IDENTIFICATION AND EVALUATION OF ENDOPHYTES FROM TROPICAL TUBER CROPS AGAINST *Colletotrichum gloeosporioides* (PENZ.) SACC. CAUSING ANTHRACNOSE IN GREATER YAM (*Dioscorea alata* L.)

by

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(2013-09-110)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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DECLARATION

I, hereby declare that this thesis entitled **"IDENTIFICATION AND EVALUATION OF ENDOPHYTES FROM TROPICAL TUBER CROPS AGAINST *Colletotrichum gloeosporioides* (PENZ.) SACC. CAUSING ANTHRACNOSE IN GREATER YAM (*Dioscorea alata* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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Dedicated to my dear parents

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LIST OF ABBREVATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
A230	Absorbance at 230 nm wavelength
A260	Absorbance at 260 nm wavelength
A280	Absorbance at 280 nm wavelength
BLAST	Basic local alignment search tool
bp	Base pair
C	Cytosine
cfu	Colony-forming unit
cm	centimetre
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
F	Forward primer
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
ITS	Internal Transcribed Spacer
Kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter

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M	Molar
mg	milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MS	Murashige and Skoog
Mt	Metric tonnes
NA	Nutrient Agar
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical density
ORF	Open reading Frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PVP	Polyvinyl pyrrolidine
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
sec	second
sp.	Species
spp.	Species (plural)
t	Tonnes
T	Thymine
Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride

U	Enzyme units
UV	Ultra violet
V	Volt
var.	Variety
v/v	volume/volume
w/w	weight/weight

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INTRODUCTION

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1. INTRODUCTION

Yams are the perennial herbaceous vine species in the genus *Dioscorea* (family Dioscoreaceae) which is cultivated for the consumption of their starchy tubers in many temperate and subtropical world areas form edible tubers. Yams are also a hostile plant, often considered a "harmful weed", outside cultivated areas. Yams are native to southeast Asia and introduced to Africa during 16th century. There are six important staple yam species that are white yam (*D. rotundata*), water yam (*D. alata*), yellow yam (*D. cayenensis),* trifoliate yam (*D. dumetorum*), aerial yam (*D. bulbifera*) and Chinese yam (*D. esculenta*) (Ng, 1994).

Dioscorea alata also known as white yam, water yam, and purple yam, which was first cultivated in Southeast Asia. Even though not fully-fledged as African yams, it has the prime dispersal worldwide of any other cultivated yam, being cultivated in Asia, the Pacific islands, Africa, and the West Indies (Mignouna, 2003). Its extended shelf life has been credited to the texture of its flesh, which is typically not as firm as that of the other species. (Dufie*et al.,* 2013).

D. alata is affected by various diseases, the most serious of which is anthracnose/blotch disease caused by fungus *Colletotrichum gloeosporioides.* The disease causes a yield reduction of up to 80%. Symptoms appear as leaf spots to extensive blackening or dark browning of leaves and stems, eventually plants may die. Symptoms differ conferring to the age of the leaf, the amount of rain and the variety of yam.

Plants can act as a pool of many microorganisms called as endophytes. Endophytes are defined as microorganisms (mostly fungi and bacteria) which can colonize internal plant tissues without producing any apparent harm or symptomatic infection to plant hosts for all or part of their life cycle (Strobel, 2003). Endophytes have

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several advantageous effects on their hosts including host growth promotion and act as biocontrol over phytopathogens.

Endophytic bacteria usually restricted in inthercellular spaces and xylem vessels of leaves, roots, seeds, stems, fruits, ovules, and tubers (Reinhold-Hurek and Hurek, 1998, Benhizia *et al.*, 2004). Numerous endophytic bacteria having biocontrol properties which can can obstruct disease symptoms caused by viral, insect, fungal, and bacterial pathogens (Berg and Hallmann, 2006). Though, it is expected that endophytic bacteria are more appropriate biocontrol agents since they sustainably get passed to the next generation.

Endophytic fungi are vastly diverse fungi of polyphyletic group, characteristically belonging to ascomycetes and anamorphic fungi (Arnold, 2007). Fungal endophytes can produce various biologically active metabolites during host-endophyte interaction (Strobel, 2003) and aid as potential sources of novel products for exploitation in medicine, agriculture and industry (Strobel and Daisy, 2004).

Current control of anthracnose comprises the use of one of, or a combination of, the following: resistant cultivars, cultural control and chemical control. Resistant greater yam cultivars might possibly form the basis of bearable management approach for anthracnose; yet, resistance breeding is hindered by worries about the variability of the pathogen (Green *et al.*, 2000). The exhaustive use of fungicides causes buildup of toxic compounds which might hazardous to humans and the environment, and also in the development of resistance of pathogens. In sight of this, identification and application of biological control agents (BCAs) appears one of the auspicious approaches (Cook, 1985). Biocontrol microorganisms are naturally occurring nonpathogenic microorganisms which can compete with the pathogen for nutrients or prevent pathogen multiplication by producing antibiotics or toxins, thereby reduce disease occurrence. Hence to develop a bio intensive management of the disease a preliminary work has been performed with the following objectives:

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- To isolate endophytes from different tropical tuber crops.
- To test the efficacy of the potential endophytes against *Colletotrichum gloeosporioides* causing greater yam anthracnose.
- Confirmation of colonization of endophytes in greater yam through biotechnological approaches.

