Piriformospora indica mediated response in taro (*Colocasia esculenta* (L.) Schott) with special emphasis to growth and leaf blight incidence

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by

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THESIS

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KERALA, INDIA

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DECLARATION

I hereby declare that this thesis entitled "***Piriformospora indica*** **mediated response in taro (*Colocasia esculenta* (L.) Schott) with special emphasis to growth and leaf blight incidence"** 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, associate ship, fellowship or other similar title, of any other university or society.

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LAKSHMIPRIYA P.

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LIST OF ABBREVATIONS

◦C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromolar
bp	Base pair
cm	Centimetre
CTAB	Cethyltrimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
Kbp	Kilo base pair
Kg	Kilogram
M	Molar
Mg	Milligram
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometre
OD	Optical Density

PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PVP	Polyvinyl pyrrolidine
PEG	Polyethyl Glycol
RNase	Ribonuclease
Rpm	Revolution per minute
RT	Room Temperature
s	Second
TrisHCl	Tris (Hydroxyl Methyl) aminomethane hydrochloride
U	Enzyme unit
UV	Ultraviolet
V	Volt
V / V	volume/ volume
W / V	weight/ volume

INTRODUCTION

1. INTRODUCTION

Taro [*Colocasia esculenta* (L.) Schott], a member of the Araceae family is an oldest cultivated crops grown for its edible leaves and corms (Coates *et al.,* 1988). Probably it is native to south eastern Asia and when it spread to Pacific islands; it became a staple crop, cultivated for its large, starchy, spherical underground tubers. Leaves and corms of taro are also accredited to have certain medicinal values like reducing pulmonary congestion, tuberculosis, ulcers, and fungal infections (Misra and Sriram, 2002). The plant is susceptible to variety of diseases in which leaf blight caused by *Phytophthora colocasiae* Rac. Which causes yield loss of about 25-50 percent is the major concern in all taro growing countries, including India, (Raj *et al.,* 2008)

Most primary infections of hosts initiated by *Phytophthora* species originate from soil borne inoculum (Andrivon, 1995). *Phytophthora* can survive in soil in the absence of host as chlamydospores, mycelia, sporangia, zoospore cysts, or oospores (Quitugua and Trujillo, 1998). The fungal inoculum as spores spread to adjacent plants and nearby plantations by wind driven rain and dew. The fungus can also remain alive on soil for about three weeks after harvesting and the disease can also be spread by using previously infected planting material (Jackson, 1999). Once established, this is the main cause of sudden spread of the disease throughout a wide area.

Several methods for managing taro leaf blight have been recommended but the use of tolerant cultivars seems to be the most ideal and economical method. The disease spread can be effectively managed by several fungicides such as, 0.2% metalaxyl + mancozeb (Ridomil MZ-72), 0.2% captafol (Foltaf) (Bhattacharyya and Saikia1996), Bordeaux mixture and 0.25% mancozeb.

Due to the mounting concern over environment pollution and hazardous effects of chemical pesticides, biological management of diseases is getting more importance than chemical methods. The chemicals that are being used for controlling plant diseases enters in to the food web and cause damage to the life directly or indirectly at various points of food web (Frampton *et al.,* 2006).

Biological control of plant diseases is the management of plant disease by reducing the pathogen inoculum with the help of beneficial antagonistic microbes. Some of the effective management practices using biocontrol agents involve fungus and bacteria. Rhizobacteria isolated from *Colocasia* rhizosphere soil have been reported to have the ability to inhibit the mycelial growth of *P. colocasiae* under *in vitro* condition. Three species of *Trichoderma viz. T. asperellum*, *T. longibrachiatum* and *T. harzianum* have been found to possess biocontrol potential against the taro leaf blight pathogen. (Nath et al., 2014). The most potent strain (TR7) was identified as *T. harzianum* and was able to control *P. colocasiae* under *in vivo* condition also.

Piriformospora indica is a beneficial endophytic root-colonizing fungus isolated from the rhizospheres of *Prosopsis juliflora* and *Zizipus nummularia* of Thar Desert by Prof. Ajith Varma and group, School of Life Sciences, Jawaharlal Nehru University. It has a wide host range including plants belonging to Pteridophyta, Bryophyta, Gymnosperm and Angiosperm. Morphologically, the fungal hyphae are hyaline, thin walled, irregularly septated and also form pear shaped chlamydospores. The fungus colonizes both inter- and intracellularly in rhizodermis and cortical areas of the root, and does not invade the aerial parts and endodermis of the plant. Similar to arbuscular mycorrhizal fungi, the fungus promotes nutrient uptake, and imparts resistance against biotic (insects and other pathogenic microorganisms) and abiotic stresses (salinity, drought, heat and cold). Further, it stimulates enhanced production of biomass, seed production and early flowering in its host plants. In contrast to AMF, *P. indica* can grow axenically. It can be cultured in different substrates like PDA, Aspergillus medium etc.

P. indica can enhance resistance of inoculated plants against several pathogens. The biotic stress protection by *P.indica* were first indicated in barley. The fungus-colonized plants were also resistant against *Blumeria graminis* infection and *Fusarium culmorum* in barley (Waller *et al.*, 2005). A similar phenomenon was also observed for many other plant species and pathogen isolates, like *Fusarium verticillioides* in *Zea mays*, *Pseudocercosporella herpotrichoides* in *Triticum aestivum*, and *Verticillium dahlia*e in *Solanum*

lycopersicum (Serfling *et al.,* 2007; Kumar *et al.,* 2009; Fakhro *et al,.* 2010). During abiotic stress in barley, wheat and maize, production of reactive oxygen species and antioxidants were also observed. Root pathogens could be inhibited directly by antagonistic activities of the *P. indica* (Franken *et al.,* 2012). Various defence related genes were upregulated in barley, upon powdery mildew infection in *P. indica* colonized plants compared to the control plants and this would be attributed to the of resistance against the disease in barley (Molitor *et al.,* 2011).

P. indica has a broad host range and showed pronounced growth-promotional effects in many plant species. The insoluble phosphates in the planting medium were mobilized and the phosphorus was translocated to the host in an energy-dependent manner (Yadav *et al.,* 2010). More than 90% survival rate was rendered by *P. indica* for tissue culture plantlets as a biological hardening agent when transferred to fields. Transgenic manipulation of the fungal component was opened up through the successful isolation of regenerative protoplasts of *P. indica* and it can also improve the symbiosis of the fungal component. *P. indica* can be axenically cultivated on economically feasible synthetic media which makes it suitable for mass scale production for agro-forestry and horticulture application.

P. indica promotes growth of plants in terms of germination, enhanced fruiting, seedling growth, early flower setting and increase in the content and quality of value added products. *P. indica* can induce antioxidant enzymes, abiotic stress-responsive genes and defence related proteins to confer tolerance in different plant species like *Arabidopsis thaliana* (Sherameti *et al.,* 2008), Chinese cabbage (Sun *et al.,* 2010) and *Hordeum vulgare cv. Ingrid* (Baltruschat *et al.,* 2008). In *Oncidium* orchid the regulation of growth and other developmental processes were elicited through miRNAs by *P. indica* (Trivedi *et al.,* 2013).

There are many studies on the potential of *P.indica* to promote growth and induce resistance against biotic and abiotic stresses in many crops. However, the potential of this versatile fungus is yet to be explored in tropical tuber crops. Pathogen suppression potential of *P. indica* has been tapped to manage *Phytophthora* infection in other crops (Curry and Diehl, 2004). The present study

was formulated with the intention of understanding the capability of *Piriformospora indica* in mediating growth and conferring leaf blight resistance in taro.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Tuber crops

Root and tuber crops are third important food crops after cereals and legumes. They are staple and subsidiary food for the one-fifth of the world population. About one- third of the world's dietary calories were contributed by them and can be considered as the important source of raw materials for industrial products and animal feed (ICAR-CTCRI, 2015). The full potential of these staple crops are being realized in tuber crops growing areas and they would contribute to energy and nutrient requirements for the increasing population. India has a rich genetic diversity of several tuber crops like aroids, cassava, yams, sweet potato and many minor tubers (Ajaybanik, 2015). All these crops must be conserved and used in a sustainable manner for further improvement of these tuber crops.

2.2 Taro (*Colocasia esculenta*)

Taro is a tropical aroid with about 1000 cultivars and an important staple crop for millions of people in developing countries. Two taro types are commonly cultivated in India viz., *C. esculenta* var. *esculenta* and *C. esculenta* var. *antiquorum*. The whole plant has got variety of uses. They are mainly cultivated for its edible corms, leaves and also for its medicinal properties. The various medicinal uses are, reducing tuberculosis, pulmonary infections, ulcers and microbial infections (Misra and Sriram, 2002).

2.3 Taro leaf blight

The major devastating disease of taro is the leaf blight disease caused by *Phythophthora colocasiae* which results in 25-50 percent yield loss. (Raj *et al.*, 2008). Leaf lamina is mainly damaged by the fungus. The pathogen causes post-harvest rot of the corm and petiole rot in susceptible varieties. The infection is generally observed on the portion of the leaves where water collects. The first sign of the infection is the formation of water soaked lesions which spread and turn into brown spots. During night, large volumes of sporangia form round the

margin of the lesions and they will have a powdery white appearance. Depending on the weather conditions, the entire leaf area will be destroyed within 3-5 days after the appearance of initial symptoms. During intermittent rains with temperature around 28°C and under cloudy weather conditions, the disease spreads at tremendous speed and the entire field gives a blighted appearance (Misra *et al.*, 2008). From the lesions, yellow to brown coloured exuded droplets dries out during the day and produces dark brown, hard deposits, which is a characteristic of the disease (Singh et al. 2012). Taro leaf blight is a very destructive disease, which reduces the photosynthetic leaf area and thus, the number of functional leaves will be reduced. Leaf blight disease spreads over long distance by the use of infected planting material. Even though the mycelial stage lasts only for about 5 days, the spores can prevail in the soil for about 3 months. Hence it is recommended not to plant new tubers on the same land for a minimum of three weeks, plant new tubers away from older infected ones and corms or suckers which can harbor the pathogens must not be used or kept along with the new ones (Jackson, 1999).

2.4 Leaf blight resistant varieties

Several methods for the management of leaf blight of taro have been recommended but the use of tolerant cultivars seems to be the most ideal and economical method. In 1987, two superior varieties were released from ICAR-Central Tuber Crops Research Institute for general cultivation under the names ‘Sree Reshmi’ and ‘Sree Pallavi’. Again in 2001, a taro leaf blight resistant variety ‘Muktakesi’ was released from Regional Centre of ICAR-CTCRI (Pillai *et al.*, 1993). Misra (1988) screened 43 cultivars of taro and identified cvs. ‘Jankhri’ and ‘Muktakeshi’ as highly leaf blight tolerant varieties. The National Agricultural Research Institute (NARI) of Papua New Guinea released three new varieties of taro named NT 01 (NARI Taro 01), NT 02 and NT 03 which yields high, display good resistance to leaf blight disease, perform well under different climate and are of good cooking and eating quality. Many cultivars of taro tolerant to leaf blight have been reported from India. Deshmukh and Chibber (1960) have

identified var. ‘Ahina’ as resistant to blight. The number of sporangia produced on Ahina was less when compared to the susceptible variety. The size of the infected lesion increases more slowly in the resistant variety than in susceptible variety. Later, Paharia and Mathur (1964) screened 20 varieties at Shimla and found var. ‘Poonam Pat’ as immune, ‘Sakin V’ as resistant and another seven varieties as moderately resistant to blight.

2.5 Fungicidal control

Management of taro leaf blight by fungicides is a common method used by the farmers. The disease can be effectively controlled by using different combinations of a variety of fungicides. Jackson et al., (1980) could get very good control of the taro leaf blight with copper oxychloride and it gave better control of *P. colocasiae* than mancozeb. Agarwal and Mehrotra (1987) evaluated six fungicides against taro leaf blight and found Deraosan 65W as the most effective one inhibiting *P. colocasiae* mycelial growth which was followed by Difolatan 80W and Fytolan. The disease spread can be substantially decreased by using Ridomil MZ-72, foltaf, Bordeaux mixture and mancozeb (Bhattacharyya and Saikia, 1996). The effect of cytokinin benzyl amino purine (BAP) on *P. colocasiae* mycelia was studied by Mishra *et al.* (2008). The results showed that benzyl amino purine has got inhibitory effect on the pathogen and further reduction of the disease was also observed during pot trials.

2.6 Biocontrol agents

Repeated spraying of fungicides is needed to effectively control the taro leaf blight, since the disease spreads during monsoon. This will cause a negative impact on the environment. It can harm the beneficial organisms and the fungicide can persist in the environment for a long time. Hence, it is recommended to use ecofriendly means of disease management like using biocontrol agents (Latha *et al.,* 2009). *Rhizoctonia solani, Fusarium oxisporum, Phytophthora colocasiae* and *Pythium* sp. are the most important phytopathogens of vegetable crops like tomato, cabbage, brinjal, taro and sugar beet responsible for damping off diseases

(Curry and Diehl, 2004). The inhibitory effect of three species of *Trichoderma* and *Pseudomonas* on the population of pathogens, *R. solani, F. oxisporum, P. colocasiae* and *Pythium* sp. in soil was studied *in vitro* by Jena (2012). Three species of *Trichoderma* viz. *T. viride, T. harzianum, T. pseudokoningii* and *Pseudomonas fluorescens* could effectively control all the pathogens and of which, *P. fluorescens* was most effective in controlling pathogen population in soil. The combination of all these bio-control agents was more effective than that of individual application. Three species of *Trichoderma viz. T. asperellum, T. longibrachiatum* and *T. harzianum have* biocontrol potential against the taro leaf blight pathogen (Nath *et al.,* 2014). The most potent strain, *T. harzianum* was able to control *P. colocasiae in vivo* also.

Veena *et al.* (2013) studied the effect of vermicompost and vermiwash in managing taro leaf blight and collar rot of elephant foot yam. Vermicompost/vermiwash treated plants showed less than 10% taro leaf blight and collar rot incidence. Yield increase was also noted in both crops with the application of vermicompost. Phylloplane microflora of *C. esculenta* in relation to *P. colocasiae* was studied by Narula and Mehrotra (1981) and found *Myrothecium roridum,* 2 bacterial isolates and 3 *Streptomyces* spp as antagonistic to *P.colocasiae* in dual culture plates. The bacteria reduced the disease incidence up to 43% under *in vivo* condition also. *Streptomyces albidoflavus* reduced infection by 93% followed by *S. diastaticus* (76%).

2.7 Induced systemic resistance

Sahoo *et al.* (2009) assessed the differential expression of antioxidative enzymes, their isozymes and phenolics and polyphenol oxidase activity in taro. Under induced blight condition, the activity of superoxide dismutase and guaiacol peroxidase was also observed to be increased when compared with control. Increase in antioxidant enzymes was elevated (67–92%) in the resistant genotypes than that of the susceptible genotype (21–29%). The amount of phenolics increased during disease.

Misra *et al.* (2008) studied the biochemical alterations in taro plants upon infection by *Phytophthora colocasiae* in a resistant (Muktakeshi) and a susceptible (Telia) cultivar and changes in peroxidase, L-phenylalanine ammonia-lyase, β-1, 3- glucanase, total sugar and total phenol in healthy and infected plants were evaluated.

The differentially expressed genes in taro during *P. colocasiae* infection were identified by Sharma *et al.* (2009). Northern blot analysis, high throughput DNA sequencing, Suppressive subtractive hybridization (SSH), cDNA libraries, and bioinformatics were employed to identify the defense-related genes. Two putative resistance genes (chitinase and lipid transfer proteins) and a transcription factor were identified among the upregulated sequences. Overall expression of defence related genes were heightened in Muktakeshi (resistant) compared to UL-56 (susceptible).

2.8 *Piriformospora indica*

Piriformospora indica is a member of the order Sebacinales and has shown extreme versatility in its ability to promote plant growth and its mycorrhizal associations. The fungus has got pear shaped chlamydospores and is isolated from Thar Desert of Rajasthan. It is a Basidiomycete which resembles Arbuscular Mycorrhizal Fungi in many aspects. *P. indica* colonizes members of bryophytes, pteridophytes, gymnosperms and angiosperms and is widely distributed as a symptomless root endophyte (Varma *et al.*, 2012). The fungus has got a broad host spectrum and has shown pronounced growth-promotional, disease resisting effects in inoculated plants. It also protects the plants from abiotic stresses like drought, salinity, cold etc. It can also enhance the secondary metabolite production in medicinal plants. Sateesan *et al.*, (2012) reported enhanced production of asiaticoside by *P.indica* colonized *Centella asiatica* plants. Increased level of Podophyllotoxin from *Linum album* colonized by *P. indica* has been reported by Baldi *et al.* (2008). *P. indica* can be successfully used as a potent plant growth promoting endosymbiont.

2.8.1 Culturing of the fungus

P.indica can be cultured on several synthetic and complex media and it can grow under solid and liquid culture conditions. In liquid shaking cultures, the mycelia aggregates to form globose like balls. The composition of the nutrients in the media strongly determines the morphology of the mycelium. During the infection experiments with populus hybrid (Esch5), a more aggressive behavior against the plant was shown by *P.indica*, which was actually because of the media conditions provided during the experiments (Kaldorf *et al.*, 2005). Yadav et al. (2010) used Jaggery prepared from sugarcane extract (*Saccharum officinarum*) for culturing the fungus and was successful in it and, optimum chlamydospore production was obtained with 4% jaggery. The plant growth promoting properties was maintained by the biomass. The Jaggery yielded 16-18 g/l of the fungal biomass when compared to Hill-Kaefer medium which yielded 12-15 g/l of the fungal biomass,. Anith et al., (2015) found that coconut water, a waste product from the coconut industry, can also be used as a medium for cultivation of *P.indica*.

2.8.2 Interaction of *P. indica* with different hosts

Piriformospora indica have the ability to interact with the roots of many plant species of different taxa which is considered as one of its specific features. It can interact with a wide range of hosts, including bryophytes, pteridophytes, gymnosperms and a large number of monocots and dicots (Fakhro *et al.*, 2010). The fungus can grow inter- and intracellularly and forms pear shaped fluorescent chlamydospores within the root cortex in the rhizosphere zone, and the endodermis and the aerial parts of the plants were not invaded. The fungus enhance nutrient uptake, allows plants to survive under several abiotic stress like water, temperature and salt stresses, and confers systemic resistance to heavy metal ions, toxins, insects and other pathogenic organisms.

2.8.3 Induced systemic resistance by *P. indica*

Similar to AM fungi, the fungus not only induces resistance against many soil-borne and root pathogens but also impart systemic resistance to different foliar pathogens after colonizing on/in roots of wide variety of plant species (Qiang *et al.,* 2012). Besides growth promoting effects, *P. indica*-colonized wheat and barley plants showed enhanced resistance against root pathogenic fungi *Cochliobolus sativus* and *Fusarium culmorum* causing root rot diseases (Waller *et al.,* 2005). *P. indica* can also provide protection against several leaf pathogens. In barley, the leaf spot pathogen *Blumeria graminis* was controlled by applying *P. indica.* In *P. indica* colonized plants, numbers of sheath layers and hydrogen peroxide concentrations were increased after *B. graminis* attack which proved that root colonization causes induction of systemic resistance or priming of the host plant (Serfling *et al.,* 2007). *P. indica* induces systemic resistance against the leaf pathogen *Blumeria graminis* in barley roots (Molitor *et. al.,* 2011). Twenty two transcripts were differentially expressed twofold, which includes pathogenesis-related genes and genes encoding heat-shock proteins, in leaves in *P. indica* root colonized plants after *B. graminis* inoculation compared with non-colonized plants. A faster induction of defence related genes were observed by detailed expression analysis after *B. graminis* inoculation between 8 and 16 hpi, which suggests that priming of these genes is an important mechanism of *P. indica*-induced systemic disease resistance. The direct antagonistic effect of *P. indica* has been demonstrated on several phytopathogenic root fungi like *R. solani*, *R. bataticola*, *F. oxysporum*, *F. udum*, *F. solani* and *Phytophthora nicotianae* var. *parasitica* due to antibiosis and production of lytic enzymes such as chitinases and glucanases (Johnson *et al.,* 2013). Kumar et al. (2009) demonstrated the bioprotection performance of *P. indica* against the root pathogen *F. verticilloides* in maize and is related to the increased activity of the antioxidant enzymes like catalase, glutathione S transferase, glutathione reductase and superoxide dismutase in the *P.indica*-colonized plants.

