Journal of Root Crops, 2016, Vol. 42 No. 2, pp. 107-114
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

Piriformospora indica, a Cultivable Endophyte for Growth Promotion and Disease Management in Taro (*Colocasia esculenta* (L.)

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Received: 5 November 2016; Accepted: 30 November 2016

Abstract

Taro leaf blight caused by *Phytophthora colocasiae* is the most destructive disease which causes about 50% yield loss. Use of resistant varieties, adopting cultural methods, application of fungicides and use of biological agents are the prevailing management practices against leaf blight. *Piriformospora indica* is an endophytic mycorrhiza like fungus which has shown pronounced growth promotional and disease suppressing activities in many crops. A study was conducted in ICAR- CTCRI, Sreekariyam to explore the potential of *P. indica* colonization in taro to promote growth and leaf blight resistance. The colonization ability of *P. indica* in taro varieties Sree Kiran and Muktakeshi was studied by Trypan blue staining, chitin specific WGA-AF 488 staining and also by amplifying species specific TEF1 gene. An increase in all the growth parameters was noted in the colonized plants compared to control plants in pot culture experiment. There was a substantial decrease in the disease incidence in colonized plants of both the varieties. During different stages of infection, about 57.6, 50.7 and 84.3% decrease in PDI was observed in *P. indica* colonized Sree Kiran and in case of Muktakeshi, reduction was 39.9, 56.2 and 72.5% over control. Increase in the activity of defense enzymes like chitinase, β-1, 3 glucanase and total phenol was observed in *P. indica* colonized plants compared to un-inoculated during initial hours of infection. Suppression Subtractive Hybridization was carried out to identify the differentially expressed genes in the colonized plants upon *P. colocasiae* infection. Various genes like Senescence associated genes, cytochrome P450, Delta (12) Oleic acid desaturase and calcium dependent protein kinase that take part in different defense related pathways were identified upon Blast2Go program. The study confirms that *P. indica* can be effectively utilized for growth promotion as well as an eco-friendly management strategy to combat taro leaf blight incidence.

Key words: Taro, *P. indica*, disease management, growth promotion

Introduction

Taro [*Colocasia esculenta* (L.) Schott], a member of the Araceae family is an oldest cultivated crop grown for its edible leaves and corms (Coates et al., 1988). Leaf blight caused by *Phytophthora colocasiae* Rac. causes yield loss of about 25-50 percent and is the major concern in all taro growing countries, including India (Raj et al., 2008). Several management practices are recommended to control taro leaf blight which includes cultural practices, application of fungicides, use of bio-agents and incorporation of organic amendments like vermicompost/vermiwash (Veena et al, 2015). 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.

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 and can be cultured in different substrates like PDA, Aspergillus medium etc.

The biotic stress protection by *P.indica* was first indicated in barley. The fungus colonized plants were also resistant against *Blumeria graminis* infection and *Fusarium culmorum* in barley (Waller et al., 2005). 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 defense 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).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.

Materials and Methods

Biological materials

Piriformospora indica culture was obtained from Amity Institute of Microbial Sciences, Amity University. The culture was maintained on PDA slants/ plates at room temperature (28±2ºC).

Taro varieties, Sree Kiran and Muktakeshi which were released by ICAR- CTCRI and maintained in the field gene bank were used for the present study.

Colonization study

a. Trypan blue in lacto phenol staining

Roots were collected from Sree Kiran and Muktakeshi plants grown in *P.indica* incorporated sterilized potting mixture. Staining was carried out as per the protocol by Philip and Hayman (1970). Slides were prepared from roots excluding the axial one and mounted in DPX mountant and the colonization was analysed under microscope (Nikon Eclipse E200, Nikon Corporation, Japan).

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

WGA-AF 488 staining was carried out by the procedure of Satheesan et al., 2012. 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 followed by washing 5 min in 1X phosphate buffer saline (PBS, pH 7.4). 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). 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 488 nm wavelength and detected at 500 – 600 nm and the images were captured at different depths.

c. Plant genomic DNA isolation and PCR analysis

Fresh roots were collected from both *P. indica* colonized Muktakeshi and Sree Kiran. The total genomic DNA was isolated from roots using a Genomic DNA isolation Kit (Fermentas) following manufacturer's protocol. The DNA obtained was analysed for its quality and quantity using a 0.8 % agarose gel electrophoresis. The high quality DNA thus obtained was used for PCR analysis.

