R., Zhuo, B., Sun, Q., Nelson, J. C., Leach, J. E., and Hulbert, S. H. 2002. Diversity in nucleotide binding site-leucine rich repeat genes in cereals. *Genome Res.* 12: 1871-1874.

Bailey, J. A. and Jeger, M. J. 1992. *Colletotrichum*. In: *Biology, pathology and control*. CAB International, Wallingford, 388p.

Bartlett, J. M. S. and Stirling, D. 2003. A Short History of the Polymerase Chain Reaction. In: Bartlett, J. M. S. and Stirling, D. (eds.), *PCR Protocols,* NJ Humana Press, Totowa, pp. 3-6.

Bent, A. F., Kunkel, B. N., Dahlbeck, D., Brown, K. L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B. J. 1994. RPS2 of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes. *Sci.* 265: 1856-1860.

Bertini, L., Leonardi, L., Caporale, C., Tucci, M., Cascone, N., Di-Berardino, I., Buonocore, V., and Caruso, C. 2003. Pathogen responsive wheat PR4 genes are induced by activators of systemic acquired resistance and wounding. *Plant Sci.* 164: 1067-1078.

Beyer, K., Binder, A., Boller, T., and Collinge, M. 2001. Identification of potato genes induced during colonization by *Phytophthora infestans*. *Mol. Plant Pathol.* 2: 125-134.

Bhandari, M. R., Kasai T., and Kawabata, J. 2003. Nutritional evaluation of wild yam (*Dioscorea* spp.) tubers of Nepal. *Food Chem.* 82: 619-623.

Bhandari, M. R. and Kawabata, J. 2005. Bitterness and toxicity in wild yam (*Dioscorea* spp.) tubers of Nepal. *Plant Food Hum. Nutr.* 60: 129-135.

Bradbury J. H. and Holloway, W. D. 1987. *Chemistry of Tropical Root Crops*. Australian Centre for International Agricultural Research. Canberra, Australia, pp. 101-119.

Brosius, J. and Gould, S. J. 1992. "Genomenclature": A comprehensive (and respectful) taxonomy for pseudogenes and other "junk DNA". *Proc. Natl. Acad .Sci. USA* 89: 10706-10710.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinology* 25: 169–193.

Butler, E. J. 1918. Fungi and Disease in Plants. In: *Fungi and Disease in Plants*. Calcutta, India, pp. 36-39.

Cannon, P. F., Damm, U., Johnston, P. R., and Weir, B. S. 2012. *Colletotrichum*: Current status and future directions. *Stud. Mycol.* 73: 181-213.

Cannon, S. B., Zhu, H., Baumgarten, A. M., Spangler, R., May, G., Cook, D. R., and Young, N. D. 2002. Diversity, distribution and ancient taxonomic relationships within the TIR and non-TIR NBS-LRR resistance gene subfamilies. *J. Mol. Evo.* 54: 548-562.

Choudhuary, S. 1963. A disease of *Zea mays* caused by *Colletotrichum gloeosporioides* (Ces.). *Indian J. Agric. Sci.* 6: 77-84.

Collinge, M. and Boller, T. 2001. Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by *Phytophthora infestans* and to wounding. *Plant Mol. Biol.* 46: 521-529.

Compton, T. 1990. Degenerate primers for DNA amplification. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.), *PCR protocols, a guide to methods and applications*. Academic Press, London, UK, pp. 39-45.

Coursey, D. G. 1967. Yam storage: A review of yam storage practices and of information on storage losses. *J. Stored Products Res.* 2: 229-244.

Coursey D. G. 1976. The origins and domestication of yams in Africa. In: Jack, R. H., Jan, M. J., and Ann B. L. (eds.), *Origin of African plant domestication*. The Hague, Netherlands, pp. 383-408.Coursey, D. G. 1982. *Traditional*

tropical root crop technology–some interactions with modern science. Institute of development studies, IDS Bulletin- 13, pp. 12-20.

Coursey, D. G. 1983. Yams. In: Chan, H. T. (ed.), *Handbook of tropical foods.* Marcel Dekker Inc, New York, US, 555p.

Craufurd, P. Q., Summerfield, R. J., Asiedu, R., and Varaprasad, P. V. 2001. Dormancy in Yams. *Exp. Agri.* 37: 147-181.

Crute, I. R. and Pink, D. A. C. 1996. The genetics and utilization of pathogen resistance in plants. *Plant Cell,* 8: 1747-1755.

Dangl, J. L., Dietrich, R. A., and Richberg, M. H. 1996. Death don't have no mercy: Cell death programs in plant-microbe interactions. *Plant Cell* 8: 1793-1807.

Dangl, J. L. and Jones, J. D. 2001. Plant pathogens and integrated defence responses to infection. *Nat.* 411: 826-833.

Das, R., Das, K., and Suryanarayana, N. 2005. A new anthracnose disease in muga food plant, som (*Persea bombycina* K.) in Assam. *Indian J. Seric.* 44: 134-135.

David, P., Chen, N. W., Pedrosa-Harand, A., Thareau, V., Sévignac, M., and Cannon, S. B. 2009. A nomadic subtelomeric disease resistance gene cluster in common bean. *Plant Physiol.* 151: 1048-1065.

Dean, R., J. A., Van Kan, Z. A., Pretorius, K. E., Hammond-Kosack, A., Di Pietro, P. D., Spanu, J. J., Rudd, M., Dickman, R., Kahmann, J., and Foster, G. D. 2012. The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13: 414-430.

Deng, C. and Davis, T. M. 2001. Molecular identification of the yellow fruit color (c) locus in diploid strawberry: A candidate gene approach. *Theor. Appl. Genet.* 103: 316-322.

Dieffenbach, C. W., Lowre, T. M., and Dveksler, G. S. 1993. General concepts for PCR primer design *Genome Res.* 3: 30-37.

Ebenebe, A. C. 1982. *Role of toxic production by Colletotrichium gloeosporioides in the Fungicidal Control of the Onion Twister Disease.* Paper presented 12th annual Conference, N.S.P.P., Benin. [Online]. Available at: http://www.istrc.org/

Ellis, J., Dodds, P. and Pryor, T. 2000. Structure, function and evolution of plant disease resistance genes. *Curr. Opinion Plant Biol,* 3: 278-284.

Ellis, J. G., Lawrence, G. J., Luck, J. E., and Dodds, P. N. 1999. Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. *Plant Cell* 11: 495-506.

Ennin, S. A., Otoo, E., and Tetteh, F. M. 2009. Ridging, a mechanized alternative to mounding for yam and cassava production. *West Afr. J. Appl. Ecol.* 15: 490-511

Erlich, H. A. 1989. *PCR technology: principles and applications for DNA amplification.* In: IRL Press at Oxford Univ. Press, Oxford, UK, 68p.