2.8.4 Growth promotion

The interaction of *P. indica* with host plant roots is accompanied by an enormous uptake of phosphoroua and nitrogen from the soil. Overall biomass production of different plants, like mono- and dicots, trees, terrestrial orchids, medicinal plants like *Bacopa monniera, Artemesia annua* (Sharma and Agrawal, 2013), and several economically important crops was observed after *P. indica* colonization. Inoculation with the co-culture or a mixture of *P. indica* and *Bacillus pumilus* promoted tomato seedling growth significantly when compared with individual application of the two biological agents (Anith *et al*., 2015).

2.8.4.1 Mechanisms of growth promotion by *P. indica*

P. indica promotes plant growth through several ways. It increases the uptake of nutrients via expression of plant genes like Nitrate reductase, starch degrading enzymes etc. Expression of fungal genes like PiPT helps in phosphate mobilization. Several transcriptional factors are expressed in both plant and fungus called as Homeodomain TF's. *P. Indica* induces increased production of auxin and cytokinin that also leads to plant growth. The *P. indica* colonized *Arabidopsis thaliana* showed enhanced root growth, leading to stunted and highly branched root systems. Sirrenberg *et al.* (2007) identified a diffusible factor which mimicked IAA that resulted in enhanced growth of *A. thaliana.*

2.8.4.1.1 Nitrogen uptake and starch mobilization

The enzyme involved in nitrogen metabolism and starch mobilization are increased in plants colonized with *P. indica* (Sherameti *et al.*, 2005). An elevation in NADH/NADPH dependant nitrate reductase enzyme that converts nitrates into nitrite was observed in plants that resulted in improved growth. The enzyme glucan dikinase functions in facilitation of starch degradation and mobilization. A homeodomain transcription factor was identified which responds to the fungus and binds to the *P. indica*-responsive promoter regions like *Nia2*, *SEX1*, and 2-nitropropane dioxygenase genes. These results suggest that some common

regulatory elements and *trans*-factors controls the expression of *P. indica*-responsive target genes.

2.8.4.1.2 Phosphorus transport

Phosphorous constitutes up to 0.5% of the dry weight of plant cell and is one of the most essential mineral nutrients for plant growth and development. It plays diverse structural, regulatory and energy transfer roles (Bieleski *et.al.,* 1983 and Schachtman *et.al.,* 1998). Phosphorous is present mainly in the form of sparingly soluble complexes in soil and are not directly accessible to plants. Plants that have mycorrhizal associations in their roots acquire phosphates by the absorbance through the hairy hyphae extended beyond root depletion zones of the mycorrhizae. Plants can either uptake directly by its own transporters or indirectly through mycorrhizal associations (Kumar *et al.,* 2011). In several plant and fungal species, high affinity phosphate transporters have been identified and also characterized. The plants are, *Arabidopsis, Medicago truncatula*, *Lycopersicon esculentum*, *Solenum tuberosum* and the fungus are, *Saccharomyces cerevisiae*, and *Neurospora crassa* (Yadav *et al.,* 2010). The *PiPT* protein complex consists of twelve transmembrane helices that are divided into two halves which is connected by a large hydrophilic loop in the middle. *PiPT* was expressed and localized in the external hyphae of *P. indica* colonized maize plant root, which suggests that initial site of phosphate uptake from the soil.was done by external hyphae.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Location

The study entitled "*Piriformospora indica* mediated response in taro (*Colocasia esculenta* (L.) Schott) with special emphasis to growth and leaf blight incidence" was conducted at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during October 2015- June 2016. Details regarding the experimental materials and methodology are elaborated in this chapter.

3.2 Morpho - cultural characteristics of *Piriformospora indica*

To study the cultural characteristics, *P. indica* was cultured on PDA medium and the cultural characters like colony morphology, spore production etc were studied. To study the mycelial characters, 2% plain agar was prepared and 15 ml molten medium was poured into sterilized Petri dishes. Upon cooling, sterilized cellophane membrane was kept over the medium and mycelial disc of *P.indica* was inoculated at the centre of the membrane. The plates were incubated for 5 days at 28°C. The membrane was removed carefully, stained with cotton blue lacto phenol and the mycelial morphology was studied under microscopic field.

3.3 Culture maintenance and standardization of media for the multiplication

Piriformospora indica culture was obtained from Dr. K. N. Anith, Professor, Department of Agricultural Microbiology, College of Agriculture, Vellayani. The culture was originally obtained from Amity Institute of Microbial Sciences, Amity University. The culture was maintained on PDA slants/ plates at room temperature.

P. indica was allowed to grow on different media, Potato Dextrose Broth, Malt Extract Broth and 4% jaggery broth. Mycelial discs of 10 mm were cut from actively growing culture of *P. indica* grown on PDA plates and were inoculated in to Erlenmeyer flasks containing 150 ml of media and was incubated for two

weeks at 28°C with 120 rpm in an orbital shaker. The mycelia were harvested by filtration through cheese cloth and were mixed with sterilized sand @ 1% w/v.

3.4 Colonization study

Mycelial mass mixed with sterilized sand was added to pots in such a way that the pot which containing approximately 2.5 kg of potting mixture should get 1% w/v of *P.indica* inoculum. Taro cormels were surface sterilized using 1% sodium hypochlorite solution for 10 min. After rinsing with sterile distilled water (2 times), the cormels were dipped in 10% jaggery solution. The cormels were then planted in the pots. The pots were kept in a net house conditions and growth was observed daily. After three weeks, the plants were uprooted and the roots were excised and examined for fungal colonization if any under microscopic field.

3.4.1 Trypan blue in lacto phenol staining

Staining was carried out as per the protocol by Philip and Hayman (1970). Roots were collected excluding the axial one, rinsed well in distilled water and boiled with 10% KOH for softening the tissue which was followed by neutralization with 2% HCl. The roots were cut in to 1cm long pieces and stained with 0.5% trypan blue (Lobachemi) in lactophenol (Appendix I) for 10 minutes. The tissues were washed in lactophenol solution for 15 minutes to remove excess stain. Slides were prepared from these samples and mounted in DPX mountant and the colonization was analysed under microscope (Nikon Eclipse E200, Nikon Corporation, Japan).

3.4.2 WGA-AF 488 (Wheat germ agglutinin- Alexa flour 488) staining and confocal imaging

WGA-AF 488 staining was carried out with slight modifications of the procedure of Wright (1984). Both the control and the *P. indica* co-cultivated plant roots were collected and the roots were fixed in trichloroacetic acid (TCA) fixation solution containing 0.15% (w/v) TCA in 4:1 (v/v) ethanol/chloroform (Appendix II) followed by washing 5 min in 1X phosphate buffer saline (PBS, pH

7.4) (Appendix III). The roots were boiled for 1 min with 10% KOH and neutralized in 1X PBS. Thereafter, the root tissues were transferred to staining solution containing 100 μg ml^{-1} WGA-AF 488 (Invitrogen, Oregon, USA) dissolved in 1X PBS (pH 7.4) (Appendix IV). After overnight incubation, the roots were destained by incubating overnight in PBS. The samples were viewed by confocal laser imaging on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany) at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The conjugated WGA-AF 488 was excited at 488nm wavelength and detected at 500 – 600 nm and the images were captured at different depths.

3.4.3 DNA extraction and PCR amplification

3.4.3.1 DNA extraction

Fresh roots were collected from both *P. indica* colonized Muktakeshi and Sree Kiran. The roots were washed thoroughly in sterile water to remove dirt and the total genomic DNA was isolated from roots using a Genomic DNA isolation Kit (Fermentas) following manufacturer's protocol. Briefly, 80 mg of root tissue was ground in liquid nitrogen with mortar and pestle, and the powder was added to a 1.5 ml micro centrifuge tube and re-suspended in 200 μL of TE buffer. From the sample, 200 μL was mixed with 400 μL of lysis solution and incubated at 65°C for 5 min. Then the sample was incubated for 10 minutes at 65°C with occasional inverting of the tube. Immediately,600 μL of chloroform was added and gently emulsified by inversion (3-5 times) and the sample was centrifuged at 10,000 rpm for 2 min. 720 μL of sterile deionized water was added with 80 μL of supplied 10X concentrated precipitation solution to prepare the precipitation solution. The upper aqueous phase that contains DNA was transferred to a new tube and 800 μL of freshly prepared precipitation solution was added and mixed at room temperature for 1-2 min and centrifuged at 10,000 rpm for 2 min. Supernatant was removed completely and was dissolved in 100 μL of NaCl solution by gentle vortexing. After the pellet was completely dissolved, 300 μL of

cold ethanol was added and the DNA was precipitated (10 min at -20°C). The precipitated DNA was centrifuged at 1000 rpm at room temperature. Ethanol was removed and the pellet was washed with 70% cold ethanol and DNA was dissolved in 100 µL of sterile deionized water. The DNA obtained was analysed for its quality and quantity using a 0.8 % agarose gel electrophoresis.

3.4.3.2 Polymerase Chain Reaction

DNA of *P. indica* was amplified using species specific primer as previously described (Satheesan *et al.,* 2012). Primer sequence and reaction mix optimized is listed below.

PiTEF forward primer: TCGTCGCTGTCAACAAGATG

PiTEF reverse primer: GAGGGCTCGAGCATGTTGT

The reaction mix consists of the following:

Sterile water	19 µL
Taq buffer	2.5 µL
dNTP mix	0.5 µL
Taq polymerase	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNA	1.5 µL
Total volume	25 µL

The components were mixed by gentle vortexing (Labnet vortex mixer, USA) and PCR amplification was performed in an Agilent Sure Cycler 8800

(Agilent Technologies, USA). The PCR regime consisted of 2 minute at 94°C, 35 cycles of 30 sec at 94°C, 1 minute at 55°C and 1 minute 30 sec at 72°C, and finally 8 minute at 72°C. Amplified products were resolved on a 1.5% agarose (Himedia) gel containing 0.5 µg ml^{-1}(0.5 µL) ethidium bromide and photograph was scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA). The amplification products were stored at -20 $^{\circ}$C. The amplified products were purified to remove excess primers and nucleotides using a Nucleospin® gel and PCR cleanup kit (Macherey Nagel).

3.4.3.3 DNA elution and sequencing

The DNA bands were observed in a UV-transilluminator and the bands were carefully cut and its weight was measured using a weighing balance (Shimadzu) and transferred into a clean tube. Elution was carried out using NucleoSpin® Gel and PCR Clean-up kit (Macherey - Nagel). For each 100mg gel slice it was recommended to add 200 µL of buffer NT1. Though the weight was 45 mg, 100 µL of buffer NT1 was added and the tube was incubated at 50^{0}C for 5-10 minutes with intermittent gentle vortexing. After the gel was completely dissolved, 700µL of the solution was added to NucleoSpin® Gel and PCR Clean-up Column placed inside the Collection Tube (2 mL). The tube was centrifuged for 30 seconds at 11,000 x g. The flow-through was discarded and the column was placed back into the collection tube and 700µL of wash buffer was added and centrifuged for 30 s at 11,000 x g. After discarding the flow through, the column was placed back to the collection tube and centrifuged again to remove remaining wash buffer. Then the silica membrane was dried for 5-10 minutes. The column was placed inside a new 1.5ml tube. 25 µL elution buffer was carefully added to the membrane and the tube was centrifuged for 1 minute. The flow through was saved as the first elute. Second elute was collected by repeating the same step with 15 µL elution buffer. The eluted DNA was checked on 0.8% agarose gel containing 0.5 µg ml^{-1}ethidium bromide and photograph was scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA) and sequenced with the

same primers as for the PCR amplifications. The sequencing was performed in Applied Biosystems® 3500 Genetic Analyzer, Life Technologies at RGCB.

3.5 Dual culture/direct confrontation study

The ability of *P.indica* to inhibit mycelial growth of *Phytophthora colocasiae* was studied. Five mm mycelial discs were taken from the edges of actively growing cultures of *P. indica* and *P. colocasiae* and placed 3 cm apart in PDA plates and incubated at room temperature for 7 days and were observed for inhibition.

The culture filtrate was also tested for its inhibitory effect on mycelial growth of *P. colocasiae.* Sterilized paper discs (5 mm) were kept at four corners on a PDA plate. By using a micropipette, culture filtrate was added over each disc until the paper discs got saturated. Five mm mycelial disc of *P.*indica was inoculated at the center of the plate. The plates were incubated at room temperature for 7 days and were examined daily for inhibition if any.

3.6 Plant growth promotion study

In order to study the effect of *P. indica* in plant growth, Sree Kiran and Muktakeshi were planted in grow bags containing approximately 20 kg potting mixture. Initially, ¾ th portion of the pots were filled with potting mixture and added two kg *P.*indica inoculum mixed in sterile sand (@1%w/v) to it. The cormels were planted in soil in such a way that the entire cormels were covered with *P.*indica mixed soil. After planting, a layer of potting mixture was again put over the cormels. Treatment details are given below. Each treatment was replicated in 10 grow bags.

Design - Factorial CRD

The experiment was conducted in an area, where taro leaf blight incidence occurs every year. The plants were allowed to get infected naturally.

Sree Kiran, released by ICAR- CTCRI and susceptible to taro leaf blight

T1- Plants with *P. indica*

T2- Plants with *P. colocasiae*

T3- Plants with *P. indica* + *P. colocasiae*

T4- Control

Muktakeshi, released by RC of ICAR - CTCRI and tolerant to taro leaf blight

T5- Plants with *P. indica*

T6- Plants with *P. colocasiae*

T7- Plants with *P. indica* + *P. colocasiae*

T8- Control

Various growth parameters of taro plants were measured

a. Number of days taken for sprouting
b. Sprouting (%)
c. Height of the plant (cm)
d. Number of leaves
e. Leaf length (cm)
f. Leaf breadth (cm)

Observations c, d, e and f were taken at monthly intervals.

Parameters measured during harvest were as follows

a. Root length (cm)
b. Root weight (g)
c. Total weight (g)
d. Shoot weight (g)
e. Weight of cormels (g)
f. Weight of mother corm (g)
g. Number of cormels

Disease incidence in taro was recorded three times (month of July) at an interval of one week using 1-6 scale.

1. No symptom
2. < 10%

3. 11-25 %

4. 26-50%

5. 51-75%

6. > 75%

PDI was calculated using the formula

$$PDI = \frac{\text{Total score}}{\text{(Number of leaves assessed x Maximum score)}} \times 100$$

Statistical analysis of the growth parameters and disease incidence were done by using the SAS system.

3.7 Sporangia production of *Phytophthora colocasiae* and challenge inoculation

3.7.1 Leaf disc method for sporangia production

Leaf disc method for sporangia production was carried out as per the protocol by Nath *et al.* (2016). Five leaf discs (5 × 5 cm) of taro variety, Sree Kiran were washed and floated in sterile distilled water in 200-mm Petri Dishes and mycelial disc of 5mm was excised from the margins of actively growing cultures of *P. colocasiae* and was used for inoculation. Plates were then covered with lids containing moistened filter paper to maintain high humidity. Then it was incubated for 4 days in dark at 25^{o}C. The leaves were observed for sporangial production under microscope. The infected region was excised and again submerged in sterile water. The whole unit was incubated at 4^{o}C for 30 minutes and was transferred to room temperature. A drop of the suspension was observed under microscope for zoospore release. This zoospore suspension was used to artificially inoculate the taro plants.

3.7.1.1 Challenge inoculation of *P. colocasiae*

Ten plants were kept as replicates for each treatment. T2, T3, T6, T7 plants were challenge inoculated spore suspension of *P. colocasiae.* The fully opened top most leaf was selected for challenge inoculation for challenge inoculation, a small piece of cotton was kept over the leaf (2 months old) and

50µL of the zoospore suspension was added onto the cotton and was sealed with cellophane tape. The plants were transferred to a humid chamber and sterile water was sprayed daily to maintain the humidity. The leaf samples were taken at different time intervals, 0, 3, 6, 12, 24, 48 and 96 hrs after inoculation. Separate plants were used to take leaves at different time intervals.

3.8 Defense enzyme quantification and differential gene expression

Sree Kiran and Muktakeshi were planted in sterilized potting mixture in pots (2.5 kg). The treatment details are described below

Sree Kiran (Susceptible)

T1- Plants with *P. indica*

T2- Plants with *P. colocasiae*

T3- Plants with *P. indica* + *P. colocasiae*

T4- Control

Muktakeshi (Tolerant)

T5- Plants with *P. indica*

T6- Plants with *P. colocasiae*

T7- Plants with *P. indica* + *P. colocasiae*

T8- Control

P.indica inoculum was applied at the rate of 1% w/v. After two months of planting, the T2, T3, T6 and T7 plants were challenge inoculated Leaf samples were collected at different intervals, 0, 3, 6, 12, 24, 48 and 96th hours post inoculation (hpi).

3.9 Enzyme assay

3.9.1 Chitinase assay

1g of leaf was homogenized in 5 ml of 0.05 M sodium acetate buffer of pH 5.0 (Appendix V) at 4°C. The extract was filtered using a cheese cloth and was

centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was used as enzyme source.

3.9.1.1 Preparation of colloidal chitin

Colloidal chitin was prepared as per the protocol by Berger and Reynold (1958). 5g chitin (Sigma Aldrich) was mixed with 30 ml concentrated HCl and was incubated overnight at 4°C. 250 ml 50% chilled ethanol was prepared and the overnight incubated mix was added slowly to the chilled ethanol by constant stirring. The mixture was centrifuged at 10,000 rpm at room temperature and the pellet was saved. The pellet was washed thoroughly using sterile distilled water three times and centrifuged. The resulting pellet was weighed and was dissolved in sterile distilled water to make a concentration of 7 mg/ ml.

The reaction mixture contained colloidal chitin solution (7mg/ ml), 1.0 ml of sodium acetate buffer of pH 5.2 and 1 ml of diluted sample. The reaction mixyure was incubated at 50°C for 1 hr, the released reducing sugar was measured as N-acetylglucosamine (NAG) equivalents by the Somogyi- Nelson method (1952). One unit of chitinase activity was defined as the amount of enzyme that produces 1 mg of NAG per hour under given assay conditions.

3.9.1.2 Somogyi method

Solution A: 25g anhydrous sodium carbonate, 25g sodium potassium tartrate and 200g sodium sulphate were dissolved in 800 ml distilled water and made up to 1000ml.

Solution B: Dissolved 30g copper sulphate pentahydrate in 200 ml distilled water containing 4 drops of concentrated sulphuric acid.

Solution C: Dissolved 50g ammonium molybdate in 900ml distilled water and added 42ml of concentrated distilled water. 6g sodium arsenate heptahydrate was separately dissolved in 50 ml water and the solution was added to the solution of ammonium molybdate. The volume was adjusted to 1 liter. The solution was boiled to 55°C for complete dissolution.

Solution D: 1ml of solution B was added to 25ml solution A.

Solution E: Solution C was diluted 5 fold (50ml made upto 250ml) with distilled water.

0.2ml aqueous sample containing up to 50mg of reducing sugar equivalents were taken in test tubes. 0.5ml solution D was added to it and mixed well in a vortex mixer. Then the tubes were kept in boiling water for 20 minutes and were cooled to room temperature. The tubes were stirred for about 10 seconds until the CO_2 was completely released. 3ml of solution E was added to each tube and vortexed for 10 seconds. The solution was allowed to stand for 10 minutes and was stirred again. The absorbance was measured at 520nm using distilled water as blank. The enzyme activity was defined as the mg sugar released/ g fresh tissue/ hr. The calculations are given below.

Test = 1ml colloidal chitin (7mg/ml) +1ml buffer + 1ml enzyme extract

Total =3ml

Incubation time = 1 hour

Constant factor = 345µg

Sample taken for Somogyi method = 0.2ml

Amount of reducing sugar in 0.2ml sample = OD of test x 345µg

Amount released by 3ml assay system = OD of test x 345 x 3/0.2

= OD of test x 345 x 15µg

Amount released by 1000µL enzyme extract = OD of test x 345 x 15µg

Amount released by 5ml i.e., 1g tissue = OD of test x 345 x 15µg x 5/1

Sugars/ g tissue/h =OD of test x 345 x 45/1000mg

$= \underline{\text{OD of test x 15.525 mg}}$

3.9.2 β- 1, 3 glucanase assay (Koga *et al.*, 1988)

1g of leaf was homogenized in 5 ml of 0.05 M sodium acetate buffer of pH 5.0 at 4°C. Then the extract was filtered using a cheese cloth and was centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was taken as the enzyme source. The assay mixture contained 900µL of diluted sample and 100µL of laminarin (Sigma Aldrich) solution (100mg/ml) in 1 ml sodium acetate buffer of pH 5.2. The mixture was incubated at 40°C for 30 minutes and the released reducing sugar was measured as glucose equivalents by Somogyi method. One unit of β- 1, 3 glucanase activity can be defined as under given assay conditions the amount of enzyme that produces 1mg of NAG per hour.