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: 5'TCGTCGCTGTCAACAAGATG3'

PiTEF reverse primer: 5'GAGGGCTCGAGCATGTTGT3'

The components were mixed as follows. 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 and mixedby 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 °C. The amplified products were purified to remove excess primers and nucleotides using a Nucleospin® gel and PCR cleanup kit (Macherey Nagel). The purified product was sequenced with the same primers as for the PCR amplifications. The sequencing was performed using Applied Biosystems® 3500 Genetic Analyzer, Life Technologies at RGCB.

Plant growth promotion study

In order to study the effect of *P. indica* on plant growth, Sree Kiran and Muktakeshi were planted in grow bags with *P. indica* mixed sterile soil as 1% w/v. The experiment was conducted in an area, where taro leaf blight incidence occurs every year. The plants were allowed to get infection naturally. The treatments were T1- Sree Kiran with *P. indica,* T2- Sree Kiran with *P. colocasiae,* T3- Sree Kiran with *P. indica* + *P. colocasiae,* T4- Sree Kiran , T5- Muktakeshi with *P. indica,* T6- Muktakeshi with *P. colocasiae,* T7- Muktakeshi with *P. indica* + *P. colocasiae* and T8- Muktakeshi. The experimental design adopted was Completely Randomized Design with 10 replications (single plant).

Various growth parameters viz., number of days taken for sprouting, percentage of sprouting, height of the plant, number of leaves, leaf length and leaf breadth were recorded. Among these parameters, height of the plant, leaf number and length and breadth of the leaves were recorded at monthly intervals. The parameters viz., root length, root weight, total weight, shoot weight, weight of cormels, weight of mother corm and number of cormels were recorded at the time of harvesting of the crop.

Disease incidence was recorded three times (in the month of July) at an interval of one week using 1-6 scale and PDI was calculated using the formula, Sum of all numerical grades x 100

Total no of leaves counted x Max grade

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

Defense enzyme quantification

Muktakeshi and Sree Kiran were planted as per the previously mentioned combinations and after two months of planting, the T2, T3, T6 and T7 plants were challenge inoculated with spore solution of *Phytophthora colocasiae* prepared by leaf disc method of sporangia production (Nath et al., 2016). Leaf samples were collected at 0, 3, 6, 12, 24, 48 and 96 hours post inoculation (hpi) and Chitinase (Somogyi., 1952), β-1,3 glucanase (Koga et al., 1988), peroxidase (Haard and Marshal, 1976), Phenyl alanine ammonia lyase (PAL) (Sadasivam and Manickam, 1997) and total phenol content were quantified.

Monitoring differential gene expression

Subtractive suppression hybridization assay was carried out using Clontech PCR-Select cDNA Subtraction Kit (Clontech, USA) as per the manufacturer's protocol for identifying the differentially expressed genes in *Phytophthora colocasiae* challenge inoculated plants. Initially, both mRNA populations were converted 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 are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA. 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). 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 sequenced using Applied Biosystems® 3500 Genetic Analyzer, Life Technologies at RGCB. The sequences thus obtained were subjected to similarity search and the annotation was carried out using the Blast2Go software.

Results and Discussion

Colonization of *P. indica* in the roots of taro

Results of colonization studies adopting various methods showed successful colonization of *P. indica* in the roots of Muktakeshi and Sree Kiran. Trypan blue staining of the colonized roots indicated the presence of chlamydospores inside the root cells of both the varieties (Fig. 1). Confocal laser imaging of the *P. indica* colonies using chitin specific WGA-AF 488 provided evidence that the fungus colonizes mostly on the outer epidermal layers of root cells (Fig. 2). PCR analysis using PiTEF1 primers produced an amplicon of 250 bp from the genomic DNA of the colonized roots which again confirmed the successful colonization of the fungus in both taro varieties (Fig. 3). This is the first report of successful colonization of *P.indica* in tropical tuber crops. *P. indica* is widely distributed as a symptomless root endophyte, and it colonizes members of bryophytes, pteridophytes, gymnosperms and angiosperms. (Varma et al., 2012).

