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Exploration of Endophytes from Tropical Tuber Crops against *Colletotrichum gloeosporioides* Causing Anthracnose in Greater Yam (*Dioscorea alata* L.) *In Vitro*

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Abstract

The study was aimed to identify and characterize the endophytes from tropical tuber crops against *Colletotrichum gloeosporioides* causing anthracnose in greater yam. The pathogen was isolated from anthracnose infected greater yam leaves and its virulence and identity were confirmed. The fungal and bacterial endophytes were isolated from greater yam (vars. Sree Keerthi, Sree Karthika and acc. nos., Da 251 and Da 262), taro (Muktakeshi), Chinese potato var. and arrow root var. Endophytes were screened against the pathogen by dual culture method. Among 139 endophytes isolated, morphologically distinct 37 bacterial and 37 fungal endophytes were selected for *in vitro* screening. Three bacterial endophytes with antifungal index of 88.6, 85.7 and 84.6% and four fungal endophytes with antifungal index of 67.1, 55.7, 49.3 and 45.3% were obtained. Potential bacterial and fungal endophytes were identified using 16S rRNA and ITS1 - ITS4 primers, respectively. Using molecular techniques, the potential endophytes were identified. Among the three bacterial endophytes, one was *Bacillus cereus and* two were *Bacillus subtilis.* The fungal endophytes were identified as *Penicillium citrinum, Phanerochaete australis, Curvularia pseudobrachyspora and Diaporthe batatas*. There is great potential in utilizing the endophytes such as *Bacillus cereus* and *Bacillus subtilis* for managing the disease. This could be a novel, ecofriendly and effective biocontrol strategy for managing anthracnose disease in greater yam.

Key words: *Dioscorea alata*, anthracnose, endophytes, biocontrol

Introduction

Yam (*Dioscorea)* is a monocotyledonous polyploid and clonally propagated crop under the family Dioscoreaceae. The important consumable yam species include *D. alata* L., *D. esculenta*, *D. rotundata Poir.*, *D. cayenensis*, *D. dumetorum*,., *D. bulbifera* L., and *D. trifida*. Among these, *Dioscorea alata* had more advantages compared to others because of its high yield, ease of propagation (through generation of bulbils and reliability of sprouting), early growth for weed suppression, and long storability of tubers. Moreover, the low glycemic file and high fiber content makes greater yam a useful, potential and nutritionally appreciated crop (Sari et al., 2013).

One of the major constraints in yam production could be the fungal infestation which causes a great loss on the production of yam worldwide and anthracnose is the major among them. Anthracnose disease of yam is caused by the fungus, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., an asexual facultative parasite belonging to the family Phyllachoraceae of the division Ascomycota. Anthracnose is a "complex disease" having the presence of various pathogenic fungi on the yam phylloplane which is required for the development of significant symptoms (Amusa et al., 1997). Yet, Abang (2003) reported that a

single isolate of *C. gloeosporioides* can cause 100% leaf abscission and premature death of up to 76% of inoculated plants under controlled environment conditions. The symptom appear as small brown lesion on young leaves which later on turn to dark brown or black spots which coalesces to large spots leading to leaf blight on the adaxial surface on leaf lamina (Winch et al., 1984). The disease occurrence is more during rainy season, as rain flashes spread their spores (conidia) to nearby areas resulting in maximum disease incidence.

Anthracnose can be controlled by adopting two management strategies by growing resistant varieties and use of chemicals. Chemical method is not effective during long rainy periods, difficult and cost intensive. Development of resistant variteies needs more time frame. Growing resistant varieties requires generation of disease-free plants which takes time. The combination of fungicide (carbendazim) and biocontrol agent (*Trichoderma asperellum*) were reported to be effective in managing the disease by 66% (Jeeva et al., 2016). However, no other effective biointensive methods have been developed which is the major concern in context to pollution free environment.

The term endophyte (Gr. endon: within; phyton: plant) was first coined in 1866 by De Bary which was defined as a microorganism such as fungi or bacteria that expends either the complete or part of its lifecycle within any internal part of a living plant, whose infections are unobtrusive and infected host tissues are at least transiently symptomless (Stone et al., 2000). These endophytic microorganisms are promising sources of novel natural products for exploitation in agriculture, medicine, and industry. There is a lot of potential in utilizing endophytes to protect plants from diseases. Various reports suggest that these endophytes can have the ability to control plant pathogens (Krishnamurthy and Gnanmanickam, 1997) by various mechanisms like antibiosis, induction of systemic resistance etc.

Bacterial endophyte *Bacillus* spp. are known to produce a secondary metabolite like cyclic lipopeptides having antifungal activity Ongena and Jacques (2008). Endophytes, *B. cereus* and *B. thuringiensis* can adversely affect the growth of various pathogens like *Phytophtora*, *Pythium* spp etc. (Silo-Suh et al., 1998). Similarly, fungal endophytes produce various enzymes which play the crucial role in the biodegradation and hydrolysis processes which are vital mechanisms against pathogenic infection (Sunitha et al., 2013). The present study was therefore designed for the screening of potential endophytes from tropical tuber crops against *C. gloeosporioides* causing anthracnose in greater yam and identification through molecular method.

Materials and Methods

Isolation of *Colletotrichum gloeosporioides*

The pathogen was isolated from the symptomatic leaves of anthracnose infected greater yam (var. Orissa Elite) showing necrotic spots from different fields of ICAR-CTCRI. Leaves were washed under running tap water. Infected regions of 5 mm^2 sizes along with some healthy tissue were excised. A standard procedure of surface sterilization (Anjum and Chandra, 2015) was done with 2.5% sodium hypochlorite solution for two minutes followed by 70% alcohol for one minute and washed repeatedly thrice in sterile distilled water to remove the traces of sodium hypochlorite and alcohol. Then surface sterilized tissues were transferred to sterile Petri dishes containing potato dextrose agar (PDA) medium with 100 µl (100 mg ml^{-1}) ampicillin under aseptic conditions and incubated at 25 ± 2°C for 5-7 days in BOD incubator after removing water using sterile filter paper. After 7 days a loop full of fungal culture was taken on a glass slide and examined under light microscopy at 40X magnification for the presence of conidia and conidiophore. After confirmation, the cultures were purified by single spore isolation technique and maintained as different isolates on PDA slants.