The calculations are given below.

Test = 1ml buffer+100µLlaminarin solution (10mg/ml) + 900µL enzyme extract

Total = 2ml

Incubation time = 30 minutes

Constant factor = 345µg

Sample taken for Somogyi method = 0.2ml

Amount of reducing sugar in 0.2ml sample = OD of test x 345µg

Amount released by 2ml assay system = OD of test x 345 x 2/0.2

= OD of test x 345 x 10µg

Amount released by 900µL enzyme extract = OD of test x 345 x 10µg

Amount released by 5ml i.e., 1g tissue = OD of test x 345 x 10µg x 5/0.9

Sugars/ g tissue/h = OD of test x 345 x 100 x 5x 60/9 x 30µg

= OD of test x 345/9 mg

= OD of test x 38.33 mg

3.9.3 Determination of Phenyl alanine ammonia lyase (PAL) activity (Sadasivam and Manickam, 1997)

1g sample was homogenized with 5ml of 0.1M sodium borate buffer (pH 7) (Appendix VI) containing 0.1g polyvinyl pyrrolidone. Then the homogenate was centrifuged at 20,000g for 15 minutes at 4˚C. The supernatant was used as enzyme source for the assay. PAL activity can be defined as the rate of conversion of L- phenyl alanine to transcinnamic acid at 290nm. 0.4ml of enzyme extract was taken in a test tube and was incubated with 0.5ml of 0.1M borate buffer of pH 8.8 and 0.5ml of 12mM Phenyl alanine for 30 minutes at 30˚C. The amount of transcinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1} Cm^{-1}

9.63/ millimole = $9.63 \times 10^{-3} \times 10^{-3}$ per nanomole

Enzyme activity = nanomole/minute/gram tissue

3.63×10^{-6} nm is equivalent to 1unit

Total volume of the assay system =2.8ml

The enzyme activity per 30 minute =OD x 2.8 x 5/ ($9.63 \times 10^{-6} \times 0.4 \times 1g$)

Enzyme activity per minute = OD x 2.8 x 5 x 10^6 x 10 / (9.63 x 4 x 30)

= 121149.2 x OD nM/min/g tissue

3.9.4 Peroxidase assay (Haard and Marshal, 1976)

Two types of assay systems were used, test and control. The test system consisted of 0.5ml 20mM guaiacol (the substrate), 0.1ml of 12.3mM hydrogen peroxide (the hydrogen donor), 2.9ml of citrate phosphate buffer (0.1M, pH 7) (Appendix VII) and 0.5ml of enzyme extract.

In the control system, 3.5ml of citrate phosphate buffer and 0.5ml enzyme extract alone was taken. The test and control system were incubated at room

temperature for 20 minutes and 2 minutes in boiling water. The absorbance was measured at 436nm.

The peroxidase activity was expressed as one unit of enzyme activity which represents OD/hr/g tissue. The specific activity of enzyme was expressed as enzyme activity units per mg protein.

Calculation:

Test reading = (T-C) = R

0.5 ml gives an OD change of R in 20 minute

OD change per hour = R x 60/20

=R x 3 by 0.5ml enzyme

0.5g tissue/2.5ml buffer

Activity in 2.5ml buffer = R x 3 x 2.5/0.5 ml

$$= \underline{R \times 15 \text{ units}}$$

Activity/g wet tissue = R x 15 x 1.0/0.5g

$$= \underline{R \times 30 \text{ units}}$$

3.9.5 Estimation of total phenol

Reagents used: 80% methanol, FCR, saturated solution of sodium carbonate. 0.5-1g of the sample was homogenized with 10ml of 80% methanol and was mixed thoroughly by agitation for 15 minutes at 70˚C. One ml of this extract was added to 5ml distilled water. 250µL of folin ciocalteau reagent was added to the solution and the solution was incubated at 25 ˚C. After a minute, 1ml of saturated solution of sodium carbonate and 1ml distilled water were added and the reaction mixture was incubated at 25 ˚C for 1 hour. The absorbance of the developed blue colour was measured using a spectrophotometer at 725nm. A

standard curve was prepared using different concentrations of catechol viz. 0.25, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5 µg/ml.

3.10 Monitoring for genes upregulated/ downregulated

3.10.1 Validating differential expression of chitinase and glucanase

Chitinase and glucanase genes were selected for monitoring the upregulation and down regulation. Reported degenerate primers were identified for both chitinase and glucanase genes. Primers were also designed based on the sequence information of various plant species available in the NCBI database. It was worth mentioning that no sequence information was available for the taro chitinase and glucanase genes in the database. The primer information is given below.

About 100-150 sequences of chitinase and glucanase genes from different plant species were retrieved from the NCBI database. Majority of the sequences were from *Solanum tuberosum* (U02607.1), *S. lycopersicum* (FJ849060.1), *Capsicum annum* (DQ073918.1), *Nicotiana tabaccum* (X16938.1), *Cucumis melo*, *Allium sativum* (M94105.1), *Musa acuminata* (AY532919.1), *Citrus* (XM_006492366.2), *Nephenthes* (JN867633). The sequences were aligned using Clustal W program (Higgings and Sharp, 1988). Regions showing similarity were identified and primer pairs were designed based on these conserved regions for both chitinase and glucanase.

The generated primers are as follows

1Chi F1	TGTCTCGGCAGAACAATGTG
1Chi R1	CCATCCTCCAGTAGTTTC
1ChiR2	CAGTAGTTTCATGGGAGGTT
1ChiR3	TGGGGCCTCGTCCGAAATAT
2ChiF2	GGGCCATGTGGAAGAGCC
2ChiR4	CCAGGGAGGCGATTGGCT
2ChiR5	GTCACCAGGACTAACTCCAA

Two primers specific for *Arabidopsis* chitinase gene were also used
AtF1-GGATCTATATATATCTTTCATGCC
AtR1-CTCTCGTACTAAATAGCAGCCT
AtF2-CAACATCAAAATGTTGACTCCC
AtR2-GATGTTTTTGTTAGCAAGTGAGG

Glucanase specific primers:

Glu 1 F	ACAGGCACATCTTCACTTACC
Glu 1 R	CGAGCAAAGGCGAATTTATCC
Glu 2 F	TACATTGCTGTAGGAAATGA
Glu 2 R	GTAAACAGCATCCAAGATGGCG
Glu 3 F	AATAGGTGTTTGCTATGGAAT
Glu 3 R	GTAAACAGCATCCAAGATGGCG
Glu 4 F	ACATTGCTGTAGGAAATGAAG
Glu 4 R	GTAAACAGCATCCAAGATGGCG
Glu 5 F	GTTCCCGCCATGAGAAACAT
Glu 5 R	GTAAACAGCATCCAAGATGGC
Glu 6 F	GTTCCCGCCATGAGAAACAT
Glu 6 R	ATCCAAGATGGCGTCAAAAAG

DNA was isolated from both Sree Kiran and Muktakeshi leaves using CTAB method. 100mg leaves were homogenized in liquid nitrogen and pre-warmed 1ml extraction buffer (Appendix VIII) was added and ground again. The sample was transferred to 2ml Eppendorf tubes and 5µL proteinase K was added and the whole content was mixed in vortex mixer. The tube was incubated at 37ºC for 30 minutes followed by incubation at 65ºC for 30 minute. The content of the tubes were centrifuged at 8000rpm for 10 minutes at room temperature. The supernatant was transferred to fresh Eppendorf tubes. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to the tubes and mixed by gentle inversion. The tubes were centrifuged at 8000rpm for 10 minutes at room temperature. After transferring the supernatant to new tube, 200µL of 2M NaCl

with 4% PEG was added and the tubes were incubated at 4ºC for 15 minutes. Following incubation, the tubes were centrifuged at 8000rpm for 10 minutes at room temperature. The supernatant was transferred to new tube and 2/3 volume of isopropanol was added to the tubes and the tubes were centrifuged at 8000rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed with 500µL 75% ethanol and the tubes were centrifuged at 8000rpm for 10 minutes. The pellet was air dried and was dissolved in 50µL TE buffer. The DNA was treated with 3µL of RNase (10mg/ml) and incubated at 37ºC for 30 minutes. Then the samples were stored at -20ºC.

PCR was carried out using the glucanase and chitinase primers mentioned above. The PCR was done using an Emerald master mix (Takara). The amplified products were visualized using 2% agarose gel. The bands were cut and DNA was eluted and was sequenced at RGCB.

3.10.2 Suppression Subtractive Hybridization

SSH was carried out to identify the differentially expressed genes in *P. colocasiae* challenge inoculated plants.SSH was carried out using Clontech PCR-Select cDNA Subtraction Kit (Clontech, USA) as per the manufacturer's protocol. Subtractive hybridization is a powerful technique used to compare two populations of mRNA for obtaining clones of genes that are expressed in one population but not in the other. First, both the mRNA populations are reverse transcribed into cDNA: the cDNA that contains specific (differentially expressed) transcripts as **tester** (plant with colonized *P. indica* and infected with *P. colocasiae*), and the reference cDNA as **driver** (plants with *P. colocasiae* infection). Tester and driver cDNAs will be hybridized, and then the hybridized sequences are removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA.

3.10.2.1 RNA isolation and cDNA synthesis

RNA was isolated from the samples using Ambion PureLink® RNA Mini Kit. cDNA synthesis was carried out using the reagents provided in the kit.

The driver, tester, and the Control Poly A+ RNA (from human skeletal muscle), the following components were mixed in a sterile 0.5 ml microcentrifuge tube.

Per rxn :	
Poly A+ RNA (2µg)	2–3 µL
cDNA Synthesis Primer (10 µM)	1 µL 1 µL

For the control synthesis, 2 µL of the skeletal muscle control poly A+ RNA was added. Then it was made up to a final volume of 4 µL using sterile water. The contents were mixed and flashed briefly in a microcentrifuge. The mix was incubated at 70°C for 2 min in a thermal cycler and suddenly cooled on ice for 2 minutes.

The following reagents were added to each reaction:

Per rxn :	
5X First-Strand Buffer	2 µL
dNTP Mix (10 mM each)	1 µL
Sterile H_2O	1 µL
DTT (20 mM)	1 µL
SMARTScribeReverse Transcriptase (100 units/µL)	1 µL 1 µL

The tubes were vortexed, centrifuged and incubated for 1.5 hr at 42°C in a thermal cycler. The first strand synthesis was terminated by placing the tubes on ice. After the synthesis of first strand cDNA, second strand cDNA synthesis was carried out immediately.

3.10.2.2 Second strand cDNA synthesis

Following procedure was carried out with each first-strand tester, driver, and the control skeletal muscle cDNA.

The following components were added to the first-strand synthesis reaction tubes (containing 10 µL):

per rxn	
Sterile H_2O	48.4 µL
5X Second-Strand Buffer	16.0 µL
dNTP Mix (10 mM)	1.6 µL
20X Second-Strand Enzyme Cocktail	4.0 µL

The contents were mixed and centrifuged. The final volume was 80 µL. The tubes were incubated for 2 hr at 16°C in a thermal cycler. 2 µL of T4 DNA Polymerase was added and gently vortexed. Then the tubes were incubated at 16°C for 30 min in a thermal cycler. Second-strand synthesis was terminated by adding 4 µL of 20X EDTA/glycogen Mix. The ds cDNA was purified and precipitated using the phenol: chloroform: isoamyl alcohol method. The precipitated DNA was dissolved in 50 µL of sterile H_2O and stored at -20 ^{o}C.

3.10.2.3 Rsa I digestion and adaptor ligation

The following procedure was carried out with each experimental ds tester and driver cDNA, and with control skeletal muscle cDNA. Shorter, blunt-ended ds cDNA fragments are generated in this step which are optimal for subtraction and required for adaptor ligation.

The following reagents were added to each reaction:

per rxn	
ds cDNA	43.5 µL
10X Rsa I Restriction Buffer	5.0 µL
Rsa I (10 units/µL)	1.5 µL

The contents were mixed gently and centrifuged. The tubes were incubated at 37°C for 1.5 hr. 2.5 µL of 20X EDTA/Glycogen Mix was added to terminate the reaction. Digest was purified and precipitated using the phenol: chloroform: isoamyl alcohol method. The precipitated DNA was dissolved in 5.5 µL of sterile H_2O and stored at -20 ^oC.

In order to identify the differentially expressed genes in *P. indica* colonized plants, only the forward subtraction was carried out. Experimental tester cDNA 1 and tester cDNA 2 were prepared by diluting 1 µL of each Rsa I-digested experimental cDNA with 5 µL of sterile H_2O.

Control skeletal muscle tester cDNA was prepared by diluting the φX174/Hae III Control DNA with sterile H_2O to a final concentration of 150 ng/ml. 1 µL of control skeletal muscle cDNA was mixed with 5 µL of the diluted φX174/Hae III Control DNA (150 ng/ml). The ligation master mix was prepared by mixing the reagents as per the first table.

For each experimental tester cDNA and for the control skeletal muscle tester cDNA, the reagents in Table 2 was combined in the order shown.

Per rxn		
Sterile H_2O	3 µL	
5X Ligation Buffer	2 µL	
T4 DNA Ligase	1 µL	
Component	**Tester 1-1***	**Tester 1-2***
Diluted tester cDNA	2 µL	2 µL
Adaptor 1 (10 µM)	2 µL	—
Adaptor 2R (10 µM)	—	2 µL
Master Mix	6 µL	6 µL
Final volume	10 µL	10 µL

In a fresh microcentrifuge tube, 2 µL of Tester 1-1 and 2 µL of Tester 1-2 was mixed to produce unsubtracted tester control. The tubes were centrifuged briefly, and were incubated at 16°C overnight. 1 µL of EDTA/glycogen was

added to stop ligation reaction. The ligase enzyme was inactivated by heating at 72°C for 5 min. the samples were stored at -20^{o}C.

3.10.2.4 First and second hybridization

For each of the experimental and skeletal muscle subtractions, the reagents in the table were mixed in 0.5 ml tubes in the order shown.

Component	**Tester 1-1***	**Tester 1-2***
Rsa I-digested DrivercDNA	1.5 µL	1.5 µL
Adaptor 1-ligated Tester 1-1*	1.5 µL	—
Adaptor 2R-ligated Tester 1-2	—	1.5 µL
4X Hybridization Buffer	1.0 µL	1.0 µL
Final volume	4.0 µL	4.0 µL

Samples were incubated at 98°C for 1.5 min in a thermal cycler followed by 68°C for 8 hr. For second hybridization, the following reagents were added into a sterile tube:

per rxn	
Driver cDNA	1 µL
4X Hybridization Buffer	1 µL
Sterile H2O	2 µL

Samples were incubated at 98°C for 1.5 min in a thermal cycler.

The tube of freshly denatured driver was removed from the thermal cycler. The following procedure was used to simultaneously mix the driver with hybridization samples 1 and 2 to ensure that the two hybridization samples mix together only in the presence of freshly denatured driver. The micropipettor was set at 15 µL. The pipette tip was used to touch the sample containing hybridization sample 2. The entire sample was drawn partially into the pipette tip. The pipette tip was removed from the tube, and a small amount of air was drawn into the tip, creating a slight air space below the droplet of sample. Same procedure was repeated with the tube containing the freshly denatured driver. The pipette tip

should contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket. The entire mixture was transferred to the tube containing hybridization sample 1 and was mixed thoroughly and centrifuged briefly. The reaction was incubated at 68°C overnight. 200 µL of dilution buffer was added and mixed by pipetting. The tubes were heated at 68°C for 7 min in a thermal cycler and stored at –20°C.

3.10.2.5 Primary and secondary PCR amplification

1 µL of each diluted cDNA was transferred (i.e., each subtracted sample and the corresponding diluted unsubtracted tester control) into an appropriately labeled tube. 1 µL of the PCR control subtracted cDNA was added into an appropriately labeled tube. PCR master mix was prepared for the 5 reactions as follows.

Reagent	**Per Rxn**	**5 Rxn Mix***
Sterile H_2O	19.5 µL	117 µL
10X PCR reaction buffer	2.5 µL	15 µL
dNTP Mix (10 mM)	0.5 µL	3.0 µL
PCR Primer 1 (10 µM)	1.0 µL	6.0 µL
50X Advantage cDNA Polymerase Mix	0.5 µL	3.0 µL

The reagents were mixed and 24 µL was aliquot into each tube containing the diluted cDNA. The reaction mix was incubated at 75°C for 5 min in a thermal cycler to extend the adaptors. And thermal cycling was commenced immediately. 94°C for 25 sec, 27 cycles: 94°C for 10 sec, 94°C for 25 sec, 27 cycles: 94°C for 10 sec, 66°C for 30 sec , 72°C for 1.5 min.

8µL from the reaction was used to analyze on agarose gel. 3µL was taken and was diluted with 27µL sterile water and from this; one µL was transferred to appropriately labelled tubes. The secondary PCR master mix was prepared as follows and 24 µL of master mix was added to the tubes.

Sterile H_2O	18.5 µL
10X PCR reaction buffer	2.5 µL
Nested PCR primer 1 (10 µM)	1.0 µL
Nested PCR primer 2R (10 µM)	1.0 µL
dNTP Mix (10 mM)	0.5 µL
50X Advantage cDNA Polymerase Mix	0.5 µL
Total volume	24.0 µL

Thermal cycling was commenced immediately as per the following cycle parameters.

Cold Lid	**Hot Lid**
10–12 cycles: • 94°C for 30 sec • 68°C for 30 sec • 72°C for 1.5 min	10–12 cycles: • 94°C for 10 sec • 68°C for 30 sec 72°C for 1.5 min

8µL from the above reactions was checked on 1% agarose gel and the rest was stored at -20^{o} C. The secondary PCR product was purified using the Genejet PCR purification Kit (Thermoscientific) and the purified product was used to clone onto *E. coli* DH5α strain. Cloning was carried out using Instaclone PCR cloning Kit (Thermofisher).

3.10.2.6 Cloning and colony PCR

Cloning of the subtracted tester was carried out using Instaclone PCR cloning Kit (Thermofisher) in *E. coli* DH5α strain as per the manufacturer's protocol. The transformed cells were plated on LA+Amp+X gal and were incubated overnight at 37^{o}C. The transformed colonies (white colonies) were picked using sterile tooth pick and was streaked on LA+Amp+X gal plate after drawing the grid to make the master plate. The colony PCR was carried out by

using M13 specific primers. Based on the colony PCR result, the colonies were selected and plasmid isolation was carried out using Genejet plasmid miniprep kit (Fermentas) using the manufacturers protocol and the plasmid was outsourced for sequencing.

3.10.2.7 Sequence annotation

The sequence result was subjected to similarity search and the annotation was carried out using the Blast2Go software. Blast2GO is a bioinformatics software tool for the automatic, high-throughput functional annotation of novel sequence data (genes and proteins). It makes use of the BLAST algorithm to identify similar sequences to then transfers existing functional annotation from yet characterized sequences to the novel one. The functional information is represented via the Gene Ontology (GO), a controlled vocabulary of functional attributes. The Gene Ontology, or GO, is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species. Sequence blast was carried out using the blast tab. the mapping and annotation was done with the help of the GO Graph and statistics section. The combined graph of process function and cell component was drawn under Go graph option

RESULTS

4. RESULTS

The results of the study entitled "*Piriformospora indica* mediated response in taro [*Colocasia esculenta* (L.) Schott] with special emphasis to growth and leaf blight incidence" carried out at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016 are presented in this chapter.

4.1 Morpho- cultural characteristics of *Piriformospora indica*

Piriformospora indica was cultured on Potato Dextrose Agar (Plate 1). Most of the mycelium grew under the media and the mycelium grown over the agar was white in colour which turned to cream yellow in colour upon ageing. Spore formation/sporulation was observed as white powder over the mycelial mat. The colony had wet appearance and took 7 days to grow completely as a dense mat and cover 90 mm Petri dish.

P. indica was cultured on cellophane membrane kept over water agar and the hyphal morphology was studied by staining the mycelium with cotton blue lactophenol (Plate 2). The mycelium was branched and sporulating structures observed on hyphal tip. Hyphae were dimitic in nature (thick and thin mycelium were observed). The hyphal cells were thin walled, hyaline and septate. Mycelium grew well under solid and liquid culture (broth) conditions. In liquid media with continuous shaking in an orbital shaker, the mycelial aggregates resembling globose balls were formed (Plate 3). No basidial formation was noted in culture.

4.2 Standardization of the media for mass production of *P. indica*

Potato Dextrose Broth (PDB), Malt Extract Broth (MEB) and 4% jaggery broth were evaluated for mycelial mass production (Plate 4). After 10 days of incubation, the mycelial aggregates were harvested and weighed. Maximum mycelial mass weight was obtained with 4% Jaggery broth followed by MEB (Table 1). Based on the result, 4% jaggery broth was selected to mass multiply *P. indica* for further studies.