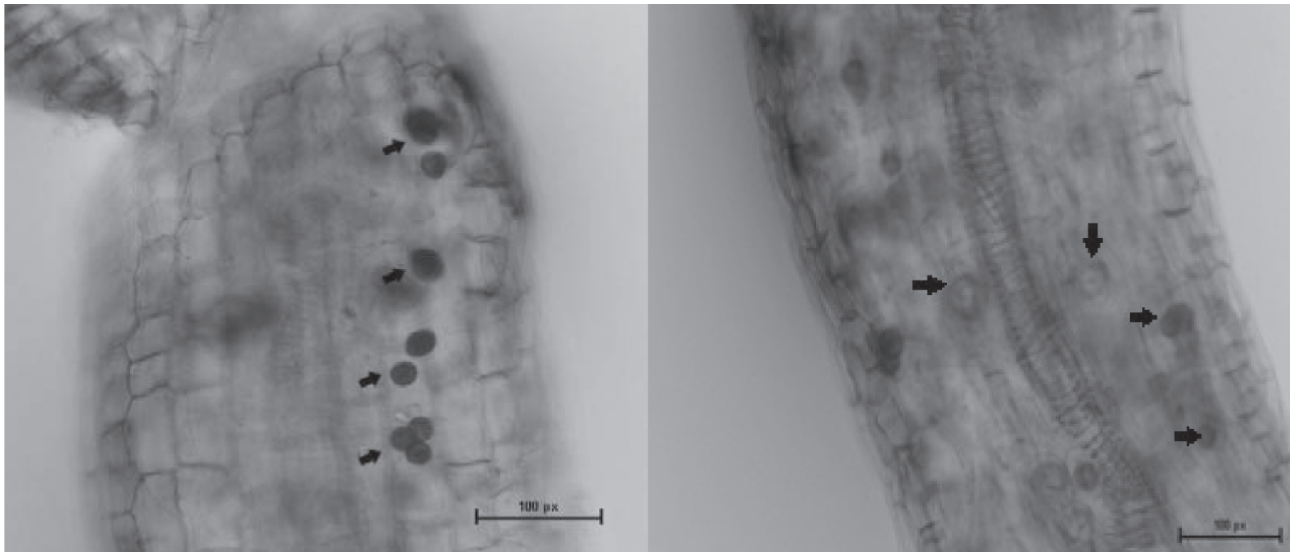

Fig.1. Trypan blue stained *P. indica* in the roots of Sree Kiran and Muktakeshi

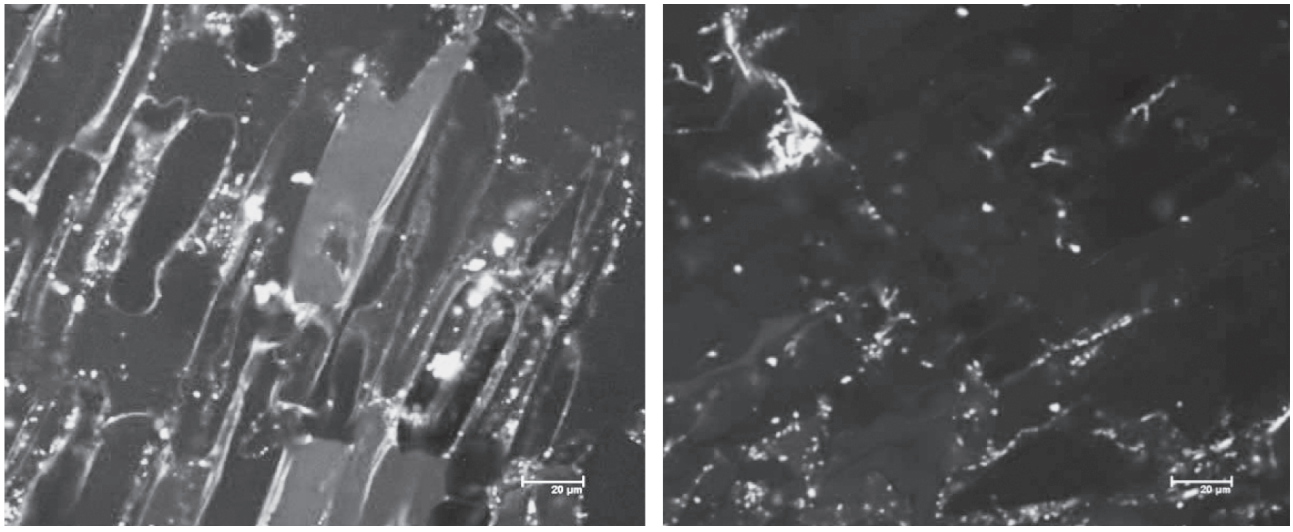

Fig.2. Confocal laser image of WGA-AF 488 stained *P. indica* in Sree Kiran and Muktakeshi roots

