DIFFERENTIAL RESPONSE OF RESISTANT GENE ANALOGUES (RGAs) AGAINST *Phytophthora colocasiae* CAUSING LEAF BLIGHT IN TARO (*Colocasia esculenta*).

by

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(2014-09-108)

THESIS

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fulfillment of the requirement

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DECLARATION

I hereby declare that the thesis entitled "Differential response of resistant gene analogues (RGAs) against *Phytophthora colocasiae* causing leaf blight in taro (*Colocasia esculenta*)" 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.

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Jyothi Lekshmi O. B.

Dedicated to my beloved family

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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
Kb	Kilo bases
kg	Kilogram

L	Litre
m	Meter
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MS	Murashige and Skoog
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
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
ppm	Parts per million
PVP	Polyvinylpyrrolidine

INTRODUCTION

1. INTRODUCTION

Root and tuber crops are the most important food crops after cereals and have found an important place in the dietary habits of small and marginal farmers especially in the food security of tribal population. Tropical root and tuber crops including cassava, sweet potato, yam and aroids are enjoyed as vegetables, used as raw materials for small-scale industries, and consumed as staple foods especially in the less developed countries (Ravi *et al.*, 1996). Root and tuber crop production is about 887 million tonnes globally from about 67 million hectares with an average yield of 13.2 t ha^{-1} (FAOSTAT, 2017).

Taro (*Colocasia esculenta* (L.) Schott.), an important tropical tuber crop, a member of the monocotyledonous family Araceae with nearly 1000 cultivars, grown for its edible corms, and leaves, serves as a staple food or a subsistence crop for millions of people in developing countries of Asia, Africa, Caribbean and Pacific Islands. It is the fourteen most consumed vegetable worldwide with about 10.2 million tonnes produced globally from about 1.7 million hectares with an average yield of 6 t ha^{-1} (FAOSTAT, 2017) and is considered as a rich source of carbohydrates, minerals, proteins and vitamins. Taro is also used as a traditional medicine with root extract used to treat rheumatism and acne, while leaf extract is used for blood clotting at wound sites, neutralizing snake poison and as a purgative medicine.

Taro and its cultivation apparently had originated in the region of South Central Asia, probably in India or Malaysia (Keleny *et al.*,1962; Onwueme *et al.*,1978). Taro is now widely consumed throughout the world and is of great significance in many places such as the Caribbean, Hawaii, the Solomons, American Samoa, West Samoa, Philippines, Fiji, Mauritius, Sri Lanka, India, Nigeria, Indonesia, New Hebrides, Tonga, Niue, Papua, New Guinea, Egypt and others. Millions of people in these areas

depend on taro as staple food and it has also been introduced in the Southern United States as supplement to potatoes.

In India, the mostly cultivated two types of taro are *C. esculenta* var. *esculenta* (Dasheen type) and *C. esculenta* var. *antiquorum* (Eddoe type). The eddoe type has small corm and large cormels, where as in dasheen type, the corm is large and cormels are small. The main area under cultivation of this crop is located in the northern and eastern states of India. The crop , especially *C. esculenta* var. *esculenta*, has very high yield potential and showed tuber yield of 30-50 t ha^{-1} during a survey of major taro growing areas (Misra, 1999).

Taro leaf blight caused by *Phytophthora colocasiae,* an oomycete water mould, is one of the most destructive diseases of taro resulting in severe yield reductions and plant death. The pathogen causes circular, water soaked, necrotic spots on the leaves, followed by the collapse of the plant. Under favourable conditions, the entire field will be devastated within few weeks of the onset of infection. The disease is prevalent in all taro growing regions of the globe including India causing yield reduction of the magnitude of 30-50% (Jackson, 1999; Lebot *et al.*, 2003; Misra *et al.*, 2008; Singh *et al.*, 2012).

Taro leaf blight is a threat to food security and economy in those countries where taro is a major staple and an export commodity. Several approaches have been advocated to control disease. Cultural practices like crop rotation and shifting of planting time have been unsuccessful in protecting the crop. Metalaxyl and mancozeb based fungicides have proved effective in controlling the disease but waxy leaf surface and occurrence of disease during rainy season makes fungicidal spray ineffective (Misra, 1999). Moreover, the fungicide sprays are too costly to be afforded by marginal farmers and the development of resistance against the fungicides is another major threat. Therefore, exploiting the natural host resistance is thought to be the most promising, non-conventional and ecologically friendly

approach to mitigate leaf blight disease. For the fulfillment of this approach, a clear understanding of the molecular mechanisms involved in host resistance to pathogen and about the high genetic diversity existing among the various taro genotypes with respect to their susceptibility or resistance to leaf blight is required.

The molecular communication between plant and pathogen commence almost immediately after the pathogen makes contact with the plant surface, which suggest that resistance (R) gene product recognises a specific avirulence gene product specified by the pathogen (gene-for-gene principle) (Flor *et al.*, 1971). During the last 15 years, a total of 70 different resistance genes showing resistance to major plant pathogen had been isolated, cloned and characterised in different plants. Approximately 75% of these resistance genes belong to NBS-LRR gene family. The NBS domain consists of around 3000 amino acids and four conserved motifs named as P loop (kinase 1 a), kinase 2, kinase3a, and GLPLAL motif. The LRR region consists of many leucine rich sequences which promots recognition of avirulence gene product (Jia *et al.*, 2000). A key 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 (Grant *et al.*, 1995).

The PCR-based strategies has proven as an excellent tool for identification, cloning and mapping of resistance gene family members analogues with the use of degenerate or specific primers for conserved motifs of different NBS–LRR genes. Through this approach, successful cloning of putative NBS–LRR resistance gene analogues (RGA) from many crops like soybean (Yu *et al.*,1996), wheat (Huang *et al.*, 2003), barley (Seah *et al.*, 1998), rice (Mago *et al.*, 1999), sugar cane (Que *et al.*, 2010), maize (Xiao *et al.*, 2006), turmeric (Joshi *et al.*, 2007), taro (Nath *et al.*,2013) and greater yam (Saranya *et al.*, 2017) was possible. RGA strategy is very useful in marker development and identification of quantitative trait loci for plant disease resistance genes, and for diversity and evolutionary studies of R genes.

Hence the objectives of this work were formulated to isolate, characterize and sequence the Resistant Gene Analogues in taro against *Phytophthora colocasiae* and to study the gene expression profiling of resistant and susceptible taro cultivars upon leaf blight infection.

REVIEW OF LITERATURE

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. 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.

2.2 TARO (*Colocasia esculenta*)

Taro is a herbaceous plant of family Araceae which consists of a central corm from which cormels, roots, and the shoot arise. The shoot consists mainly of the leaves which arise in a whorl from the apex of the corm. Plant height is determined by the height of the leaves, ranges from 1-2 metre. Each leaf consists of a long erect petiole and a large lamina. The attachment of the petiole to the lamina is not at the

edge of the lamina, but at some point in the middle of the lamina. This form of attachment (peltate leaf) is a diagnostic feature which generally distinguishes taro from tannia and the other edible aroids. Leaf lamina in taro is large, thick, entire and glabrous. Its shape varies greatly from almost round to ovate with a notch at the base and a pointed tip. Three main veins radiate from the point of attachment of the petiole, and several prominent lateral veins originate from the main vein. The petiole may be more than one metre long; thick along its entire length, but thicker at the base than near the attachment of lamina. The base of the petiole, where it is attached to the corm, is flared out so that it clasps around the apex of the corm. The petiole is solid throughout its length, but it is replete with large air spaces. These air spaces presumably function as conduits for aeration of subterranean organs when the plant is grown under swampy or flooded conditions (Misra *et al.*, 2005). Flowering in taro is only occasional, and confined to a few cultivars under natural condition. The root system of taro is fibrous and is confined mostly to the top layers of the soil. The roots arise from the lower portions of the corm.

2.3 TARO DISEASES

Taro leaf blight and viral diseases are important constraints in taro cultivation resulting in severe yield reductions and plant death. The main effect of virus infection is a reduction in corm size and quality, with yield loss up to 20%. There are currently five viruses reported to infect taro with varying distribution in taro growing region and throughout the Pacific Islands i.e. *Dasheen mosaic virus* (*DsMV*), *Taro bacilliform virus* (*TaBV*) is a badnavirus, *Colocasia bobone disease virus* (*CBDV*), *Taro vein chlorosis virus* (*TaVCV*) and *Taro reovirus* (*TaRV*) (Revill *et al.*, 2005). Other important taro disease include the taro soft rot, which is caused by several species of *Pythium*, which is soil borne and attacks the roots and corm. Infected plants display wilting and chlorosis of the leaves as well as the proliferation of roots at the base of the shoot; the corm becomes soft and decayed and the plant often dies.

Sclerotium rot is caused by *Sclerotium rolfsii*, which causes stunting of the plant, rotting of the corm and formation of numerous spherical sclerotia in the corm. *Cladosporium* leaf spot is caused by *Cladosporium colocasiae* where brown spots appear on the older leaves.

2.4 TARO LEAF BLIGHT

Leaf blight of taro, caused by *Phytophthora colocasiae*, is the most destructive disease of *Colocasia esculenta*. It was first reported from Java in 1900 and this disease alone brought a decline in taro cultivation and production (30-50%) in the Solomon Islands, Papua New Guinea, Philippines, Indonesia, China, Malaysia, Japan, India and countries of Africa and Caribbean. Butler and Kulkarni (1913) reported leaf blight of taro for the first time in India, which had been observed in various places in India since 1905 causing serious damage. To date, taro leaf blight has been recorded in a number of countries in the Pacific region, most recently in Nigeria and West Africa (Bandyopadhyay *et al.*, 2011). The first symptoms of *Phytophthora* blight of taro appear as small, dark brown flecks or light brown spots on the upper leaf surface of taro. These early spots often occur at the tips and edges of leaves at the site of water accumulation, which enlarge rapidly, become circular, zonate, and purplish-brown to brown in colour. On the lower leaf surface, spots have a water-soaked, or dry gray appearance. As the spots increase in size they coalesce and quickly destroy the leaf within 3-5 days after the initial symptoms, depending on the weather conditions. Dead leaves often hang on their long petioles like flags.

The normal longevity of a healthy leaf is about 40 days. The disease significantly reduces the number of functional leaves and can lead to yield reductions of the magnitude of 50% (Jackson, 1999). Inoculum in the form of spores is spread by wind-driven rain and dew to adjacent plants and nearby plantations. The disease can also be spread on taro planting material and the fungus has been reported as remaining alive on planting tops for about three weeks after harvest (Jackson, 1999).

Planting material is the most likely source of the pathogen in new countries and the means for its rapid spread within a country, once established. In addition to yield losses due to the reduced leaf area in diseased plants, there is also a corm rot caused by *P. colocasiae.* Fortunately, *P. colocasiae* does not have a wide host range. *Xanthosoma* is immune to taro leaf blight; but *Amorphophallus* has been reported to be infected by *P. colocasiae* (Misra *et al.*, 2008). Raciborski (1900), in Java, was the first person to study taro leaf blight disease and was the first to name the causal pathogen.

2.4.1 History of Taro Leaf Blight

Leaf blight has become a limiting factor for taro production in the Solomon Islands, Hawaii, Fiji, Western Samoa and in India causing up to 50% yield loss (Graham, 1965; Gollifer and Brown, 1974; Jackson *et al.*, 1980; Misra, 1999; Misra *et al.*, 2008). The geographic distribution of this disease is probably restricted to South-East Asia and the Pacific areas (Holliday, 1980). The occurrence of leaf blight of taro has been reported from different countries.

In addition to leaf blight, *P. colocasiae* causes a serious post-harvest decay of corms (Jackson and Gollifer, 1975). In India also, leaf blight is reported to be a serious disease in many areas such as Kangra valley of Punjab which is now in Himachal Pradesh (Luthra, 1938), Assam (Chowdhury, 1944), Bihar (Anonymous, 1950), Himachal Pradesh (Paharia and Mathur, 1961) and other states (Thankappan, 1985; Misra, 1999).

There is limited information on the origin of *P. colocasiae* and the magnitude of the area of origin is yet to be defined (Zhang *et al.*, 1994). It is suspected that Southeast Asia may be the centre of origin of *P. colocasiae* given that it is the world's centre of origin for many wild and cultivated varieties of taro (Ko, 1979). One of the indications of the centre of origin of a fungus such as *Phytophthora* is the

existence of an A1/A2 mating type ratio of about 1:1. However, it is believed that only A1 mating type has been found in India (Narula and Mehrotra, 1980) while recently both mating types were observed in India (Misra *et al.*, 2011; Nath *et al.*, 2014). The presence of both mating types together in taro fields not only increases the potential of oospore production and genetic recombination of *P. colocasiae* but also may answer the question of *P. colocasiae* origin. However, extensive studies need to confirm the origin of this pathogen, probably more isolates need to be collected from southern part of India where A2 mating type has been reported and until then the status still remains unclear.

2.4.2 Crop Loss

Phytophthora blight of taro appears as small, water-soaked spots that increase in circumference and also spread to healthy plants. The entire leaf area is destroyed within 3-5 days after the initial symptoms depending on the weather conditions. Under cloudy weather conditions with intermittent rains and temperature around 28°C, the disease spreads at tremendous speed and the entire field gives a blighted appearance. This disease is reported to have destroyed taro plantings in Papua New Guinea, both in the islands of Manus and Bougainville (Packard, 1975). Yield losses of 25-50% are common in Solomon Islands (Gollifer and Brown, 1974; Jackson *et al.*, 1980) and in Hawaii (Bergquist, 1974) due to infected plants having three functional leaves instead of the more usual number of six or seven. Jackson and Gollifer (1975) found that the infected leaves collapse within 20 days of unfurling compared to 40 days in healthy leaves. They have also found 30-40% loss in tuber yield when the attack was recorded in 40 to 70 days old crops. Leaf blight adversely affects dry matter production through destruction of leaf area as expressed in terms of disease severity. A decrease in crop growth, in turn, reduces tuber yield. Out of 128 representative fields of taro tested during the 1988 monsoon season, 94% of the fields were infected by leaf blight with 78.38% of fields having more than 80% incidence.

During 1989, out of 164 taro fields 92% showed blight infection with 81.75% of fields showing more than 80% incidence.

In India, Misra (1999) conducted elaborate field trials to assess yield losses caused by taro blight. One set of trials was conducted in the farmer's field at Salepur in Odisha (India) using a local variety and another set was conducted at the farm of Regional Centre of CTCRI, Bhubaneshwar using two varieties, one tolerant 'Jankhri' and the other susceptible 'Telia'. Spraying with mancozeb (0.2%, one to five sprays) was done to obtain variation in disease severity. One plot with 5 sprayings was considered as a check plot and yield loss was calculated over this. During 1988, yield loss of 45.20% was recorded in the farmer's field while on the farm, a yield loss of 50.46% was recorded in the *Phytophthora* leaf blight susceptible variety and 35.94% in the *Phytophthora* leaf blight resistant cultivar (Misra, 1991 and 1999). During 1989, 39.41% yield loss was recorded in a local variety in the farmer's field, whereas on the farm, the susceptible variety showed 55.17% yield loss compared to 29.30% in the tolerant cultivar. Interactions effect between varieties and treatment were highly significant (Misra, 1991 and 1999). During 1990, yield loss of 28.75% was recorded in the farmer's field, whereas on the Institute farm, 46.75% yield loss was recorded in the susceptible variety and 22.75% in the tolerant variety (Misra, 1990 and 1999).

It is evident that the yield losses caused by *P. colocasiae* are substantial and adequate attention is required to manage leaf blight before undertaking taro cultivation. Use of tolerant cultivars, in areas known for high blight severity, can alone considerably reduce the damage caused by leaf blight disease.