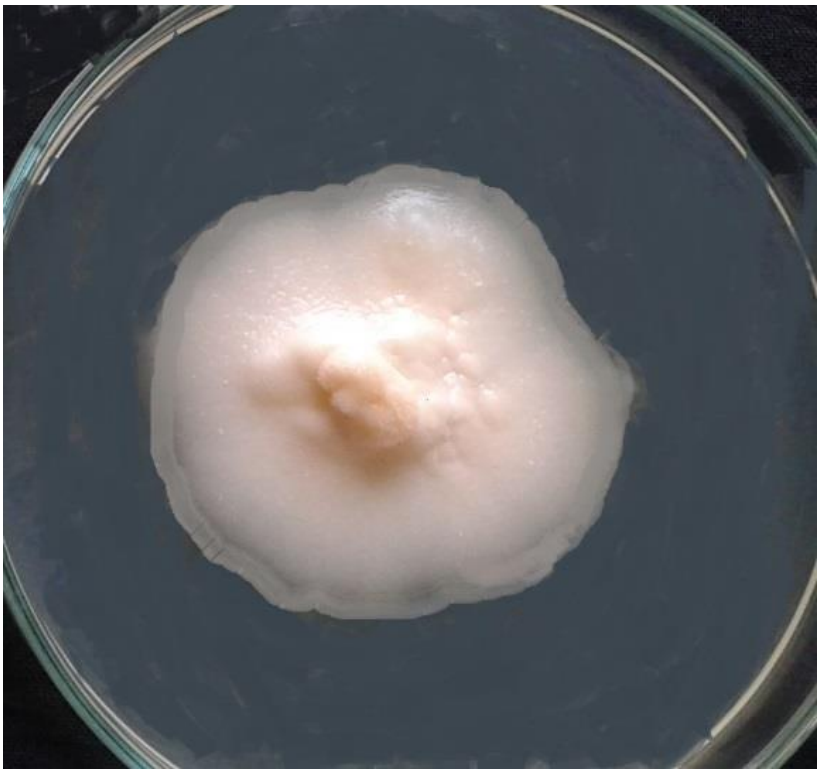

Plate 1. *P. indica* cultured on PDA

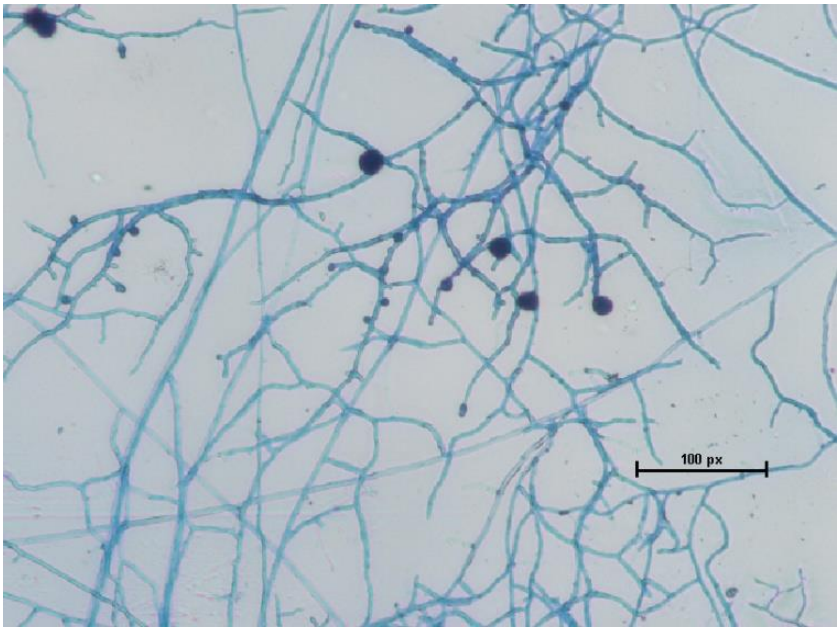

Plate 2. *Piriformospora indica* grown on cellophane membrane, stained with lactophenol cotton blue

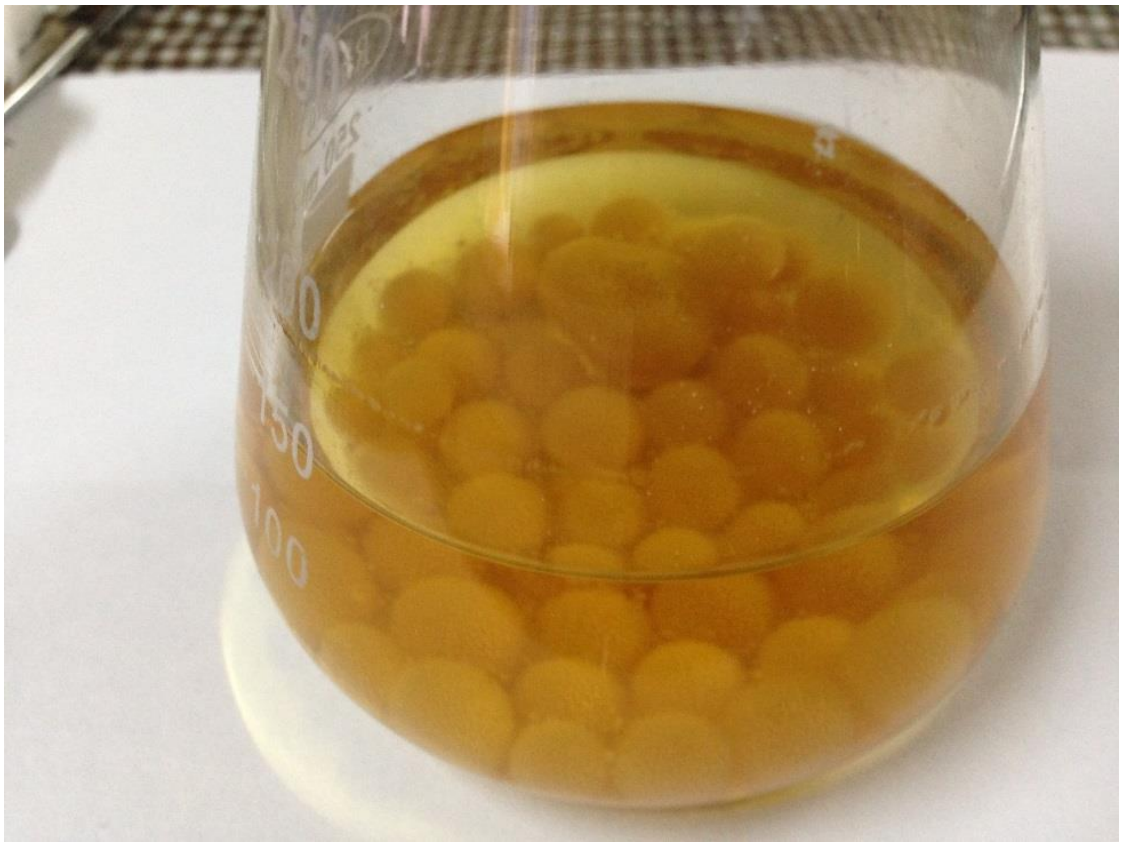

Plate 3. *P. indica* mycelial aggregates in PDB

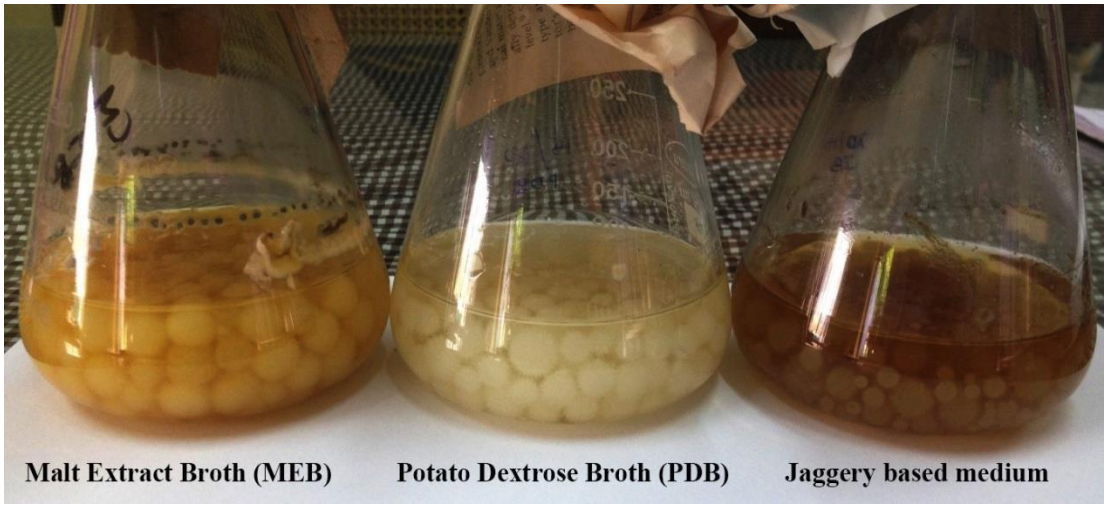

Plate 4. *P. indica* cultured on different broths

Table.1 Effect of media on mycelial growth of *P.indica*

Media	Yield (g)/ 100 ml media *
Potato Dextrose Broth (PDB)	22
Malt Extract Broth (MEB)	24
4% Jaggery broth	28

* mean of 10 flasks

4.3 Colonization study

4.3.1 Trypan blue in lacto phenol staining

The colonization study was carried out after 2 months of planting Sree Kiran and Muktakeshi in *P. indica* incorporated soil. Two types of staining procedure were adopted and confirmed the colonization of *P. indica* in both the varieties. Staining with a general dye Trypan Blue indicated the presence of *P. indica* inside the root cells of Sree Kiran and Muktakeshi. Spores of *P. indica* were observed inside the cortical cells of both the varieties (Plate 5). Dark blue coloured chlamydospores were seen inside root cells. In some cells, light coloured spores were also observed.

4.3.2 WGA-AF 488 (Wheat germ agglutinin- Alexa flour 488) staining and confocal imaging

Chitin specific WGA-AF 488 provided evidence that the fungus colonizes the taro root and produce hyphae intra and intercellularly in both Sree Kiran and Muktakeshi (Plate 6). It was noted that, *P. indica* could colonize the cortical and epidermal cells of the root. Lesser colonization was observed towards the centre of the root.

4.3.3 DNA isolation and PCR amplification

DNA was isolated from the *P. indica* colonized Sree Kiran and Muktakeshi and PCR was carried out using the *P. indica* specific TEF gene primers. PCR analysis

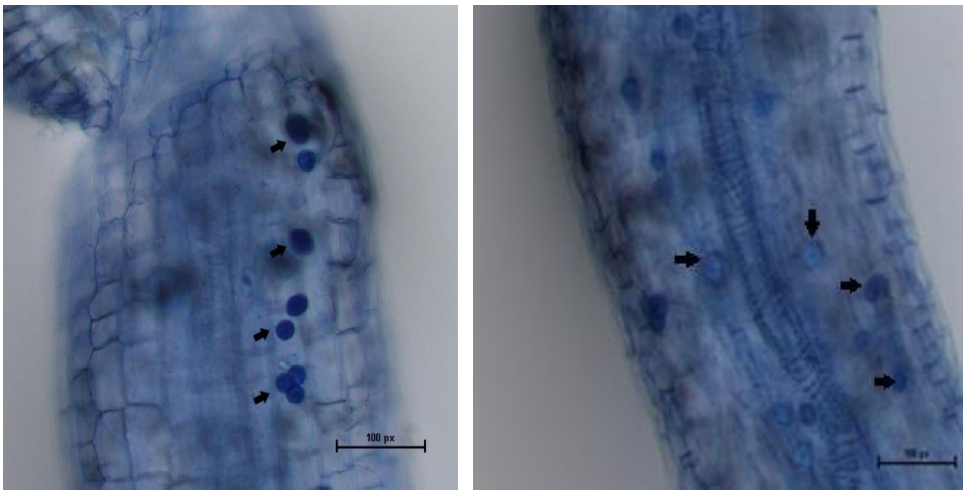

Plate 5. Trypan blue stained *P. indica* in the roots of Sree Kiran and Muktakeshi

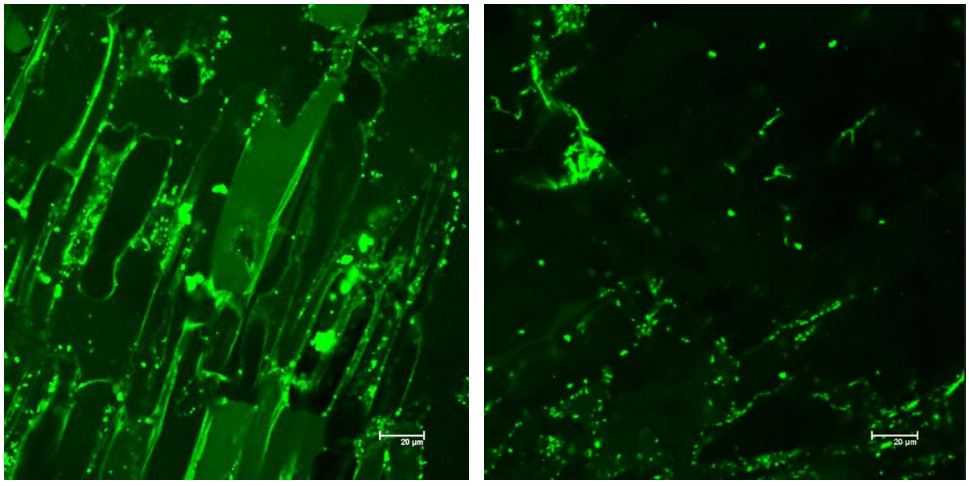

Plate 6. Confocal laser image of WGA-AF 488 stained *P. indica* in Sree Kiran and Muktakeshi roots

using *P. indica* specific gene PiTEF1 produced an amplicon of about 220bp from the genomic DNA isolated from the colonized roots of both Sree Kiran and Muktakeshi (Plate 7). These results confirmed the effective root colonization of *P. indica* in taro roots.

4.3.3.1 DNA elution and sequencing

The DNA band obtained after PCR amplification was eluted using NucleoSpin® Gel and PCR Clean-up kit (Macherey- Nagel) and was checked in 0.8% Agarose gel and the eluted product was outsourced for sequencing. The sequenced result was obtained as electropherogram resulting from capillary sequencing in .abi format. The sequencing of the amplified products confirmed the presence of *P. indica*. The sequence obtained were subjected to BLAST search in the NCBI database and results showed 100% similarity to *P. indica* sequences available in the database (Figure 1).

4.4 Dual culture study

Dual culture study was carried out using *P. indica* and *P. colocasiae.* The mycelia of *P. indica* could inhibit the mycelial growth of *P. colocasiae* and the pathogen could not over grow *P. indica* (Plate 8). However, the culture filtrate did not show any direct effect on mycelial growth of *P. colocasiae* (Plate 9).

4.5 Plant growth promotion study

This study was conducted in open condition and sufficient distance was maintained among the plants of different treatments. However, during heavy rainfall, sporangia and zoospores of *P.colocasiae* spread by rain splash and windblown rain and all the plants got infected. Therefore the treatments, T1 (Sree Kiran with *P.indica*) and T3 (Sree Kiran with *P.indica* + *P.colocasiae*) were combined and taken as **T3**. Treatments, T2 (Sree Kiran with *P.colocasiae*) and T4 (Sree Kiran -control) were combined and taken as **T2**. Similarly T5 (Muktakesi with *P.indica*) and T7 (Muktakesi with *P.indica* + *P.colocasiae*) were combined and taken as **T7**. Treatments, T6 (Muktakesi with *P.colocasiae*) and T8

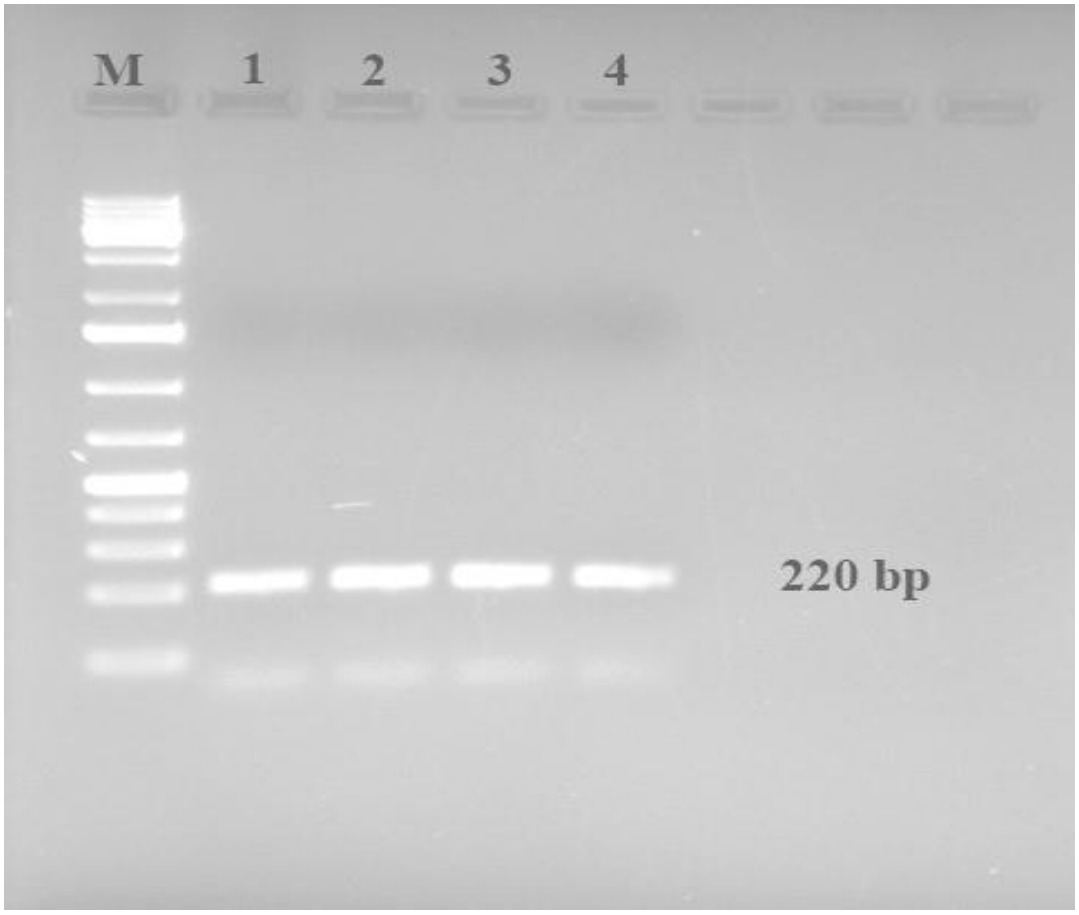

Plate 7. Confirmation of *P. indica* colonization by PCR using PiTEF1 primers. M: 1Kb plus marker, Lane 1 & 2: Amplification from Sree Kiran roots, Lane 3 & 4: Amplification from Muktakeshi roots.