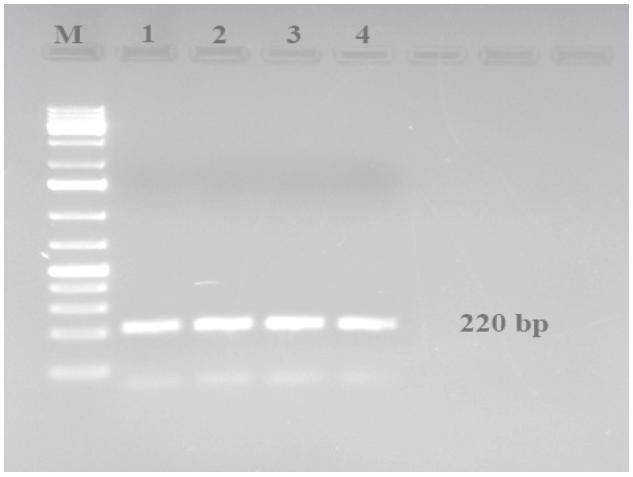

Fig.3. Confirmation of *P. indica* colonization by PCR using PiTEF1 primers. M: 1Kb plus marker, Lane 1 & 2: Amplification from Sree Kiran roots, Lane 3 & 4: Amplification from Muktakeshi roots.

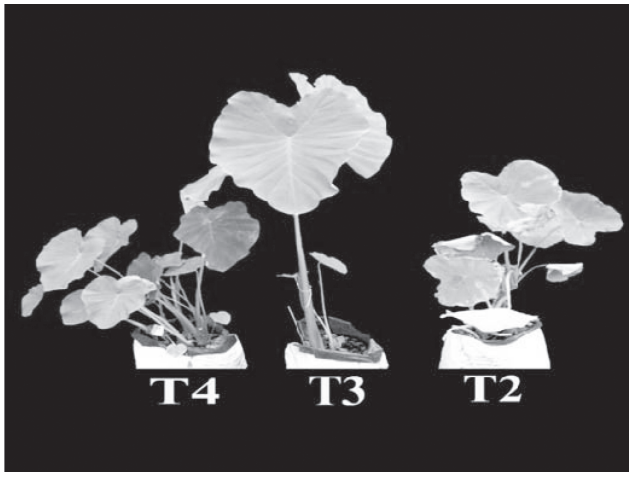

Fig.4. Comparison of disease incidence between Sree Kiran control (T4), Sree Kiran + *P. indica* + *P. colocasiae* (T3) and Sree Kiran +*P. colocasiae* (T2)

Growth promoting and disease suppressing effect of *P. indica* in taro varieties

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.* The fungus has not shown any negative effect on the plant growth or any other parameters studied. Sahay et al. (1998) and Singh (2004) observed that maize plants exhibited enhanced growth upon inoculation with *P. indica*. Treatment with *P. indica* enhanced seed germination and increased seed production in *Oryza sativa, Brassica oleracea, Tridax procumbans* etc. (Kumari *et al.,* 2004). The severity of the disease was recorded 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 (Fig 4. and Fig 5.). The PDI reduction (Fig. 6) was more in susceptible variety, Sree Kiran. During the different stages of infection, 57.6, 50.7and 84.3 % decrease in PDI were observed in *P. indica* colonized Sree Kiran when compared to the control plants. In the case of Muktakeshi, reduction in PDI was 39.9, 56.2 and 72.5% over control. Pre-treatment of Arabidopsis roots with *P.indica* could protect plants from *Verticillium* infection, the plants survived better and the production of *Verticillium dahliae* microsclerotia was dramatically reduced (Sun et al., 2014). *The P.indica* colonized plants were more resistant to *Blumeria graminis* infection in shoots and *Fusarium culmorum* in roots (Waller et al. 2005).