2.4.3 *Phytophthora colocasiae*

Raciborski was the first who described the causal organism of leaf blight of taro as *P. colocasiae* in 1890 from Indonesia. The mycelium is hyaline, coenocytic and inter- or intracellular. The haustoria are slender, long and unbranched. The

growth of the fungus is optimum at pH 6.5 and 28°C. Sporangia are formed at the end of short, unbranched or sparingly branched sporangiophores at the edge of the lesions. They are ovoid to ellipsoid; lemon- or pear-shaped with a distinct narrow apical plug (semi-papillate), average 40-50 x 23 µm, and have a length to width ratio of 1.6:1. The sporangiophores are very slender, unbranched and extremely narrow at the tip and measure up to 50 µm in length. They germinate directly or indirectly depending on the weather conditions. When indirect (20-21°C) as many as 12 remiform, biflagellate zoospores are released, which convert to cysts and germinate after 30 min (Misra, 1999). Thick-walled, round, hyaline chlamydospores are also produced, especially in old cultures (Thankappan, 1985; Misra, 1999).

The zoosporangiophores are slender, unblemished and extremely narrow at the tip and measure up to 50 µm. Misra (1999) observed that zoosporangial length was over 100 µm and the width was over 50 µm. Depending on the weather conditions, the zoosporangium is capable of producing another zoosporangium. It may germinate directly by producing one or more germ tubes or by producing zoospores. Zoosporogenesis (indirect zoosporangial germination), like in most other species of *Phytophthora*, starts with the fusion of cleavage vesicles, which occurs almost spontaneously with the release of zoospores. Chilling the zoosporangia at 4°C for 10 min induces zoospore cleavage. Immediately after cleavage, the apical exit pore plug material balloons outwards to form a discharge vesicle that varies in size. The zoospores get expelled from the discharge vesicle and break through the thin plastic wall to escape. Within 20 min of the release, the zoospores encyst having a rather thick cell wall. The cysts germinate within 30 min of their encystment. The cysts are more damaging from a disease point of view as they are produced in large numbers, are small in size and light in weight compared to zoosporangia. The abundant production of zoosporangia, zoospores and cysts make *P. colocasiae* a devastating pathogen.

Sexual reproduction of this heterothallic species depends on the presence of both A1 and A2 mating types. Hormones produced by one mating type stimulate production of antheridia (male) and oogonia (female) in the opposite mating type. Each antheridium attaches to the base of an oogonium, surrounding its stalk-like attachment (amphigynous).The nuclei in these organs undergo meiosis and the haploid nucleus from the antheridium unites with a haploid nucleus in the oogonium, forming a diploid oospore 18-30 µm (average 23 µm) in diameter.

P. colocasiae produces pectolytic enzymes like polygalacturonase, pectin methyl transeliminase and poly methyl galacturonase and these enzymes may play a major role in the pathogenesis of *C. esculenta. Amorphophalluspaeonnifolius* and black pepper were also reported to be the host for *P. colocasiae* (Paharia and Mathur, 1961).

Besides *P. colocasiae* few other species of *Phytophthora viz.*, *P. arecae* (Coleman) Peth., *P. palmivora* Butler, *P. parasitica* Dast. var. *pipernia* Dast., *P. nicotiana* Bredade Ham. var. *parasitica* Dastur, have been reported to infect taro in other countries. However, the role of these species in the severity and damage or epidemiological aspects is not known.

2.4.4 Biology and ecology

P. colocasiae has survival devices that are less simple than those of *P. infestans*. The disease spread is by sporangia and zoospores that are carried by splash between plants and plantings. Butler and Kulkarni (1913) found that the fungus survives in rhizomes. Since oospores and chlamydospores are reproduced, persistence of soil infectivity should not be a mystery. In Philippines, the survival of *P. colocasiae* in the soil has been reported through oospores, in the corm or leaf tissue left in the field after harvest (Gomez, 1925). Saprophytic survival of *P. colocasiae* and the role played by alternate host has been studied by Gollifer *et al.* (1980) in

Solomon Island, where taro is grown all-year round. They found that the inoculum in the soil remained viable only for a few days. Perennation between crops is affected by short-lived propagules and possibly by mycelium with petiole lesions. Propagating material in the form of decapitated taro tops of infected crops carries the short-lived inoculum to new taro fields. In India, the situation is different where corms are stored and serve as the seed material for next season crop. Narula and Mehrotra (1984) studied the saprophytic survival of *P. colocasiae* in Indian soil. During rainy season, *P. colocasiae* is present in naturally infested soil and it can be detected in soils only up to the end of September by leaf baiting technique. Under these conditions, when *P. colocasiae* does not seem to survive much longer freely in the soils or in the infected dead leaf tissues, the corm-borne inoculum of *P. colocasiae* would have much more importance in the recurrence of the disease. *P. colocasiae*, like other foliar *Phytophthora*, seems to have a poor competitive saprophytic ability in soil (Narula and Mehrotra, 1984). Another important source of survival is self-sown taro plants, which grow as wild plants near ponds or compost pits (Misra and Chowdhury, 1997). Besides, the pathogen can also survive on many collateral hosts, which have been found to be natural hosts of *P. colocasiae* (Thankappan, 1985).

2.4.5 Epidemiology

Leaf blight is observed in severe form in areas having high relative humidity and frequent rainfall, whereas warmer areas having little rainfall and relative humidity are comparatively free from this disease. The blight epidemics occur when night and day temperatures ranged between 20-22°C to 25-28°C, respectively, with a relative humidity of 65% during the day and 100% at night and accompanied by cloudy rainy weather. Under such conditions taro leaves could be damaged by blight disease in 5-7 days. Low temperature below 20°C and high temperature above 28°C prevented sporulation of the fungus and reduced severity despite high humidity and rain. The disease out-break could occur when the temperature and relative humidity

conditions are optimum for 6-8 h for three consecutive days with light rain or dew in the morning. Minimum temperature and relative humidity had a significant positive correlation with disease severity. Occasional sunlight with intermittent rain is more favorable for disease severity compared to prolonged cloudy weather with rainfall (Misra and Chowdhury, 1997).

2.5 DISEASE RESISTANCE IN PLANTS

Plants defend themselves from attack by microbes, viruses, invertebrates, and sometimes other plants. Since plants lack a circulatory system like animals, they 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.

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).

2.5.1 Plant resistance genes

Although plants are under continuous attack by pathogens, most encounters result in plant resistance and disease being the exception. Passive defence lines such

as cell walls, wax layers and chemical barriers confer broad resistance to a wide variety of pathogens. If a pathogen overcomes this first line of defence, there is a second line, which is mounted by proteins encoded by specific resistance (*R*) genes. At this level at least four fundamentally different resistance mechanisms can be recognised:

1) The *R* gene product inactivates a toxin, which is produced by the pathogen, and which normally induces necrosis or inhibits the induction of active defence responses.

2) The *R* gene product encodes a pathogenicity target. Thus, absence of this target results in plant resistance.

3) The *R* gene product primes the plant defence responses.

4) The *R* gene product mediates specific recognition of a pathogen that expresses a matching avirulence (*Avr*) gene (i.e. gene-for-gene resistance)

The understanding of the molecular structure and function of *R* genes has been crucial for plant resistance research.Until now, >140 *R* genes have been cloned in different plant species (Zhang *et al.*, 2018).

2.6 RESISTANCE GENE ANALOGUES

Resistance gene analogues (RGAs) are a large class of potential R-genes that have conserved domains and structural features. As such, RGAs can be identified from sequenced genomes using bioinformatics approaches. In the last 15 years, more than 50 plant genomes have been sequenced and assembled. Despite this great resource, only a relatively small number of R-genes have been cloned and fully characterized providing not only information on their structure, function and evolution, but also generating useful genetic resources to create novel resistant cultivars.

RGAs can be grouped as either nucleotide binding site leucine rich repeat (NBS-LRR) or transmembrane leucine rich repeat (TM-LRR). Recent findings have identified other modes of plant resistance mechanisms including pentatricopeptide repeats (PPRs) and peroxidases. NBS-LRR can be further classified as toll/interleukin receptor (TIR)-NBS-LRR (TNL) or non-TNL/coiled coil-NBS-LRR (CNL). Both TNL and CNL specifically target pathogenic effector proteins inside the host cell, termed effector triggered immunity (ETI) response. Likewise, TM-LRRs could be subdivided into two classes: receptor like kinases (RLKs) and other receptor like proteins (RLPs). RLPs and RLKs are pattern recognition receptors (PRRs) that mediate pathogen/microbe associated molecular pattern (PAMP/MAMP) triggered immunity (PTI/MTI) to allow recognition of a broad range of pathogens .PAMP/MAMPs are conserved features of most pathogens, such as chitin, flagella, and lipopolysaccharides.

2.6.1 Nucleotide Binding Site - Leucine Rich Repeat (NBS-LRR) Family

NBS-LRR is the best-known family of RGAs. The two classes of NBS-LRR are distinguished by their N terminal TIR or non-TIR domains. The non-TIR domains are most commonly coiled coil (CC) structures. Another non-TIR domain is the leucine zipper (LZ), with interspersed hydrophobic heptad repeat sequences L-X(6)-L-X(6)-L-X(6)-L . The domain combination refers to LZ-NBS-LRR proteins which are not as common but have been found in agricultural plants such as tomato and potato. At the N-terminal region lies the highly irregular and variable LRR domain. This domain is responsible for protein-protein interactions .Between the NBS and LRR domains exists a region called the ARC domain, named so because of its occurrence in APAF-1, R protein and CED-4.This ARC domain can be further divided into ARC1 and ARC2 subdomains. The ARC domain, together with the NBS domain, forms a region for nucleotidebinding.

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 Mg2+ (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).

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).

2.6.2 Receptor Like Kinase (RLK) and Receptor Like Protein (RLP) Families

RLK and RLP are main components of the first line of plant immune response triggered by microbial elicitors PAMPs or MAMPs, where the interactions between receptor and elicitor usually take place in the extracellular space (Yang *et al.*,2012). The two proteins are structurally similar with (1) a signal peptide (SP) at the beginning of N-terminus; (2) extracellular domains for perception of the microbial pattern through the leucine-rich repeats and (3) a transmembrane helix domain that can anchor RLP and RLK in the plasma membrane. RLPs differ from RLKs by the lack of an intracellular kinase domain; thus RLPs are unable to independently transduce the perceived signal into a downstream cascade. Both RLPs and RLKs are

considered PRRs that recognize elicitors such as lipids, proteins, nucleic acids, and carbohydrates.

2.7 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 the region in the DNA which 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.

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.

2.8 REAL-TIME PCR

Real-time PCR is currently considered as the most powerful and standard method for detection of plant pathogens and gene expression studies. This technique allows the monitoring of the reaction during the amplification process by the use of a fluorescent signal that increases proportionally to the number of amplicons generated and to the number of targets present in the sample. The first reported method of real-time PCR was done by using ethidium bromide intercalation and a modified thermocycler to irradiate the samples with ultraviolet (UV) light and then fluorescent signal was detected with a charged coupled device (CCD) camera. The main drawbacks of that approach were the use of carcinogen and equal detection of non – specific PCR products in the measured fluorescent signal.

Today, the widely used two types of fluorescent reporters in real time PCR are SYBER Green and Taqman probes. There are many advantages of real-time PCR over conventional PCR, including that this system does not require the use of post PCR processing (e.g. agarose gel electrophoresis), avoiding the risk of carryover contamination and reducing assay labour and material costs. Due to its improved sensitivity, specificity, accuracy and reproducibility, this technique allows the detection of slight variations in the gene expression (Bustin *et al.*, 2010).

2.8.1 Endogenous Controls

The most commonly used method of data normalization is the use of stably expressed reference genes as endogenous controls. This is done to ensure the reliability of target gene expression when results are expressed in terms of relative quantification using the popular comparative CT method ($2^{-\Delta\Delta CT}$ method).The genes which are involved in the basic cellular processes like 18S rRNA, actin, tubulin are used as house keeping genes or reference genes.

2.9 GENE EXPRESSION STUDIES

Identification of genes whose expression is evoked or inhibited in response to pathogen infection can provide an improved understanding of the molecular mechanisms related to resistance and susceptibility and provide a foundation for biotechnology approaches to improve disease resistance in plants (Casado-Diaz *et al.*, 2006). The NBS-LRR disease resistance genes are basally expressed in plant tissues but upon pathogen detection, the expression of these genes is up-regulated in order to initiate defense responses. 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.

Natural variations in disease resistance present in different cultivars can serve as a valuable resource for dissecting the molecular mechanisms of host resistance to a specific pathogen. Hu *et al.* (2017) has studied about the expression of 12 NBS-encoding genes in response to pathogen infection by *A. alternata* in two apple cultivars and found that all the genes were involved in the host response against pathogen infection. The gene expression profile showed that, 3 out of 12 NBS-encoding genes were observed to be highly expressed and can be used as valuable candidates for breeding resistant apple cultivars through genetic engineering.

Plasmopara viticola causes downy mildew in grapevine. The investigational studies on natural resistance has isolated a CC-NBS-LRR-type R gene from Chinese wild grapevine. The results of quantitative real time PCR showed the rapid and high gene expression in the resistant variety after the pathogen infection and transient gene expression in susceptible variety (Zhang *et al.*, 2018).

Homology based computational approach was also used for identifying potential NB-LRR genes in plants and this was found successful in identifying 40 resistance gene homologues with unique domains in switchgrass, which is an

important bioenergy crop. The results of the study in rust-resistant and rust-susceptible switchgrass cultivar have indicated that the expression of some of these resistance gene homologues was developmentally regulated (Frazier *et al.*, 2016)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The study entitled "Differential response of resistant gene analogues (RGAs) against *Phytophthora colocasiae* causing leaf blight in taro" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during October 2018 – August 2019. Details relating to the experimental materials and methodology adopted for the study are detailed in this chapter.

3.2 AMPLIFICATION OF RGA FROM TARO

3.2.1 Sample Collection

The leaf samples used in this study were collected from the taro varieties Sree Kiran (susceptible), and Muktakeshi (tolerant) from tissue culture raised plants of the above varieties and that growing in fields at ICAR - Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram.

3.2.2 Isolation of DNA

Young leaves of *Colocasia esculenta* plants, Sree Kiran (susceptible) and Muktakeshi (resistant) were collected for DNA isolation. CTAB method was used to isolate genomic DNA from taro leaf samples.

3.2.2.1 CTAB Method of DNA Extraction (Sharma *et al.*, 2008)

Young leaf tissues (100 mg) were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. Followed that 1 ml of extraction buffer (pre-warmed at 65 °C) (Appendix III) was added to the samples and it was ground once again in the buffer. All the samples were transferred to sterile 2.0 ml eppendorf tubes and 5

µL Proteinase K (10 mg ml^{-1}) was added. The tube was incubated in 37 ºC for 30 min and then at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for another 30 min with frequent swirling. Samples were centrifuged at 8,000 g (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 10 min at 28 °C and supernatant was transferred to fresh eppendorf tube. Equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) were added and mixed by gentle inversion for 30 - 40 times. The samples were centrifuged at 8,000 g for 10 min at 28 °C and then transferring the aqueous layer or supernatant into a fresh tube. 200 µL of 2 M NaCl solutions containing 4% PEG was added and incubated for at least 15 min at 4 °C increased the recovery of DNA yield. The samples were centrifuged at 8,000 g for 10 min at 27 °C and the supernatant was transferred to a fresh tube and precipitated with 0.66 volume of isopropanol. The precipitates were collected and washed twice with wash solution (Appendix III). The nucleic acid pellet obtained was air-dried at 37 °C for 30 min After the ethanol was removed completely, it was dissolved in appropriate amount of TE buffer (50 µL). The nucleic acid dissolved in TE buffer were treated with RNase (10 mg ml^{-1}), incubated at 37°C for 30 min and stored at -20 °C until use.

3.2.2.2 Agarose Gel Electrophoresis

The most common method used to assess the integrity of nucleic acid preparation is to run an aliquot of the sample on a agarose gel stained with ethidium bromide (EtBr). 0.8% agarose gel was prepared in 1X TAE buffer (Appendix III), then heated in microwave oven until completely melted. After cooling the solution, 0.5 µl of ethidium bromide (10 mg ml^{-1}) was added. It was then poured into casting tray containing sample comb and allowed to solidify at room temperature. Then the combs were removed followed by placing gel into the electrophoresis chamber and covered with buffer. An aliquot of the DNA sample (3 µl) mixed with the loading dye (2 µl) was loaded in each well of the gel. The gel was run at 50 Vcm^{-1} (BIO RAD Power Pac HV, USA) for 30 min. The gel was then visualized under UV light and the

image was documented using the Gel Doc System(Alpha Imager, Alpha Innotech, USA).