Etejere, E. O. and Bhat, R.B. 1986. Traditional and modern storage methods of underground root and stem crops in Nigeria. *Turrialba.* 36: 33-37.

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D. G., Daniels, M. J., and Parker, J. E. 1999. EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. USA,* 96: 3292-3297.

FAO 1985. FAO production yearbook. Food and Agriculture Organization, United Nations, Rome, pp. 80-124.

FAO 1994. Tropical Root and Tuber Crops. In: Onwueme, I. C. and Charles, W B. (eds.), *Production, Perspectives and future Prospects*. FAO Plant Production and Protection Paper Rome, Italy, 126p.

FAO 1998. Storage and processing of roots and tubers in the tropics. FAO. Rome, Italy, 786p.

FAO 2002. Production Yearbook, Collection FAO Statistics, no. 170. FAO, Rome, Italy, 159p.

FAO 2012. Food and Agricultural Organization Statistics Food data and Agriculture Organisation of the United Nations. [Online]. Available at: http://faostat.fao.org/

FAO 2014. FAOSTAT database [Online]. Available at: http://faostat.fao.org/ [10. August 2014].

Farr, D. F., Aime, M. C., Rossman, A. Y., and Palm, M. E. 2006. Species of *Colletotrichum* on Agavaceae. *Mycol. Res.* 110: 1395-1408.

Flor, H. H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopath.* 9: 275-296.

Freeman, S., Shabi, E., and Katan, T. 2000. Characterization of *Colletotrichum acutatum* causing anthracnose of Anemone (*Anemone coronaria* L.). *Appl. Environ. Microbiol.* 66: 5267-5272.

Freeman, W. M., Walker, S. J., and Vrana, K. E. 1999. Quantitative RT-PCR: Pitfalls and potential. *Biotechniques* 26: 112-124.

Gabriel, D. W. and Rolfe, B. G. 1990. Working models of specific recognition in plant microbe interactions. *Annu. Rev. Phytopath.* 28: 365-391.

Gamiette, F., Bakry, F., and Ano, G. 1999. Polyploidy and sterility inrelation to sex in *Dioscorea alata* L. (*Dioscoreaceae*) conventional chromosomes counting. *Genet. Resour. Crop Evolution.* 46: 19-27.

Gao, Y., Xu, Z., Jiao, F., Yu, H., Xiao, B., Li, Y., and Lu, X. 2009. Cloning structural features, and expression analysis of resistance gene analogs in Tobacco. *Mol. Biol. Rep.* 37: 345-354.

Gautam, A. K., Avasthi, S., and Bhadauria, R. 2012c. *Additions to New Plant Fungal Disease during 21st Century in India: An Update for 2000-2011.* Lambert Academic Publishing, Germany, 20p.

Gedil, M. and Sartie, A. 2010. Perspectives on molecular breeding of Africa's main staple food crops- cassava and yams. *Aspects Appl. Biol.* 96: 123-135.

Graham, M., Marek, L., and Shoemaker, R. 2002. Organization, expression and evolution of a disease resistance gene cluster in soybean. *Genet.* 162: 1961-1977.

Grant, M. R., McDowell, J. M., Sharpe, A. G., Torres, Z. M., Lydiate, D. J., Dangl, J. L. 1998. Independent deletions of a pathogen-resistance gene in Brassica and Arabidopsis. *Proc. Natl. Acad. Sci. USA*, 95: 15843–15848.

Green, K. R. 1994. Studies on the Epidemiology and Control of Yam Anthracnose. PhD thesis, University of Reading, UK, 163p.

Green, K. R. and Simons, S. A. 1994. Dead skin on yams (*Dioscorea alata*) caused by *Colletotrichum gloeosporioides. Plant Pathol.* 43: 1062-1065.

Green, K. R. 1998. Distribution and severity of foliar diseases of yam (*Dioscorea* spp.) in Nigeria. In: Akoroda, M. O. and Ekayanake, I. J. (eds.), *Root Crops and Poverty Alleviation.* Proceedings of the 6th Triennial Symposium of the International Society of Tropical Root Crops-Africa

Branch, 1995; Lilongwe. ISTRC-AB/Government of Malawi/IITA, pp. 439-444.

Green, K. R., Abang, M. M., and Iloba, C. 2000. A rapid bioassay for screening yam germplasm for response to anthracnose. *Trop. Sci.* 40: 87-99.

Grennan, A. K. 2006. Plant response to bacterial pathogens overlap between innate and gene-for-gene defense response. *Plant Physiol.* 142: 809-811.

Guo, Y. L., Fitz, J., Schneeberger, K., Ossowski, S., Cao, J., and Weigel, D. 2011. Genome-wide comparison of nucleotide-binding site-leucine-rich repeat-encoding genes in Arabidopsis. *Plant Physiol.* 157: 757-769.

Gupta, S. K., Jarial, K., and Kansal, S. 2009. *Colletotrichum gloeosporioides* causing anthracnose in bell pepper seed crop: a new record from Himachal Pradesh. *J. Plant Dis. Sci.* 4: 126-127.

Gururani, M. A., Venkatesh, J., Upadhyaya, C. P., Nookaraju, A., Pandey, S. K., and Park, S. W. 2012. Plant disease resistance genes: Current status and future directions. *Physiol. Mol. Plant* 78: 51-65.

Gutierrez, N., Giménez, M. J., Torres, A. M., Atienza, S. G., Avila, C. M., and Palomino, C. 2012. Up-regulation of resistance gene analogs (RGA) in chickpea in the early response to *Fusarium* wilt. *Euphytica* 186: 793-804.

Hahn, S. K. 1984. Tropical root crops: Their improvement and utilization. I.I.T.A. In: *Int. Inst. Trop. Agr., Conference Paper 2.* Ibadan, Nigeria, pp. 140-186.

Hahn, S. K. 1995. Yams: *Dioscorea* spp. (Dioscoreaceae). In: Smaarttand, J. and Simmonds, N. W. (eds.), *Evolution of crop plants,* Longman scientific and Technical, UK, pp. 112-120.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41: 95-98.

Hammond-Kosack, K. E. and Jones, J. D. 1996. Disease resistance gene-dependent inducible plant defense mechanisms. *Plant Cell* 8: 1773-1791.

Hammond-Kosack, K. E. and Jones, J. D. 1997 Plant disease resistance genes. *Annu. Rev. Plant Physiol. Mol. Biol.* 48: 575-607.

Han, S. K., Osiru, D. S. O., Akoroda, M. O., and Oto, J. A. 1987. Yam production and its future prospects. *Outl. Agric.* 16: 109-118.

Harrison, B. D. and Roberts, I. M. 1973. Association of virus-like particles with internal brown spot of yam (*Dioscorea alata*). *Trop. Agric.* 50: 335-340.