Defense enzyme quantification

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 in the T3 plants (Sree Kiran + *P. indica* + *Phytophthora colocasiae*). The highest activity was noted at 6 hpi (9.6 mg sugar released / g tissue) and the least activity was at 48 hpi (4.05 mg sugar released / g tissue). The glucanase enzyme activity 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 zero hour till 48 hour. In T3, the maximum activity was recorded at 24th hour (1.91 mg sugar released / g tissue) and in T7; maximum activity was obtained at 6th hour (1.686 mg sugar released/ g tissue). Eventhough the PAL activity was found to be increased in T3 plants during 3rd and 6th hpi, no significant increase was observed in T7 plants. The peroxidase activity was also not increased in the *P. indica* colonized plants. As a non-specific line of defense, the total phenol content 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 and 12 hour after infection. 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

Fig.5. Comparison of disease incidence between Mukthakeshi control (T8), Mukthakeshi + *P. indica* + *P. colocasiae* (T7) , Mukthakeshi +*P. colocasiae* (T6)

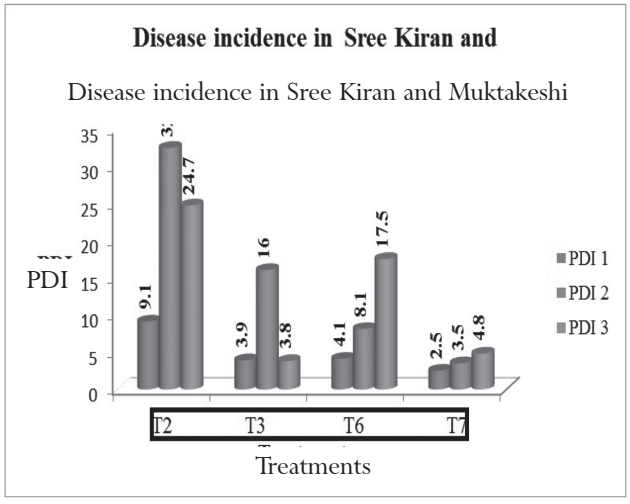

Fig.6. Percentage disease index of Sree Kiran and Muktakeshi. T2 - Sree Kiran+ *P. colocasiae*, T3 - Sree Kiran + *P. indica* + *P. colocasiae*, T6 - Muktakeshi+ *P. colocasiae*, T7 - Muktakeshi+ *P. indica* + *P. colocasiae*

antioxidant enzymes like catalase, peroxidase, superoxide dismutase, glutathione reductase and glutathione S transferases were increased in the colonized plants upon pathogen infection. The induction of these defense enzymes by *P.indica* may be interfering in pathogen establishment and resisting the spread of the pathogen.

Induction of defence- related genes in colonized plants

Differentially expressed genes in *P. indica* colonized taro plants upon infection with *P. colocasiae,* was identified by adopting suppression subtractive hybridization. Various genes that take part in different defense related pathways were obtained upon annotation. The sequencing result obtained was annotated using Blast2Go software. The sequences were analyzed and the cellular, molecular and biological functions were determined. Five genes were identified and their functions were determined out of which four were coming under different defense related pathways. The genes identified are senescence associated genes, cytochrome P450, Delta (12) oleic acid desaturase FAD2, calcium-dependent protein kinases (CDPKs). 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. 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). These genes are expressed in plants during various biotic and abiotic stresses.

Conclusion

The utilization of *P.indica* in the present study to promote growth as well as suppress pathogenic fungi was the first study in tuber crops. The establishment of root

colonization of taro roots by the endophytic fungus, *P. indica* provides one more option to tackle the most devastating disease of taro. *P. indica* was able to promote growth and increased yield in both the varieties, Sree Kiran and Muktakeshi of taro. *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. The chitinase and glucanase activity were found increasing in initial hours of pathogen attack. Upon suppression subtractive hybridization, five genes that indirectly take part in defense responses were identified. The major functions of the identified genes were, production of phytoalexins, programmed cell death of the pathogen infected cells of plants, activation of enzymes in various pathways like Jasmonate, ethylene signalling and finally activate the Pathogenesis Related (PR genes). Studies on compatibility of *P*. indica with other management strategies will help in including *P. indica* in IDM strategies for taro leaf blight management in future.

Acknowledgement

The authors are grateful to Amity Institute of Microbial Sciences, Amity University for providing the culture of *P.indica*. They are also thankful to Dr. S. K. Chakrabarti, Director, ICAR- Central Potato Research Institute, Shimla and Dr. G. Padmaja, Emeritus Scientist, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram for the help and support provided throughout the work.

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