3.2.2.3 Nanodrop spectrophotometer analysis

The absorbance of DNA samples at 260, 280 and 230 nm were recorded to determine the quantity and quality of DNA isolated. The optical density (OD) was measured using NanoDrop spectrophotometer (DeNovix DS-11). The spectrophotometer was calibrated to blank (zero absorbance) with 1µl of sterile 1X TE buffer. Then the concentration of each DNA samples (1µl) was recorded. The quality of DNA preparation was determined by analyzing the A260/A280 and A260/A230 readings.

3.2.3 PCR amplification of RGAs using degenerate primers

The conserved region between P-loop and GLPL of R-genes in *Colocasia esculenta* were targeted using degenerate primers reported earlier (Nath *et al.*, 2013).

3.2.3.1 Gradient PCR for standardizing annealing temperature

Two sets of degenerate primers were used for the amplification of RGAs in *Colocasia esculenta*.

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

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

RGA 2F (forward primer) sequence: 5'GGTGGGGTTGGGAAGACAACG 3'

RGA 2R (reverse primer) sequence: 5'CCACGCTAGTGGCAATCC 3'

To standardize the annealing temperature of the primer pair used, gradient PCR was performed for each primer set. The isolated DNA sample of taro 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

The reaction mix was prepared, vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the programme was set with an initial denaturation at 94°C for 5 min, followed by 42 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 amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were resolved on a 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and photograph was scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA).

3.2.3.2 PCR amplification of RGAs

After standardizing the annealing temperatures of the primer pairs, the taro DNA samples were subjected to PCR using same reaction mixture as described in section 3.2.3.1. PCR was carried out in Agilent Technologies sure Cycler 8800 (USA) and the same cycling conditions were set as given in section 3.2.3.1 with an annealing temperature of 52.5°C for RGA1 and 49°C for RGA2. The amplified PCR

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products were separated on agarose gel (1.5%) and viewed using the Gel Doc System (Alpha Imager, Alpha Innotech, USA).

3.2.4 Purification of PCR amplification product by gel elution method

Gel extraction of PCR fragments was attained with GeneJET Gel Extraction kit (Thermo Scientific, USA). The PCR products were seperated 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 200 µl of binding buffer was added to the gel slice and it was incubated at 50 - 60°C for 10 min and vortex the samples briefly every 2-3 min until the gel slice was completely dissolved. Then 700 µl of the solubilised gel solution was added to GeneJET purification column and was followed by centrifugation at 11,000 g for 30 seconds. The column was placed back into the same collection tube after discarding the flow-through. The remaining samples were added and the centrifugation step was repeated. After addition of 700 µl of wash buffer to the column, it was centrifuged at 11,000 g for 30 seconds. Again, the flow-through was discarded and the column was placed back into the collection tube. The washing step was done twice and 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. The column was placed into a new 1.5 ml collection tube and was air dried for 10 min. The purified PCR product was eluted by adding 15-30 µl of elution buffer to the centre of the purification column membrane and incubated at room temperature for 1 min, followed by centrifugation for one min at 11,000 g. After elution, the column was discarded and the purified elute was stored at -20°C.

3.2.5 DNA SEQUENCING

The reamplified gel elutes were sequenced (Genetic Analyzer ABI 3500) at the AgriGenome Labs Pvt. Ltd., Kochi, Kerala. The sequence obtained was edited with

BioEdit Sequence Alignment Editor Programme version 7.2.5 and was compared to known RGA sequences using BLASTX algorithms against the GenBank database (http://www.ncbi.nlm.nih.gov). ExPASY translate tool was used for obtaining the deduced amino acid sequences and phylogenetic tree was constructed from BioEdit aligned sequences using MEGA version 6.06 based on neighbor-joining method. Multiple alignment of obtained amino acid sequences with available database sequences of different plant species were performed using CLUSTALX program of BioEdit software. A conserved domain database (CDD) search was performed for obtaining annotations of domains and conserved functional sites on protein sequences at the NCBI server (http://www.ncbi.nlm.nih.gov/Structure/cdd/ wrpsb.cgi).

3.2.6 Primer Designing For Gene Expression Analysis Using Real Time PCR

Primers were designed to amplify the NB-ARC conserved region present in plant R genes based on the obtained sequence information. Primer 3 plus was used to design specific primers. The primer sequences were sent to the AgriGenome Labs Pvt. Ltd., Kochi, Kerala for synthesis.

3.3 TISSUE CULTURE

The sprouted tubers of susceptible (Sree Kiran) and tolerant (Muktakeshi) varieties of taro were collected from the fields of ICAR-CTCRI. Tissue culture was done using shoot tips from sprouted tubers as explants to raise disease free taro plantlets required for studying gene expression of RGAs in resistant and susceptible taro cultivars.

3.3.1 Media preparation for tissue culture

Shoot tip culture of taro was established by using Murashige and Skoog (MS) media (HIMEDIA PT021X1L). Each pack containing 4.08 grams of MS powder was dissolved in distilled water and added sucrose (30g), PVP (0.2%) and agar (0.8%)

(Appendix I). The MS media was supplemented with plant growth regulators- 6-benzyladenine (BA) (5 mg L^{-1}) and NAA (1 mg L^{-1}) (Hegde *et al.*, 2012). Subculturing was done in half MS media without hormones. The pH was adjusted to 5.7 with 1N NaOH/1N HCl and the volume was made to 1 L. Then 15 ml of medium was poured into test tubes (150 X 25 mm tubes) and sterilized at 121°C and 15 psi pressure for 20 min. The sterilized culture tubes were stored in culture room until use.

All instruments (metal /glass) and other accessories used in the tissue culture inoculation cabinet were wrapped in aluminium foil and sterilized in autoclave as explained earlier. Forceps, scalpel blades, scissors, etc were again dipped in alcohol and flamed at the time of use.

3.3.2 Explant preparation

Taro corms were spread in moist sterile sand in aluminium trays to facilitate sprouting in a net house. Once the shoot grows up to approximately 1 cm, they were excised from the corms. The excised shoot tips were washed in running tap water for 2 hours and then placed in 2% (v/v) mild detergent solution (Labolene) for 1 hour with frequent agitation at 50 rpm. The shoot tips were then washed in running tap water for 30 min to get rid of detergent and rinsed twice in distilled water. This was followed by surface sterilization in 70% ethyl alcohol for 1 min. Outer layers of the explants were removed, trimmed and re-disinfested in 0.1% mercuric chloride containing few drops of Tween-20 for 3 min. And finally rinsed 4-5 times with sterile double distilled water and blotted dry on Whatman No.1 filter paper.

3.3.3 Inoculation and incubation

All inoculation operations were carried out under aseptic conditions inside a laminar air flow chamber, which was made sterile by alcohol wipe and UV exposure for 20 min before use. Pre sterilized instruments and glass-wares were used to carry

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out all operations. The explants blotted on sterile filter paper were then vertically inoculated with the basal cut surface in contact with the medium by using sterile forceps. The inoculated tubes were maintained at 25±1°C under 16/8 h photoperiod of around 2000 lux light intensity provided by white fluorescent tubes with 60 % relative humidity inside the tissue culture room.

3.3.4 Hardening of plants

The four weeks old regenerated shoots were aseptically transferred and subcultured on half MS basal medium. The well developed plantlets were taken out of the culture tubes after two months of subculturing with the help of forceps and avoiding any mechanical damages. Then washed with sterile water to remove traces of agar and then dipped in carbendazim (0.05%) solution for 3 min. Following fungicide treatment, the plantlets were carefully placed in disposable paper cups containing potting mixture (vermiculite + sand, 1:1).The hardened plants were initially covered with polythene bags for one week to maintain humidity. Liquid MS media without sucrose is sprayed during every three days after transplantation and watering is done as per the requirement .The plants were monitored daily for growth and when the plants are acclimatized and roots are well developed, they were transferred to small pots and treated like normal plants.

3.4 CULTURING OF *P. colocasiae*

Virulent *P. colocasiae* culture (CTCRI-PC) being maintained at ICAR- CTCRI was used for the study. Culturing was done on Carrot Agar media plates (Appendix II) and sub culturing was done after 12 days. Plates were kept in incubator at 28°C. After 12 days, a 5 mm disc obtained from the periphery of the colony, in areas of active growth, was placed at the centre of petri dishes containing media mentioned above.

3.4.1 Preparation of zoospore suspension for artificial inoculation

P. colocasiae culture was inoculated into the leaves of susceptible variety (Sree Kiran) by placing 5 mm disc on different positions on the leaf. Ten days old inoculated leaves was crushed in sterile water and kept for 15 min in 4°C.This is filtered through a clean cheese cloth and the filtrate was used as the spore suspension. Ten 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. The zoospore suspension (approx. $1x10^{-4}$ ml^{-1}) of *Phytophthora colocasiae* was sprayed on abaxial and adaxial surfaces of the taro leaves for disease induction.

3.4.2 Artificial inoculation of disease for studying gene expression

Disease inoculation using the fungal zoospore suspensions of *Phytophthora colocasiae* were carried out in Sree Kiran(susceptible) and Muktakeshi (tolerant) varieties of taro for studying the host resistance genes expression during 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. 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 the 12, 24, 36 and 48 hours after inoculation. The RNA was isolated to study the expression of RGAs.

3.5 ISOLATION OF TOTAL RNA

Leaf samples collected after 12, 24, 36 and 48 hours of pathogen inoculated plants and control plants were taken for RNA isolation. From the leaf samples, RNA was isolated using Ambion Purelink RNA Mini Kit, TRIzol based method and LiCl2 based method. The isolated RNA was stored at -80^0C.

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

Young leaf sample weighed 80-100 mg was taken for the RNA isolation. The tissue was ground into fine powder with liquid nitrogen using DEPC treated mortar and pestle and was transferred to an RNase free micro centrifuge tube along with 1ml of lysis buffer and vortexed (Labnet Vortex Mixer, USA) briefly. The samples were centrifuged at 20,000 *g* (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 7 min at room temperature and the supernatant was transferred to a fresh tube. Equal volume of 70 percent ethanol was added to the supernatant and mixed thoroughly. Then, 700 µl of the sample was transferred to the spin cartridge inserted in a collection tube. Following centrifugation at 12,000 g for 30s at room temperature, the flow through was discarded and the cartridge was reinserted into the collection tube. The above step was repeated with rest of the samples to appropriate spin cartridges. The spin cartridge was centrifuged at 12,000 g for 30s after the addition of 700 µl of the wash buffer I. The flow through was discarded and the spin cartridge was placed into a new collection tube. Then 500 µl of the 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 to repeat the above step once again. Then the empty spin column was centrifuged at 12,000 g for one minute at room temperature and the collection tube was discarded. The cartridge was inserted into a recovery tube and air dried for 10 to 15 min. Then 30 µl of the elution buffer (RNase free water) was added to the center of the spin cartridge and was incubated at room temperature for one minute followed by centrifugation at 12,000 g for one minute to obtain the first elute of RNA. Finally 20 µl of the elution buffer was added to the spin cartridge and centrifuged at 12,000g for one minute at room temperature to obtain the second elute. The quality of the isolated RNA was checked on agarose gel (1.5%) and stored at -20°C.

3.5.2 Manual Method of RNA Isolation

3.5.2.1 Trizol Method

The leaf sample (100 mg) was taken and ground into fine power with liquid nitrogen using mortar and pestle. The powdered tissue was transferred to a fresh tube to which 1 ml of TRIzol reagent (Life Technologies,USA) was added and incubated at room temperature for five min. Then 200 µl of chloroform was added, shaken vigorously and incubated at room temperature for 2-3 min. After centrifugation at 12,000 g for 15 min at 4°C, the colourless upper aqueous phase from the three layers appeared was transferred into a new tube. Following that 500 µl of ice cold isopropanol was added, incubated at room temperature for 10 min and centrifuged at 12000 g for 15 min at 4°C. The supernatant was discarded and to the RNA pellet,1ml of 75% ethanol was added and vortexed. The tubes were centrifuged at 7,500 g for five min at 4°C. This washing procedure was repeated again. The RNA pellet was air dried for 5 - 10 min and then resuspended in 30 µl of nuclease free sterile water and incubated at 55-60°C for 10 -15 min. The quality of isolated RNA was checked on 1.5% agarose gel.

3.5.2.2 Lithium Chloride Method

Hundred mg of fresh leaf sample was ground into fine powder using liquid nitrogen. The tissue powder was transferred into a sterile 2 ml centrifuge tube. One ml of extraction buffer (pre-warmed at 65 °C) (Appendix III) was added, vortexed (Labnet Vortex Mixer, USA) and incubated at 65 °C (Lab Companion BS-31 shaking waterbath, Canada) for 10 min. After incubation the tube was centrifuged at 15,000 rpm (Kubota High Speed Refrigerated Centrifuge 6500, Japan) for 15 min at 4 °C and supernatant was transferred to a fresh 2 ml tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 20,000 *g* for 10 min at 4 °C. After transferring the supernatant into fresh tube, equal volume of

chloroform: isoamyl alcohol was added and centrifuged at 20,000 *g* for 10 min at 4 °C, the supernatant was transferred to a fresh tube. Then, 0.25 volume of ice cold 10 M lithium chloride was added, mixed well and incubated overnight at 4 °C. Following centrifugation at 30,000 *g* for 30 min at 4 °C, the pellet was washed with 75 percent ethanol by centrifuged at 10,000 *g* for 10 min at 4 °C. The washing was repeated with 200 µl of 75 percent ethanol. RNA pellet obtained was air dried at 37°C for 30 min and then dissolved in 30 µl DEPC water. After incubating at 37 °C forl h, the RNA was stored at -20 °C.

3.6 EXPRESSION STUDY OF RESISTANT GENE ANALOGUES IN TARO VARIETIES.

3.6.1 First Strand cDNA Synthesis

The RNA isolated from control and pathogen inoculated leaf samples of susceptible and tolerant varieties at different intervals 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:

5x Reaction buffer	:	4µl
10mM dNTP mix	:	2µl
Oligo $(dT)_{18}$ primers	:	1 µl
Revert Aid M-MuLV RT (200 U µl-1)	:	1 µl
Ribolock RNase inhibitor (200 U µl-1)	:	1 µl
Water (nuclease free)	:	8 µl

Template RNA : 3 µl

Total volume : 20 µl

All the components 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 h followed by an extension step at 72 °C for five min.

3.6.2 Real time PCR

Quantitative real-time PCR is the most commonly used technique for gene expression studies. The cDNA of control and test samples from resistant and susceptible varieties were diluted to a final concentration of 100 ng μl^{-1} with sterile water. The real-time quantitative PCR (qPCR) was performed with the Applied Biosystems PCR system in a total volume of 20 µl. The reaction mixture consists of:

Power SYBR Green PCR Master Mix (2X) : 5µl

Forward primer : 0.2µl

Reverse primer : 0.2µl

Nuclease free water : 0.6µl

cDNA template : 4µl

The thermal cycling parameters are set at initial denaturation at 95°C for10 mins, followed by 40 cycles of denaturation at 94 °C for 15 sec, annealing at 60°C for 1 min and extension at 50°C for 2 min. The final extension was carried at 50°C for 8 mins.

The specific primer designed based on the sequence of degenerate primer RGA1 is as follows:

Taro (RGA) F : 5'CTGAGCCTTTCCTCATCTGC 3'

Taro (RGA2) R : 5'CCAAAGCCTTCACCATGACT 3'

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

ACT1F (Forward primer) : 5'GGCTGATTGTGCTGTGCTTA 3'

ACT1R (Reverse primer) : 5'CTCACTCCAAGGGTGAAAGC 3'

After the completion of the real time PCR reactions, the threshold cycle (Ct) was recorded and gene expression level was calculated using comparative Ct method or delta-delta Ct method. All samples were run in triplicates and results are presented as mean values ± SD. A melt curve analysis was performed at every run to assess the product specificity.

The relative gene expression level of resistant and susceptible varieties is represented as $2^{-\Delta\Delta CT}$ method.