Hiremath, S. V., Hiremath, P. C., and Hegde, R. K. 1993. Studies on cultural characters of *Colletotrichum gloeosporioides* a causal agent of Shisham blight. *J. Food Legumes* 26: 67-69.

Hubballia, M., Nakkeerana, S., Raguchandera, T., Ananda, T., and Renukadevi, P. 2011. Physiological characterisation of *Colletotrichum gloeosporioides*, the incitant of anthracnose disease of noni in India. *Arch. Phytopath. Plant. Prot.* 44: 1105-1114.

Huber, H. 1998. *Dioscoreaceae*: Flowering plants - monocotyledons. In: Kubitzki, K. (ed.), *The families and genera of vascular plants*. Springer-Verlag, Berlin, pp. 192-199.

Hulbert, S. H., Webb, C. A., Smith, S. M., and Sun, Q. 2001. Resistance gene complexes: Evolution and utilization. *Annu. Rev. Phytopath.* 39: 285-312.

IITA 1993. Archival Reports (1989 - 1993). In: *(Part III yam,) Dioscorea sp.*, Crop Improvement Division/ Tuber root Improvement Program, Ibadan, Nigeria, pp. 20-85.

IITA 2004. International Institute of Tropical Agriculture, *Nigerian's Cassava Industry: Statistical Handbook*, Ibadan, Nigeria. [Online]. Available at: http://www.iita.org/

IITA 2009. International Institute of Tropical Agriculture. *Yam production in Africa.* Ibadan, Nigeria. [Online]. Available at: http://www.iita.org/cms/details/yam_project_details.aspx?zoneid=63&articleid=2 68.

IITA 2013. International Institute of Tropical Agriculture. *Healthy yam seed production.* IITA Publications. Ibadan, Nigeria. [Online]. Available at: http://www.iita.org/2013-press-releases/

Ike, P. C. and Inoni, O. E. 2006. Determinants of yam production and economic efficiency among small-holder farmers in South-eastern Nigeria. *J. Cent. Eur.Agric.* 7: 337-342.

Innes, R. W., Ameline-Torregrosa, C., Ashfield, T., Cannon, E., Cannon, S. B., and Chacko, B. 2008. Differential accumulation of retroelements and diversification of NB-LRR disease resistance genes in duplicated regions following polyploidy in the ancestor of soybean. *Plant Physiol.* 148: 1740-1759.

Izekor, O. B. and Olumese, M. I. 2010. Determinants of yam production and profitability in Edo State, Nigeria. *Afr. J. Gen. Agric.* 6: 205-221.

Johal, G. S. and Briggs, S. P. 1992. Reductase activity encoded by the HM1 disease resistance gene in maize. *Sci.* 12: 985-987.

Jones, J. D. and Dangl, J. L. 2006. The plant immune system. *Nat.* 444: 323-329.

Joshi, R. K., Kar, B., Mohanty, S., Subudhi, E., and Nayak, S. 2012. Molecular cloning, characterization, and expression analysis of resistance gene candidates in *Kaempferia galanga* L. *Mol. Biotechnol.* 50: 200-210.

Kampke, T., Kieninger, M., and Mecklenburg, M. 2001. Efficient primer design algorithms. *Bioinformatics* 17: pp. 214-215.

Kanazin, V., Marek, L. F., and Shoemaker, R. C. 1996. Resistance gene analogs are conserved and clustered in soybean. *Proc. Natl. Acad. Sci. USA* 93: 11746–11750.

Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24: 447-463.

Knepper, C. and Day, B. 2010. From Perception to Activation. In: *The Molecular-Genetic and Biochemical Landscape of Disease Resistance Signaling in Plants*. The Arabidopsis Book / American Society of Plant Biologists, 12 p.

Kumar, V., Gupta, V. P., Babu, A. M., Mishra, R. K., Thiagarajan, V., and Datta, R. K. 2001. Surface ultrastructural studies on penetration and infection process of *Colletotrichum gloeosporioides* on mulberry leaf causing black spot disease. *J. Phytopath.* 149: 629-633.

Kunkel, B. N. 1996. A useful weed put to work: Genetic analysis of disease resistance in *Arabidopsis thaliana*. *Trends Genet.* 12: 63-69.

Kwok, S., Chang, S. Y., Sninsky, J. J., and Wang, A. 1994. A guide to the design and use of mismatched and degenerate primers. *PCR Methods Appl.* 3: 39-47.

Lamark, J. 1792. *Dioscorea cayenensis*. In: *Encyclopedia Methodioque,* Paris, p. 233.

Landschulz, W. H., Johnson, P. F., and McKnight, S. L. 1998. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Sci.* 240: 1759-1762.

Latunde-Dada, A. O. 2001. *Colletotrichum*: Tales of forcible entry, stealth, transient confinement and breakout. *Mol. Plant Pathol.* 2:187-198.

Lebot, V., Trilles, B., Noyer, J. L., and Modesto, J. 1998. Genetic relationships between *Dioscorea alata* L. cultivars. . *Genet. Res. Crop Evolution* 45: 499-508.

Lebot, V. 1999. Biomolecular evidence for plant domestication in Sahul. *Genet. Res. Crop Evolution* 46: 619-628.

Lebot, V., Malapa R., Molisade, T., and Machad J. L. 2005. Physicochemical characterization of yam (*D. alata* L.) tubers from Vanuatu. *Genet. Res. Crop Evolution* 3: 1-15.

Leister, D., Ballvora, A., Salamini, F., and Gebhardt, C. 1996. A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. *Nat. Genet.* 14: 421-428.

Lexa, M., Horak, J., and Brzobohaty, B. 2001. Virtual PCR. Bioinformatics. 17: 192-193.

Liu, J. J. and Ekramoddoullah, A. K. M. 2003. Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola*) *Mol. Genet. Genome.* 270: 432-441.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR Method. *Methods* 25: 402-408.

Lodhi, M. A., Guang-Ning, Z., Weeden, F. N., and Reisch, B. I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and Ampelopsis. *Plant Mol. Biol. Rep.*12: 6-13.

Long, S. and Staskawiu, B. 1993. Prokaryotic plant parasites. *Cell* 73: 921-935.

Lu, G., Cannon, P. F., Reid, A., and Simmons, C. M. 2004. Diversity and molecular relationships of endophytic *Colletotrichum* isolates from the Iwokrama Forest Reserve, Guyana. *Mycol. Res.* 108: 53-63.

Mago, R., Nair, S., Mohan, M., 1999. Resistance gene analogues from rice: cloning, sequencing and mapping. *Theor. Appl. Genet.* 99: 50-57.

Maharana, T. 1993. Productivity and quality study in *Dioscorea* of Orissa. Ph.D thesis, Orissa University of Agriculture and Technology, Bhubaneswar, Orissa, India, 311 p.