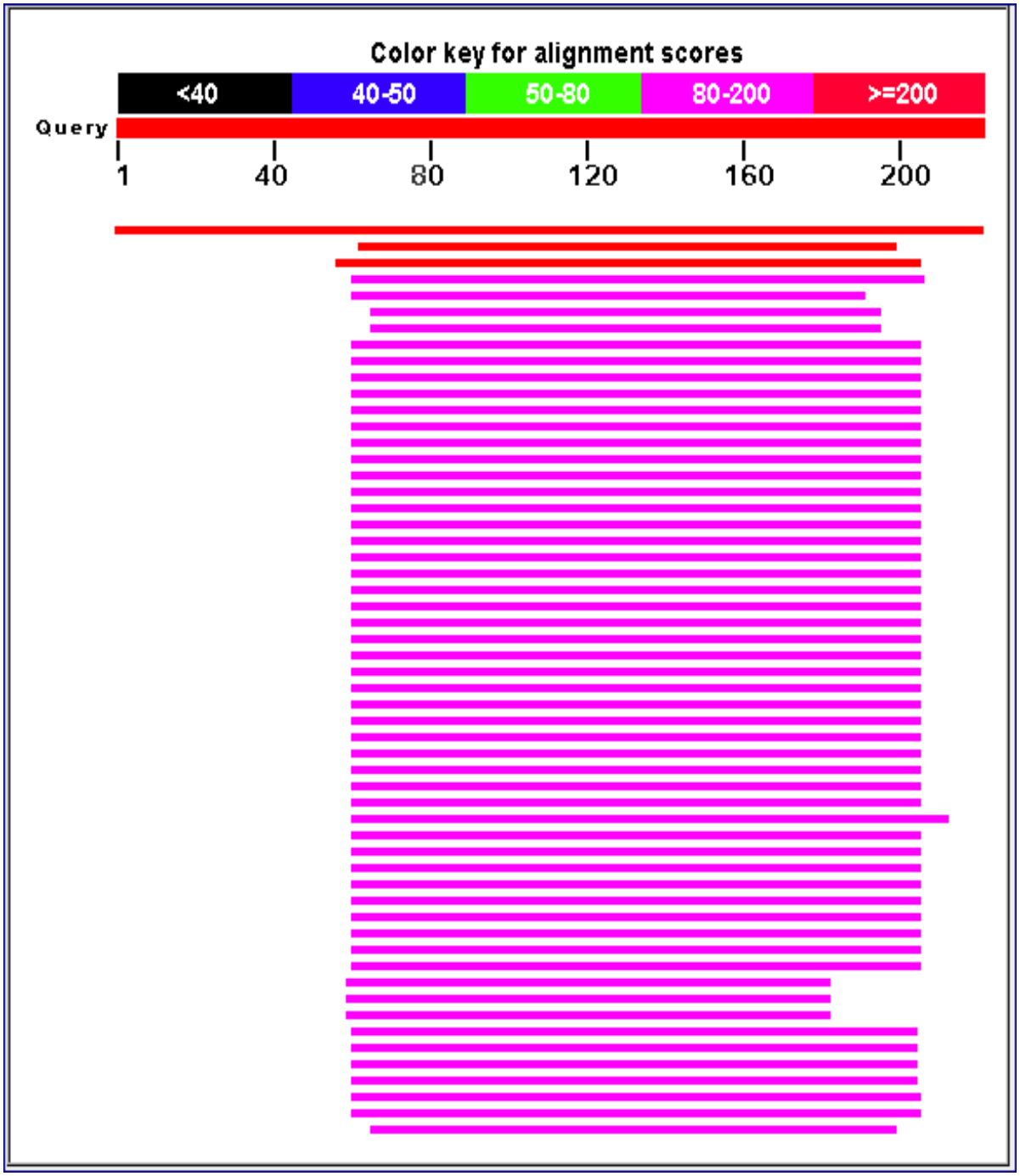

Figure 1: Blast analysis of *P. indica* TEF1 sequences

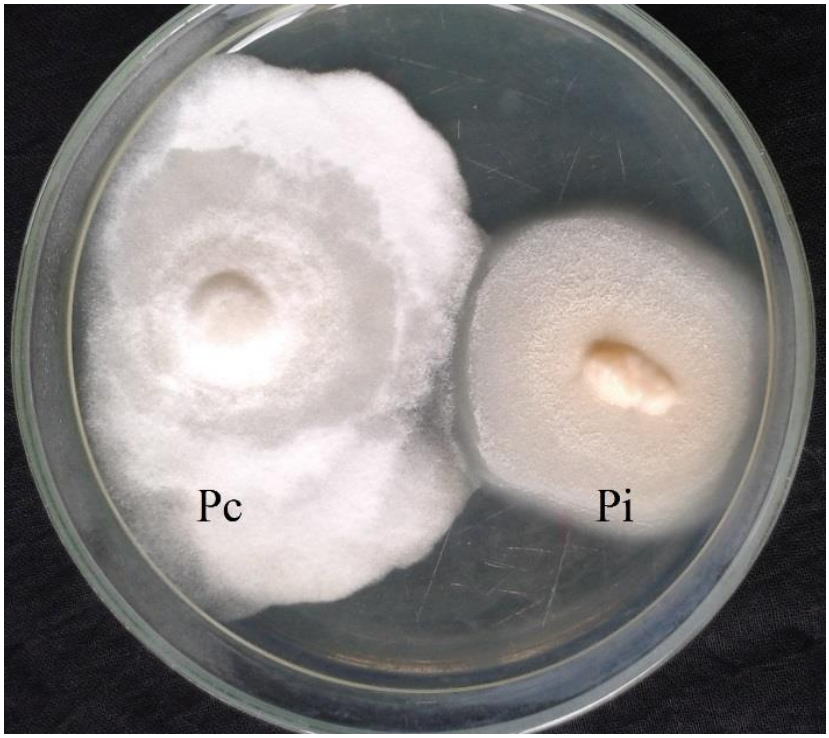

Plate 8. Dual culture study of *P. indica & P. colocasiae*

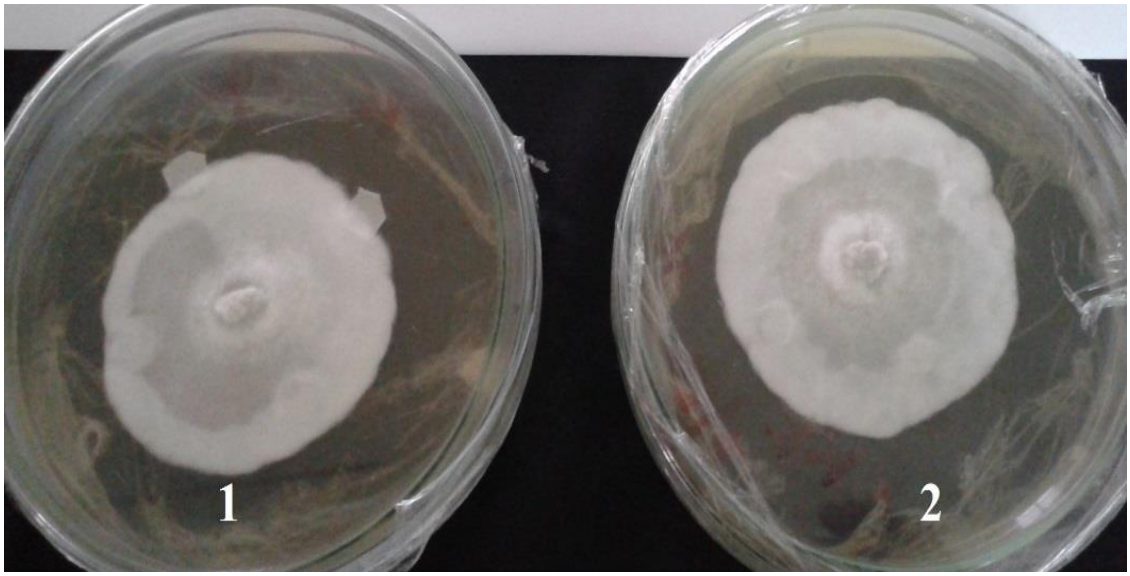

Plate 9. Effect of culture filtrate on *P. colocasiae.* 1- Control 2- With culture filtrate

(Muktakesi -control) were combined and taken as **T6**. Comparison between *P. indica* colonized and non- colonized plants in terms of disease resistance and growth promotion was made. Comparison was made between **T2** (Sree Kiran + *P.colocasiae*) and **T3** (Sree Kiran + *P.indica* + *P.colocasiae*). Similarly, comparison was made between **T6** (Muktakesi + *P.colocasiae*) and **T7** (Muktakesi +*P.indica* + *P.colocasiae*).

For plant growth promotion study, Sree Kiran and Muktakeshi were planted as indicated in materials and methods and various growth parameters were recorded at regular intervals. A summary of the result is given in Table. 2a& 2b. Plate numbers 10 and 11 shows the growth promotion by *P. indica* in Sree Kiran and Muktakeshi. Average number of days taken for sprouting in *P. indica* applied Sree Kiran was reduced to 9 from 10 in the control plants. In Muktakeshi, *P. indica* colonized plants sprouted 6 days earlier than that of control plants. Various growth parameters like height of the plant, leaf area and number of leaves were recorded four times during the crop period. Both the varieties showed growth promotion in terms of plant height, number of leaves, leaf length and leaf breadth consequent to incorporation of *P.indica*. Increase in height ranged from 17.3 to 39.7% and 4.7 to 44.6% in Sree Kiran and Muktakeshi respectively during different growth period.

Other parameters like number of cormels, weight of cormels and mother corm, root length, shoot weight and total weight of plants were recorded during harvest (Table. 2c). All the above mentioned growth parameters were found to be increasing in the *P. indica* colonized plants than that of control plants. However, the result was not statistically significant. Comparison between *P. indica* colonized and non colonized plants in terms of disease incidence is shown in Plate 12 and Plate 13. The severity of the disease was measured three times at an interval of one week during the period of disease incidence Significant reduction in the disease was observed in *P. indica* colonized plants than that of the non-colonized plants (Figure 2). The PDI reduction was more in susceptible variety, Sree Kiran. During the different stages of infection, 57.6, 50.7 and 84.3 %

Plate 10. *P. indica* colonized and control plant (Sree Kiran)

Plate 11. Control and *P. indica* colonized plants (Muktakeshi)

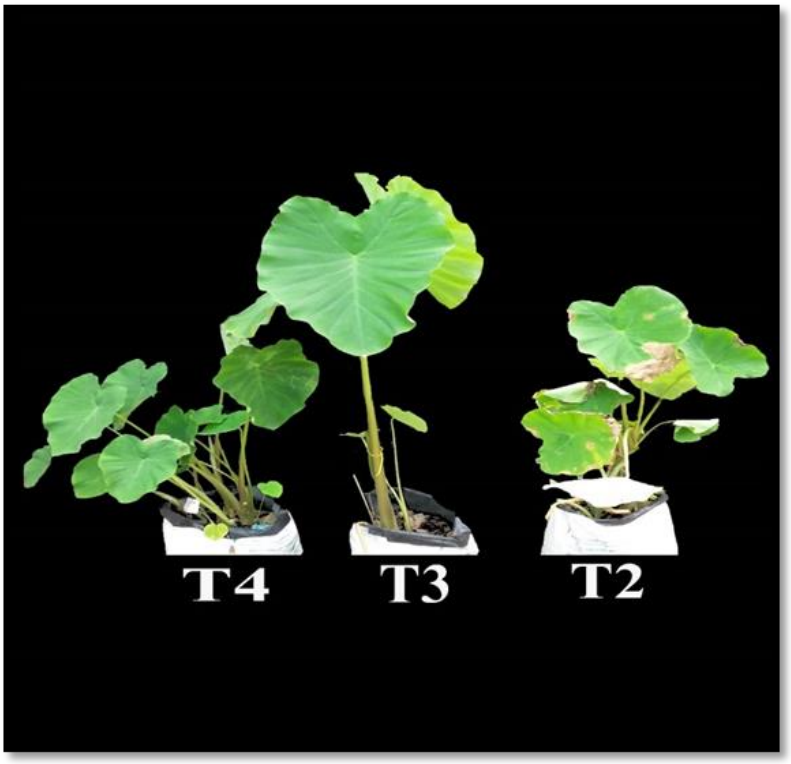

Plate 12. Comparison of disease incidence between Sree Kiran control (T4), Sree Kiran + *P. indica* + *P. colocasiae* (T3) and Sree Kiran +*P. colocasiae* (T2)

Plate 13. Comparison of disease incidence between Mukthakeshi control (T8), Mukthakeshi + *P. indica* + *P. colocasiae* (T7) , Mukthakeshi +*P. colocasiae* (T6)

decrease in PDI was observed in *P. indica* colonized Sree Kiran when compared to the control plants. In case of Muktakeshi, reduction in PDI was 39.9, 56.2 and 72.5% over control.

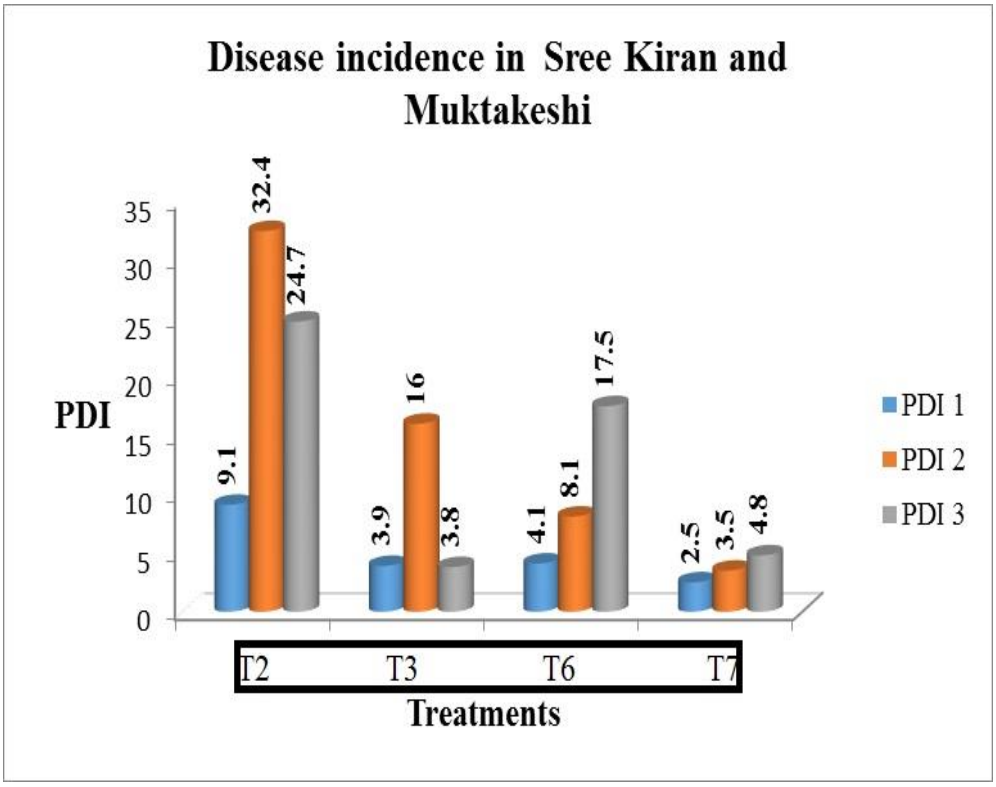

Figure 2. Percentage disease index of Sree Kiran and Muktakeshi

T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T6 – Muktakeshi+ *P. colocasiae*, T7 – Muktakeshi + *P. indica* + *P. colocasiae*

Table 2a. Effect of *P.indica* on growth parameters of taro varieties

Treatment	Days for sprouting	Height 2 MAP (cm)	Height 3MAP (cm)	Height 3(cm)	Height 4(cm)	No. of leaves 1	No. of leaves 2	No. of leaves 3	No. of leaves 4
T2	10.0 C	21.3 A	34.3 B	59.9 B	81.6AB	3.6 A	5.1 B	5.5 C	4.8 B
T3	9.0 C	28.2 A	56.9 A	88.4 A	98.7 A	4.1 A	4.5 B	6.5BC	5.4AB
T6	26.6 A	3.6 B	56.3 A	60.0 B	65.7 B	0.5 B	7.9 A	8.3 B	6.0AB
T7	20.8 B	6.5 B	51.0 A	55.6 B	69.0 B	0.8 B	8.8 A	11.3A	7.4 A

T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P.colocasiae*, T6 – Muktakeshi+ *P. colocasiae*, T7 – Muktakeshi + *P. indica* + *P.colocasiae*

Table 2b. Effect of *P.indica* on growth parameters of taro varieties

Treatment	Leaf Breadth 1(cm)	Leaf Breadth 2 (cm)	Leaf Breadth 3 (cm)	Leaf Breadth 4 (cm)	Leaf length 1(cm)	Leaf length 2(cm)	Leaf Length 3 (cm)	Leaf Length 4 (cm)
T2	13.5 A	16.6 A	23. AB	25.4AB	14.5A	17.4B	25.0 B	27.3AB
T3	14.6 A	21.2 A	27.7 A	29.3 A	16.1A	22.3AB	30.8 A	31.3 A
T6	1.4 B	16.4 A	17.0 C	19.5 C	1.75B	23.9 A	23.2 B	24.2 B
T7	2.25 B	18.1 A	18.7 BC	23.0 BC	2.8 B	24.6 A	24.3 B	26.3 AB

T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P.colocasiae*, T6 – Muktakeshi+ *P. colocasiae*, T7 – Muktakeshi + *P. indica* + *P.colocasiae*

Table 2c. Effect of *P.*indica on growth parameters of taro varieties

Treatment	Root Length (cm)	Root weight (g)	Shoot weight (g)	Total weight (g)	No of cormels	Weight of cormels (g)	Weight Mother Corm (g)
T2	34.8 A	11.9 B	54.0 A	250.0 B	4.3 B	49.0 B	134.0 A
T3	34.9 A	12.9 B	63.0 A	315.0 B	5.0 B	105.5 B	134.0 A
T6	40.8 A	22.8 A	51.0 A	416.0 A	5.9 AB	203.0 A	133.0 A
T7	46.1 A	24.6 A	59.0 A	454.0 A	7.4 A	239.0 A	137.0 A

T2 – Sree Kiran + *P. colocasiae,* T3 – Sree Kiran + *P. indica* + *P.colocasiae,* T6 – Muktakeshi+ *P. colocasiae,* T7 – Muktakeshi + *P. indica* + *P.colocasiae*

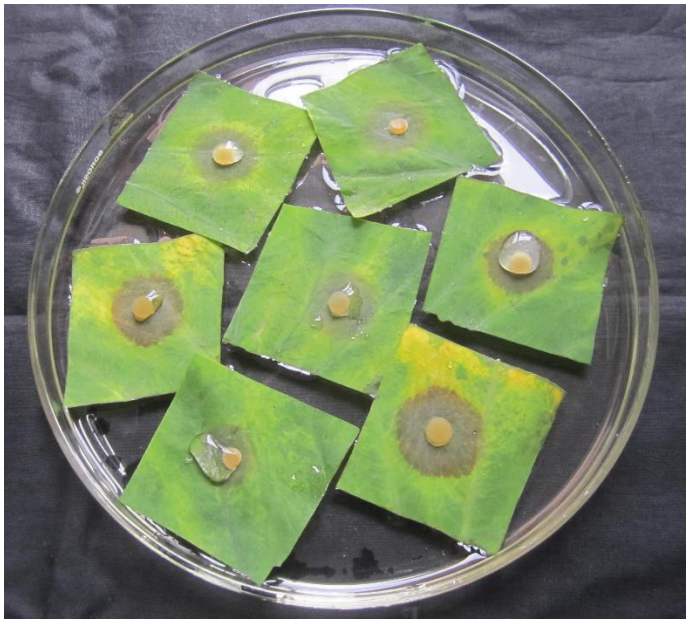

Plate 14. Leaf disc method of sporangia production

Plate 15. Taro leaf bit infected by *P. colocasiae* for sporangia production

4.5 Defense enzyme quantification and differential gene expression

After two months of planting, T2, T3, T6 and T7 plants were challenge inoculated with zoospore suspension prepared by the leaf disc method of sporangial production (Plate. 14, 15). The enzyme assays were carried out using standardized established protocols. There was a steady increase in the chitinase activity in *P. indica* colonized plants upon challenge inoculation with *P. colocasiae.* Starting from 0 till 96 hpi, significant increase in chitinase activity was noted (Figure 3 & 4) in the T3 plants (Sree Kiran + *P.indica* + *Phytophthora colocasiae*). The highest activity was noted at 6^{th} hpi (9.6 mg sugar released / g tissue) and the least activity was at 48^{th} hpi (4.05 mg sugar released / g tissue). After 6 hours, the activity was gradually decreased in all the infected plants. In T7 plants (Muktakeshi + *P.indica* +*Phytophthora colocasiae*), a peak in the enzyme activity was noted at 12^{th} hour after challenge inoculation (9.25 mg sugar released / g tissue).

The glucanase enzyme activity (Figure 5 & 6) was elevated in T3 (Sree Kiran + *P.indica* +*Phytophthora colocasiae*) as well as T7 plants (Muktakeshi plants + *P.indica* + *Phytophthora colocasiae*) over plants challenge inoculated with *Phytophthora colocasiae* from the 0^{th} hour till the 48^{th} hour. In T3, the maximum activity was recorded at 24^{th} hour (1.91 mg sugar released / g tissue) and in T7; maximum activity was obtained at 6^{th} hour (1.686 mg sugar released / g tissue).

Even though the PAL activity (Figure 7 & 8) was found to be increased in T3 plants during 3rd and 6th hpi, no significant increase was observed in T7 plants. The peroxidase activity (Figure 9 & 10) was also not increased in the *P. indica* colonized plants.

As a non-specific line of defense, the total phenol content (Figure 11 & 12) was found increasing in both T3 and T7 plants when compared to all other plants. In all the *P. indica* colonized plants the increased level of enzyme activity was observed during 6^{th} and 12^{th} hour after infection.