ΔCt = Ct (target gene) – Ct (reference gene)

$\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (control)

RESULTS

4. RESULTS

The results of the study entitled "Differential response of resistant gene analogues (RGAs) against *Phytophthora colocasiae* causing leaf blight in taro", carried out during the period of October 2018-August 2019 at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram are presented in this chapter.

4.1 SAMPLE COLLECTION

Leaf samples used for DNA isolation of taro varieties, Sree Kiran (susceptible) and Muktakeshi (resistant), were collected from the fields of ICAR-CTCRI. The taro field with leaf blight infection as well as the disease symptoms observed in the net house is shown in Plate 1. The susceptible and resistant varieties used for the study is shown in Plate 2.

4.2 AMPLIFICATION OF RESISTANT GENE ANALOGUES FROM TARO

4.2.1 DNA isolation

For standardizing the amplification of resistant genes, the genomic DNA was extracted using CTAB method (Sharma *et al.,* 2008) from the young leaf samples of resistant (Muktakeshi) and susceptible (Sree Kiran) cultivars collected from the ICAR- CTCRI fields. The isolated DNA was run on 0.8% agarose gel and the bands were visualized under UV light and the image was documented using Alpha Imager (Alpha Innotech, USA) (Plate 3).

The quantity and quality of isolated DNA was estimated using a NanoDrop spectrophotometer (DeNovix DS-11) and the obtained results were shown in Table 1.

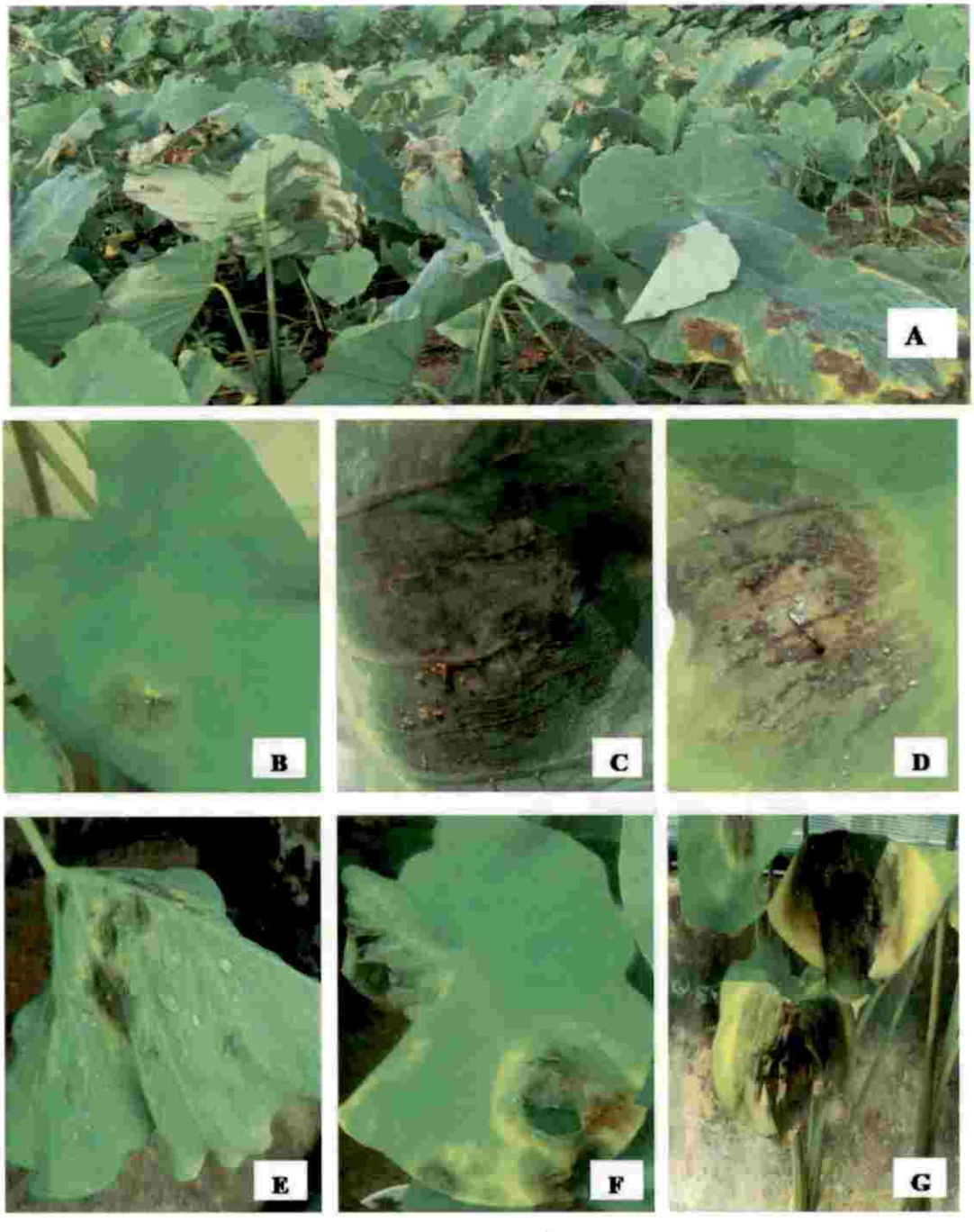

Plate 1. Symptoms of taro leaf blight disease. (A) Field view of taro with leaf blight infection. (B) Early stage of leaf blight. (C) Closer view of the symptom with exudates- abaxial side. (D) Adaxial side (E) Multiple lesions (F) Blight (G) Destruction of entire leaf.

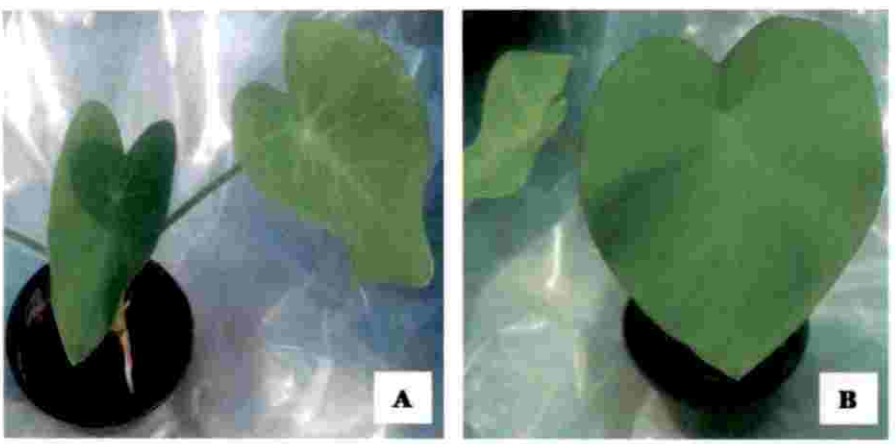

Plate 2. A: Resistant (Muktakeshi) and B: susceptible (Sree Kiran) varieties used for the study.

Plate 3. DNA isolated from taro varieties Muktakeshi and Sree Kiran. Lane 1 and 2: DNA of Muktakeshi, Lane 3 and 4: DNA of Sree Kiran.

Table 1. Concentration and the purity ratio of DNA estimated using nanodrop spectrophotometer.

Sl.No.	Sample	Concentration (ng μl^{-1})	A260/280	A260/230
1	DNA 1 (Muktakeshi)	3750	1.98	1.88
2	DNA 2 (Muktakeshi)	3520	1.96	1.9
3	DNA 3 (Sree Kiran)	1315	2.4	2.09
4	DNA 4 (Sree Kiran)	1623	2.2	2.07

4.2.2 Gradient PCR for optimizing annealing temperature

The annealing temperatures of the two degenerate primer (RGA 1 and RGA 2) pairs used for amplification of RGAs was standardized by conducting a gradient PCR from 47°C-54°C.The optimum annealing temperature of primer pairs RGA 1 and RGA 2 was 48.8°C and 52.2°C respectively ,where the amplification was good (Plate 4 and 5).

4.2.3 PCR amplification of RGAs using degenerate primers

The optimum annealing temperature of the two sets of degenerate primers, RGA1 and RGA2 were found to be 48.8°C and 52.2°C(Plate 6 and 7).The DNA samples of susceptible and tolerant varieties were amplified with the primer pairs and the PCR conditions were mentioned in 3.2.3.1. The amplicons of RGA1and RGA2 were 500 and 620 bp size respectively, when separated on a 1.5% agarose gel. The PCR product was gel eluted and given for sequencing.

4.3 DNA SEQUENCING AND ANALYSIS

The gel elutes of PCR products were reamplified, purified and sent to AgriGenome Labs Pvt. Ltd, Kochi, along with their respective primers. The results were obtained in the form of electopherogram resulting from capillary sequencing in .aib format.

The results of obtained sequence were initially analyzed and edited using BioEdit Sequence Alignment Editor Program version 7.2.5 and the similarity search was done using online BLASTx program of NCBI. The presence of resistance gene analogues was identified on both susceptible and resistant varieties on the basis of sequence analysis (Fig.1 and Fig.2). BLASTx is a BLAST search tool which translates the input nucleotide query into amino acid sequence and the six-frame conceptual translation product was compared against the protein database.

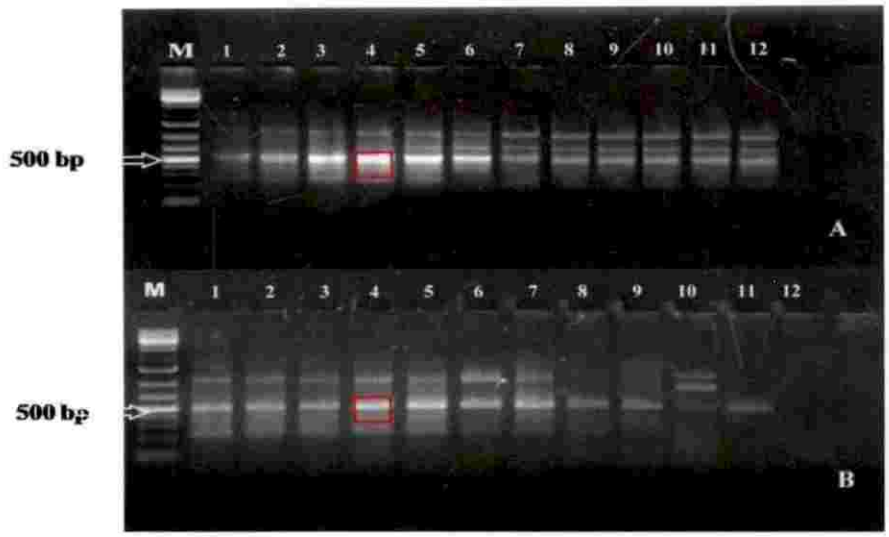

Plate 4. Gradient PCR (47°C-54°C) to optimize anneling temperature of primer RGA 1 (A) Muktakeshi (B) Sree Kiran. Lane 4: Optimum annealing temperature - 48.8°C. M: 1kb plus DNA marker.

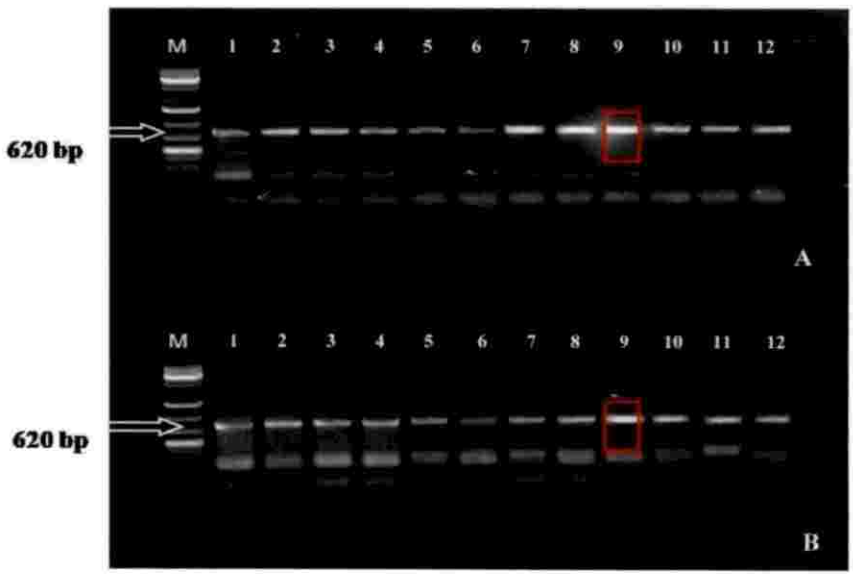

Plate 5. Gradient PCR (47°C-54°C) to optimize annealing temperature of RGA 2 primer pair (A) Muktakeshi (B) Sree Kiran Lane. 9: Optimum annealing temperature - 52.2°C. M: 1kb plus DNA marker.

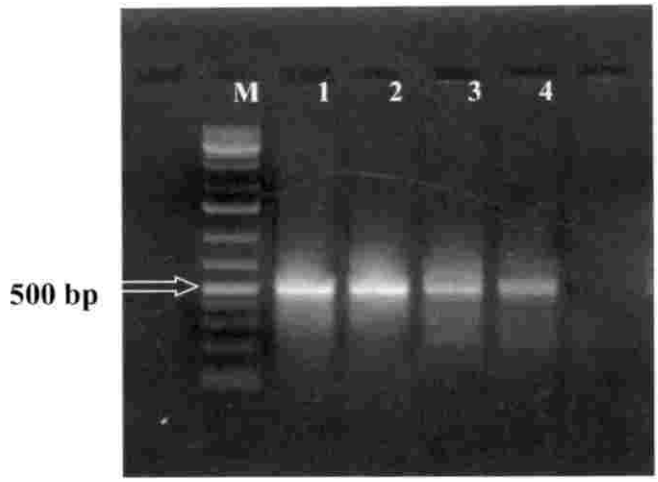

Plate 6. PCR product of Muktakeshi (Lane 1 and 2) and Sree Kiran (Lane 3 and 4) using degenerate RGA 2 primer separated on agarose gel. M: 1kb plus DNA marker.

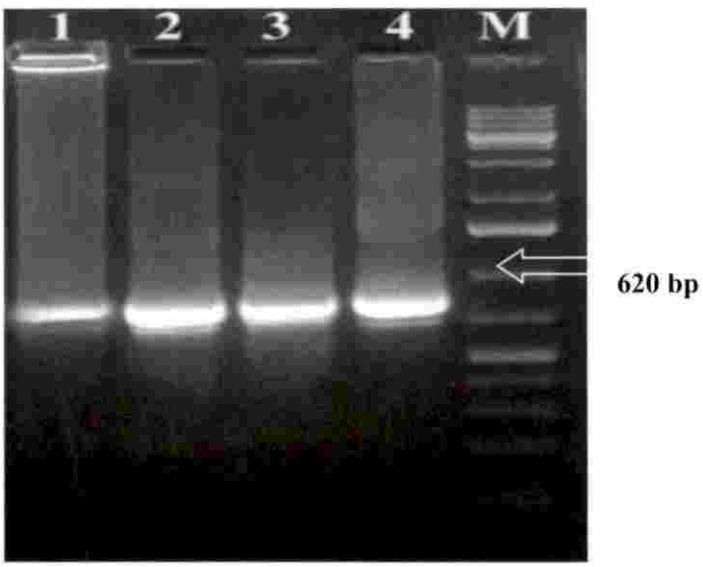

Plate 7. PCR product of Sree Kiran (Lane 1 and 3) and Muktakeshi (Lane 2, 4) using degenerate RGA 2 primer separated on agarose gel. M: 1kb plus DNA marker.