Pathogenicity testing and molecular confirmation of pathogen

The isolates of the pathogen were tested on detached healthy greater yam leaves (var. Orissa Elite) in order to find the virulent one. For pathogenicity test, 15 µl of spore suspension ($5x10^5$ spores ml^{-1}) was sprayed on the leaf surface and incubated at 26°C in BOD incubator for 8 days (Hong and Hwang, 1998). To maintain humidity for the development of disease, sterile distilled water was sprinkled and the moistened filter paper was placed on the base and inner surface of the lid and it was observed daily for symptom development.

Genomic DNA extraction

The virulent isolate selected by pathogenecity test was grown for two days in a potato dextrose broth (PDB) and the mycelia were harvested. The total genomic DNA

was extracted from the harvested mycelia using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee (1996). The purity and quantification of the total DNA were measured using NanoDrop spectrophotometer (DeNovix DS-11) and agarose electrophoresis. Then DNA was stored at -20°C until further use.

PCR amplification using species specific primers

The Primer pairs CgsF1 (GGCGGGTAGGGT CTCCGTGAC) / CgsR1 (TTTGAGGGC CTACATC AGCT) (Raj et al., 2013) were used for PCR to confirm the pathogen as *C. gloeosporioides*. PCR assays were performed in an automated temperature cycling device (Agilent tech.), using 2 µl of total DNA, 2.5 µl of 10X KAPA Taq buffer (with 25 mM $MgCl_2$) and 0.5 µl of dNTP mixture (10 mM), 1 µl of each primer (10 µM), 0.2 µl of KAPA Taq DNA Polymerase (5U/µl) along with 18.8 µl PCR grade water in a total volume of 25 µl. The amplification cycling program consisted of a 2-min initial denaturation at 94°C, followed by 35 cycles of a 30 sec at 94°C, a 40 sec annealing at 62°C, and a 40 sec extension at 72°C, with a final extension at 72°C for 5 min. Amplified products were resolved on a 1.5% agarose gel stained with ethidium bromide (0.5 µl $ml^{-1)}$ and image was visualized by Gel Doc System (Alpha Innotech Corporation, San Leandro, CA, USA).

Isolation of bacterial and fungal endophytes

The leaves, stem and root of greater yam (var. Sree Keerthi, Sree Karthika, and Acc. Nos., Da 251 and Da 262), taro (Muktakeshi), Chinese potato and arrow root were collected for the isolation of endophytes. The samples were excised into small sections (5 mm^2 long) using a sterile scalpel. The endophytes were isolated from surface disinfected samples for the elimination of epiphytic microorganisms. The surface disinfection was performed using the standard procedure (Anjum and Chandra, 2015) with slight modifications in the following steps: washing in distilled water followed by sodium hypochlorite 4% (v/v) (2 min), ethanol 70% (1 min) and two washings with sterile distilled water. Each isolation procedure was done in triplicate for each cultivar. Bacterial endophytes were isolated by placing the samples in nutrient agar (NA) incubated at 37°C for 24 and fungal endophytes were isolated in potato dextrose agar (PDA) containing ampicillin (100 mg ml^{-1}), incubated at 26°C for 5 days. To confirm the disinfection protocol, aliquots of the sterile water used in the final rinse were plated in NA and PDA and the plates were examined for the presence or absence of microorganisms which serve as the control. The endophytes obtained were pure cultured and morphological characteristics such as colony color, shape, margin, optical property, texture and growth rates were recorded.

In vitro screening of endophytes against *Colletotrichum gloeosporioides*

The isolated endophytes were screened against the pathogen by dual culture technique (Cherif *and* Benhamou, 1990) in PDA. The control plate was maintained without antagonist. The treatments were replicated thrice and incubated at 28 ± 2 °C. The growth of the pathogen towards the endophyte antagonist and inhibition zone was measured until control plates attained full growth.

The growth inhibition of the pathogen was calculated by using the following formulae (Bae et al., 2011)

Growth inhibition (%) $GI = [(dc - dt)/dc] \times 100$, where dc and dt represent the fungal growth diameter in control and treated sample respectively. Based on the growth inhibition the potential bacterial and fungal endophytes were identified.

Molecular identification of potential endophytes

Genomic DNA extraction

The genomic DNA extraction of the potential bacterial and fungal endophytes were done as described by Wilson (2001) and Knapp and Chandlee (1996), repectively. The potent bacterial isolates were grown overnight in nutrient broth and pelleted by centrifugation at maximum speed of 12000 rpm for 5 min. The pellets were then washed twice with TE buffer (10-mM Tris-Cl, 1-mM EDTA, pH 8.0). The total genomic DNA of the isolated strains was extracted using the SDS denaturing method. The potent fungal endophytes were grown in potato dextrose broth for 2 days and the DNA was isolated from the mycelial mat obtained after incubation. The quantity and the purity of the total DNA were measured using NanoDrop spectrophotometer (DeNovix DS-11) and agarose electrophoresis. Then DNA was stored at -20 °C until further use.

PCR amplification of potent endophytes

PCR amplification of bacterial DNA

To identify the potent bacteria using 16S rRNA targeting primers, 16S - 23S rRNA intervening sequence 8F (5' AGA GTT TGA TCC TGG CTC AG 3'), 1492R (5' CGG CTA CCT TGT TAC GAC TT 3') primers were used (Frank et al., 2008). The PCR mix consisted of 2 µl of total DNA, 2.5 µl of 10X KAPA Taq buffer (with 25 mM $MgCl_2$), and 0.5 µl of dNTP mixture (10 mM), 1 µl of each primer (10 µM), 0.2 µl of KAPA Taq DNA Polymerase (5U μl^{-1}) , 18.8 µl of PCR grade water, in a total volume of 25 µl. The amplification cycling program was initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 90°C for 20 sec, annealing temperature at 55.5°C for 45 seconds and extension at 72°C for 1.30 min. The final extension was carried out at 72°C for 8 min.