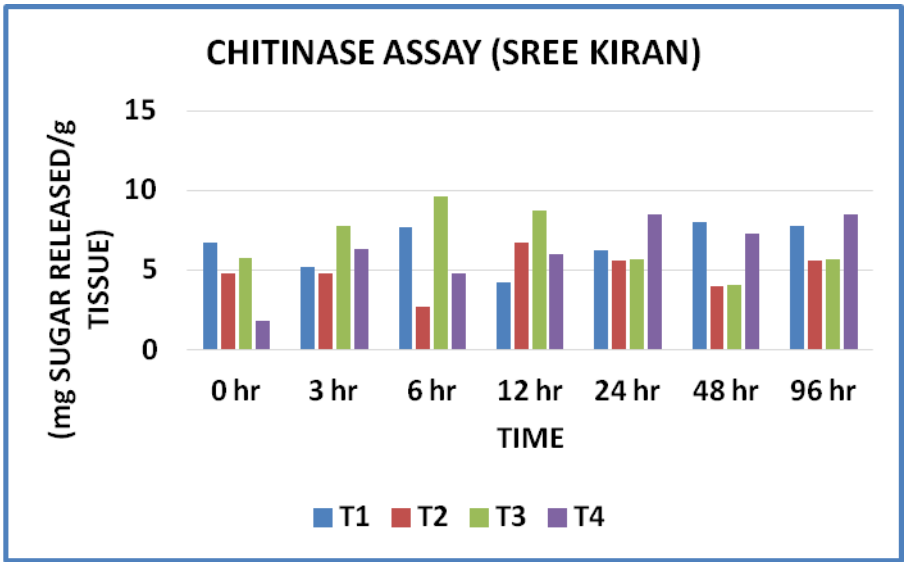

Figure 3. Chitinase activity in Sree Kiran

T1 – Sree Kiran + *P. indica*, T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T4 – Control

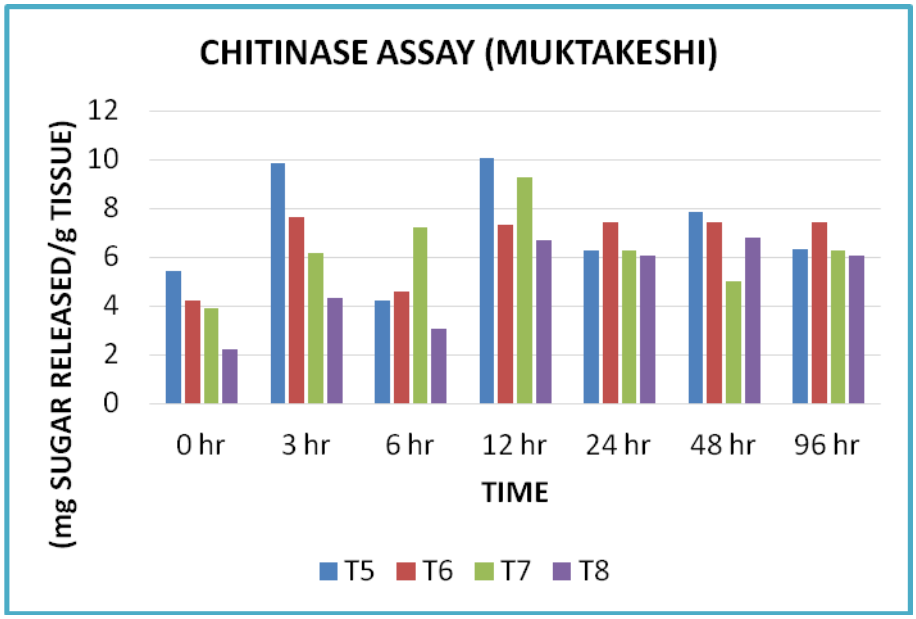

Figure 4. Chitinase activity of Muktakeshi. T5 – Mukthakeshi+ *P. indica*, T6 – Mukthakeshi+ *P. colocasiae*, T7 – Mukthakeshi + *P. indica* + *P. colocasiae*, T8 - Control

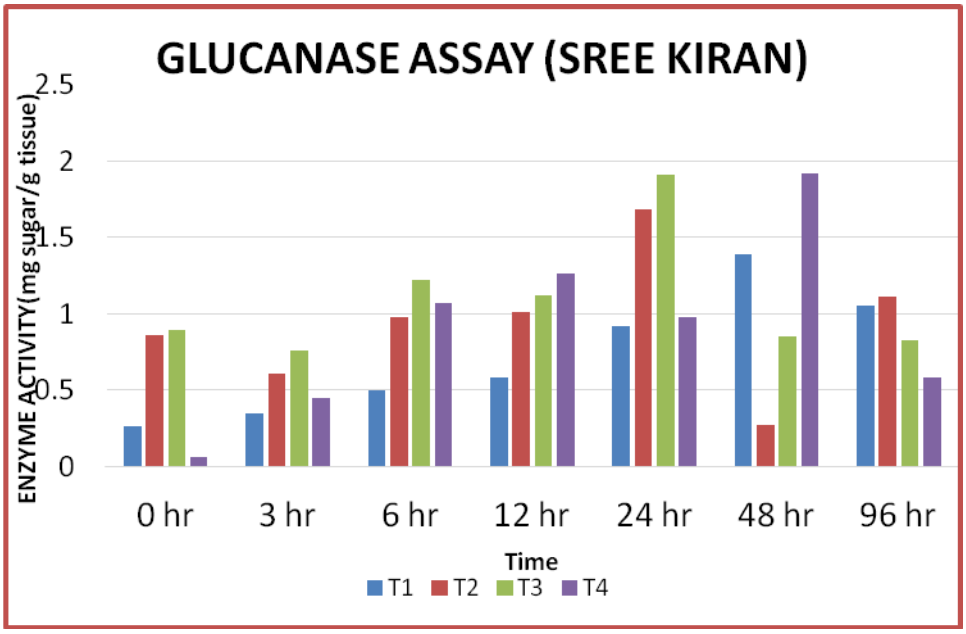

Figure 5. Glucanase activity of Sree Kiran

T1 – Sree Kiran + *P. indica*, T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T4 – Control

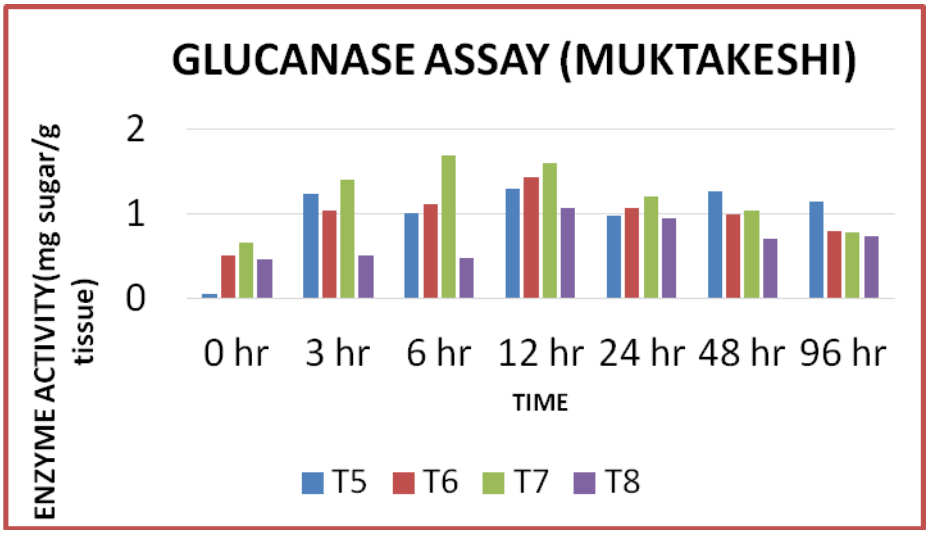

Figure 6. Glucanase activity of Muktakeshi.T5- Muktakeshi+*P. indica*, T6- Muktakeshi + *P. colocasiae*, T7- Muktakeshi + *P. indica* + *P. colocasiae*, T8- control

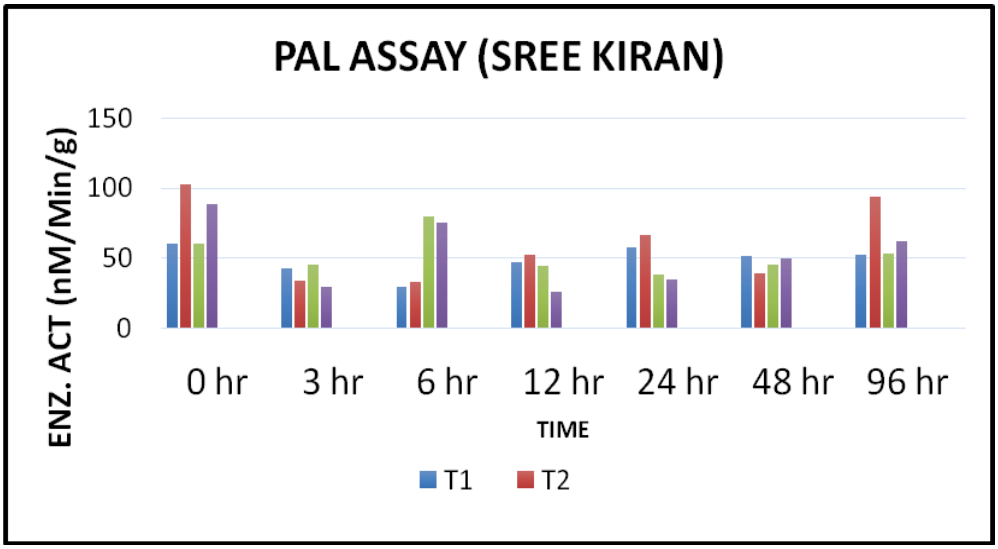

Figure 7. PAL activity of Sree Kiran

T1 – Sree Kiran + *P. indica*, T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T4 – Control

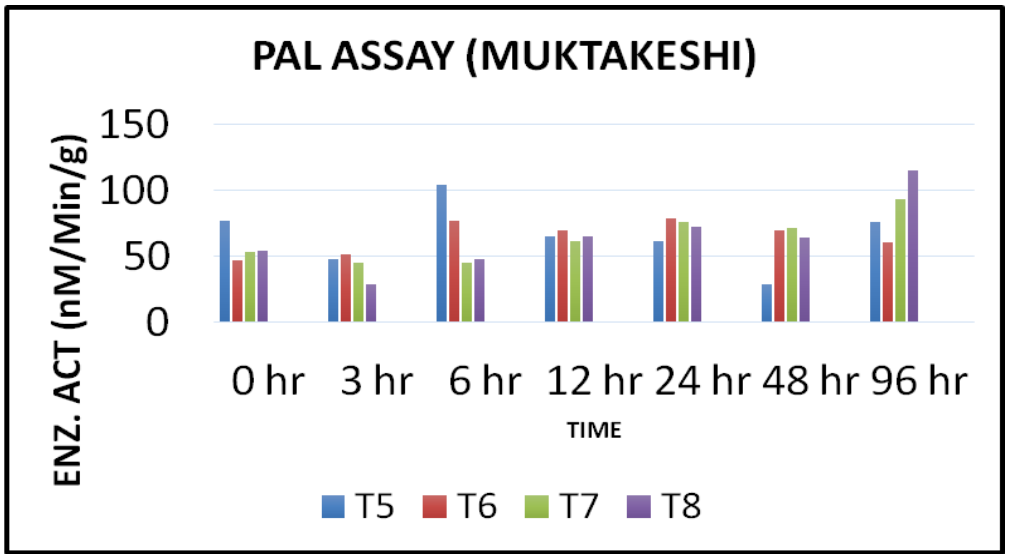

Figure 8. PAL activity of Muktakeshi

T5- Muktakeshi+*P. indica*, T6- Muktakeshi + *P. colocasiae*, T7- Muktakeshi + *P. indica* + *P. colocasiae*, T8- control

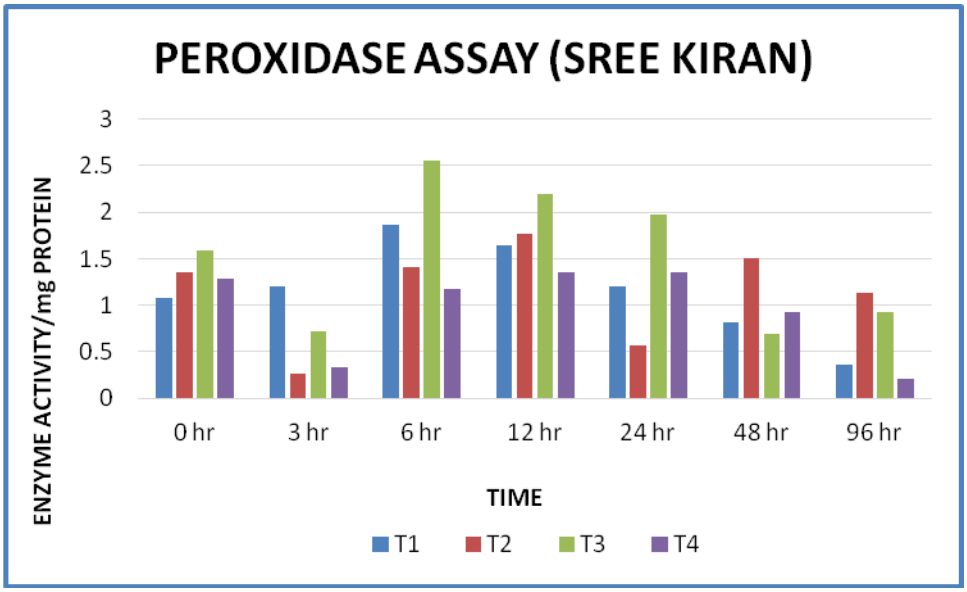

Figure 9. Peroxidase activity of Sree Kiran

T1 – Sree Kiran + *P. indica*, T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T4 – Control

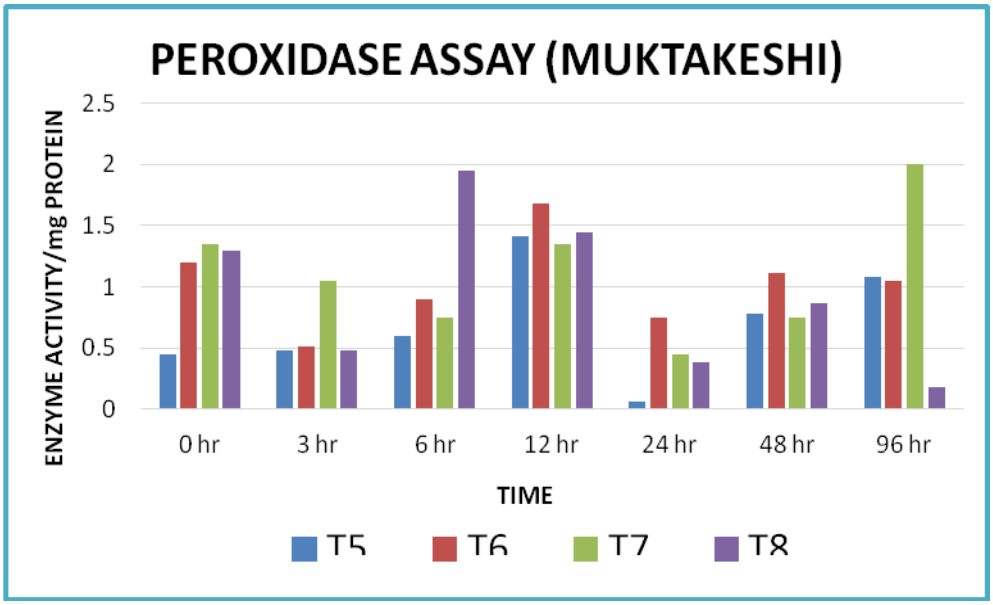

Figure 10. Peroxidase activity of Muktakeshi

T5- Muktakeshi+*P. indica*, T6- Muktakeshi + *P. colocasiae*, T7- Muktakeshi + *P. indica* + *P. colocasiae*, T8- control

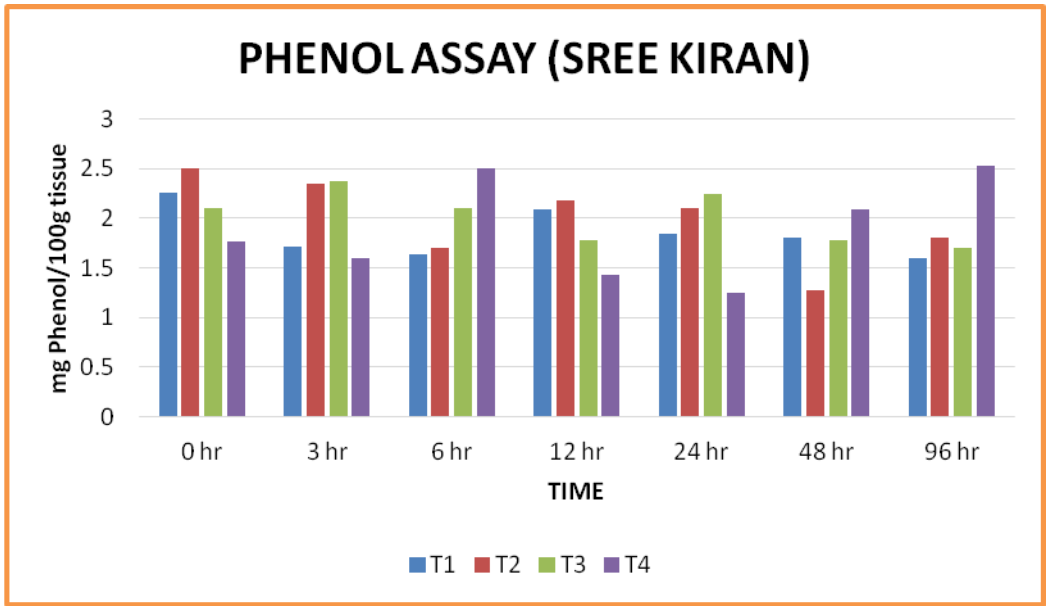

Figure 11. Total phenol content of Sree Kiran

T1 – Sree Kiran + *P. indica*, T2 – Sree Kiran + *P. colocasiae*, T3 – Sree Kiran + *P. indica* + *P. colocasiae*, T4 – Control

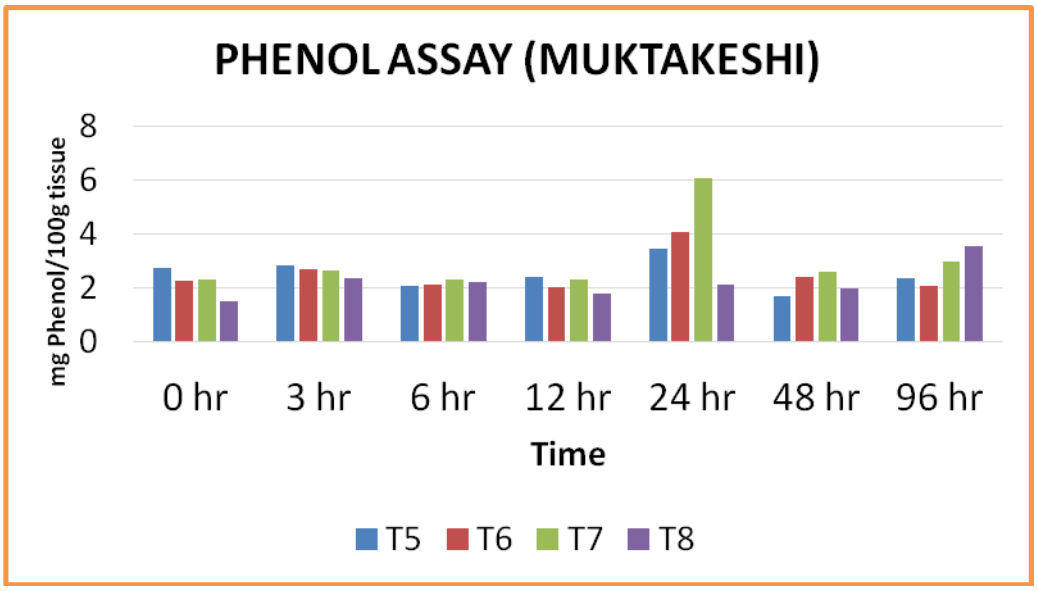

Figure 12. Total phenol content of Muktakeshi

T5- Muktakeshi+*P. indica*, T6- Muktakeshi + *P. colocasiae*, T7- Muktakeshi + *P. indica* + *P. colocasiae*, T8- control

4.5.1 Monitoring for genes upregulated/ downregulated

Among the chitinase and glucanase primers used, the chitinase primer pair 2ChiF2 & 2ChiR5 was able to amplify the taro DNA (Plate 16). But the amplification was not specific because the amplification resulted in multiple bands due to the non-specificity of the primer. However, the obtained bands were cut, DNA was eluted and was sequenced. Similar sequences were searched using BLAST. But none of the sequences were of chitinase. So, the particular objective of monitoring the relative expression of gene was abandoned and proceeded to study the differentially expressed genes through Suppression Subtractive Hybridization method.