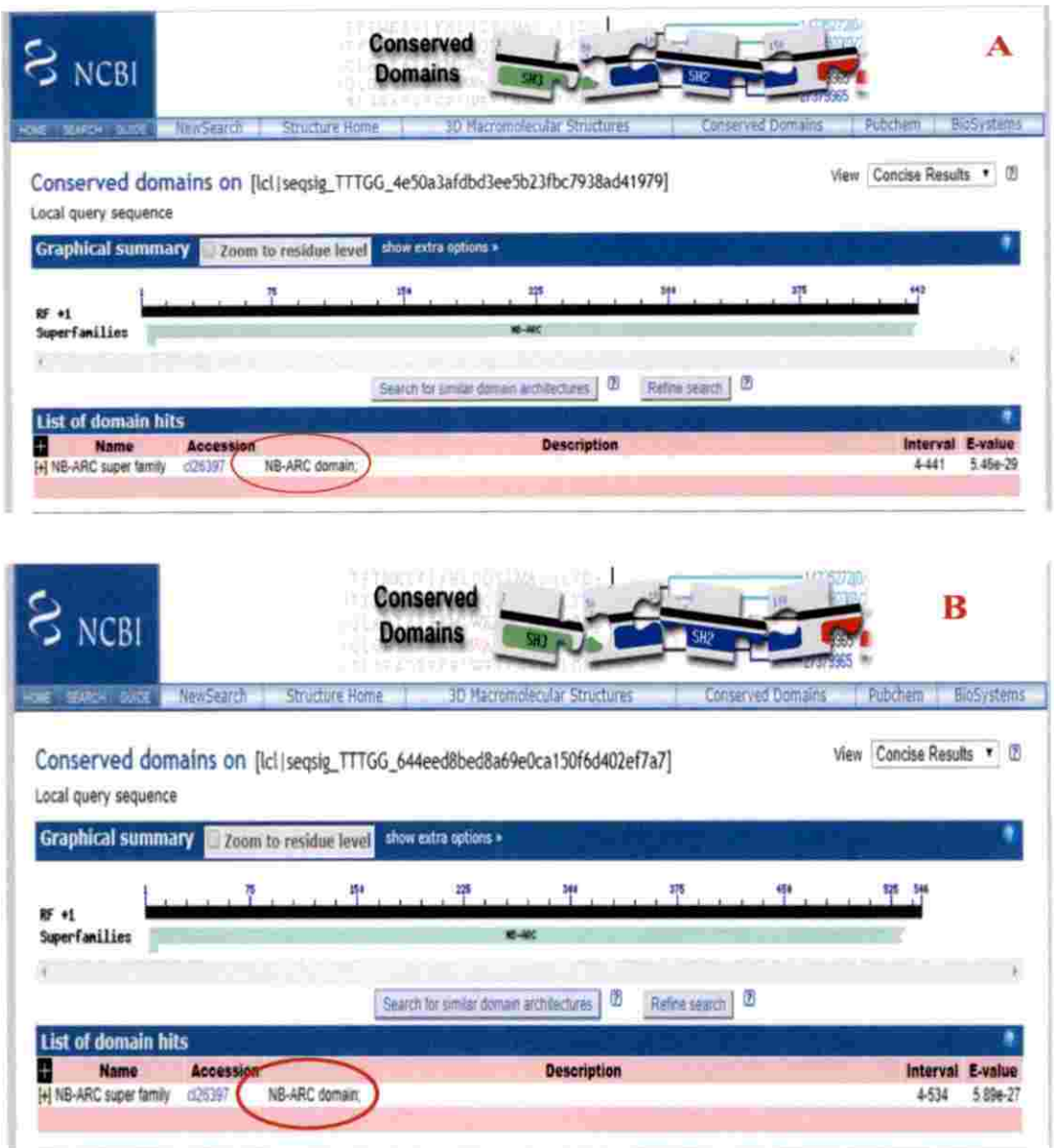

Fig 1. Conserved domain database (CDD) search results of RGA 1 PCR product (A) Muktakeshi (B) Sree Kiran.

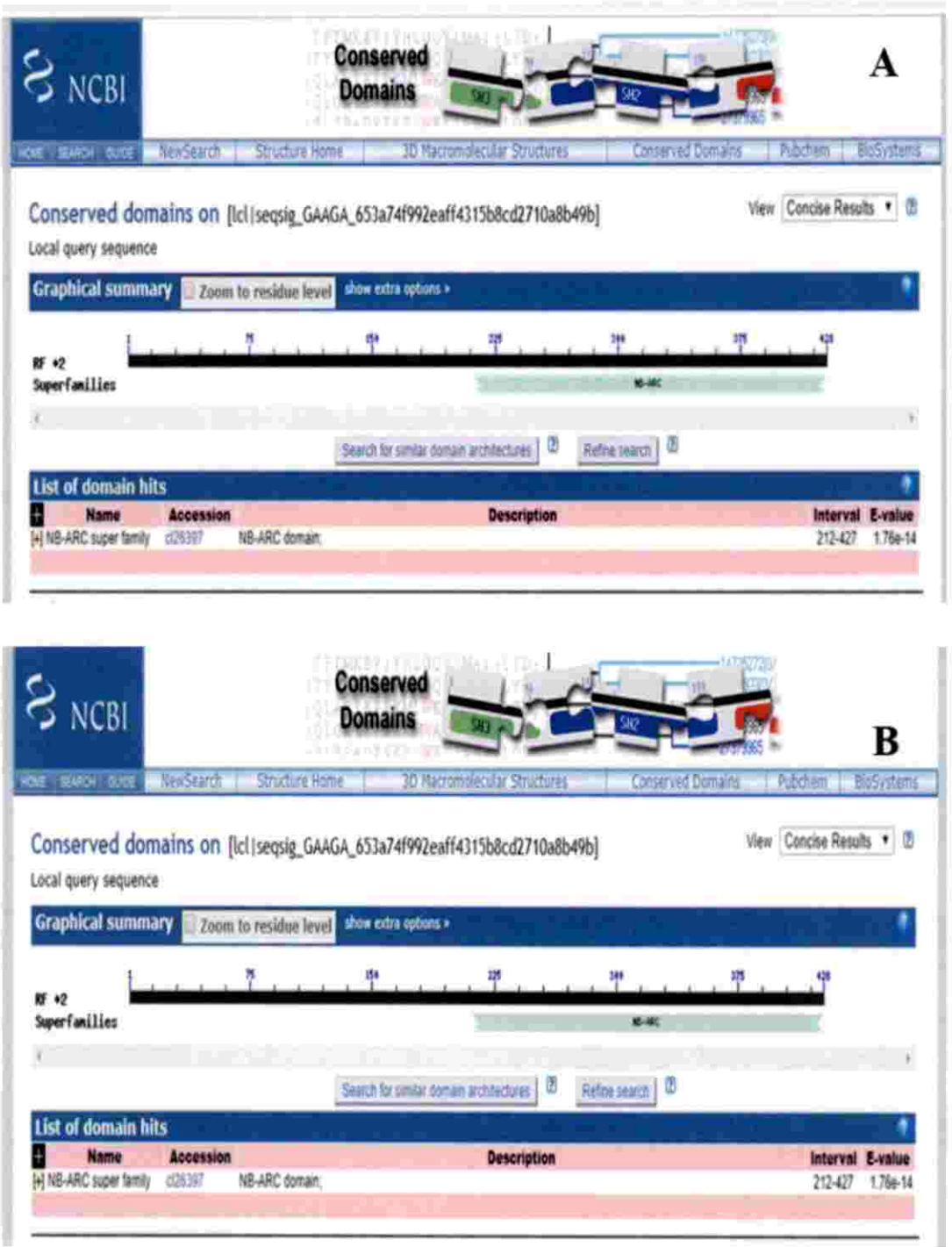

Fig 2. Conserved domain database (CDD) search results of RGA 2 PCR product (A) Muktakeshi RGA (B) Sree Kiran RGA

The BLAST analysis of the obtained sequences showed maximum similarity to NBS-LRR disease resistance protein of *Panax notoginseng* (a medicinal plant) other than NBS LRR disease resistance protein of *Colocasia esculenta* (Table 2). Conserved Domain search tool at NCBI (http://www.ncbi.nlm.nih.govStructure/cdd/wrpsb.cgi) was used to confirm the presence of NB-ARC domain in all the obtained sequences of both the varieties.

The phylogenetic tree was constructed with amino acid sequences of the obtained RGA sequences of Muktakeshi and Sree Kiran along with similar sequences of BLAST analysis (Fig 3). The tree was constructed using MEGA X by Neighbor-Joining method and using 1000 bootstrap replicates.

4.4 PRIMER DESIGNING

Specific primers were designed for the amplification of the NB-ARC conserved domain of the resistance gene analogues in taro varieties Muktakeshi and Sree Kiran based on the sequence analysis. Specific primers for amplifying taro resistance gene analogue were synthesized from AgriGenome Labs Pvt. Ltd.

The analysis of primers using FastPCR programme showed that the primers did not exhibit any hairpin formation and 3' complementarity and had good GC content and annealing temperature. The synthesized specific primer pair is given below:

T RGA F (Forward primer) sequence : 5'CTGAGCCTT TCCTCATCTGC 3'

T RGA R (Reverse primer) sequence : 5'CCA AA CCT TCACCATGACT3'

4.4.1 Gradient PCR for Tm optimization

The annealing temperature of the specific primer was optimized by using gradient PCR set from 50-60 °C and the optimum annealing temperature for better amplification was observed at 55 °C (Plate 8).

Table 2. Amino acid sequence identity between obtained RGAs and other plant species.

Primer	Variety	Similar sequences identified	Accession No	RGA sequence identity (%)
RGA 1	Muktakeshi	NBS LRR disease resistance protein (*Colocasia esculenta*)	AOD75225.1	98.24
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ9633O.1	77.91
		Disease resistant protein homolog (*Arabidopsis thaliana*)	AAB61691.1	45.93
		Disease resistant protein product (*Vitis vinifera*)	CB127879.1	47.65
	Sree Kiran	NBS LRR disease resistance protein (*Colocasia esculenta*)	AOD75222.1	97.65
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ96329.1	46.63%
		Disease resistant protein homolog (*Arabidopsis thaliana*)	AAB61689.1	46.51%
		RCa9 (*Manihot esculenta*)	AA038220.1	47.06%
RGA2	Muktakeshi	NBS LRR diseaseresistance protein (*Colocasia esculenta*)	AOD75225.1	59.28%
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ9633O.1	51.79%
		NBS/LRR Resistance protein-like protein (*Theobroma cacao*)	AAL009991	40.48%

		Putative disease resistance protein isoform XI (*Cinnamomum micranthum*)	RWR90802.1	42.51%
	Sree Kiran	NBS LRR diseaseresistance protein (*Colocasia esculenta*)	AOD75225.1	60.45%
		NBS LRR disease resistance protein (*Panax notoginseng*)	QBZ9633O.1	51.69%
		CC-nbs-lrr-Resistance protein – like protein (*Theobroma cacao*)	EOY25574.1	41.01%
		Disease resistance protein At5g63020 isoform XI(*Citrus sinensis*)	XP00648460.1	38.33%

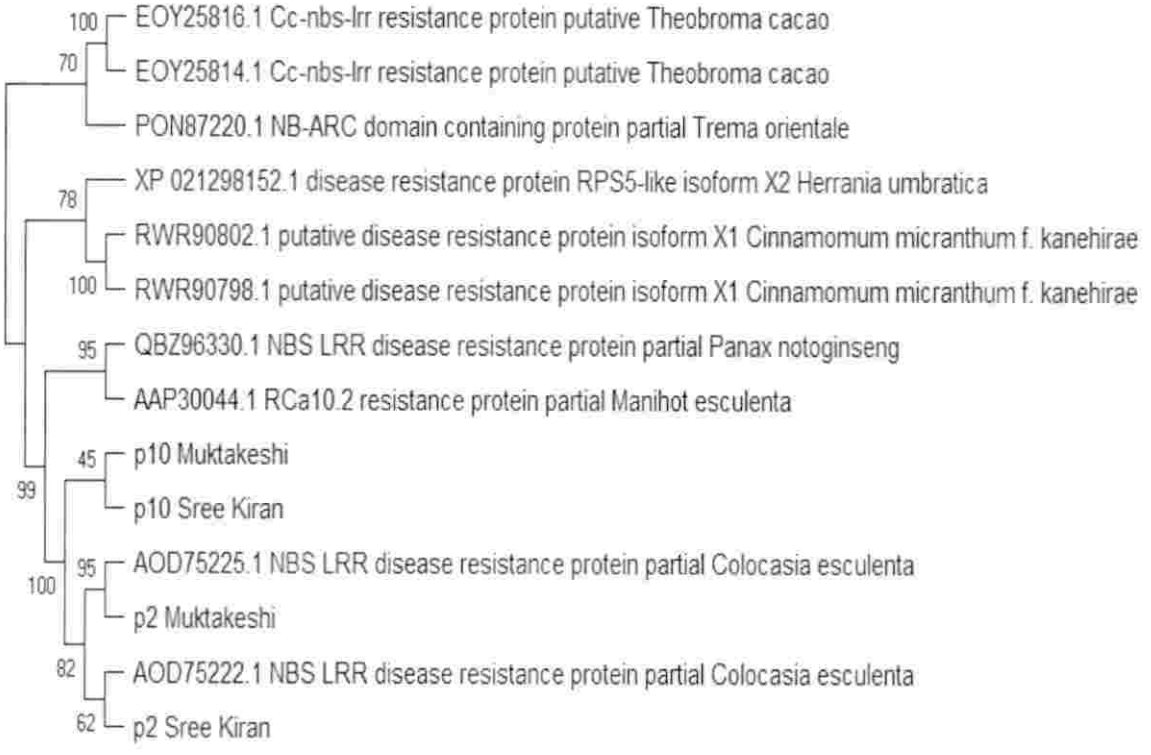

Fig 3. Phylogenetic tree constructed based on the amino acid sequences of the obtained RGA sequences of Muktakeshi and Sree Kiran along with other similar plant species available in the NCBI database. The tree was constructed using MEGA X by Neighbor-Joining method. The numbers shown next to the branches are the percentage of replicate based on bootstrap analysis (1000 replications).

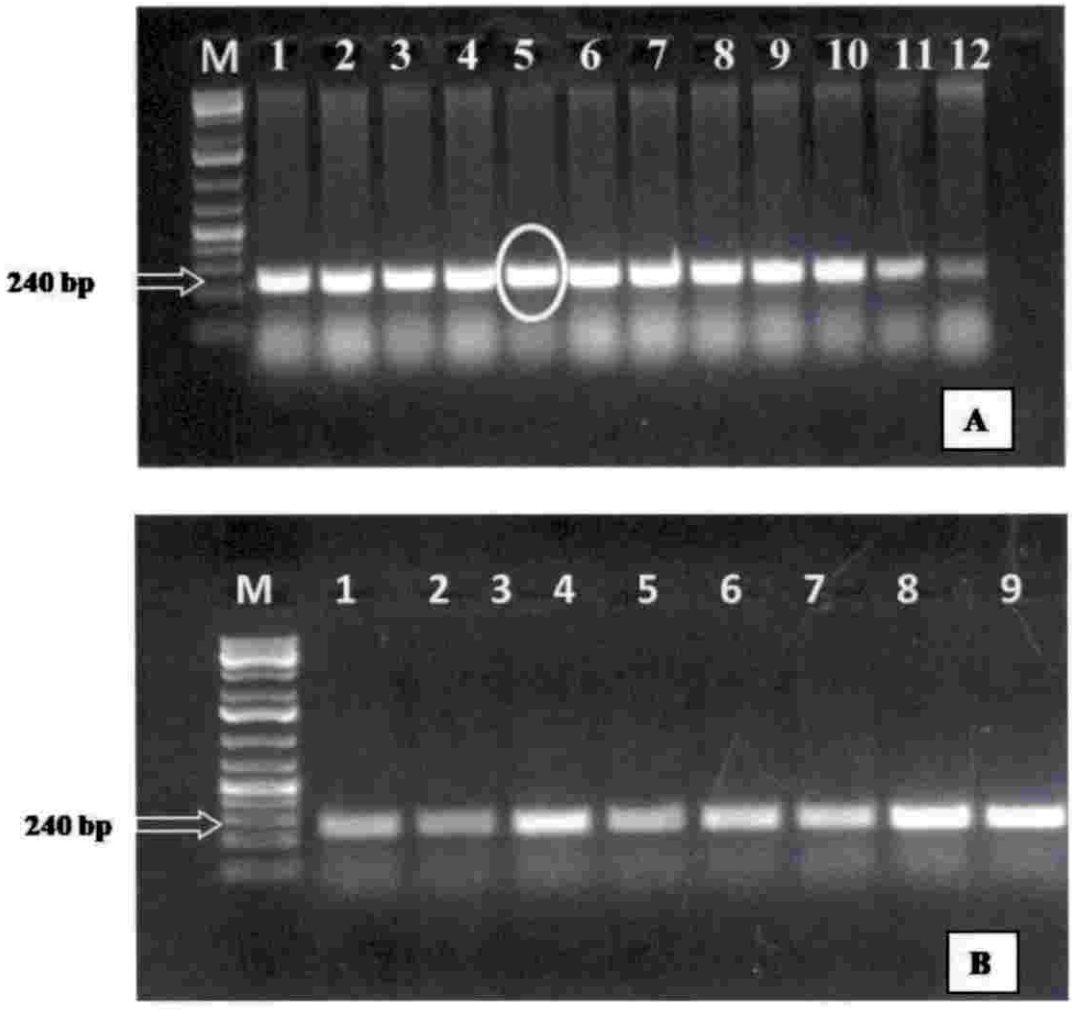

Plate 8. (A) Gradient PCR (50°C - 60°C) to optimize annealing temperature of newly designed primer T RGA. Lane 5: Optimum annealing temperature – 55.5°C. (B) PCR product of specific primer T RGA. M: 1kb plus DNA marker.