The PCR amplification was also done using RecA primers, RecAF: 5'TGAGTGATCGTCAGGCAGCCT 3'and RecAR: 5' TTCTTCATAAGAATACCACG AACCGC 3' (Guo et al., 2015) to confirm species of potent bacteria. The PCR mixture was optimized as 2 µl template DNA,12.5 µl of 2X PCR Master Mix (Emerald Amp GT PCR TAKARA BIO INC), 0.5 µl of each primer (10 µM), and 9.5 µl of PCR - grade water in a total volume of 25 µl. The annealing temperature was optimized by gradient PCR. The amplification cycling program was initial denaturation at 95°C for 2 min followed by 35 cycles denaturation at 90°C for 20 sec, annealing temperature at 55-66°C for 30 seconds and extension at 72°C for 1.30 min. The final extension was carried out at 72°C for 8 min. The PCR amplified products along with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

PCR amplification of fungal DNA using ITS1 and ITS4 targeting primers

The ITS1-5.8S-ITS2 region of ribosomal DNA from antagonist fungal endophytes were amplified with ITS1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3') primers (White et al., 1990). PCR mixture used was as same as described earlier. The samples were amplified using the PCR conditions initial 94°C for 3 min followed by 35 cycles denaturation at 94°C for 30 seconds, annealing temperature at 53°C for 30 seconds and extension at 72°C for 1 min. The final extension was carried out at 72°C for 8 min. The PCR amplified products were resolved on agarose gel (1.5%) and viewed using Alpha Imager.

All the amplicons from bacterial endophytes and fungal endophytes were purified and sequenced by Agri Genome (Kerala). Sequences were aligned using Geneious pro 11.1.4 software, and nBLAST searches were performed using the GenBank Internet server (http://www.ncbi.nlm.nih.gov) for comparison with other isolates deposited in the public databases, to identify the species taxon of each isolate. Sequences that showed more than 98% similarity were considered as belonging to the same taxonomic unit. The sequences attained were deposited to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) to allow public access.

Statistical analysis

The data were statistically analyzed using The SAS System. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) Duncan's multiple range tests were used to assess the differences among the factor levels studied. Diameters of inhibition zone values in graphs are presented in the form of mean ± standard deviation (SD).

Results and Discussion

Isolation of pathogen

The pathogen was isolated from the infected leaves of greater yam and the mycelia growth was observed after two days of incubation in PDA. The isolate was identified and confirmed as *Colletotrichum gloeosporioides* based on the morphological descriptions of the conidia under 40X magnification in light microscopy (Fig. 1).

Pathogenicity tests indicated that all the isolates caused similar anthracnose symptoms and the most virulent one was selected. Leaves were inoculated with *C. gloeosporioides* at a concentration of 5×10^5 spores $ml^{-1.}$ The symptoms were initiated after three days of inoculation.

Molecular confirmation of pathogen

The DNA from the pathogen having a concentration of 350 ng μl^{-1} was subjected to PCR amplification. The PCR reaction using species specific primers pairs, CgsF1

(GGCGGGTAGGGTCTCCGTGAC)/ CgsR1 (TTTGA GGGCCTACATCAGCT) (Raj et al., 2012) yielded amplicons of ~300 bp when resolved on agarose gel which confirmed that the isolate was *C. gloeosporioides*

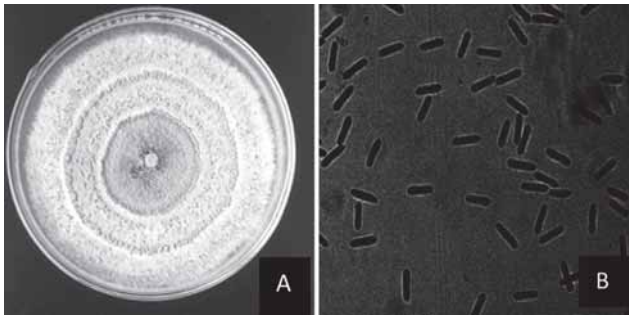

Fig. 1. (A) Seven days old culture of *C. gloeosporioides* on PDA media. (B) Spores of *C. gloeosporioides* observed under microscope (40X).

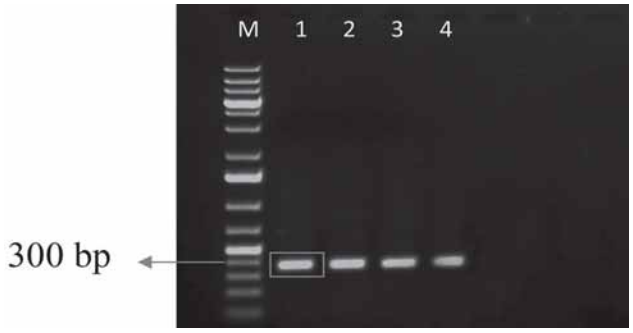

Fig. 2. PCR amplification of *C. gloeosporioides* using specific primer M- kb plus ladder. Lane 1-4 PCR product of *C.gloeosporioides*

Isolation of bacterial and fungal endophytes

Endophytes were isolated from various tuber crops such as greater yam, Chinese potato, arrow root and taro. A total of 139 endophytes were isolated, of which 65 were bacterial and 74 fungal endophytes. These endophytes were morphologically distinguished and distinct isolates of 37 bacterial and 37 fungal endophytes were selected. The morphological characteristics and growth rate of selected bacterial endophytes and fungal endophytes are shown in Table 1 and Table 2 respectively.

The deleterious effects of certain pathogenic organisms prevented by endophytes were reported by many researchers (Khare et al., 2018; Kumar et al., 2017). Berg et al. (2005) reported that endophytes from potato plants showed antagonistic activity against several fungi and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas.* Some of the endophytic isolates are reported to produce antibiotics and siderophores (Ajit Kumar Passari et al., 2016). Endophytic bacteria interact more closely with the host plant and therefore, could be an efficient biological control agent in sustainable crop production. An important step in isolation of endophytes is surface disinfection of plant parts which aims to remove the external microorganisms. The sterilized water used for surface disinfection was cultured on NA and PDA plates. If there were no colonies found within 3 days, then

Table 1. Morphological characteristics of bacterial endophytes

Sl no.	Name of isolates	Shape	Margin	Elevation	Size	Optical property	Grams reaction
1	DaSkL1	Circular	Entire	Flat	Small	Opaque	Positive
2	DaSkL2	Circular	Entire	Flat	Small	Opaque	Positive
3	DaSkL3	Circular	Entire	Raised	Small	Opaque	Positive
4	DaSkS1	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive
5	DaSkR1	Circular	Entire	Flat	Small	Opaque	Positive
6	DaSiL1	Irregular	Lobate	Flat	Moderate	Opaque	Positive
7	DaSiR1	Rhizoid	Lobate	Flat	Moderate	Opaque	Positive
8	Da251R1	Irregular	Undulate	Flat	Moderate	Opaque	Positive
9	Da251R2	Rhizoid	Lobate	Flat	Moderate	Opaque	Positive
10	Da262L1	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive
11	Da262L2	Circular	Entire	Raised	Small	Opaque	Positive
12	Da262S1	Circular	Entire	Flat	Punctiform	Transparent	Positive
13	Da262S2	Circular	Entire	Convex	Small	Opaque	Negative
14	Cs1L1	Circular	Entire	Flat	Small	Opaque	Negative
15	Cs1R1	Circular	Entire	Flat	Punctiform	Opaque	Positive
16	Cs2S1	Circular	Entire	Raised	Small	Opaque	Positive
17	Cs3S1	Irregular	Undulate	Flat	Small	Opaque	Positive
18	Cs3S2	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive

19	Cs3R1	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive
20	SrL1	Circular	Entire	Convex	Small	Opaque	Positive
21	SrL2	Irregular	Lobate	Flat	Moderate	Opaque	Positive
22	SrL3	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive
23	SrL4	Irregular	Lobate	Flat	Small	Opaque	Positive
24	SrS1	Circular	Entire	Flat	Small	Transparent	Negative
25	SrS2	Circular	Entire	Flat	Punctiform	Opaque	Positive
26	SrR1	Irregular	Lobate	Flat	Small	Opaque	Positive
27	SrR2	Rhizoid	Rhizoid	Flat	Moderate	Opaque	Positive
28	SrR3	Irregular	Lobate	Flat	Small	Opaque	Positive
29	SrR4	Circular	Entire	Raised	Moderate	Opaque	Positive
30	SrR5	Circular	Entire	Raised	Small	Opaque	Positive
31	SrR6	Circular	Entire	Convex	Small	Opaque	Positive
32	MaL1	Circular	Entire	Flat	Punctiform	Opaque	Positive
33	MaS1	Circular	Entire	Raised	Small	Opaque	Positive
34	Ma R1	Circular	Entire	Raised	Small	Opaque	Negative
35	Ma R2	Irregular	Lobate	Flat	Small	Opaque	Positive
36	Ma R3	Circular	Entire	Flat	Small	Opaque	Positive
37	Ma R4	Circular	Entire	Flat	Small	Opaque	Positive

Table 2. Morphological characteristics of fungal endophytes

Sl no.	Name of isolates	Shape	Color	Size on 5th day	Growth rate	Texture
1	DaSkFL1	Round	Creamy white	5	Medium	Cottony
2	DaSkFL2	Irregular	Greyish white	7.2	Fast	Powdery
3	DaSKFL3	Irregular	Greenish white	6.3	Fast	Cottony
4	DaSkFS1	Irregular	Creamy white	6.2	Fast	Cottony
5	DaSkFR1	Irregular	Greyish white	4	Slow	Cottony
6	DaSiFL1	Irregular	Greyish white	5	Medium	Powdery
7	DaSiFS1	Irregular	Blackish brown	4.5	Slow	Cottony
8	DaSiFS2	Irregular	Creamy white	5.4	Medium	Cottony
9	DaSiFR1	Irregular	Greyish white	7.3	Fast	Cottony
10	Da251FL1	Round	Greyish white	5	Medium	Powdery
11	Da251FR1	Round	Greyish white	6.4	Fast	Powdery
12	Da251FR2	Round	Creamy white	7.3	Fast	Cottony
13	Da262FS1	Round	Blackish brown	5.8	Medium	Cottony
14	Da262FS2	Round	Greyish white	5	Medium	Cottony
15	Da262FS3	Irregular	Greyish white	5	Medium	Cottony
16	Cs1FL1	Round	Greyish white	5.4	Medium	Powdery
17	Cs1FS1	Round	Creamy white	5	Medium	Cottony
18	Cs1FR1	Irregular	Blackish brown	7.2	Fast	Powdery
19	Cs2FL1	Round	White	7.4	Fast	Cottony
20	Cs3FS1	Irregular	Blackish brown	6.4	Fast	Cottony
21	SrFL1	Irregular	Greyish white	7.2	Fast	Powdery
22	SrFL2	Irregular	White	5	Medium	Cottony
23	SrFS1	Irregular	Creamy white	7.4	Fast	Powdery
24	SrFS2	Irregular	Greyish white	5.3	Medium	Cottony
25	SrFS3	Round	Creamy white	6.4	Fast	Cottony
26	SrFS4	Round	Creamy white	5	Medium	Cottony
27	SrFS5	Round	Creamy white	7	Fast	Cottony
28	SrFS6	Round	Greyish white	7.4	Fast	Powdery
29	SrFR1	Round	Blackish brown	6.4	Fast	Cottony
30	SrFR2	Round	Greyish white	5.4	Medium	Powdery
31	MaFL1	Round	White	6.4	Fast	Cottony

32	MaFL2	Irregular	Greyish white	5	Medium	Cottony
33	MaFS1	Irregular	Greyish white	5.1	Medium	Cottony
34	MaFS2	Irregular	Greyish white	5.7	Medium	Powdery
35	MaFR1	Round	Creamy white	4.9	Slow	Powdery
36	MaFR2	Irregular	Creamy white	5	Medium	Powdery
37	MaFR3	Irregular	Creamy white	5	Medium	Cottony

disinfection procedure was done appropriately to confirm that the isolates obtained were endophytes.

In vitro screening of endophytes against *Colletotrichum gloeosporioides*

The antagonistic effects of bacterial and fungal endophytes on *C. gloeosporioides* were calculated as inhibition of mycelial growth by dual culture method. Mycelial inhibition of bacterial endophytes is presented in Table 3 and Fig. 3 and fungal endophytes in Table 4 and Fig. 4. Among the endophytes screened, the bacterial isolate from arrow root leaf (MaL1), Chinese potato stem (SrS1) and root (SrR2) exhibited maximum antifungal index of 88.6%, 85.7% and 84.6% over control,