4.5.2 Suppression Subtractive Hybridization

4.5.2.1 RNA isolation and cDNA synthesis

RNA was isolated from *P. indica* colonized Sree Kiran with *P. colocasiae* infection and Sree Kiran with only *P. colocasiae* infection by using Ambion PureLink® RNA Mini Kit. The isolation of RNA was successful and was of high quality. Since RNA is less stable than DNA, first strand cDNA synthesis was carried out soon after RNA isolation. The gel image of cDNA synthesized is shown in Plate 17. All the steps starting from cDNA synthesis was carried out using Clontech PCR-Select cDNA Subtraction Kit as per the manufacturer's protocol. Second strand cDNA synthesis was carried out immediately after synthesizing the first strand.

4.5.2.2 Rsa I digestion and adaptor ligation

The ds cDNA was digested with Rsa1 restriction enzyme to generate blunt ends and adaptor was ligated to the tester to form 2 tester populations. The gel image of Rsa 1 digested cDNA is shown in Plate 18. The ligation efficiency test was carried out in order to check whether the adaptors have ligated to the blunt ends of cDNA. The ligation was successful and proceeded to the next step.

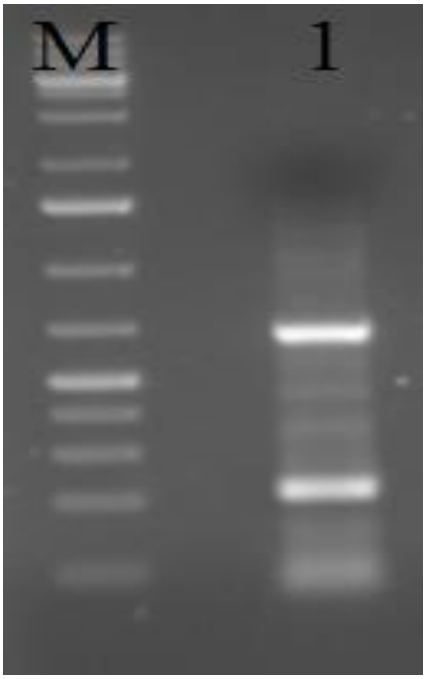

Plate 16. Amplification of Chitinase gene using primer pair 2ChiF2 &2ChiR5

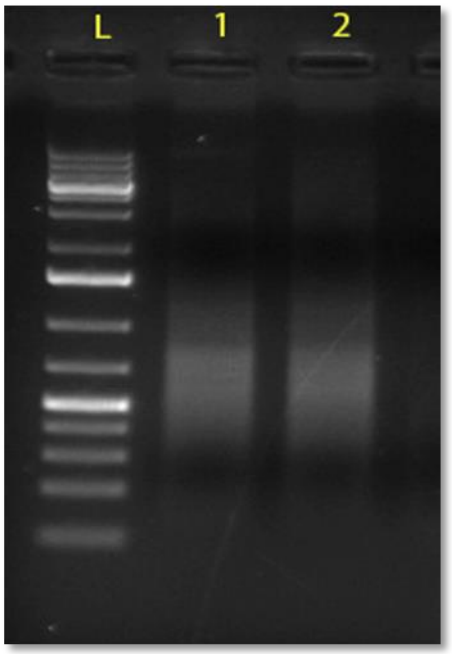

Plate 17. cDNA synthesized from Tester and Driver samples. L- 1Kb plus Marker, lane 1-Tester cDNA, lane 2- Driver cDNA

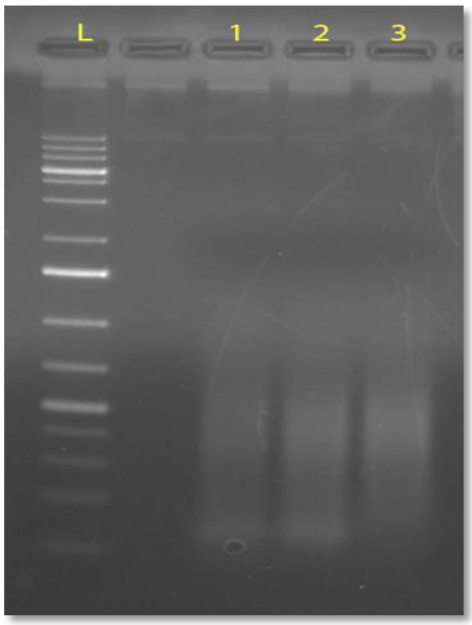

Plate 18. Rsa 1 digested Tester (Lane 1), Driver (Lane 2) and kit control (Lane 3), L- 1Kb plus DNA marker

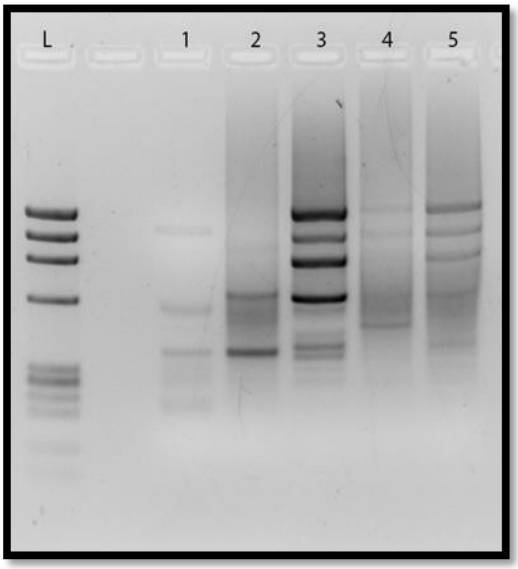

Plate 19. Primary PCR amplification. L – Hae III digest size markers, lane 1 – forward subtracted experimental cDNA, lane 2 – unsubtracted tester control, lane 3 – subtracted control skeletal muscle cDNA, lane 4 – unsubtracted tester control for the control subtraction, lane 5 – PCR control subtracted cDNA

4.5.2.3 Primary and secondary PCR amplification

After carrying out the second hybridization, the commonly expressed genes in tester and driver were hybridized and in order to amplify and to enrich the differentially expressed genes, primary (Plate 19) and secondary PCR (Plate 20) was carried out. The PCR was carried out using primers specific for the adaptor sequence. After the second PCR, the forward subtracted experimental tester cDNA was purified.

4.5.2.4 Cloning and colony PCR

Cloning was carried out using Instaclone PCR cloning Kit (Thermofisher) in *E. coli* DH5α strain as per the manufacturer's protocol. The transformed colonies were selected based on blue white screening (Plate 21). The white colonies were picked and a master plate was prepared and along with it, colony PCR was also carried out (Plate 22).

4.5.2.5 Sequence annotation

The sequencing result obtained was annotated using Blast2Go software. The sequences were analyzed and the cellular (Figure 13), molecular (Figure 14) and biological functions (Figure 15) were determined. Five genes were identified and their functions were determined out of which four were coming under different defense related pathways. The genes are listed below.

- Senescence associated genes
- Cytochrome P450
- Delta (12) oleic acid desaturase FAD2
- Calcium-dependent protein kinases (CDPKs)

These genes are expressed in plants during various biotic and abiotic stresses. Table 3 shows the annotated result that produced significant alignments.

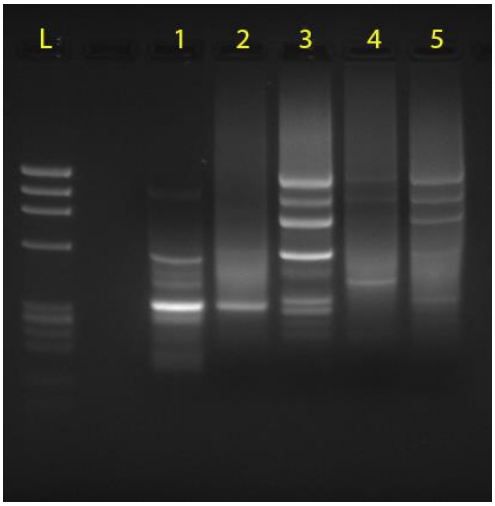

Plate 20. Secondary PCR amplification. L – Hae III digest size markers, lane 1 – forward subtracted experimental cDNA, lane 2 – unsubtracted tester control, lane 3 – subtracted control skeletal muscle cDNA, lane 4 – unsubtracted tester control for the control subtraction, lane 5 – PCR control subtracted cDNA

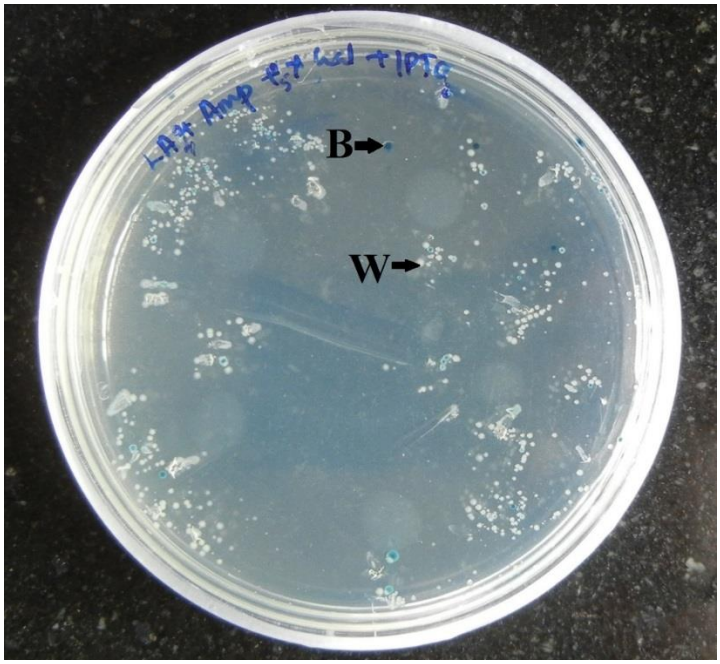

Plate 21. Blue white screening of the transformed colonies

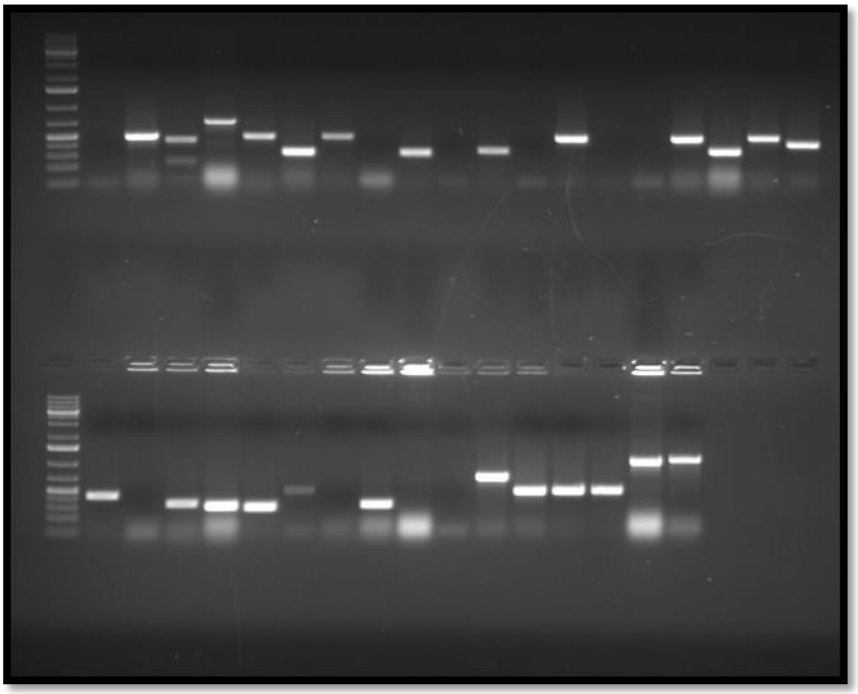

Plate 22. Colony PCR amplification

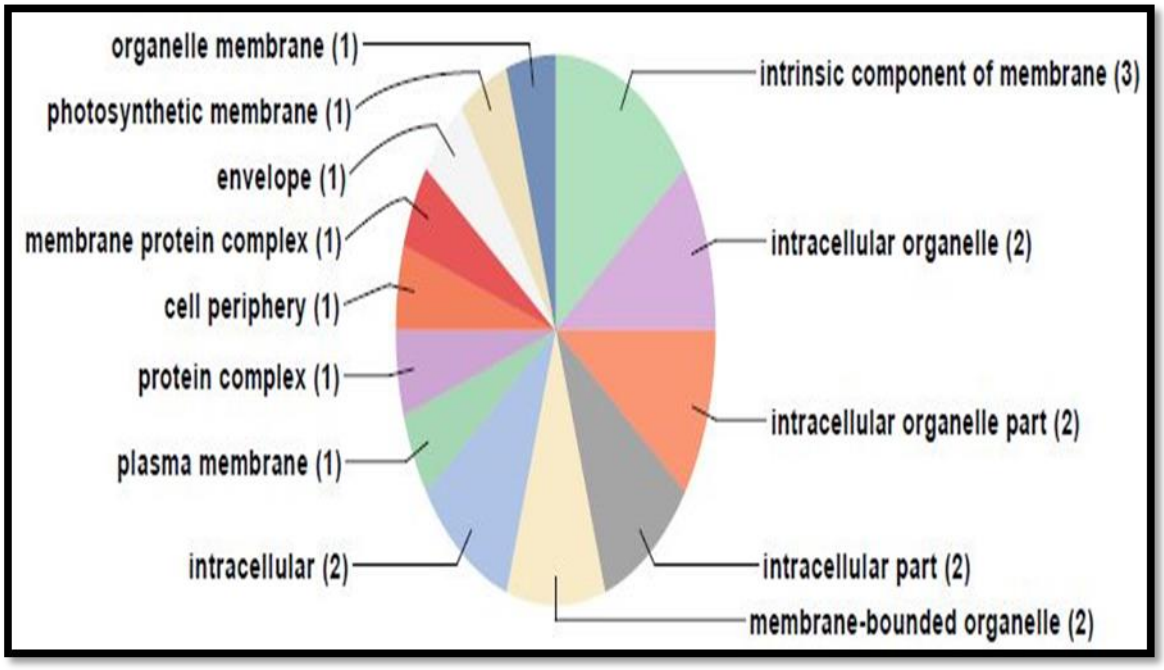

Figure 13. Cellular functions of annotated genes

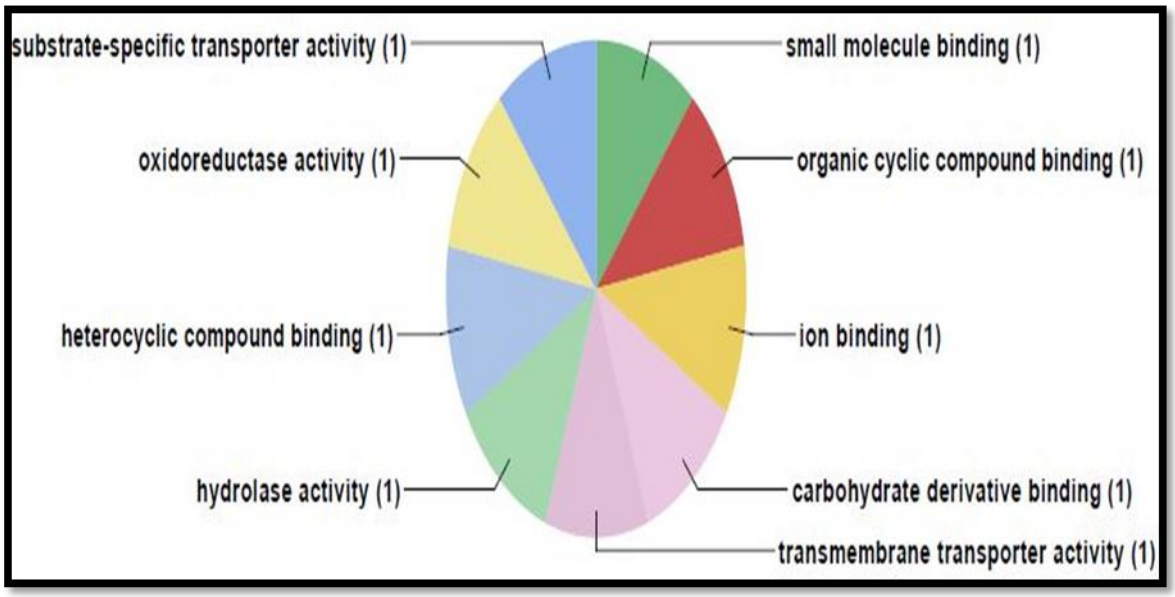

Figure 14. Molecular functions of annotated genes

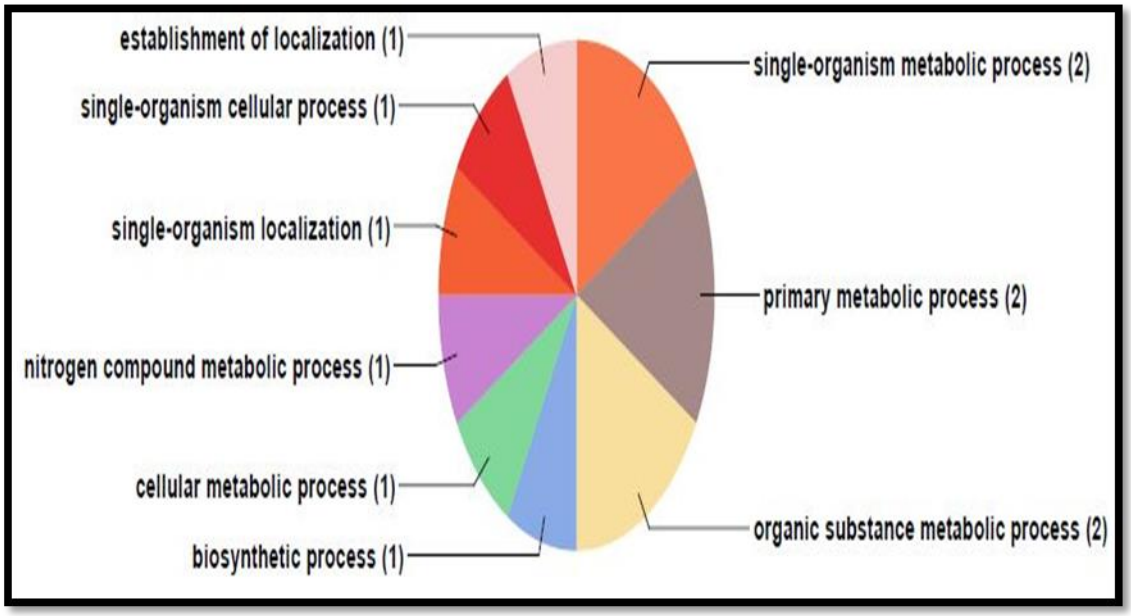

Figure 15. Biological functions of annotated genes

Table 3. Sequence producing significant alignments

Sequence identity	**Sequence producing significant alignments**	**E value**	**Similarity**	**Accession**
TSSHPi 1	Cytochrome P450-like TBP protein [*Lilium longiflorum*]	8.69E-13	100%	ABO20848.1
TSSHPi 2	Senescence-associated protein, putative [*Medicago truncatula*]	2.18E-49	75.5%	KEH16988.1
TSSHPi 3	ATP synthase CF0 B subunit (chloroplast) [*Colocasia esculenta*]	8.53E-14	97.3%	AEK48392.1
TSSHPi 4	Delta(12) oleic acid desaturase FAD2-like [*Solanum tuberosum*]	2.05E-11	71.7%	XP_006365798.1
TSSHPi 5	*Triticum aestivum* clone WC7-50-39 calcium-dependent protein kinase 3 like protein (CDPK3) mRNA	0.89	100%	JX119169.1

DISCUSSION

5. DISCUSSION

Taro [*Colocasia esculenta* (L.) Schott] is the most widely cultivated species in the Araceae family which are used as vegetables for their corms, leaves, and petioles. It is a perennial, tropical plant primarily grown as a root vegetable for its edible starchy corm, and as a leaf vegetable. Leaf blight of taro, caused by *Phytophthora colocasiae* Raciborski, is the most destructive disease of taro. Various management strategies of taro leaf blight are cultural practices, use of resistant varieties, use of fungicides and use of biological agents. Among the management strategies, cultivation of the resistant varieties is most valued. Disease resistance is associated with the activation of a wide range of defense related genes that serve to prevent pathogen infection (Sahoo *et al.*, 2007).