4.5 TISSUE CULTURE OF TARO

4.5.1 Explant preparation and inoculation of shoot tip

Shoot tips were taken from sprouted tubers of taro varieties, Muktakeshi and Sree Kiran and cultured on Murashige and Skoog media supplemented with growth hormones, 6-benzyladenine (BA) (5 mg L^{-1}) and NAA (1 mg L^{-1}). Twenty five shoot tips of both the varieties were cultured, from which 12 plants of Muktakeshi and 10 plants of Sree Kiran were raised respectively.

Shoot buds started appearing after 14 days of explant inoculation. After 4 weeks, shoots with the length of not less than 3-4 cm were aseptically transferred to rooting media. The plantlets were subcultured in half strength MS media without hormones. Eighteen plants of Muktakeshi and fifteen plants of Sree Kiran were produced after subculturing.

4.5.2 Hardening

The well developed plantlets after 3 months were carefully taken out from the culture tube, washed properly to remove the media and after finally dipped in fungicide (0.05% Carbendezim) and planted in paper cups containing sterile potting mixture. Only six plants of Muktakeshi and five plants of Sree Kiran survived after hardening. Since the percentage of survival was very low for both the varieties, plants raised from uninfected and surface sterilized tubers, maintained under controlled conditions were used for further studies. Plate 9 shows the different stages of micropropagation of taro. Fig 4 shows the details of number of shoot tips inoculated, number of mother plants raised, number of plants subcultured and hardened.

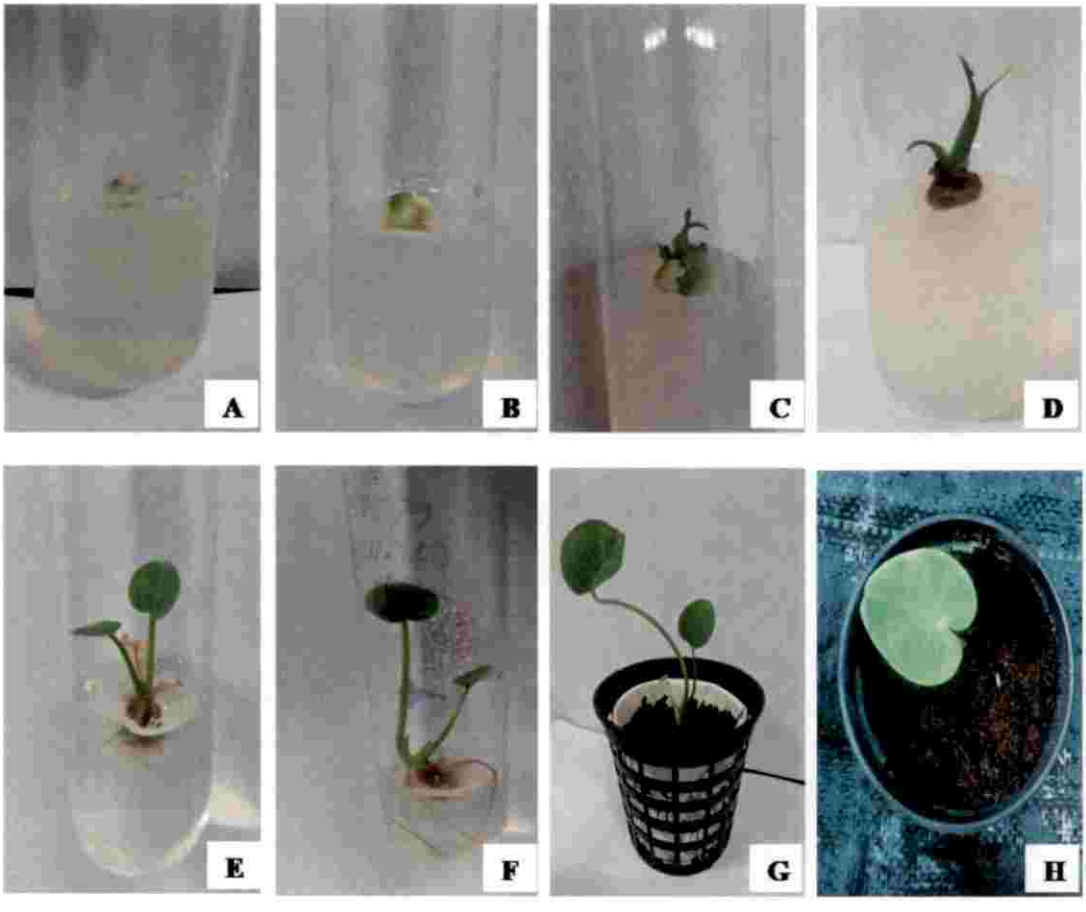

Plate 9. Micropopagation of taro: different growth stages (A) Inoculation of explant after sterilization. (B) Explant turns green after 1week. (C and D) Initiation and elongation of shoot development. (E and F) Initiation and elongation of adventitious root. (G and H) Matured plantlets in plastic pots containing potting mixture.

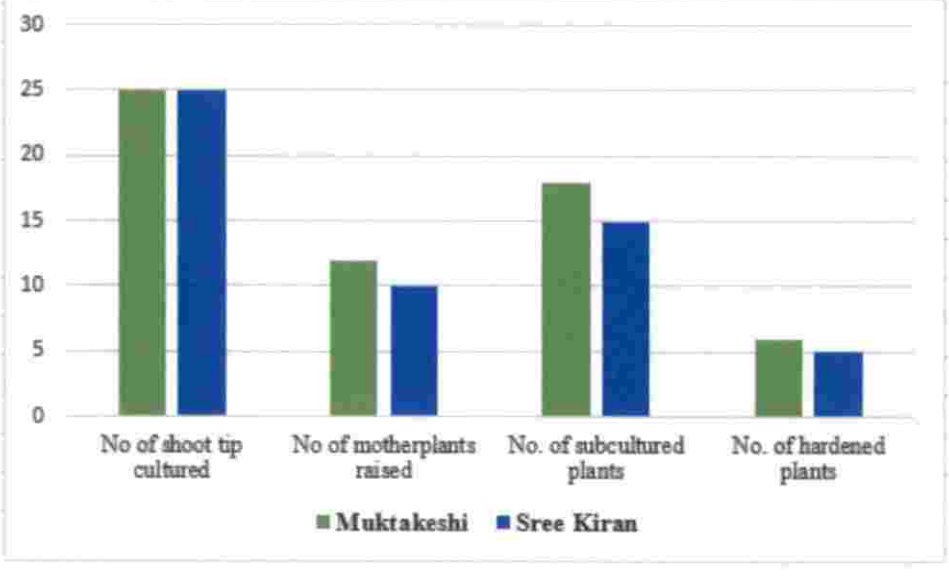

Fig 4. Details of tissue culture raised taro plants.

4.6 CULTURING OF *P. colocasiae*

P. colocasiae, causal organisam of taro leaf blight was subcultured on carrot agar media plates. Growth of mycelia was found within 24 hours of inoculation. Sub culturing was done after 12 days and plates were kept in incubator at 28°C. Plate 10 shows the culture and sporangia of *P. colocasiae*.

Virulence of the pathogen was maintained by inoculating and reisolating the culture on fresh leaf of susceptible variety once in two months or after five subculturing (Plate 11).

4.6.1 Artificial inoculation for studying gene expression

Zoospore suspension of *Phytophthora colocasiae* as used for disease induction in Sree Kiran (susceptible) and Muktakeshi (resistant) varieties of taro for studying expression of the host resistance genes during disease development. The zoospore suspension (25µl) was applied on the abaxial and adaxial surface of the leaves and for control plants sterile water was sprayed. Leaf samples were collected after 12, 24, 36 and 48 h after pathogen inoculation and control plants of both the varieties were also taken for RNA isolation.

Symptoms initiated within 24 h of inoculation in susceptible variety and in resistant variety it took 48 h. The initial symptoms were the occurrence of light water soaked brown lesions which later developed into circular, purplish brown lesions. Bright orange coloured plant exudate oozing from the infected sites was also observed on the lower surface. The development of symptoms in the susceptible variety (Sree Kiran) was faster and severe compared to the resistant variety (Muktakeshi). The presence of yellow tissue around the lesions were also observed. As the infection spreads, the lesions coalesce and leads to the destruction of entire leaf. On the seventh day of inoculation, severity occurred in the susceptible variety

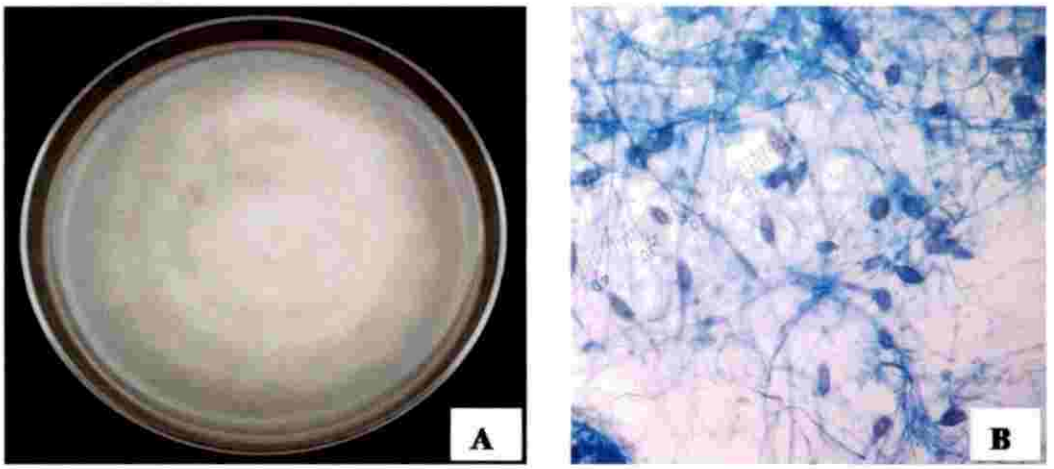

Plate 10. (A) Ten days old culture of *Phytophthora colocasiae* on carrot agar media. (B) Sporangia of *P. colocasiae* observed under microscope (10X).

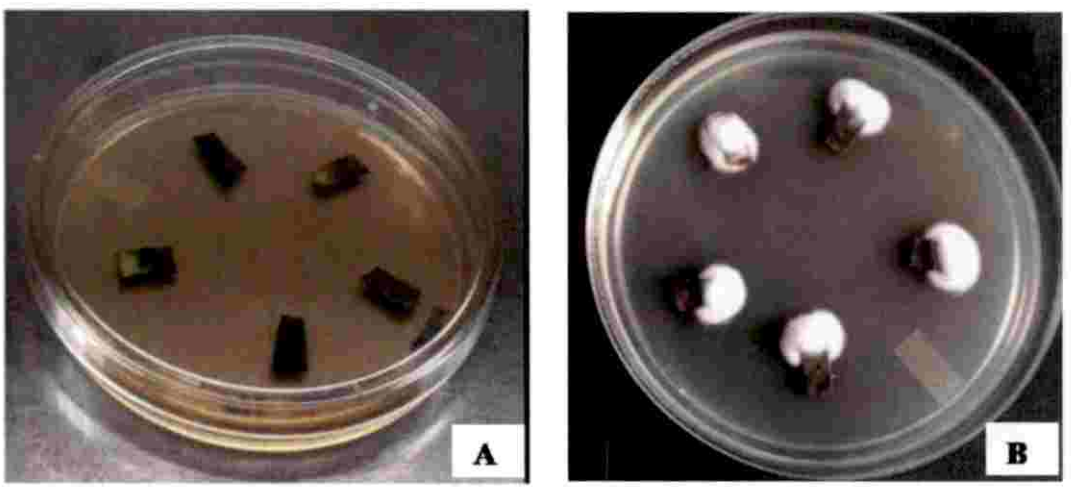

Plate 11. Reisolation of fungi from leaf to maintain virulence (A) Leaf tissue ($5mm^2$) kept in PDA (B) Growth of *P.colocasiae* from infected leaf

was 80% and to the resistant variety was 5% (Plate 12). The centre of the lesions had fallen out producing "shot –hole" appearance in resistant cultivars where as dead leaves hanged on their petioles like flags in the susceptible variety.

4.7 ISOLATION OF TOTAL RNA

RNA was isolated from both pathogen inoculated and control taro plants of both susceptible and resistant varieties after 12, 24, 36 and 48 h of inoculation. RNA was isolated using Ambion Purelink RNA Mini Kit, TRIzol based method and LiCl2 based method. Out of the above methods better results were obtained in TRIzol method and the RNA was stored at -80°C. Two distinct bands were observed when resolved on agarose gel (1.5%), which indicated no apparent RNA degradation (Plate 13). The quantity and quality of the isolated RNA on different intervals was analyzed using nanodrop spectrophotometer (Table 3).

4.8 EXPRESSION STUDY OF RESISTANT GENE ANALOGUES IN TARO VARIETIES

The isolated RNA samples of pathogen inoculated and control plants were converted into cDNA as mentioned in section 3.6.1. The concentration of the cDNA samples were quantified and diluted to a final concentration of 100 ng μl^{-1} and was used for the expression study using real time PCR. The resistance gene analogues present in the resistant and susceptible variety were targeted using designed specific primers T RGA F and T RGA R and the SYBR green PCR assay was used for studying gene expression. The relative gene expression level of resistant and susceptible varieties is studied using $2^{-\Delta\Delta CT}$ method. Actin (primers ACT F and ACT R) was used as the reference gene for the expression study. A negative control was kept for both primers with water instead of cDNA in the components.