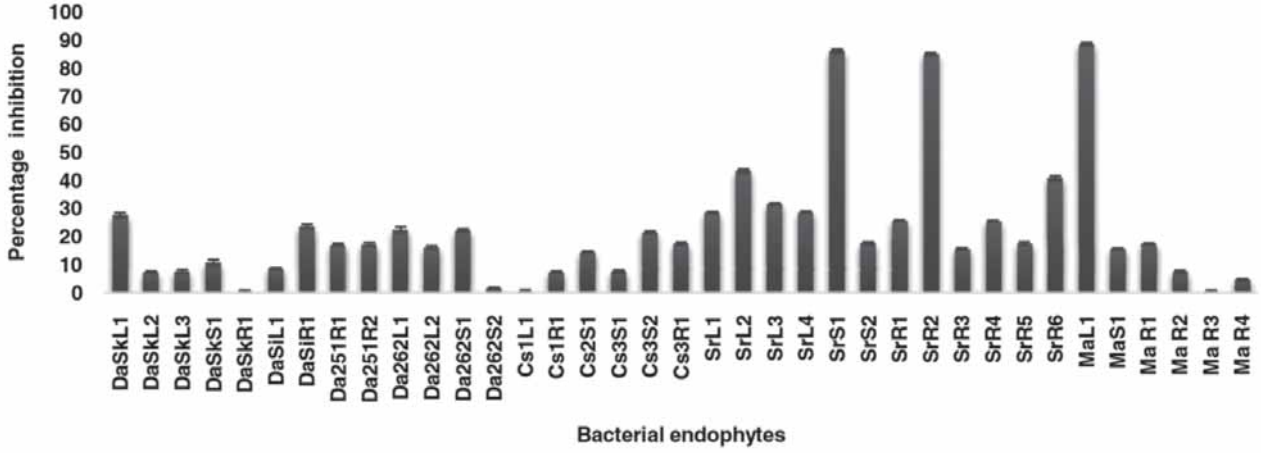

Fig. 3. Percentage inhibition of bacterial endophytes on pathogen

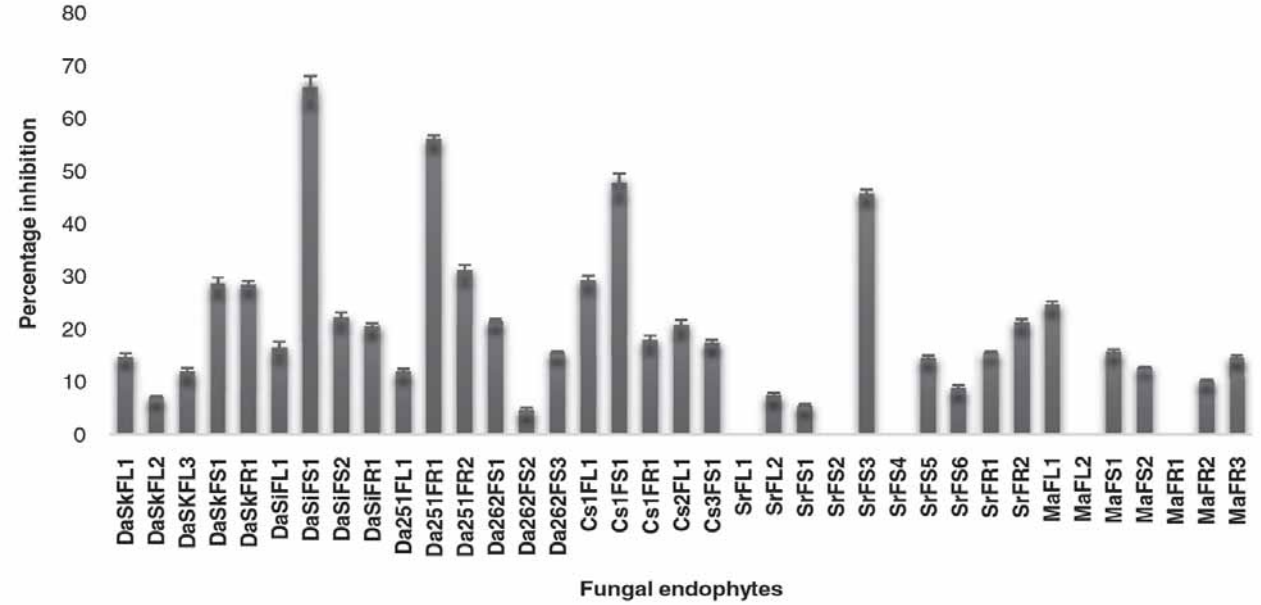

Fig. 4. Percentage inhibition of fungal endophytes on pathogen

Table 3. Mycelial inhibition of bacterial endophytes

Sl no.	Name of isolate	*Percentage inhibition** ± standard deviation	*Transformed values
1	DaSkL1	27.6^{I} ±0.81	0.55^{GF}
2	DaSkL2	7.4^{R} ± 0.28	0.27^{M}
3	DaSkL3	7.6^{R} ± 0.60	0.28^{M}
4	DaSkS1	10.7^{P} ± 1.07	0.3^{L}
5	DaSkR1	0.37^{U} ± 0.5	0.03^{P}
6	DaSiL1	8.7^{Q} ± 0.17	0.29^{M}
7	DaSiR1	23.7^{K} ± 0.72	0.50^{HI}
8	Da251R1	17.13^{M} ± 0.41	0.42^{J}
9	Da251R2	17.23^{M} ± 0.58	0.42^{J}
10	Da262L1	22.5^{L} ± 1.05	0.49^{I}
11	Da262L2	16.2^{N} ± 0.50	0.41^{KJ}
12	Da262S1	22.3^{L} ± 0.52	0.49^{I}
13	Da262S2	1.6^{T} ± 0.25	0.12^{O}
14	Cs1L1	0.3^{U} ± 0.63	0.03^{P}
15	Cs1R1	7.4^{R} ± 0.28	0.27^{M}
16	Cs2S1	14.5^{O} ± 0.25	0.39^{K}
17	Cs3S1	7.6^{R} ± 0.46	0.27^{M}
18	Cs3S2	21.6^{L} ± 0.30	0.48^{I}
19	Cs3R1	17.5^{M} ± 0.45	0.43^{J}
20	SrL1	28.6^{H} ± 0.25	0.5^{F}
21	SrL2	43.5^{E} ± 0.56	0.72^{D}
22	SrL3	31.6^{G} ± 0.25	0.59^{E}
23	SrL4	28.8^{H} ± 0.20	0.56^{F}
24	SrS1	86.2^{C} ± 0.61	1.19^{C}
25	SrS2	17.6^{M} ± 0.46	0.43^{J}
26	SrR1	25.7^{J} ± 0.11	0.53^{GH}
27	SrR2	85.1^{D} ± 0.5	1.17^{C}
28	SrR3	15.6^{N} ± 0.25	0.40^{KJ}
29	SrR4	25.6^{J} ± 0.17	0.53^{GH}
30	SrR5	17.5^{M} ± 0.49	0.43^{J}
31	SrR6	40.9^{F} ± 0.79	0.69^{D}
32	MaL1	88.6^{B} ± 0.40	1.22^{B}
33	MaS1	15.6^{N} ± 0.20	0.40^{KJ}
34	Ma R1	17.4^{M} ± 0.26	0.43^{J}
35	Ma R2	7.5^{R} ± 0.5	0.27^{M}
36	Ma R3	0.3^{U} ± 0.57	0.03^{P}
37	Ma R4	4.6^{S} ± 0.3	0.21^{N}
38	control	0	