Maheshwari, V. K., Gupta, M. N., and Agarwal, V. K. 1983. Fungi associated with yam tubers during transit and marketing. *Nat. Acad. Sci. Lett.* 6: 182.

Martin, F. W. 1976. *Dioscorea alata.* 3 volumes. Tropical yams and their potential, USDA Agriculture Handbook no. 495, 40 p.

Martin, F. W. and Rhodes, A. M. 1977. Intra-specific classification of *Dioscorea alata. Trop. Agric. (Trinidad)* 54: 1-13.

Martin, G. B., Bogdanove, A. J., and Sessa, G. 2003. Understanding the functions of plant disease resistance proteins. *Annu. Rev. Plant Biol.* 54: 23-61.

Martín, M. P. and García-Figueres, F. 1999. *Colletotrichum acutatum* and *C. gloeosporioides* cause anthracnose on olives. *Eur. J. Plant Pathol.* 105: 733-741.

Martine, J. and Cappadocia, M. 1992. Effects of growth regulators on *in vitro* tuberization in *Dioscorea alata* L. 'Brazo fuerte' and *D. abyssinica* 'Hoch'. *Plant Cell Rep.* 11: 34-35.

Maryam, G. Z., Kazhal, H., Ali, A., and Samareh, K., 2007. A semiquantitative RT-PCR assay to evaluate the expression patterns of NBS-LRR family genes in infected citrus by *Candidatus Leiberibacter* Asiaticus. *Int. J. Agric. Crop Sci.* 7: 1509-1513.

May, A. P., Whiteheart, S. W., and Weis, W. I. 2001. Unraveling the mechanism of the vesicle transport ATPase NSF, the N-ethyl maleimide- sensitive factor. *J. Biol. Chem.* 276: 21991-21994.

McDonald B. A. and Linde. L. 2002. Pathogen population genetics, evolutionary potential and research advances in disease management. *Trop. Agric.* 53 - 57.

McDowell, J., Dhandaydham, M., Long, T., Aarts, M., Go, V. S., Holubd, E., and Dangl, J. 1998. Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the RPP8 locus of *Arabidopsis*. *Plant Cell* 10: 1861-1874.

McRae, W. 1924. *Economic Botany part-III*: *Mycology*. Annual Report (1922-23) Board of Scientific Advice, India, pp. 31-35.

Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., and Young, N. D. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding super family. *Plant J.* 20: 317-332.

Meyers, B. C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R. W. 2003. Genome-wide analysis of NBS-LRR encoding genes in *Arabidopsis*. *Plant Cell* 15: 809-834.

Michelmore, R. W. and Meyers, B. C. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. *Genome Res.* 8: 1113-1130.

Mignouna, H. D., Ellis, N.T.H., Knox, M. R., Asiedu, R., and Ng, Q. N. 1998. Analysis of genetic diversity in Guinea yams (*Dioscorea* spp.) using AFLP fingerprinting. *Trop. Agric. (Trinidad)* 75: 224-229.

Mignouna, H. D., Mank, R. A., Ellis, T. H. N., Vanden-Bosch, N., Asiedu, R., Abang, M.M., and Peleman, J. 2002a. A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance. *Theor. Appl. Genet.* 105: 726-735.

Mignouna, H. D., Mank, R.A., Ellis, T.H.N., VanDenBosch, N., Asiedu, R., Ng, S., and Peleman, J. 2002b. A genetic linkagemap of Guinea yam (*Dioscorea rotundata* Poir.) based on AFLP markers. *Theor. Appl. Genet.* 105: 716-725.

Mitchell, S., Asnani, V., and Coke, L. 1989. The role of the tissue culture and mini tubers in yam propagation: Experience with yam (*Dioscorea trifida*) and yellow yam (*D. cayenensis*). In: *Proceedings of 3rd Annual National Conference on Science and Technology*, Agriculture Development for the 21st Century, Kingston, Jamaica, pp. 500-504.

Mitchell, S. A. and Ahmed, M. H. 1999. Morphological changes of *Dioscorea trifida* L. cv. Short Neck Yampie and *Dioscorea cayenensis* L. cv. Round leaf yellow yam linked to the number and size of harvested tubers. *J. Hort. Sci. Biotech.* 74: 531-539.

Moffett, P., Farnham, G., Peart, J., and Baulcombe, D. C. 2002. Interaction between domains of a plant NBS-LRR protein in disease resistance related cell death. *EMBO. J.* 17: 4511-4519.

Mondragon-Palomino, M., Meyers, B. C., Michelmore, R. W., and Gaut, B. S. 2002. Patterns of positive selection in the complete NBS-LRR gene family of *Arabidopsis thaliana*. *Genome Res.* 12: 1305-1315.

Muzac-Tucker, I., Asemota, H. N., and Ahmad, M. H. 1993. Biochemical composition and storage of Jamaican yams (*Dioscorea* sp). *J. Sci. Food Agric.* 62: 219-224.

Nath, V. S., Senthil, M., Hegde, V. M., Jeeva, M. L., Misra, R. S., Veena, S. S., and Mithun, R., 2013. PCR-based approach for mining of resistant gene analogues in taro (*Colocasia esculenta*). *Arch. Phytopath. Plant Prot.* 46: 803-812.

Ng, S. Y. C. 1992. Micropropagation of white yam (*Dioscorea rotundata* Poir) In: Bajaj, Y. P. S. (eds.), *Biotechnology in agriculture forestry*, Springer, Berlin, pp. 135-159.

Nicholson, R. L. and Moraes, W. B. C. 1980. Survival of *Colletotrichum graminicola*: Importance of the spore matrix. *Phytopath.* 70: 255-261.

Njoh, E. E., Billard C., Petro, D., Adenet, S., and Rochefort, K. 2015. Physicochemical, Nutritional and sensorial qualities of Boutou yam (*Dioscorea alata*) varieties. *J. Expe. Biol. Agric. Sci.* 3: 139-150.

Noir, S., Combes, M. C., Anthony, F., and Lashermers, P. 2001. Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees. *Mol. Genet. Genomics* 265: 654-662.

Noon, R. A. 1978. Storage and market diseases of yams. *Trop. Sci.* 20: 177-188.

Nwakiti, A. O. and Arene, O. B. 1978. Disease of yam in Nigeria. *Pest Articles News Summaries (PANS)* 24: 468-496.

Nwankiti, A. O. and Ene, L. S. O. 1984. Advances in the study of anthracnose/ blotch disease of *Dioscorea alata* in Nigeria. In: Shidler, F. S. and Rincon, H. (eds.), *Proceedings of the 6th Symposium of the International Society of Tropical Root Crops*, Lima, Peru, pp. 633-640.