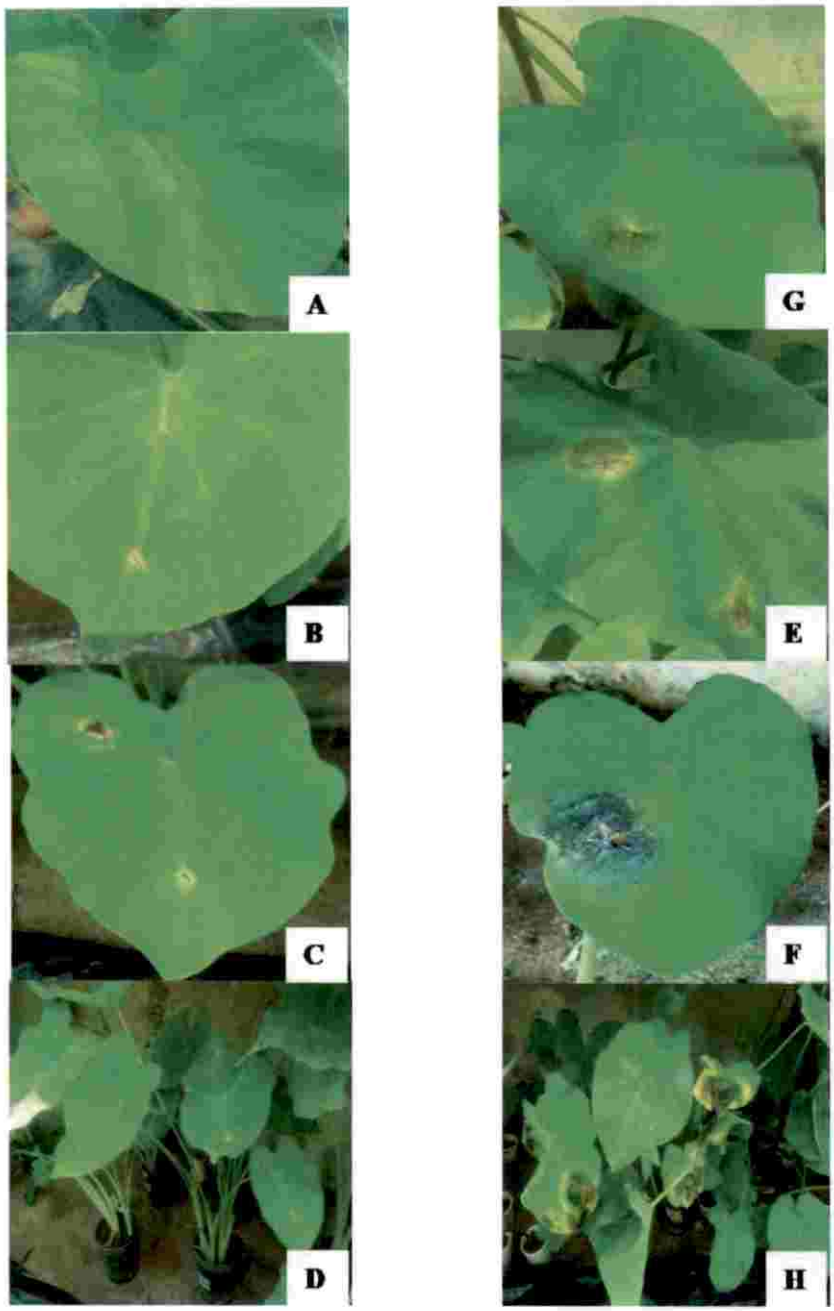

Plate 12. (A) Artificial inoculation of *P.colocasiae* on two taro varieties, Muktakeshi and Sree Kiran. (A to D): Symptoms in Muktakeshi (resistant variety) on 1st, 3rd, 5th and 7th day. (E to H): Symptoms in Sree Kiran (susceptible variety) on 1st, 3rd, 5th and 7th day.

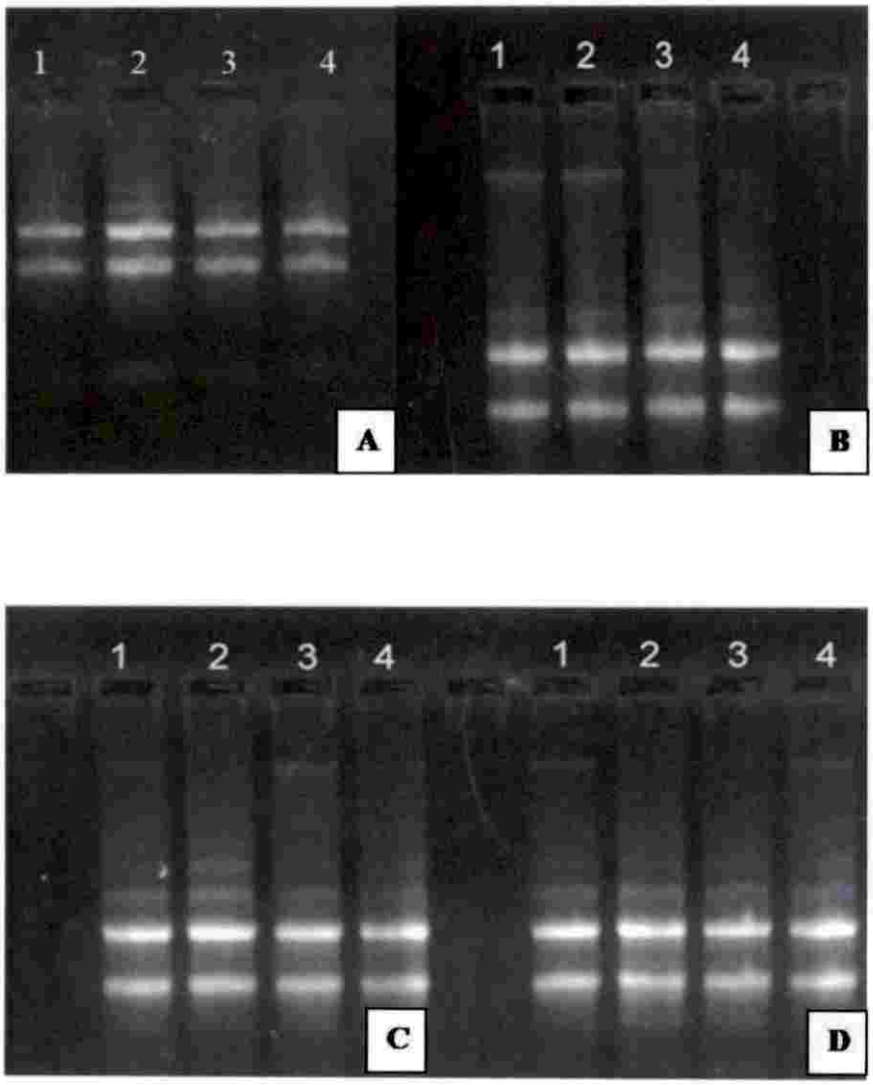

Plate 13. Total RNA isolated after 12 (A), 24 (B), 36 (C) and 48 h (D) after inoculation. Lane 1: RNA from control plant of Muktakeshi, Lane 2: RNA from control plant of Sree Kiran, Lane 3: RNA from pathogen inoculated plant of Muktakeshi, Lane 4: RNA from pathogen inoculated plant of Sree Kiran.

Table 3. Concentration and absorbance ratio of isolated RNA estimated using nanodrop spectrophotometer

Time Intervals	Sample	RNA yield(ng μl^{-1})	A260/280	A260/230
12 hours	Muktakeshi (Control)	1028	1.93	2.05
	Sree Kiran(Control)	987	2.0	2.16
	Muktakeshi	1094	1.98	2.18
	Sree Kiran	1122	2.03	2.06
24 hours	Muktakeshi (Control)	1524	2.2	2.21
	Sree Kiran (Control)	1834	2.13	2.0
	Muktakeshi	1184	2.2	2.11
	Sree Kiran	1066	1.96	2.03
36 hours	Muktakeshi (Control)	1925	2.08	1.97
	Sree Kiran (Control)	1652	1.89	2.01
	Muktakeshi	1894	2.1	2.05
	Sree Kiran	1657	2.12	2.14
48 hours	Muktakeshi (Control)	1955	2.0	1.98
	Sree Kiran (Control)	1015	1.97	2.09
	Muktakeshi	1877	2.11	2.15
	Sree Kiran	1009	2.16	2.04

The standard fluorescent amplification representing exponential growth of PCR products was observed in each cycle, yielding threshold cycle (Ct) values that ranged from 15 – 30.2 for the target (T RGA F and T RGA R) and reference (ACT F and ACT R) primers. The Ct (Cycle threshold) value is given in the logarithmic scale and inversely proportional to the quantity of cDNA. Thus highly expressed genes have low ΔCt values and low expressed genes have high ΔCt values. The fold change ($-\Delta\Delta Ct$) can be calculated by comparing the normalized expression (ΔCt) of the two conditions. The fold change, viz. the expression ratio, indicated the upregulation and downregulation of the gene when it was positive and negative respectively.

To develop improved methods for managing the taro leaf blight caused by *P. colocasiae*, it is important to understand the mechanisms involving infection, disease establishment and the interaction of the plants to infection. One approach is to study the gene expression patterns of RGAs in different taro cultivars during the course of infection. In general, the results of real time PCR showed that the target gene was up-regulated during the course of infection in both the resistant and susceptible cultivars. The reference gene (Actin) had a low ΔCt value than that of the target gene, which means that the target gene was expressed in low concentration.

In the resistant variety (Muktakeshi), the sample collected after 12 h of pathogen inoculation had a 1.42 fold change which rapidly raised to 2.89 at 24 h. The surge in the expression was maintained in the samples collected after 36 h and 48 h with 3.21 and 3.14 fold change respectively. In the susceptible variety (Sree Kiran), the sample collected after 12 h of pathogen inoculation had 0.8 fold change which increased to 1.45 at 24 h. A sudden increase in gene expression was observed in the samples collected after 36 h with 2.15 fold change, but it decreased to 1.86 fold change in the samples collected after 48 h of pathogen inoculation (Fig 5.).

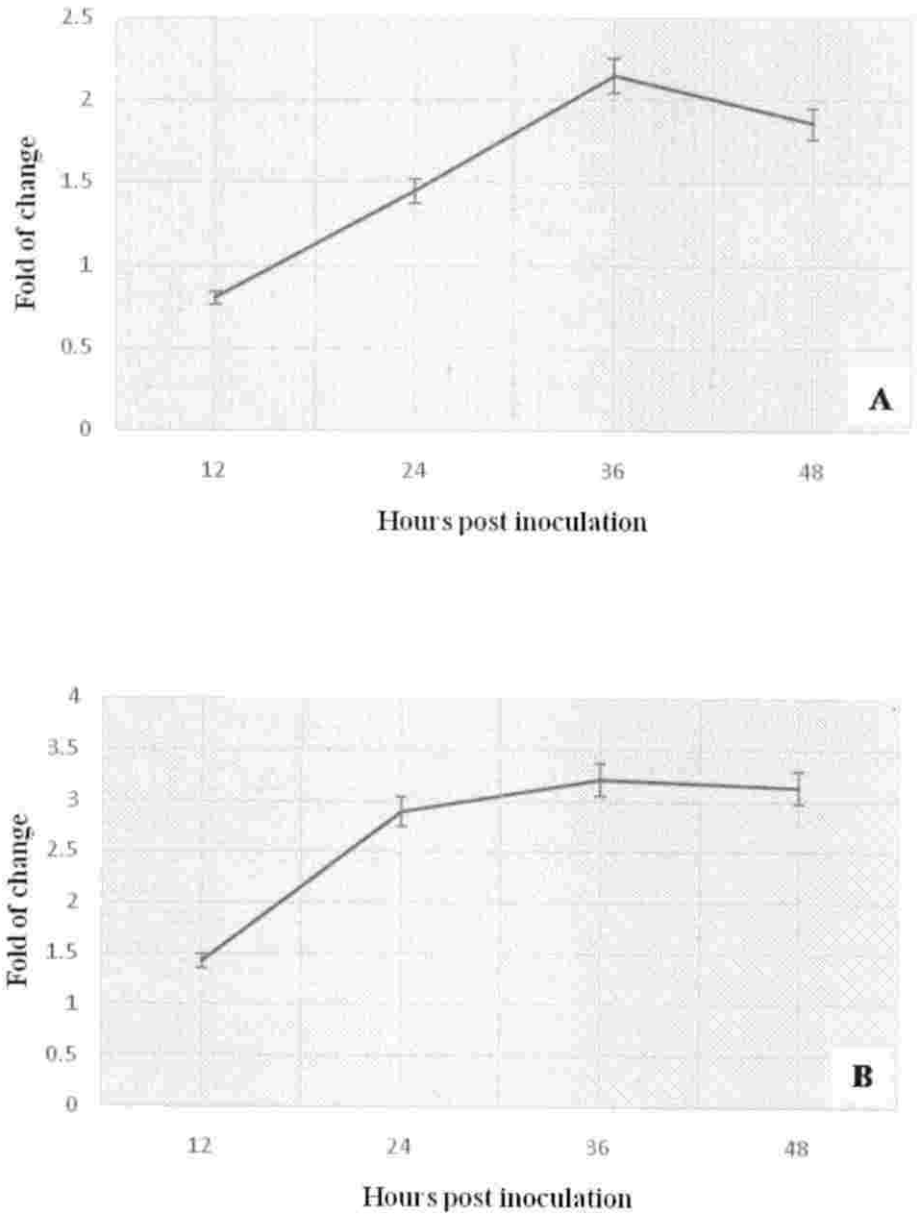

Fig 5. Relative gene expression of RGA in samples of Sree Kiran (A) and Muktakeshi (B).

The normalized gene expression was increased during early stages with a noticeable hike at 24 h after pathogen inoculation in the resistant variety and at 36 h of pathogen inoculation in the susceptible variety. The maximum fold change (3.21) was observed in Muktakeshi (resistant) sample collected after 36 h of inoculation while the minimum fold change (0.8) was recorded in Sree Kiran after 24 h of inoculation when compared to the control. The melting curve analysis at the end of the cycling reactions revealed single dissociation peak at 75°C (T RGA F and T RGA R) and 78°C (ACT F and ACT R) indicating the specific binding of the designed primers (Fig.6). The results showed the expression of gene in both varieties. However, the increase in expression varies at different time intervals and at 48 h there is a noticeable reduction in expression in susceptible varieties.

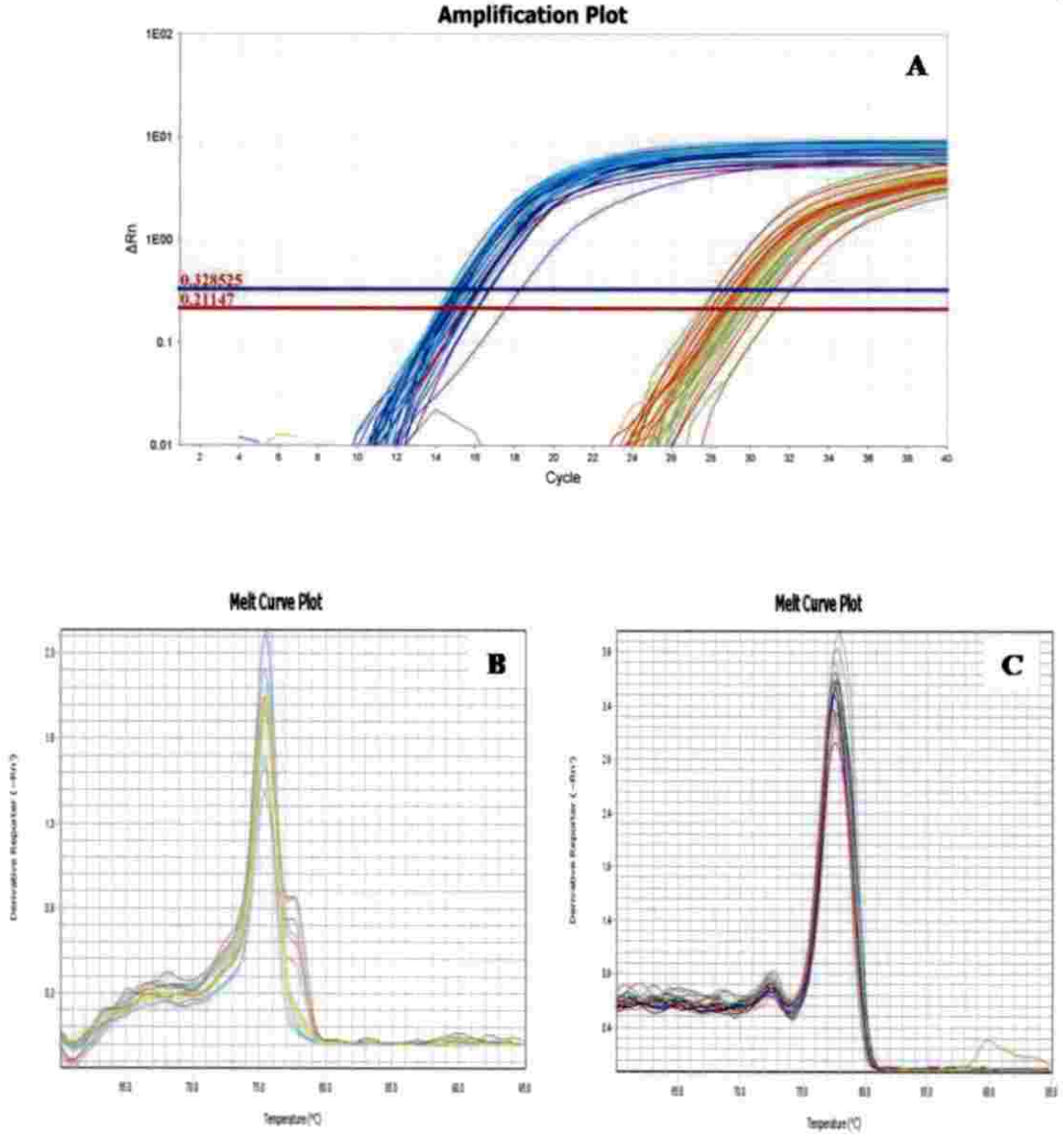

Fig 6. Amplification plot (A) and melt curve analysis of the Real-time PCR assay using the designed primer pairs (B) and reference gene Actin (C).