*mean of three replicates **Means with the same letter are not significantly different ($P < 0.05$)

Table 4. Mycelial inhibition of fungal endophytes

Sl no.	Name of isolate	*Percentage inhibition** ± standard deviation	*Transformed values
1	DaSkFL1	14.9NO ± 0.52	0.39NOP
2	DaSkFL2	7.1S ± 0.2	0.26T
3	DaSKFL3	12P ± 0.6	0.35Q
4	DaSkFS1	28.8G ± 0.98	0.56G
5	DaSkFR1	28.6G ± 0.57	0.56G
6	DaSiFL1	16.5LM ± 1.11	0.41LM
7	DaSiFS1	66B ± 2.08	0.94B
8	DaSiFS2	22.3I ± 0.87	0.49I
9	DaSiFR1	20.9J ± 0.57	0.47J
10	Da251FL1	11.9P ± 0.47	0.35Q
11	Da251FR1	56.2C ± 0.62	0.84C
12	Da251FR2	31F ± 1.00	0.59F
13	Da262FS1	21.5JI ± 0.32	0.48JI
14	Da262FS2	4.6T ± 0.35	0.21V
15	Da262FS3	15.5NMO ± 0.20	0.40MNOP
16	Cs1FL1	29.3G ± 0.75	0.57G
17	Cs1FS1	47.8D ± 1.69	0.76D
18	Cs1FR1	17.9K ± 0.76	0.43K
19	Cs2FL1	20.8J ± 0.80	0.47J
20	Cs3FS1	17.4LK ± 0.47	0.43KL
21	SrFL1	0U ± 0	0W
22	SrFL2	7.4S ± 0.41	0.27T
23	SrFS1	5.5T ± 0.20	0.23U
24	SrFS2	0U ± 0	0W
25	SrFS3	45.9E ± 0.57	0.74E
26	SrFS4	0U ± 0	0W
27	SrFS5	14.5O ± 0.40	0.39P
28	SrFS6	8.7R ± 0.47	0.30S
29	SrFR1	15.7NMO ± 0.10	0.40MNO
30	SrFR2	21.4JI ± 0.50	0.48IJ
31	MaFL1	24.8H ± 0.50	0.52H
32	MaFL2	0U ± 0	0W
33	MaFS1	15.8NM ± 0.37	0.40MN
34	MaFS2	12.8P ± 0.11	0.36Q
35	MaFR1	0U ± 0	0W
36	MaFR2	10.2Q ± 0.20	0.32Q
37	MaFR3	14.7NO ± 0.37	0.39OP
38	control	0	

*mean of three replicates **Means with the same letter are not significantly different.

respectively. The fungal endophytes from greater yam (var. Sree Keerthi) stem (DaSiFS1), (acc. no., Da251) Da251 root (Da251FR1), taro stem (Cs1FS1) and Chinese potato stem (SrFS3) had the antifungal index of 67.1%, 55.7%, 49.3% and 45.3% over control, respectively (Fig. 5 and Fig. 6). The mean antifungal index was observed maximum in bacterial endophytes compared to fungal endophytes. The most effective endophytes that inhibit pathogen growth in the dual culture showed large zone of inhibition. A microbial biocontrol agent may act through different mechanisms against pathogens by production of non-volatile antibiotics or metabolites etc during their antagonistic activity (Vinale et al., 2008).

Molecular identification of selected endophytes with potential activity

The DNA was isolated from potent bacterial and fungal endophytes by SDS and CTAB method. PCR amplified using 16S rRNA targeting and RecA primers and yielded amplicons of 1500 bp and ~ 800 bp, respectively. The fungal DNA was amplified using ITS1 and ITS4 targeting primers which yielded ~ 750 bp. The PCR products were purified and sequenced. The sequencing results were obtained as electropherogram resulting from capillary sequencing in .ab1 and FASTA format and sequences were alligned. After edition the sequences were run through the online BLAST (BLASTn) program of NCBI. Among three potent bacterial sequences, one was *Bacillus cereus* and the other two were *Bacillus subtilis*. The four potent fungal isolates, viz., DaSFS1, Da251FR1, Cs1FS1, SrFS3 were identified as *Penicillum citrinum*, *Phaenrochaete australis*, *Curvularia pseudobrachyspora* and *Diaporthe batatas*, repectively.

In the present study, two bacterial endophytes such as *Bacillus cereus* and *Bacillus subtilis* and four endophytic isolates *Penicillium citrinum*, *Curvularia pseudobrachyspora*, *Diaporthe batatas* and *Phanerochaete australis* from tropical tuber crops had antagonist activity against *Colletotrichum gloeosporioides* causing anthracnose in greater yam *in vitro*. *Bacillus* endophytes were reported to colonize the internal

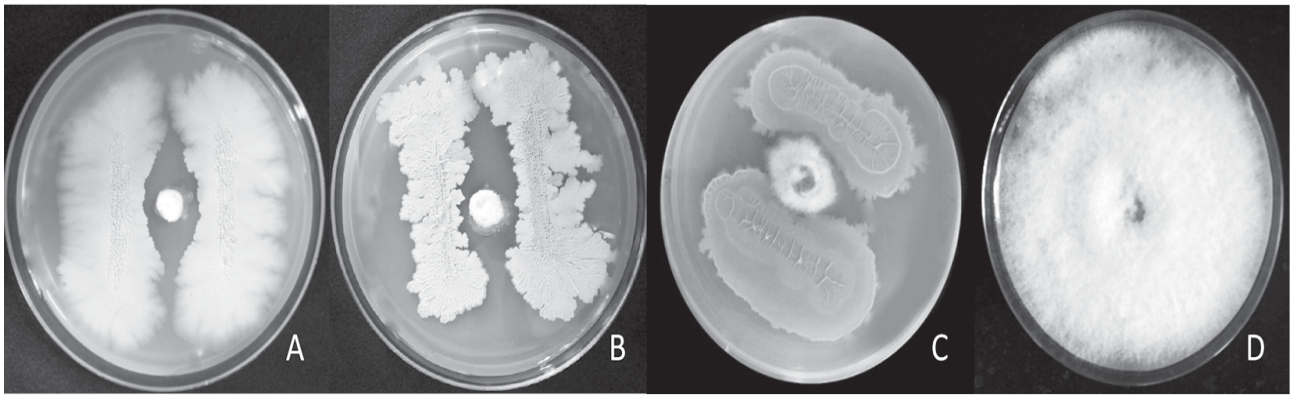

Fig. 5. Potent bacterial endophytes (A) Arrowroot leaf (MaL1) (B) Chinese potato stem (SrS1) (C) Chinese potato root (SrR2) (D) Control plate