Nweke, F. I., Okoroji, E. C., Njoku, J. E., and King, D. J. 1992. Elasticities of demand for major food items in a root and tuber-based food system, In: *Emphasis on yam and cassava in South Eastern Nigeria.* Research and Crop Management Program Research Monograph No. 11, International Institute of Tropical Agriculture, Ibadan, Nigeria, p.113.

Oh, J. W., Kong, Q., Song, C., Carpenter, C. D., and Simon, A. E. 1995. Open reading frames of turnip crinkle virus involved in satellite symptom

expression and incompatibility with *Arabidopsis thaliana* ecotype Dijon. *Mol. Plant Microbe Interact.* 8: 979-987.

O'Hair, S. 1990. Tropical Root and Tuber Crops. In: Janick, J. and Simon, J. E. (eds.), *Advances in new crops*, Timber Press, Portland, USA, pp. 424-428.

Okezie, C. E. A. 1987. Involvement of day length in the tuberization of *Dioscorea rotundata* minisetts under Nsukka conditions. In: Terry, E. R., Akoroda, M. O., Arene, O. B. (eds.), *Tropical root crops: root crops and the African food crisis.* International Development Research Centre, Ottawa, Canada, p. 53.

Okigbo, R. N. 2005. Biological control of postharvest fungal rot of yam (*Dioscorea* spp.) with *Bacillus subtilis*. *Mycopathologia* 159: 307-314.

Okoli, O. O. 1980. Dry matter accumulation and tuber sprouting in yams (*Dioscorea* spp.). *Exp. Agric.* 16: 161-167.

Olsvik, O., Wahlberg, J., Petterson, B., Uhlen, M., Popovic, T., Wachsmuth, I. K., and Fields, P. I. 1993. Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin. Microbiol.* 31: 22-25.

Onwueme, I. C. 1984. Yam. In: Goldsworthy, P. R. and Fisher, N. M. (eds.), *The physiology of Tropical field crops*, John Wiley and Sons, Chichester, pp. 569-588.

Onwueme, I. C. and Charles, W. B. 1994. Tropical root and tuber crops. In: *Production, perspectives, and future prospects*. Rome, FAO. [Online]. Available at: http://trove.nla.gov.au/

Onyeka, T. J., Pétro, D., Jacqua, G., Etienne, S., and Rubens, S. 2005a. Assessment of partial resistance to anthracnose in water yam (*D. alata*)

using tissue culture-generated whole plant. In: *Breeding for Plant Resistance to Pests and Diseases*. IOBC Bulletin, 28: 51-57.

Onyeka, T. J., Pétro, D., Etienne, S., Jacqua, G., and Ano, G. 2006. Optimizing controlled environment assessment of levels of resistance to yam anthracnose disease using tissue culture-derived whole plants. *J. Phytopath.* 154: 286-292.

Opara, L. U. 1999. Yam storage. In: Bakker, A. (ed.), *CGIAR Handbook of Agricultural Engineering*, Volume IV Agro Processing. The American Society of Agricultural Engineers, St. Joseph, USA pp. 182-214.

Orkwor, G. C. 1998. The Importance of yams. In: *Food Yams. Advances in Research.* IITA (Ibadan) and NRCRI (Umudike), Nigeria, pp. 1-12.

Osagie, A. U. and Opute, F. I. 1981. Major lipid constituents of *D. rotundata* tubers during growth and maturation. *J. Exp. Bot.* 32: 737-740.

Osagie, A. U. 1992. *The Yam in Storage.* Postharvest Research Unit, University of Benin, Nigeria, pp. 107-173.

Otoo, J. A., Osiru, S. O., Ng, S. Y., and Hahn, S. K. 1985. Improved technology for seed yam production. Ibadan, IITA, pp. 1-66.

Pan, Q., Wendel, J., and Fluhr, W. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *J. Mol. Evol.* 50: 203-213.

Parker, J. E., Coleman, M., Szabo, V., Frost, L., Schmidet, R., Biezen, E. V. D., Moores, T., Dean, C., and Jones, J. D. G. 1997. The Arabidopsis downy mildew resistance gene RPP5 shares similarity to the Toll and Interleukin-1 receptors with N and L6. *Plant Cell* 9: 879-894.

Parniske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. M., Jones, D. A., Harrison, K., Wulff, B. B. H., and Jones, J. D. G. 1997. Novel disease

resistance specificities result from sequence exchange between tandemly repeated genes at the Cf-4/9 locus of tomato. *Cell* 91: 821-832.

Passam, H. C. and Noon, R. A. 1977. Biodeterioration of Yams and Cassava during storage. *Ann. Appl. Biol.*85: 436-440.

Patel, R. V., Joshi, K. R., Solanky, K. U., and Sabalpara, A. N. 2005. *Colletotrichum gloeosporioides*: A new leaf spot pathogen of turmeric in Gujarat. *Indian Phytopath.* 58: 125-131.

Patricia, S. S., John, V., Itamar, G., and Noel, B. 2009. "Primers development and virus identification strategies". In: *Insect Pathogens: Molecular Approaches and Techniques*. CAB International, 22p.

Penzig, A. G. O. 1831. *Funghi Agrumicoli: Contribution to the study of Fungi Parassiti Degli Agrumi.* (trans. Ittalian, Michelia), Nabu Press 88p.

Perfect, S. E., Hughes, H. B., O'Connell, R. J., and Green, J. R. 1999. *Colletotrichum*: A model genus for studies on pathology and fungal-plant interactions. *Fungal Genet. Biol.* 27: 186-198.

Pettersson, E., Lundeberg. J., and Ahmadian, A. 2009. Generations of sequencing technologies. *Genomics* 93: 105-111.

Poiret, J. C. 1813. *Dioscorea rotundata.* In: *Encyclopedia Methodioque,* Suppl. III, Paris, 130p.

Raddatz, G., Dehio, M., Meyer, T. F., and Dehio, C. 2001. Prime Array: genome-scale primer design for DNA microarray construction. *Bioinformatics* 17: 98-99.

Raeymaekers, L. 1993. Quantitative PCR: Theoretical considerations with practical implications. *Anal. Biochem.* 214: 582–585.

Rani, S. G. and Murthy, K. V. M. K. 2004. Cultural and nutritional characterstics of *Colletotrichum gloeosporioides*, the causal organism in cashew anthracnose. *J. Mycol. Plant Pathol.* 34: 317-318.

Ravi, V., Aked, J., and Balagopalan, C. 1996. Review on tropical root and tuber crops: Storage methods and quality changes. *Crit. Rev. Food Sci. Nutr.* 36: 661-709.

Reckhaus, P. and Nienhaus, F. 1981. Etiology of a virus disease of white yam (*Dioscorea rotundata*) in Togo. *J. Plant Dis. Prot.* 88: 492-509.