REVIEW OF LITRATURE

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2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Roots and tubers add remarkably to sustainable development and income generation mainly in the tropical regions and considered as the most significant food crops following cereals. Cassava, potato, sweet potato and yam are considered as food security crop with high production potential. It could be grown on a extensive range of soils and can give pleasing yield even on acid soils. The major root and tuber crops are sweet potato, cassava, and yam which possess roughly 41.23 million hectares worldwide and generate 760.066 million tons annually (FAO, 2017).

The commitment of roots and tubers to the energy supply in various populations differs with the nation. The significance of these yields is apparent through their yearly worldwide creation which is around 389 million tons (FAO, 2017). Asia is the primary maker took after by Africa, Europe, and America. Asian and African areas created 43 and 33%, separately, of the worldwide generation of roots and tubers.

2.2 YAMS

Yam is a multi-species, monocotyledonous polyploid and clonally propagated crop cultivated for its starchy tubers. *Dioscorea* is imperative for food, income and socio-cultural events. Yam is considered as the staple food in West Africa, Southeast Asia, and the Caribbean regions (Liu *et al.*, 2007).

Yam is a foremost source of calories for many of the world's tropical and subtropical populations and provides wholesome benefits as protein and micronutrients. *Dioscorea* species have been broadly utilized in traditional medicines (Sari *et al.*, 2013) and as a source of steroidal precursors also. Yet yams are categorized as understudied and underutilized. Yam tubers have wide range of bioactive segments,

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to be specific, mucin, dioscorin, allantoin,polyphenols, diosgenin, and vitamins, for example carotenoids and tocopherols (Bhandari *et* al., 2003).

In India so far twenty-six species of *Dioscorea* have been accounted (Abruna *et al.*, 1981). At present thirteen *Dioscorea* species are accessible in Odisha. Out of them eleven are wild and two species are cultivated. The different wild edible yams (*Dioscorea* spp) serve as a "lifesaving" plant aggregate for the agricultural and tribal groups, in times of sustenance shortage and furthermore ethnically imperative secondary staple of tribal people in the Western Ghats.

2.2.1 Origin, taxonomy and production status of yams

Dioscoreaceae is accepted as the most punctual angiosperms among the families and presumably originated in Southeast Asia (Coursey, 1976). *Dioscorea* species were originated in three centers: Africa, Southeast Asia and South America thought about territories for autonomous yam taming and signify consider variety. The financially most imperative yam species comprise *Dioscorea alata, Dioscorea rotundata and Dioscorea cayenensis. Dioscorea alata* were originated in Southeast Asia, all the more particularly in tropical Myanmar and Thailand (Orkwor, 1998). *D. alata* seems to have been produced from local species originating in the Assam-Burma district, and spread through Thailand and Vietnam into the Pacific locale, westwards and southwards to India and Malaysia. It is developed all through the tropical world. Some properties in yams like a non-emergent cotyledon and reticulate-veining of the leaves are characteristic of dicotyledonous plants (Purseglove, 1972) which has prompted the recommendation that the genus *Dioscorea* possibly has been derived from plant forms that arose before the distinction of monocots and dicots (Degras, 1993). Around six hundred species had been portrayed under *Dioscorea,* which making it as biggest variety of the family Dioscoreaceae. The genus is further divided into several sections, under species are assigned. The section Enantiophyllum

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is the biggest as far as number of species which incorporates the vital types of *D. alata*, *D. rotundata* and *D cayenensis*.

Yams are cultivated on around 5 million hectares in around forty-seven nations in tropical and subtropical locales of the world (International Institute of Tropical Agriculture, 2010). In excess of 54 million tons of yams are delivered in Sub-Saharan Africa every year on 4.6 M Ha. More than 95% of this generation lies in a five-nation "yam belt" that incorporates Nigeria, Benin, Togo, Ghana, and Côte d'Ivoire.

2.2.2 Agronomic characteristics and nutritional value of yam.

Yams flourishes best in very much depleted sandy topsoil or sediment soil, with a normal temperature extending from 25°C to 30°C. Under cultivation, yam is generally propagated vegetatively by the utilization of either little entire tubers or bits of larger tubers. The tuber is annually organ on account of the consumable yam or might be enduring, expanding in size and continuously winding up more lignified from year to year to finally a rhizome. The aerial stem might be smoothy, thorny or sometimes hairy and might be round or square in cross-area. The leaves are of alternate or opposite, as a rule heart-molded and might be smooth or hairy. In specific species, bulbils or aerial tubers are framed in the axils of leaf.

Yam generally has a lower glycemic index compared to potato products, about 54% of glucose per 150 grams serving (Glycemic index and glycemic load for 100+ foods, Harvard Health Publications, Harvard Medical School. 2008). Greater yam contains energy sources like 27.88 g carbohydrates, 0.5 g sugar, 4.1 g dietary fibers and 1.53 g proteins. It also comprises various vitamins such as vitamin A, B1, B2C, K and minerals like calcium, iron, zinc, phosphorous etc. (USDA Nutrient Database, 2015).