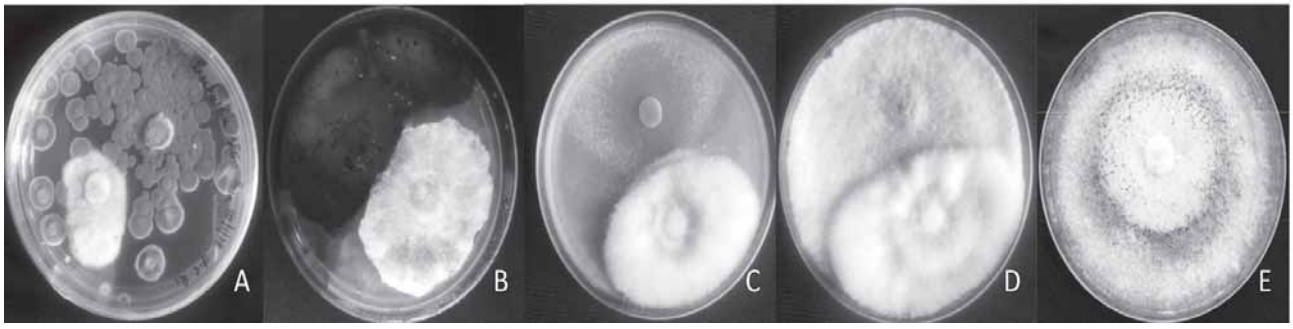

Fig. 6. Fungal endophytes having more than 45% inhibition (A) Greater yam (var. Sree Keerthi) stem (DaSiFS1), (B) Taro stem (Cs1FS1), (C) Chinese potato stem (SrFS3), (D) Greater yam (acc. no.) root (Da251R1) (E) Control plate

Table 5. Identified bacterial and fungal endophytes and their accession number

Sl no.	Isolate	Endophytes	Max score	Total score	Identity	Query cover	Accession number
1	MaL1	*Bacillus cereus*	1810	1810	100%	100%	MH747095
2	SrS1	*Bacillus subtilis*	1083	1695	91%	100%	MH747096
3	SrR2	*Bacillus subtilis*	856	1621	94%	100%	MH562713
4	DaSiFS1	*Penicillium citrinum*	232	232	100%	100%	-
5	Cs1FS1	*Curvularia pseudobrachyspora*	941	941	100%	100%	MH744769
6	SrFS3	*Diaporthe batatas*	863	863	99%	100%	MH744768
7	Da251R1	*Phanerochaete australis*	904	904	97%	100%	-

tissues of plants and found effective in the biocontrol of multiple plant diseases caused by soil borne pathogens and have been used in commercially available biocontrol products (Ongena et al., 2005). Zhang et al., (2010) isolated bacterial strains belonging to *Erwinia* and *Bacillus* species complex from yam rhizomes or tubers. However, *B. subtilis* and *B.cereus* have not been reported as biological control agents against anthracnose in greater yam. There are reports showing that *B. subtilis* and *B. cereus* were endophytes with various biological and biochemical properties which are potentially useful though the endophyte *B. cereus* have been poorly studied as biocontrol agent. Molecular techniques exhibit high sensitivity and specificity for identifying microorganisms. In the present study, the endophytic bacteria were identified using 16S rRNA primers while the fungal endophytes were identified using ITS1 and ITS4 primers. Many evidences have proved that identification of *B. subtilis* and *B.cereus* cannot rely on the limited information obtained from 16S rRNA gene analysis and biochemical and physiological assays (Oleg et al., 2004; Heather and Geraldine, 2011). Phylogenetic analysis of RecA gene, encoding the highly conserved subunit of the bacterial recombinase, proved to be significant marker for bacterial species identification (Zeigler, 2003). Milad et al.(2016) reported that RecA provided twofold more discrimination than 16S rRNA gene analysis of *B. subtilis* and *B. cereus*. In our study, *B. cereus* could be identified using RecA primers. The inhibition of *C. gloeosporioides* causing greater yam anthracnose by endophytes has not been studied yet. However, evidence can be noted in the results of Prapagdee et al. (2008) in which 53.77% prevention against *C. gloeosporioides* was obtained using *S. hygroscopicus* in Orchid plants. Shimizu et al. (2009), reported that several strains of *Streptomyces* spp. significantly reduced the number and size of spots on leaf of cucumber seedlings caused by anthracnose. Suspension of 10^8 and 10^9 cfu ml^{-1} of *Streptomyces* spp. caused reduction of disease by 79% and 93%, respectively. The gene sequences of potential endophytes, viz., *Bacillus cereus*, *Bacillus subtilis*, *Bacillus subtilis*, *Penicillium citrinum*, *Curvularia pseudobrachyspora*, *Diaporthe batatas*, *Phanerochaete australis* obtained in the present study were submitted in NCBI and accession numbers are shown in the Table 5.

Conclusion

The present study revealed that there is great potential for endophytes in tropical tuber crops which are beneficial to the plant by helping in growth promotion and also antagonistic to the pathogen associated with anthracnose disease of greater yam. These endophytes could be explored for improving greater yam growth and protecting from diseases. The endophytes could be utilized for ecofriendly management of the disease in field after evaluation. Moreover, it could also be utilized for various important diseases of tropical tuber and other crops.

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References

Abang, M. M., Stephan, W., Hodeba, D. M., Kim, R. G. and Robert, A. 2003. Molecular taxonomic, epidemiological and population genetic approaches to understanding yam anthracnose disease. *J. of Biotech*., **2**(12): 486-496.

Ajit Kumar Passari , Vineet Kumar Mishra, ,Vincent Vineeth Leo,Vijai Kumar Gupta, Bhim Pratap Singh. 2016. Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. *Microbiological Res.*, **93:** 57-73.