Reichl, U. and Kochanowksi, B. 1999. Quantitative PCR: A Survey of the Present Technology. In: Kochanowski, B. and Reischl, U. (eds.), *Quantitative PCR Protocols*. Humana Press, New Jersey, pp. 3-20.

Ren, T., Qu, F., and Morris, T. J. 2000. HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. *Plant Cell* 12: 1917-1925.

Roberto, L., Martha, T. H., Simon, P., and Jean-Luc, J., 2015. Identification and distribution of the NBS-LRR gene family in the Cassava genome *BMC Genomics* 16: 360.

Rossi, M., Araujo, P. G., Paulet, F., Garsmeur, O., Dias, V.M., Chen, H., Van, S.M.A., and D'Hont, A., 2003. Genomic distribution and characterization of EST-derived resistance gene analogs (RGAs) in sugarcane. *Mol. Genet. Genomics* 269: 406-419.

Saboki, E., Sharma, T., and Singh, B., 2013 Resistance gene analogues in mango against mango malformation. *Acta Phytopath. Ent. Hungarica* 48: 238-334.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Sci.* 230: 1350-1354.

Sanseverino, W., Hermoso, A., D'Alessandro, R., Vlasova, A., Andolfo, G., and Frusciante, L. 2012. PRGdb 2.0: towards a community-based database model for the analysis of R-genes in plants. *Nucleic Acids Res.* 41: 1167-1171.

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., Kato, T., and Nakao, M. 2008. Genome structure of the legume, *Lotus japonicus*. *DNA Res.*15: 227-239.

Saurabh, B., Kiran, S., Randhir, D., and Tanmoy, B. 2015. *Modern applications of plant biotechnology in pharmaceutical sciences.* Acadamic Press, UK, 166p.

Scott, G. J., Best, R., Rosegrant, M., and Bokanga, M. 2000a. Roots and tubers in the global food system: A vision statement to the year 2020 (including annex). A co-publication of the International Potato Center (CIP), International Food Policy Research Institut (IFPRI), International Institute of Tropical Agriculture (IITA), and International Plant Genetic Resources Institute (IPGRI). Lima, Peru, 111p.

Seah, S., Sivasithamparam, K., Karakousis, A., and Lagudah, E.S. 1998. Cloning and characterization of a family of disease resistance gene analogs from wheat and barley. *Theor. App. Genet.*97: 937-945.

Sharkey, D. J., Scalice, E. R., Christy, K. G., Atwood, S. M., and Daiss, J. L. 1994. Antibodies as Thermolabile Switches: High Temperature Triggering for the Polymerase Chain Reaction. *Nat. Biotech.* 12: 506-509.

Sharma, M. C. and Sharma, B. C. 1969. Toxic metabolites production by *Colletotrichum gloeosporioides* causing citrus die-back in India. *Indian Phytopath.* 22: 65-73.

Sharma, P. N., Kaur, M., Sharma, O. P., Sharma, P., and Pathania, A. 2005. Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chillies in the subtropical region of north-western India. *J. Phytopath.* 153: 232-237.

Shen, K. A., Meyers, B. C., Islam-Faridi, M. N., Chin, D. B., Stelly, D. M., and Michelmore, R. W. 1998. Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. *Mol. Plant Microbe Interact.* 11: 815-823.

Siebert, P. D. 1999. Quantitative RT-PCR. In: Kochanowski, B. and Reischl, U. (eds.), *Quantitative PCR Protocols*, Humana Press, New Jersey, pp. 61-85.

Simon S. A. 1993. *Epidemiology and control of yam anthracnose.* Natural Resourse Institute, UK, 361p.

Singh, A., Verma, K.S., and Mohan, C. 2006. Effect of different culture media on the growth and sporulation of *Colletotrichum gloeosporioides* causing guava anthracnose. *Plant Dis. Res.* 21: 224.

Singh, R. D., Prasad, N., and Mathur, R. L. 1966. On the taxonomy of the fungus causing anthracnose of *Dioscorea alata* L. *Ind. Phytopath.* 10: 65-71.

Song, W. Y., Pi, L.Y., Wang, G.L., Gardner, J., Holsten, T., and Ronald, P.C. 1997. Evolution of the rice Xa21 disease resistance gene family. *Plant Cell* 9: 1279-1287.

Splittstoesser, W. E., Martin F. W., and Rhodes. A. M. 1973. The amino acid composition of five species of yam (*Dioscorea*). *J. Am. Soc. Hort. Sci.* 98: 563-567.

Srinivas, T., Nedunchezhiyan, M., and Misra, R. S. 2012. *Marketing system of aroids and yams in India*. Technical Bulletin Series No. 53. CTCRI, Thiruvananthapuram, 82p.

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., and Bergelson, J. 1999. Dynamics of disease resistance polymorphism at the Rpm1 locus of Arabidopsis. *Nat.* 400: 667–671.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. *Sci.* 268: 661-667.

Sturm, J. 1798. *Deutschlands flora in nature descriptions*. (trans. Gedruckt, V.). Nrnberg. [Online]. Available at: http://www.biodiversity library.org/

Supriya, D., Manabendra, D. C., and Pranab, B. M. 2013. Micropropagation of *Dioscorea alata* L. through nodal segments. *Afr. J. Biotech.* 12: 6611-6617.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evolution* 30: 2725-2729.

Terauchi, R. and Kahl, G. 1999. Mapping of the *Dioscorea tokoro* genome: AFLP markers linked to sex. *Genome Res.* 42: 752-762.

Tetteh, J. P. and Saakwa, C. 1994. *Prospects and constraints to yam production in Ghana*. Symposium on Tropical Root Crops in a Developping Economy. ISHS Acta Horticulture, 380: 355-359.

Thellin, O., Zorzi, W., Lakaye, B., Borman, B.D., Coumans, B., Hennen, G., Grisar, T., Igout, A., and Heinen, E. 1999. Housekeeping genes as internal standards: Use and limits. *J. Biotech.* 75: 291-295.

Thouvenel, J. C. and Fauquet, C. 1979. Yam mosaic, a new potyvirus infecting *Dioscorea cayenensis* in the Ivory Coast. *Ann. Appl. Biol.* 93: 279-283.

Tian, Z. D., Liu, J., Wang, B. L., and Xie, C. H. 2006. Screening and expression analysis of *Phytophthora infestans* induced genes in potato leaves with horizontal resistance. *Plant Cell Rep.* 25: 1094-1103.

Traut, T. W. 1994. The functions and consensus motifs of nine types of peptide segments from different types of nucleotide-binding sites. *Eur. J. Biochem.* 222: 9-19.

Treche, S. and Delpeuch, F. 1982. *Dioscorea dumetorum*. In: Miège, J. and Lyonga, S.N, (eds.), Ignames. Clarendon Press, Oxford, UK, 411p.