DISCUSSION

5. DISCUSSION

Taro [*Colocasia esculenta* (L.) Schott] is an important tropical tuber crop, belonging to the family *Araceae* with wide distribution in tropics. Leaf blight caused by *Phytophthora colocasiae*, oomycete plant pathogen, is the most destructive disease affecting the leaves and petioles of taro plants (Raciborski, 1900). Thus results in extensive damage of the foliage and also causes extensive post-harvest decay of corms. Considering the economic and medicinal importance of taro, it is high time to develop an effective leaf blight management strategy. Exploiting the natural host resistance is possibly the most economical and environment friendly approach to mitigate leaf blight, since the disease coincides with intermittent rainfall which affects the efficiencies of fungicides.

Most plant-pathogen interactions are complex intertwined mechanism which involves the presence the resistance (R) gene product that recognize a pathogen (Avr) gene product resulting in defence activation (gene-for-gene model) (Flor *et al.*, 1971). Resistance gene analogues (RGAs) are a group of potential R-genes with conserved domains and motifs, which has a significant role in pathogen's resistance. Information concerning the sequences of large number of R genes as well as the genomic location and organization with in the plant genome, can facilitate the understanding of the mechanisms underlying host resistance to pathogens. This will be of great value for crop improvement strategies associated with disease resistance. Taking into account, the present study was undertaken to isolate the resistance gene analogues in taro and to study the difference in expression of RGAs during leaf blight infection in resistant (Muktakeshi) and susceptible (Sree Kiran) varieties. PCR amplification with degenerate primers was the method used for isolating RGAs in taro.

The PCR-based strategies by using degenerate primers designed from the NBS region of already reported disease resistance genes has been proved as an excellent

tool for cloning many resistance gene like sequences in several other plant species (Yu *et al.*, 1996; Xiao *et al.*, 2006; Nath *et al.*, 2013). The degenerate primers reported by Aswati and Thomas (2007), which targets the P-Loop and GLPL conserved domains of NBS-LRR gene are used for this study. A common feature of degenerate primers is the co-amplification of non specific DNA fragments along with desired fragment and is observed in many plant species (Mago *et al.*, 1999; Lopez *et al.*, 2003; Nath *et al.*, 2013).

Two sets of degenerate primer pairs (RGA1 and RGA2) were used in the present study which resulted in amplicons of size 500 and 600 bp. The amino acid sequence analysis of the obtained four sequences showed the presence of RGAs when compared with other known sequences in the database. To visualize the relatedness between the obtained taro RGAs and R-genes of other plant species in the database, a phylogenetic tree was constructed using MEGAx by Neighbor-Joining method. The cluster analysis of phylogenetic tree supported the classification of the taro RGAs into the non-toll interleukin receptor (non-TIR) subclass since they were all clustered with other non-TIR types of NBS sequences. No TIR-type of RGA sequences were identified from any of the taro RGAs studied. This is in agreement with the previous reports on RGAs suggesting the lack of 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 at the N terminus, the TIR proteins vary notably in plant species and is assumed that the loss of TIR domain from monocots have occurred during the divergence of the monocots and dicots (Pan *et al.*, 2000).

Taro leaf blight occurs in severe form during intermittent rainfall which makes spraying fungicides or application of other agents difficult. At present chemical and bio control methods are being used for management which has not managed the disease satisfactorily. Development and use of resistant varieties are the best economic approach to fight taro leaf blight disease and this can be achieved by identifying novel R-genes for disease management. Till now, the major group of R

genes successfully identified and cloned from a large number of plant species belong to NBS-LRR family. In most of the plants studied so far, the major function of NBS-encoding genes was proved to be pathogen recognition and thus involved in disease resistance (Dangl, 2001; Mchale *et al.*, 2006). Due to the low transcript levels of RGAs, the expression levels are still under discussion and only few literatures are available regarding R-gene expression (Graham et al., 2002). The previous studies on R-gene expression demonstrates the low levels of gene expression before pathogen attack (Hulbert *et al.*, 2001; Hammond-Kosack and Jones, 1997) when compared to induced or up-regulated gene expression followed by pathogen inoculation (Wang *et al.*, 1999; Hu *et al.*, 2017).

The difference in gene expression in the resistant taro cultivar (Muktakeshi) was compared with that of susceptible taro cultivar (Sree Kiran). Whole plants raised from surface sterilized tubers were used for the assays, where as similar studies were done in detached leaves by other researchers (Wang *et al.*, 1999; Tian *et al.*, 2006). The plants were maintained under sterile conditions in order to avoid interaction with other pathogens and was also monitored to avoid wound responses not coupled with the infection progress. These are taken into special care, since many pathogenesis or defense related genes are also induced followed by abiotic stress like wounding (Vignutelli *et al.*, 1998; Bertini *et al.*, 2003). Leaf blight disease was induced by artificial inoculation of *P. colocasiae* for studying the resistant gene expression in taro cultivars. The appearance of leaf blight symptom was delayed in resistant cultivars and the rate of infection spread was also slow as compared to susceptible cultivars (Misra and Singh, 1991; Misra and Chowdhury, 1997). Natural variations in disease resistance present in different cultivars can serve as a valuable resource for mining the molecular mechanisms of plant- pathogen interaction (Jing *et al.*, 2013).

To study the differential gene expression of RGAs in susceptible and resistant varieties of taro, a real time PCR was performed using both pathogen inoculated and

pathogen un-inoculated control plants maintained under controlled conditions. RNA isolated after 12, 24, 36 and 48 h of pathogen inoculation was used for expression study. The obtained sequence information was used to design specific primers for real time PCR using Primer 3 plus software, which is the first report in taro. The cDNA concentration was normalized prior to expression study using real time PCR as the yields of the product may vary. Real time PCR is an established and fast technique for quantitative gene expression studies. Since RT-qPCR is a sensitive technique, it is used for studying the low transcript levels of RGAs. The relative gene expression level of resistant and susceptible varieties is studied using $2^{-\Delta\Delta CT}$ method.

In short, the results of real time PCR showed that the target gene was up-regulated during the course of infection in both the resistant and susceptible varieties. But the difference was that the hike in expression upon pathogen infection was found earlier in resistant variety than the susceptible variety and the level of expression (fold change) was also found to be more in the resistant variety. The normalized gene expression was increased with a noticeable hike in the sample collected after 24 h of pathogen inoculation of the resistant variety and a gradual increase in the sample collected at 36 h of pathogen inoculation in the susceptible variety. In tobacco, many RGAs was found to show induced expression after infection with either *Tobacco mosaic virus* (TMV) or the tobacco black shank pathogen (*Phytophthora parasitica var. nicotianae*) (Gao et al., 2009). The increase in transcript level observed after 24 h of pathogen inoculation on Muktakeshi seems to indicate their role in early pathogen recognition, thereby makes it resistant variety. The results could be utilized to study the resistance in different taro lines after validation with more cultivars or lines with known resistance

Expression of RGAs in other species had also shown a similar expression pattern i.e. low gene expression prior to pathogen attack and induced expression subsequent to pathogen attack (Hulbert *et al.*, 2001; Wang *et al.*, 1999; Yoshimura *et al.*, 1998). A similar up-regulated gene expression pattern was observed in chickpea

RGAs due to *Fusarium* wilt (Gutierrez et al., 2012). The molecular mechanisms associated with resistance and susceptibility of plants could be better understood by identifying genes whose expression is either upregulated or downregulated in response to pathogen infection and can be used to modify the biotechnological approaches to improve disease resistance in plants (Casado-Diaz *et al.*, 2006).

As a conclusion, in the present study the candidate gene tested behaved differently in the resistant and the susceptible genotype in response to *P. colocasiae*, which indicates the role of RGAs in the resistance response against the pathogen. Thus, the identified RGAs and expression pattern could be used as a good start point for further studies such as candidate gene mapping for taro leaf blight.

SUMMARY

6. SUMMARY

The study entitled "Differential response of resistant gene analogues (RGAs) against *Phytophthora colocasiae* causing leaf blight in taro" was carried out during October 2018-August 2019 at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram. The objectives of the study was to isolate, characterize and sequence the Resistant Gene Analogues in taro against *Phytophthora colocasiae* and to study the gene expression profiling of resistant and susceptible taro cultivars upon leaf blight infection.

PCR based amplification of RGAs was carried out using two sets of reported degenerate primers, which targets the P-Loop and GLPL conserved domains of R genes. DNA was isolated from young leaf samples and Resistance Gene Analogues (RGAs) were amplified through PCR. PCR amplification of RGAs in genomic DNA of taro resulted in amplicons of the predicted size 500 bp and 620 bp based on previous reports on RGA sequences. The reamplified gel elutes were given for sequencing. The similarity search of the obtained results was done by using online BLASTx program of NCBI and the presence of resistance gene analogues was identified on both susceptible and resistant varieties. Conserved Domain search tool at NCBI was used to confirm the presence of Nucleotide Binding-ARC domain in all the obtained sequences. The phylogenetic tree was constructed using MEGA X by Neighbor-Joining method with amino acid sequences of the obtained RGA sequences of Muktakeshi and Sree Kiran along with similar sequences in the database.Based on the nucleotide sequence information of taro RGAs, specific primers were designed using Primer3 plus online software and the product size was 240 bp.

Two taro varieties Sree Kiran (susceptible) and Muktakeshi (resistant) were used for studying the resistant gene expression pattern. Shoot tip culture of taro varieties Muktakeshi and Sree Kiran were established on Murashige and Skoog media supplemented with growth hormones, 6-benzyladenine (BA) (5 mg L^{-1}) and

NAA (1 mg L^{-1}) with an idea of utilizing tissue culture plants for the study. Since the percentage of survival was very low for both the varieties, plants raised from uninfected, surface sterilized tubers maintained under controlled conditions were used for pathogen inoculation, RNA isolation and gene expression study. These plants were inoculated with zoospore suspension of *Phytophthora colocasiae* for disease induction to study the expression in both susceptible and resistant varieties during disease development. Leaf samples were collected after 12, 24, 36 and 48 h of pathogen inoculation from inoculated as well as control plants of both the varieties for RNA isolation. RNA was isolated using Ambion Purelink RNA Mini Kit, TRIzol based method and LiCl2 based method, in which better results were observed in TRIzol based method.

cDNA was prepared out of the RNA isolated from samples collected after 12, 24, 36 and 48 hours of pathogen inoculated plants and control plants of both varieties. The resistance gene analogues present in the resistant and susceptible variety were targeted using designed specific primers and gene expression was studied by the SYBR green PCR assay. Actin was used as the reference gene for the expression study. The relative gene expression level of resistant and susceptible varieties was studied using $2^{-\Delta\Delta CT}$ method. A negative control was kept for both primers with water instead of cDNA in the components.

In general, the results of real time PCR showed that the target gene was up-regulated during the course of infection in both the resistant and susceptible cultivars. But the hike in expression upon pathogen infection was earlier in resistant variety than the susceptible variety and the level of expression (fold change) was also found to be more in the resistant variety. The normalized gene expression was increased with a noticeable hike in the sample collected after 24 h of pathogen inoculation of the resistant variety and a gradual increase in the sample collected at 36 h of pathogen

inoculation in the susceptible variety. The findings could be validated using more varieties and candidate gene mapping will help to find out resistance in taro.

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APPENDICES

APPENDIX I

TISSUE CULTURE

Murashige and Skoog (MS) media (1 L) supplemented with 6-benzyladenine (BA) and 1-Naphthalene acetic acid (NAA)

MS media powder (HIMEDIA PT021X1L) - 4.08 g
Sucrose - 30 g
10X Calcium chloride - 10 ml
Polyvinylpyrrolidone (PVP) - 2.0 g
Agar (0.8%) - 8 g
BA (5 mg ml^{1}) - 1 ml
NAA (1 mg ml^{-1}) - 1 ml

Dissolve the components (except agar) in 900 ml distilled water and adjust the pH to 5.7 with 0.1N HCl/ 0.1N NaOH. Make up the volume to 1 L and add 8 grams of agar and after melting transfer to test tubes. Sterilize the media at 121°C, 15 psi pressure for 20 minutes.

BA (5 mg ml^{-1}) – 10 ml

Dissolve 50 mg of BA in a small volume of 1N NaOH and transfer to a volumetric flask. Make up the volume to 10 ml with distilled water and mix well. Aliquot and store at 4°C.

NAA (1mg ml^{-1}) – 10 ml

Dissolve 10 mg of NAA in a small volume of 1N NaOH and transfer to a volumetric flask. Make up the volume to 10 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) – 2.04 g
Sucrose – 15g
10X Calcium chloride – 10 ml
Polyvinylpyrrolidone (PVP) – 2.0 g

Dissolve in 900 ml distilled water and adjust the pH to 5.7 with 0.1N HCl/ 0.1N NaOH. Make up the volume to one litre and add 4 g of agar 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 ***P. colocasiae***

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.

Carrot Agar (CA) pH 7.0

Carrot - 250g
Agar - 20g

APPENDIX III

TARO DNA AND RNA ISOLATION

CTAB DNA Extraction Buffer

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

Wash solution

15 mM ammonium acetate in 75% (v/v) of ethanol

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 or covered with Aluminium foil and stored at room temperature.

Gel loading dye (6X)

Bromophenol blue (w/v) - 0.25%
Xylene cyanol FF (w/v) - 0.25%
Glycerol - 50%
EDTA (pH 8.0) - 10 mM
Dissolve the components in nuclease free water and store at -20°C

CTAB RNA Extraction Buffer

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

All the reagents are prepared in DEPC treated water.

ABSTRACT

DIFFERENTIAL RESPONSE OF RESISTANT GENE ANALOGUES (RGAs) AGAINST *Phytophthora colocasiae* CAUSING LEAF BLIGHT IN TARO (*Colocasia esculenta*)

By

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(2014-09-108)

Abstract of Thesis

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9. ABSTRACT

Taro (*Colocasia esculenta* (L.) Schott.), an important tropical tuber crop with high nutritive, and medicinal potential, is ranked fourteen among the most consumed vegetable worldwide. Leaf blight caused by *Phytophthora colocasiae*, is one of the most destructive diseases of taro leads to severe yield loss up to 30-50%. The main objective of this study was the gene expression profiling of resistant gene analogues (RGAs) in resistant and susceptible taro cultivars upon leaf blight infection. For isolating the taro RGAs, PCR-based strategy with degenerate primers was used, and the obtained sequences showed similarity with other RGA sequences in the NCBI database, which categorised them into the NBS-LRR class of gene family. The conserved domain search has proved the presence of Nucleotide Binding-ARC domain in all the sequences. A phylogenetic tree constructed with the obtained taro RGAs and RGAs of other plant species in the database grouped them into the non-toll interleukin receptor (non-TIR) subclass of NBS sequences. RGA specific primer was designed based on sequence information, which is the first report in taro. The expression profiles of RGAs in Muktakeshi (resistant) and Sree Kiran (susceptible) genotypes in response to leaf blight infection determined by the SYBR green PCR assay demonstrated that, taro RGAs were up-regulated during the course of infection in both the resistant and susceptible cultivars. The normalized gene expression had shown a noticeable hike in the sample collected after 24 h of pathogen inoculation in the resistant variety, whereas in the susceptible variety it was observed at 36 h. Although, RGAs were expressed in both resistant and susceptible control plants, increased fold change was observed in the test plants following pathogen inoculation. The relative gene expression level of resistant and susceptible varieties was studied by using $2^{-\Delta\Delta CT}$ method and actin was used as the reference gene. These RGAs could be used as a good start point for further studies such as candidate gene mapping for taro leaf blight.