Piriformospora indica, a member of the order Sebacinales, is extremely versatile in its mycorrhizal associations and its ability to promote plant growth. *P. indica* is widely distributed as a symptomless root endophyte, and it colonizes members of bryophytes, pteridophytes, gymnosperms and angiosperms. (Varma *et al*., 2012). Similar to arbuscular mycorrhizal fungi, this fungus promotes plant growth, increases the resistance of colonized plants against fungal pathogens and their tolerance to abiotic stress (Harman, 2011).

The present study was undertaken to understand the effect of *P. indica* in promoting growth and inducing taro leaf blight disease resistance in taro. Two staining procedures were undertaken to establish *P. indica* colonization in taro root. Further confirmation of colonization was done by amplifying the species specific TEF gene. This is the first report of successful colonization of *P.indica* in tropical tuber crops. The confirmation of successful colonization of *P. indica* in *Centella asiatica* was done by amplifying Pitef1 gene of *P. indica* from the whole genomic DNA isolated from the roots by Satheesan *et al.*, (2012). The fungus hasn't shown any negative effect on the plant growth or any other parameters studied. The fact that *P. indica* has a broad host range, which is not only confined to vascular plants but also to colonized mosses, implies that this fungus has

evolved highly effective colonization strategies (Qiang *et al.,* 2012). Root colonization by *P. indica* is characterized by germination of chlamydospores at the cortical cells and further growing of hyphal mat. Intercellular and intracellular hyphae are formed during colonization that helps in exchange of nutrients and minerals.

Growth parameters, number of days for sprouting, leaf area, height of plants, root length, root weight, number and weight of cormels etc. were increased in *P. indica* colonized taro when compared to the control plants. However the results were not statistically significant. It may be due to the insufficient colonization of *P.indica* in taro roots. The quantity of inoculum supplied to taro plants might haven't sufficient for proper colonization. There is a chance that the rhizosphere condition existing in taro plants needs modification to facilitate the colonization. The dose of inoculum was fixed in the study based on previous reports. Further studies are required to standardize the optimum inoculum load required by taro plants to ensure significant growth promotion. Treatment with *P. indica* enhanced seed germination and increased seed production in *Oryza sativa, Brassica oleracea, Tridax procumbans* etc. (Kumari *et al.,* 2004). Increase in biomass was observed in *O. sativa, Zea mays, T. procumbans, Nicotiana tabaccum, P. vulgaris, Solanum nigrum, Brassica nigra, Saccharum officinarum and Spinacea oleracea.* Sahay *et al*. (1998) and Singh (2004) observed that maize plants exhibited enhanced growth upon inoculation with *P. indica* and the fungus was able to colonize the root cortex.

Apart from the root colonization and mycelial inhibition expressed by *P.indica,* the culture filtrate of the fungus also showed growth promoting activity. There is a need to completely characterize the culture filtrate, to pin point the active ingredients involved in plant growth promotion.

Percentage disease index (PDI) was calculated three times during the period of infection at an interval of one week. *P.indica* colonized taro plants were able to delay the onset of disease incidence for a week and could significantly reduce the infection in susceptible as well as tolerant varieties. During the different stages of infection, 57.6 %, 50.7% and 84.3 % decrease in PDI was observed in *P. indica*

colonized Sree Kiran compared to the control plants. About 39.9%, 56.2% and 72.5 % decrease in PDI was observed in *P. indica* colonized Muktakeshi at different infection stages. *P. indica* induces disease resistance in various plants against foliar and root pathogens. The beneficial endophytic fungus *P.indica* reduces *Verticillium* mediated disease development in Arabidopsis. Pre-treatment of Arabidopsis roots with *P.indica* protected plants from *Verticillium* infection, the plants survived better and the production of *Verticillium dahliae* microsclerotia was dramatically reduced (Sun *et al.*, 2014).

The fungus-colonized plants were more resistant to *Blumeria graminis* infection in shoots and *Fusarium culmorum* in roots (Waller et al. 2005).The results obtained in present study is in complete agreement with the studies conducted earlier in other crops with *P.indica.* It is evident that the plants with *P. indica* colonization enhanced disease resistance and for the first time, the resistance offered by *P. indica* against taro leaf blight pathogen was established.

It was reported by Misra *et al.* (2008), that during the infection by *P. colocasiae,* enzymes like PAL, peroxidase, chitinase, β-1,3- glucanase and the total phenol content were increased and the total sugar content of the plant decreased. In present study, to check whether the *P. indica* colonization enhances/supresses the levels of these enzymes, the enzyme assay was carried out. The assay of various defense enzymes showed that, there was an increase in the activity of chitinase and β-1.3- glucanase in the *P. indica* colonized Sree Kiran and Muktakeshi during initial stages of infection. β -1,3-glucanase was markedly induced on infection with *P. colocasiae* in *P. indica* colonized Muktakeshi (T7) and Sree Kiran (T3) over its infected control starting from the 0^{th} hour till the 48^{th} hour. The PAL activity was found to be increased in T3 during 3^{rd} and 6^{th} hour. About 2 fold increase in the PAL activity was observed at 6^{th} hour after infection and gradually decreased over time. Even though there was an increase in the PAL activity in *P.colocasiae* infected Muktakeshi (T6), no significant increase was observed in the *P. indica* colonized plants. There was a steady increase in the peroxidase activity in T3 plants when compared to the T2 plants. There was two fold increase in the peroxidase activity in T7 plants during 3rd and 96^{th} hour. As a

result of unspecific mechanisms to eliminate the pathogen, the total phenol content was f increased in the infected plants. But the quantity was slightly increased in the *P. indica* colonized plants. Kumar *et al.* (2009) reported that there was an enhanced activity of several antioxidant enzymes in Maize colonized by *P. indica* upon infection by the root pathogen *Fusarium verticilloides.* The levels of many antioxidant enzymes like catalase, peroxidase, superoxide dismutase, glutathione reductase and glutathione S transferases were increased in the colonized plants upon pathogen infection. The visible symptoms of *Phytophthora* infection is seen in taro within 24-48 h of inoculation. The results of the study clearly showed that there was a significant increase in all these defense inducing enzymes during initial hours of inoculation in *P.indica* colonized plants. Elevation of these enzymes during initial hours of pathogen attack may be interfering in pathogen establishment in host and further spread of the pathogen. These *P.indica* induced increase in enzymes may be conferring resistance to *P.colocasie* infection in taro. These enzymes may be conferring resistance to *P.*colocasiae attack. The induction of these defense enzymes by *P.indica* may be interfering in pathogen establishment and resisting the spread of the pathogen.

Based on the enzyme assay it can be concluded that there is an elevation in the chitinase and glucanase enzyme activity in the *P. colocasiae infected P. indica* colonized plants compared to the infected control plants. These two genes were selected for monitoring the differential expression. In *P. indica* colonized roots, expression of various defense- related genes like Pathogenesis related (PR) genes, ethylene signaling compounds, ethylene targeted transcription factors and the antioxidant system of plants are found to be activated upon pathogen attack (Kumar *et al.,* 2009).The two genes selected here for studying the differential expression are highly variable among different plant species and no information regarding taro chitinase and glucanase was available in the databases. Amplification of these two genes using the various designed primers were unsuccessful in taro because of the high variation of genes between species.

In order to identify the differentially expressed genes in *P. indica* colonized plants upon infection with *P.colocasiae,* suppression subtractive hybridization

was carried out. Various genes that take part in different defense related pathways were obtained upon annotation. The sequence analysis was carried out by Blast2Go software. The various functions of the annotated genes were determined and classified in to cellular, molecular and biological functions. Most of the proteins were intrinsic component of either intracellular organelle or plasma membrane of the cell. Their molecular functions include, substrate specific transporter activity, oxidoreductase and ion binding activity, hydrolase activity, heterocyclic compound binding activity and transmembrane transporter activity. The proteins were part of certain biosynthetic and metabolic pathways. About five genes were identified, in which four of them has got defense related activities.

The first gene was showing 100% similarity with Cytochrome P450 of *Lilium longiflorum*. Cytochrome P450 is the largest enzymatic protein family which has got various functions like, biosynthesis of sterols, triterpenes, modification of shikimate pathway, synthesis of oxylipin derivatives and alleneoxides in octadecanoid and Jasmonate pathways. The major function of this protein complex in defense related activity is the production of phytoalexins. Phytoalexins are the antimicrobial compounds that are produced during pathogen attack. Members of this superfamily are involved in multiple metabolic pathways with distinct and complex functions, playing important roles in a vast array of reactions. As a result, numerous secondary metabolites are synthesized that function as growth and developmental signals or protect plants from various biotic and abiotic stresses (Jun *et al.,* 2015). Identification of genes differentially expressed in a resistant reaction to *Fusarium oxysporum* in *Lilium regale* by SSH was done by Rao *et al.* in 2013. One among the different genes involved in disease defence includes cytochrome P450.

Senescence associated genes codes for several enzymes like lipases, RNases, proteases, mineral transporters, transcription factors related to antioxidant enzymes etc. Plant innate immunity against invasive biotrophic pathogens depends on the intracellular defence regulator enhanced disease susceptibility1 (EDS1). In *Arabidopsis thaliana* EDS1 interacts in vivo with another protein, Senescence-Associated Gene101 (SAG101). Together with

Phytoalexin-Deficient4 (PAD4), a known EDS1 interactor, SAG101 contributes intrinsic and indispensable signaling activity to EDS1-dependent resistance. The combined activities of SAG101 and PAD4 are necessary for programmed cell death triggered by the Toll-Interleukin-1 Receptor type of nucleotide binding/leucine-rich repeat immune receptor in response to virulent pathogen isolates and in restricting the growth of normally virulent pathogens (Feys *et al.*, 2005).

Delta (12) oleic acid desaturase FAD2 is an enzyme that desaturases the extrachloroplast lipids. The FAD2 gene appears to be important in the chilling sensitivity of plants since polyunsaturated membrane phospholipids are essential for maintaining cellular function and plant viability at lowered temperatures and during biotic stress (Pirtle *et al.*, 2001).

Calcium-dependent protein kinases (CDPKs) comprise a large family of serine/threonine kinases in plants and protozoans. It may function as a potential sensor that decodes and translates the elevation of calcium concentration into enhanced protein kinase activity and subsequent downstream signalling events. CDPKs activate the proteins that take part in Jasmonic acid and ethylene signalling pathways which finally activate the pathogenesis related genes. In *P. indica* colonized plants when challenge inoculated with *P. colocasiae,* all the above mentioned genes were found to be differentially expressed when compared to the non colonized plants. These genes may be playing active role in resisting *P. colocasiae* infection in *P. indica* colonized taro plants.

The present study suggests that *P. indica* can successfully colonize taro roots and can impart growth promotion. Moreover, it can help the plant to fight against leaf blight which is the most devastating disease in taro. Few differentially expressed genes were identified and they haves got functions in the disease defence pathways. This was the first attempt to exploit the potential of *P.indica* for growth promotion and imparting disease resistance in tropical tuber crops. There is a need to standardize the inoculum dose, rhizosphere condition required etc for the optimum colonization of *P.indica* in taro roots. It will help in tapping the full potential of *P.indica* for the organic cultivation of taro. Compatibility of

P.indica with bio- pesticides, other bio- agents and fungicides may be worked out to enable to include this versatile fungus in IDM strategies to manage taro leaf blight. Large scale experiments are required to validate the growth promotion as well as disease suppression potential of *P.indica* in field condition. *P.*indica can be effectively utilized for growth promotion and as an eco- friendly management strategy to combat taro leaf blight incidence.

SUMMARY

6. SUMMARY

The objective of the study entitled "*Piriformospora indica* mediated response in taro [*Colocasia esculenta* (L.) Schott] with special emphasis to growth and leaf blight incidence" was to understand the effect of *P. indica* colonization on growth and leaf blight incidence in taro and to study the differential expression of pathogen related genes in susceptible and tolerant varieties of taro, consequent to application of *P. indica.* The significant findings of the above studies are summarized in this chapter.

The colonizing ability of *P. indica* in taro varieties Sree Kiran and Muktakeshi was established by staining procedures, trypan blue staining and WGA-AF488 staining and confocal imaging. Further confirmation of colonization was done by amplifying the species specific TEF gene. This is the first report of successful colonization of *P.indica* in tropical tuber crops

The growth promotional activity of *P. indica was* studied and various growth parameters were monitored. The days taken for the colonized plants for sprouting were reduced and it can be significantly observed in Muktakeshi plants. The colonized plants took an average of 20 days for sprouting against 26 days in control plants. Other growth parameters, total weight of plants, number of leaves, leaf area and yield were increased due to *P.indica* colonization. However, the result was not statistically significant. There is a need to standardize the dose of *P.indica* inoculum and rhizosphere conditions required for proper colonization in taro.

The disease index was recorded three times at an interval of one week. *P.indica* colonized taro plants were able to delay the onset of disease incidence for a week and could significantly reduce the infection in susceptible as well as tolerant varieties. During the different stages of infection about 57.6, 50.7and 84.3 % decrease in the PDI was observed in *P. indica* colonized Sree Kiran compared to the control plants and in case of Muktakeshi, 39.9, 56.2 and 72.5 % PDI reduction was observed over control.

The activity of defense enzymes like chitinase, glucanase, peroxidase, PAL and total phenol was estimated at 0, 3, 6, 12, 24, 48 and 96 hour post inoculation. The chitinase and glucanase activity was found increasing in initial hours of pathogen attack. As a primary defense response, the total phenol content in the colonized plants was found to be increased when compared to the non colonized plants. The visible symptoms of *Phytophthora* infection is seen in taro within 24-48 h of pathogen inoculation. The results of the study clearly showed that there was a significant increase in all these defense inducing enzymes during initial hours of inoculation in *P.indica* colonized plants. The induction of these defense enzymes by *P.indica* may be resisting the pathogen attack, delaying the establishment and reducing the severity of the disease incidence. In order to monitor the differential expression of chitinase and glucanase gene in the colonized plants, several primers were designed based on the conserved domains and PCR amplification was carried out. The primers were non- specific and multiple bands were obtained after amplification. So,the sequence didn't give the expected result.

For identifying the differentially expressed genes in colonized plants on *P. colocasiae* infection, Suppression Subtractive Hybridization was carried out using infected *P, indica* colonized plants as tester and the infected non colonized plants as driver. After sequencing, the results were analysed and annotated using Blast2Go program and five genes were identified which are differentially expressed. The cellular, biological and molecular functions were determined and charts were drawn using the same software. The identified genes were, cytochrome P450, Senescence associated gene, Delta (12) oleic acid desaturase FAD2 and Calcium-dependent protein kinases (CDPKs) which are expressed in plants during various biotic and abiotic stress. The major function of Cytochrome 450 protein complex in defense related activity is the production of phytoalexins. Senescence associated proteins along with certain defense regulators leads to programmed cell death of the plants which will arrest the spread of virulent pathogens. Delta (12) oleic acid desaturase FAD2 and Calcium-dependent protein kinases (CDPKs) are highly expressed in plants under abiotic and biotic stress.

CDPKs activates various proteins that take part in several defence related pathways like Jasmonate, ethylene signalling and finally activate the Pathogenesis Related (PR genes).

It can be summarized that, *P. indica* can successfully colonize taro plants and can enhance plant growth. It enables/induces the plant to fight against leaf blight which is the most devastating disease in taro. The differentially expressed genes, which have got functions in the disease defence pathways, were identified. This was the first attempt to exploit the potential of *P.indica* for growth promotion and imparting disease resistance in tropical tuber crops. Studies are required on the areas of inoculum dose, rhizosphere condition required etc to tap the full potential of *P.indica* for organic cultivation of taro. Compatibility of *P.*indica with other management strategies may be studied and this can help in including *P.indica* in IDM strategies for taro leaf blight management. Large scale experiments are required to validate the growth promotion as well as disease suppression potential of *P.indica* in field condition.

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APPENDICES

APPENDIX I

0.5% Trypan blue in lactophenol (100 ml)

Trypan blue (Lobachemi)- 0.5 g

Dissolve in 90 ml sterile distilled water, make upto 100 ml.

Store at room temperature.

APPENDIX II

Trichloro acetic acid solution (100 ml)

Ethanol - 80 ml

Chloroform - 20 ml

Mix both to form 100 ml ethanol - chloroform solution

TCA - 0.15 g

Dissolve TCA in 800 ml ethanol- chloroform solution and make up the volume to 100 ml.

APPENDIX III

Phosphate buffer saline (PBS) pH- 7.4 (10X) (1000 ml)

Sodium Chloride - 80 g

Dibasic Sodium Phosphate - 11.6 g

Monobasic Potassium Phosphate - 2.4 g

Potassium Chloride - 2 g

Sodium Azide - 2 g

Dissolve in 900 ml water; adjust pH with HCl and make upto 1 L

PBS - 1X (1000 ml)

100 ml (10X) PBS + 900 ml distilled water

APPENDIX IV

WGA- AF in PBS (10 ml)

WGA- AF 488- 1 mg

Dissolve in 8 ml 1X PBS and make up to 10 ml

APPENDIX V

Sodium Acetate Buffer

Prepare 50 ml 0.2M acetic acid (A) and 50 ml 0.2M Sodium acetate solution (B)

Mix 8.8 ml solution A with 41.2 ml solution B and make up to 200 ml using distilled water.

Adjust the pH to 5 using HCl.

APPENDIX VI

Sodium borate buffer

Dissolve 8g NaOH and 47g Boric acid in 900 ml water

Make up to 1 L using distilled water

Adjust pH to 7 using NaOH

APPENDIX VII

Citrate Phosphate buffer

Prepare 50 ml 0.2 M Sodium Hydrogen orthophosphate (A) and 10 ml of 0.1 M citrate (B).

Mix 43.6 ml of solution A with 6.4 ml of solution B and adjust pH to 7.0

APPENDIX VIII

CTAB extraction buffer

CTAB	-2%
PVP	-2%
Tris HCl (pH- 8)	-100mM
EDTA	-25mM
NaCl	-2M
β- Mercaptoethanol	-2%

EDTA was dissolved using sodium hydroxide pellets and pH was adjusted to 8. Then it was heated for complete dissolution of EDTA. pH of Tris was adjusted using Conc. HCl. The reagents were autoclaved and stored.

ABSTRACT

Piriformospora indica mediated response in taro (*Colocasia esculenta* (L.) Schott) with special emphasis to growth and leaf blight incidence

by

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ABSTRACT

The study entitled "*Piriformospora indica* mediated response in taro (*Colocasia esculenta* (L.) Schott) with special emphasis to growth and leaf blight incidence" was conducted at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during October 2015- June 2016 with an aim to identify the potential of *P. indica* colonization in taro plants to promote growth and leaf blight resistance.

Taro is an oldest cultivated crop grown for its edible corms, leaves and also for its broad medicinal properties. Leaf blight caused by *Phytophthora colocasiae* is the most destructive disease which causes about 50% yield loss. Use of resistant varieties and adopting cultural methods, application of fungicides and use of biological agents are the prevailing management practices to combat taro leaf blight incidence. *Piriformospora indica* is an endophytic mycorrhiza like fungi which have shown pronounced growth promotional and disease suppressing activities. In present study, the colonization ability of *P. indica* in taro varieties Sree Kiran and Muktakeshi was studied. The colonization was confirmed by various staining methods, trypan blue staining and chitin specific WGA-AF 488 staining and also by amplifying species specific TEF1 gene. In pot culture experiment to study the growth promotion and disease suppression potential, an increase in all the growth parameters was noted in the colonized plants compared to control plants. There was a substantial decrease in the disease incidence in colonized plants of both the varieties. During the different stages of infection about 57.6, 50.7and 84.3 % decrease in PDI was observed in *P. indica* colonized Sree Kiran compared to the control plants and in case of Muktakeshi, reduction was 39.9, 56.2 and 72.5 % over control plants. Induction of defense enzymes during pathogen attack in *P.indica* colonized plants was also studied. Increase in the activity of defence enzymes like chitinase, β-1, 3- glucanase and total phenol was observed in the colonized plants when compared to control plants during the initial hours of pathogen infection. Suppression Subtractive Hybridization was

carried out to identify the differentially expressed genes in the colonized plants upon *P. colocasiae* infection. Various genes that take part in different defense related pathways were identified upon annotation with Blat2Go program. The genes are, Senescence associated genes, cytochrome P450, Delta (12) oleic acid desaturase FAD2 and Calcium-dependent protein kinases (CDPKs) which are expressed in plants during various biotic and abiotic stresses. Thus the study revealed the potential of *P. indica* as an effective growth promoter as well as potential bio-control agent for taro leaf blight management.