Tschannen, A. T., Girardin, O., Nindjin, C., Dao, D., Farah, Z., Stamp, P., and Escher, F. 2003. Improving the application of gibberellic acid to prolong dormancy of yam tubrs (*Dioscorea* spp.). *J. Sci. Food Agric.* 83: 787-796.

Udoh, E. J. and Etim, N. A. 2007. Application of stochastic production frontier in the estimation of technical efficiency of cassava based farms in Akwa Ibom State, Nigeria. *Agric. J.* 2: 731-735.

Van-Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., and Genin, S. 1995. The hrp gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15: 1095-1114.

Vicente, J. and King, G. 2001. Characteisation of disease resistance genelike sequences in *Brassica oleracea* L. *Theor. Appl. Genet.* 102: 555–563.

Vidyalakshmi, A. and Divya, C. V. 2013. New report of *Colletotrichum gloeosporioides* causing anthracnose of *Pisonia alba* in India. *Arch. Phytopath. Plant Prot.* 46: 201-204.

Vignutelli, A., Wasternack, C., Apel, K., and Bohlmann, H. 1998. Systemic and local induction of an Arabidopsis thionin gene by wounding and pathogens. *Plant J.* 14: 285-295.

Vision, T., Brown, D. G., and Tanksley, S. D. 2000. The origins of genomic duplications in *Arabidopsis thaliana*. *Genome Res.* 42: 887-892.

Walbot, V. 1985. On the life strategies of plants and animals. *Trends Genet.* 1: 165-169.

Walton, J. D. 1996. Host-selective toxins: agents of compatibility. *Plant Cell* 8: 1723-1733.

Wan, H., Yuan, W., Ye, Q., Wang, R., Ruan, M., Li, Z., Zhou, G., Yao, Z., Zhao, J., Liu, S., and Yang, Y., 2012. Analysis of TIR- and non-TIR-NBS-LRR disease resistance gene analogous in pepper: characterization, genetic variation, functional divergence and expression patterns. *BMC Genomics,* 21: 1471-1553.

Wanasundera, J. P. and Ravindran, G. 1994. Nutritional assessment of yam (*Dioscorea alata*) tubers. *Plant Foods Hum. Nutr.* 46: 33-39.

Wang, B., Liu, J., Tian, Z., Song, B., and Xie, C. 2005. Monitoring the expression patterns of potato genes associated with quantitative resistance to late blight during *Phytophthora infestans* infection using cDNA microarrays. *Plant Sci.* 169: 1155-1167.

Wang, Z., Yano, M., Yamanouchi, U., Iwamoto, M., Monna, L., Hayasaka, H., Katayose, Y., and Sasaki, T. 1999. The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. *Plant J.* 19: 55-64.

Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. *Stud. Mycol.* 73: 115-180.

Wheatley, A. O., Ahmed, M. H., and Asemota, H. N. 2005. Development of salt adaptation *in vitro* greater yam (*Dioscorea alata*) plantlets. *Cell Dev. Biol. Plant* 39: 346-353.

Winch, J. E., Newhook, F. J., Jackson, G. V. H., and Cole, J. S. 1984. Studies of *Colletotrichum gloeosporioides* disease on yam, *Dioscorea alata*, in Solomon Islands. *Plant Path.* 33: 467-477.

Xiang-Li, X. and Wen-Hsiung, K. 1997. *A quantitative confined inoculation method for studies of pathogenicity of fungi on plants*. Department of Plant Pathology, Beaumount Agricultural Research Center, University of Hawaii at Manoa, Hilo, Hawaii 96720, USA, 39: 187-190.

Xiting, Z., Zhang, X., Gùo, X., Li, S., Han, L., Song, Z., Wang, Y., Li, J., and Li, M. 2016. Identification and Validation of Reference Genes for qRT-PCR Studies of Gene Expression in *Dioscorea opposita*. *Bio. Med. Res. Int.* 2016: 13-14.

Yang, J. K., Kil, H. K., Sangrea, S., Min, Y. Y., Suli, S., Moon, Y. K., Kyujung, V., and Suk-Ha, L., 2012. Genome-wide mapping of NBS-LRR genes and their association with disease resistance in soybean. *BMC Plant Biol.* 12: 139-144.

Yang, S. M., Dowler, W. M., and Johnson, D. R. 1991. Comparison of methods for selecting fungi pathogenic to leafy spurge *Plant Dis.* 75: 1201-1203.

Yang, W. W., Crow-Willard, E. N., and Ponce, A., 2008. Production and characterization of pure *Clostridium* spore suspensions. *J. Appl. Microbiol.* 106: 27-33.

Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z., Kono, I., Kurata, N., Yano, M., Iwata, N., and Sasaki, T. 1998. Expression of Xa1 a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. *Proc. Natl. Acad .Sci. USA,* 95: 1663-1668.

Yu, Y. G., Buss, G. R., and Saghai, M. A. 1996. Isolation of a superfamily of candidate disease resistance genes in soybean based on a conserved nucleotide-binding site. *Proc. Natl. Acad. Sci. USA*, 93: 11751–11756.

Yuksel, B., Estill, J. C., Schulze, S. R., and Paterson, A. H., 2005. Organization and evolution of resistance gene analogs in peanut. *Mol. Genet. Genomics* 274: 248-63.

Zaknayiba, D. B. and Tanko, L. 2013. Costs and returns analysis of yam production among small scale farmers in Karu local government area, Nasarawa State, Nigeria. *Path.* 9: 73-80.

Zhang, Y., Dorey, S., Swiderski, M., and Jones, J. D. G. 2004. Expression of RPS4 in tobacco induces an AvrRps4- independent HR that requires EDS1, SGT1 and HSP90. *Plant J.* 40: 213-224.

Zhou, T., Wang, Y., Chen, J. Q., Araki, H., Ping, Z., Jiang, K., Shen, J., and Tian, D. 2004. Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. *Mol. Genet. Genomics.* 271: 402-415.

Zipfel, C. 2008. Pattern-recognition receptors in plant innate immunity. *Curr. Opin. Immunol.* 20: 10-16.

Zoundjihekpon, J., Hamon, S., Tio-Toure, B., and Hamon, P. 1994. First controlled progenies checked by isozymic markers in cultivated yams *Dioscorea cayenensis- rotundata*. *Theor. Appl. Genet.* 88: 1011-1016.

APPENDICES

APPENDIX I

TISSUE CULTURE OF *D. alata*

Murashige and Skoog (MS) media (1 L) supplemented with IAA and Kinetin

MS media powder (HIMEDIA PT021X1L)	– 4.8 g
Sucrose	– 30 g
Polyvinylpyrrolidone (PVP)	– 1.0 g
IAA (100 mg L^{-1})	– 15 ml
Kinetin (100 mg L^{-1})	– 20ml

Dissolve in 900 ml distilled water and adjust the pH to 5.7 with 0.1N HCl/ 0.1N NaOH, and make up the volume to 1 L and transferred to test tubes. Sterilize at 121°C, 15 psi pressure for 20 minutes.