Amusa, N. A. and Ayinla, K. L. 1997. Screening cassava and yam cultivars for resistance to anthracnose using toxic metabolite of *Colletotrichum* species. *Mycological pathol*., **150**(3):137-142.

Anjum, N. and Chandra, *R. 2015*. Endophytic bacteria: optimizaton of isolation procedure from various medicinal plants and their preliminary characterization. *Asian J. Pharm. Clin. Res*., **8**(4): 233-238.

Bae, H., Roberts, D. P., Lim, H. S., Strem, M. D., Park, S. C., Ryu, C. M., Melnick, R. L. and Bailey, B. A. 2011. Endophytic Trichoderma isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant-Microbe Interact*., **24**(3): 336-351.

Berg, G., Eberl, L. and Hartmann, A. 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ. Microbiol*., **7**(11): 1673-1685.

Cherif, S. S. and Benhamou, C. S. 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* spp. on *Fusarium oxysporum* f. sp. radicans-lycopersici. *Phytopathol.*, **80**(2): 1406-1414.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. and Oslen, G. J. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA Genes. *Appl. And Environ. Microbiol.*, **74**(8): 2461-2470.

Guo, W., Cui, P. and Chen, X. 2015. Complete genome of *Bacillus* sp. Pc3 isolated from the Antarctic seawater with antimicrobial activity. *Mar. Genomics*., **20**: 1-2.

Heather, M. and Geraldine, V. A. 2011. Bacillus taxonomy in the genomic era finds phenotypes to be essential though often misleading. Infect. *Genet. Evol.*, **5**: 789-797.

Hong J K. and Hwang, B. K. 1998. Influence of inoculum density, wetness duration, plant age, inoculation method, and cultivar resistance on infection of pepper plants by *Colletotrichum* cocodes. *Plt. Dis*., **82**(10): 1079-1083.

Jeeva, M. L., Veena, S. S., Sheela, M. N., Jyothi, M. N. and Sreekumar, J. 2016. Management of anthracnose/die-back caused by *Colletotrichum gloeosporioides* in greater yam (*Dioscorea alata* L.) In: *NCTTC-2016 Conference Document. National Conference on Tropical Tuber Crops for the sustenenance and Welfare of Tribal Communities, 20-22*, October, 2016, ISRC and ICAR- Central Tuber Crops Research Institute, 38p.

Khare, E., Mishra, J. and Arora, N.K., 2018. Multifaceted interactions between endophytes and plant: developments and prospects. *Frontiers in microbiology*, **9**: 2732

Knapp, J. E. and Chandlee, J. M. 1996. RNA/DNA mini-prep from a single sample of orchid tissue. *Biotechniques.*, **21**: 54-56.

Krishnamurthy, K. and Gnanamanickam, S. S. 1997. Biological control of sheath blight of rice: induction of systemic resistance in rice by plant-associated *Pseudomonas spp. Curr. Sci*., **72**(5): 331–334.

Kumar, J., Singh, D., Ghosh, P. and Kumar, A., 2017. Endophytic and epiphytic modes of microbial interactions and benefits. In: *Plant-Microbe Interactions in Agro-Ecological Perspectives* Springer, Singapore., pp. 227-253.

Milad, M., Nezafat, N., Berenjian, A., Mobasher, M. A. and Ghasemi, Y. 2016. Identification of *Bacillus* probiotics isolated from soil rhizosphere using 16S rRNA, recA, rpoB gene sequencing and RAPD-PCR. *Probiotics* and *Antimicro. Prot*., **8**(1): 8-18.

Oleg, N. R., Christina, D., Johan, M. and Fergus, G. P. 2004. Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Microbiol. Ecol*., **48**: 249-259.

Ongena, M. and Jacques, P. 2008. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol*., **16**(3):115-125.

Ongena, M. D., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonar, P. 2005. *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Appl. Microbiol. Biot*., **67**(5): 692- 698.

Prapagdee, Benjaphorn, Akrapikulchart, U. 2008. Potential of a Soil-Borne *Streptomyces hygroscopicus* for biocontrol of anthracnose disease caused by *Colletotrichum gloeosporioides*. Orchid J. Biol. Sci., 8(10): 1187-1192.

Raj, M., Hegde, V., Jeeva, M. L., Senthil, M., Nath, V. S., Vidyadharan, P. and Archana, P. V. 2013. Molecular diagnosis of *Colletotrichum gloeosporioides* causing anthracnose / dieback disease in greater yam (*Dioscorea alata* L.). *Arch. of Phytopathol. and Plant Prot*., **46**(8): 927-936.

Sari, I. P., Lukitaningsih, E., Rumiyati, and Setiawan, I. M. 2013. Glycaemic index of uwi, gadung, and talas which were given on rat. *J. Trad. Med*., **18**(3): 127-131.

Shimizu, M., Yazawa, S. and Ushijima, Y. 2009. A promising strain of endophytic *Streptomyces* spp. for biological control of cucumber anthracnose. *J. Gen. Plant Pathol*., **75**: 27–36.

Silo-Suh, L. A., Stabb, E. V., Raffel, S. J. and Handelsman, J. 1998. Target range of Zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. *Curr. Micobiol*., **37**(1): 6–11.

Stone, J. K., Bacon, C. W. and White, J. F. 2000. An overview of endophytic microbes: endophytism defined. In: *Microbial endophytes. Bacon*, New York. pp. 3–30.

Sunitha, V. H., Nirmala, D. D. and Srinivas, C. 2013. Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. *World J. Agric. Sci*., **9**(1): 01–09.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Barbetti, M.J., Li, H., Woo, S.L. and Lorito, M., 2008. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathol*., **72**(1-3), pp.80-86.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. Innis M A, Gelfand D H , Sninsky J J and White T J (Eds.) New York. pp. 315-322.

Wilson, K. 2001. Preparation of Genomic DNA from Bacteria. *Curr. Protocols in Mol. Biol.*, **56**(1): 2.4.1-2.4.5.

Winch, J. E., Newhook, F. J. 1984. Studies of *Colletotrichum gloeosporioides* disease on yam, *Dioscorea alata*, in Solomon Islands. *Plant Pathol*., **33**(4): 467-477.

Zeigler, D. R. 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int. J. Evol. Micr.*, **53**(6): 1893-1900.

Zhang, Z. D., Xie, Y. Q., Chu, M., Gu, M. Y., Song, S. Q., Tang, Q. Y., Mao, J. 2010. Study on isolation and identification of endophytes in yam rhizome *J.* Xinjiang. *Agri. Sci*., **1**: 24.