IAA (100 mg L^{-1}) – 100 ml

Dissolve 10 mg of IAA in a small volume of 1N NaOH and transfer to a volumetric flask. Make up the volume to 100 ml with distilled water and mix well. Aliquot and store at 4 °C.

Kinetin (100 mg L^{-1}) – 100 ml

Dissolve 10 mg of Kinetin in a small volume of of 1N NaOH and transfer to a volumetric flask. Make up the volume to 100 ml with distilled water and mix well. Aliquot and store at 4 °C.

Half - Murashige and Skoog (MS) media (1 L)

MS media powder (HIMEDIA PT021X1L) – 2.4 g
Sucrose – 15g
Polyvinylpyrrolidone (PVP) – 1.0 g

Dissolve in 900 ml distilled water and adjust the pH to 5.7 with 0.1N HCl/ 0.1N NaOH, and make up the volume to one litre and transferred to test tubes. Sterilize at 121°C, 15 psi pressure for 20 minutes.

0.1N HCl (100 ml)

Transfer 0.833 ml of concentrated HCl (12 N) to a volumetric flask (100 ml) and make up the volume to 100 ml with distilled water.

0.1 N NaOH (100 ml)

Dissolve 0.4 g of NaOH in 80 ml of distilled water and transfer to a volumetric flask (100 ml). Make up the volume to 100 ml.

APPENDIX II

CULTURING OF *Colletotrichum gloeosporioides*

Potato Dextrose Agar (PDA)

Suspend 3.9 g of PDA powder (HIMEDIA) in 100 ml distilled water and boil to dissolve. Sterilize by autoclaving at 15 psi pressure, 121°C for 15 minutes.

APPENDIX III

DNA ISOLATION

CTAB Extraction Buffer

Tris-Hcl (pH 8.0)	- 100 mM	
EDTA	- 20 mM	
NaCl	- 1.4 M	
CTAB	- 2 %	
β-mercaptoethanol	- 0.2 % (v/v)	Freshly added prior to DNA extraction
PVP	- 2 % (w/v)	

TAE Buffer, 50X (pH 8.0)

Tris-base - 242 g

Glacial acetic acid - 57.1 ml

0.5 M EDTA - 100 ml

Dissolve in 600 ml of distilled water, adjust the pH to 8.0 and make up the volume to 1L.

TAE buffer, 1 X

2 ml 50 X TAE + 98 ml distilled water

Agarose Gel (0.8%)

Agarose - 1.5 g

1 X TAE Buffer - 100 ml

Ethidium Bromide (10 mg ml^{-1})

Add 1 g of ethidium bromide to 100 ml of distilled water, stirred vigorously and transferred to dark bottle and stored at room temperature.

APPENDIX IV

CLONING AND TRANSFORMATION

Luria Agar

Suspend 3.5 g luria agar (HiMedia, Mumbai) in 100 ml distilled water, boil to dissolve and sterilize the medium by autoclaving.

T solution

Thaw T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µl each of T-solution (A) and (B) in separate tube and keep on ice.

Ampicillin stock solution (50 mg ml^{-1})

Dissolve 2.5 g of ampicillin sodium salt in 50 ml of deionized water. Filter sterilize and store as aliquots at -20°C.

X-Gal stock solution (20 mg ml^{-1})

200 mg X-Gal (5-bromo-4-chloro-3-indolyl–β-D-galactopyranoside) is dissolved in 10 ml N,N-dimethylformamide. Store at -20°C in dark.

IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG (isopropyl-b-D-thiogalactopyranoside) in 50 ml of deionized water. Filter sterilize, aliquot and store at 4°C.

LB-ampicillin X-Gal/IPTG plates

Suspend 4 g Luria Agar in 100 ml distilled water, boil to dissolve and sterilize the media by autoclaving. Before poring the plates, allow the medium to cool to 55°C. Then add 1ml of ampicillin stock solution to a final concentration of 50 µg ml^{-1}. Mix gently and pour the plates. Allow the LB-ampicillin agar medium to solidify. Dry plates under UV light for 30 minutes. Add 40 µl each of X-Gal

stock solution and IPTG; spread evenly with sterile L-rod. Pre-warm the plates at 37°C for at least 20 minutes before use.

ABSTRACT

MINING OF RESISTANCE GENES ASSOCIATED WITH ANTHRACNOSE INFECTION IN GREATER YAM (*Dioscorea alata* Linn.)

SARANYA G.

(2011-09-106)

Abstract of the thesis

Submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

B.Sc.-M.Sc. (INTEGRATED) BIOTECHNOLOGY
DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM-695 522
KERALA, INDIA

2016

9. ABSTRACT

Anthracnose caused by *Colletotrichum gloeosporioides* is the major fungal disease of greater yam (*Dioscorea alata*), which is one of the important tropical tuber crops with high production and nutritive potential. For identifying the Resistance Gene Analogues (RGAs) in greater yam, degenerate primers based on the conserved motifs were used to isolate nucleotide-binding site (NBS) type sequences. Cloning and sequencing of identified NBS-type sequences called resistance gene analogues (RGAs) showed similarity to other cloned RGA sequences available in the database and the presence of conserved domains, viz. P-loop, RNBS-B, RNBS-C, Kinase-2 and GLPL, categorising them with the NBS–leucine-rich repeat class gene family. Amino acid sequence alignment of the *Dioscorea* RGAs with RGAs of other plant species grouped them with the non-Toll interleukin receptor (TIR) subclasses of the NBS sequences. The expression profiles of RGAs determined using semi quantitative Reverse Transcriptase polymerase chain-reaction in Sree Keerthi (tolerant) and Orissa Elite (susceptible) genotypes in response to anthracnose infection demonstrated that, *Dioscorea alata* RGAs were up-regulated three days after disease inoculation in the tolerant genotype, whereas in the susceptible genotype it was observed on the 5th day. In contrast, RGAs were found to be expressed in both tolerant and susceptible control plants, but the level was found to be increased in the test plants following pathogen inoculation. The reverse transcription PCR product was normalized and the efficiency was evaluated using Actin primers, which serves as reference gene. The result suggests a role of Resistance Genes Analogues in the early pathogen recognition of Sree Keerthi against *C. gloeosporioides*, which may be one of the reasons for its tolerance to anthracnose disease. These genes could be a good start point for further studies such as candidate gene mapping or understand the bases for resistance in greater yam. The isolation and expression analysis of *D. alata* RGAs have been reported for the first time in this study.