2.3 GREATER YAM

Dioscorea alata, acquainted from Asia with Africa amid sixteenth century, which is the most broadly circulated *Dioscorea* species all through the tropics. It comprises

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accessions with 2n = 40, 60 and 80 chromosomes (Arnau *et al.*, 2009). *Dioscorea alata* L. (greater yam or ubi badak) is a monocotyledonous tuber Dioscoreaceae family. Its focal points incorporate high yield, easily propagated (through generation of bulbils), early growth for weed concealment, and long storability of tubers. *Dioscorea alata* has low glycemic file and high fiber content (Sari *et al.*, 2013). Regardless of having those useful potential and great nutritional value, yet more noteworthy greater yam contains only small amount of protein (0.32 to 2%). The productivity is 40 tons for each hectare and it can grow above the sea level more than 1500 above sea level. Up to this time greater yam is just considered as a wild plant that is constrained utilization. Greater yam contains starch comprising of amylose and amylopectin up to 19-20%. Moreover, yam has other micronutrients, for example, flavonoids, saponins, and phenolic compounds that are advantageous to the body thus it can be considered as chosen tuber for day by day utilization.

India holds a rich genetic diversity of yams and around fifty distinctive *Dioscorea* species was reported, to a great extent in the west, east and northeastern districts. Greater yam (*Dioscorea alata* L.) is the most vibrant species established all through India among the *Dioscorea* species. In India, greater yam is cultivated in the states of Andhra Pradesh, Bihar, Gujarat, Kerala, Maharashtra, North Eastern states, Odisha, Tamil Nadu, Uttar Pradesh and West Bengal. The National Repository on Tuber Crops Germplasm at Central Tuber Crops Research Institute, India conserves 591 accessions of greater yam as field gene bank (ICAR-CTCRI 2017).

2.4 CONSTRAINTS TO YAM PRODUCTION AND STORAGE

Yam generation is antagonistically influenced by a few components, which incorporate restricted accessibility and cost of planting material, the surprising expense of work for tasks, for example, land preparation, staking, weeding, collecting, storage, pests and diseases. Since the planting materials are a palatable and financially critical piece of the harvest, the requirement for yam tubers as planting

material contends with the requirement for utilization. As indicated by Nweke *et al.*, (1991), planting material records for around 50 % and work for more than 40 % of the cost of production. Yam is inclined to contamination by different ailments including diseases and pests from the sprout stage through to collecting and in the wake of gathering, during storage. The harvest has low yield per hectare appeared differently in relation to other root items, for instance, cassava and sweet potato. Also, yam is hard to secure and store over extended time periods. Thus, and issues of securing accumulated yam, the costs of yam generation are high and yam is steadily losing ground to cassava and other sustenance staples.

2.5 DISEASES OF YAM

Yam diseases can be classified into two, in particular, field and storage disease. The field diseases that cause harm to yam in the field from the seedling stage to the time of gathering (Amusa *et al.*, 2003). A few pathogens causing diseases in yams, for example, anthracnose and leaf spots. In addition to foliage, spoiling of yam tubers have been discovered (IITA, 1975). Yam Mosaic Virus Disease is another serious disease which is caused by potyvirus which infects *D. alata*, *D. cayenensis*, *D. rotundata* and *D. trifida*. The symptoms vary according to host, including vein banding, curling, green-spotting etc. Water yam virus disease (*Dioscorea alata* virus) is another commonly found disease on *D. alata*. Symptoms including vein banding, chlorosis and leaf puckering. Anthracnose is a foliar disease caused by a few fungi, including *Colletotrichum* and *Glomerella* species (Onwueme and Charles, 1994). These pathogens occur in complex causing pre-reap and post-harvest diseases. In India yam anthracnose has been reported in all three edible species *D.alata, D.rotundata and D.esculenta* (unpublished). The severity is more in *D.alata* followed by *D.esculenta* and compared to African countries the infection is very less in *D.rotundata*. Concentric leaf spot disease of yam is another commonly encountered fungal foliar disease on yam in south western Nigeria (Amusa, 2000).

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Sclerotium rolfsii (Amusa, 2000) is the causal agent has been identified. Symptoms of disease including leaf spots of different sizes that form concentric rings. Greater yam is also infected by other minor leaf spots disease caused by *Cercospora, Curvularia* and *Pestalotia* species which cause very negligible yield reduction.

2.6 GREATER YAM ANTHRACNOSE

Greater yam anthracnose disease causes a significant effect on yam production around the world. The anthracnose disease was caused by pathogen which was first depicted as *Gloeosporium pestis* from yam in Fiji (Massee, 1908). Then it was stated from *D. alata* in India (Prasad and Singh, 1960) and classified as *Colletotrichum gloeosporioides*. The disease anthracnose is a "complex disease" having the presence of various pathogenic fungi on the yam which is required for the development of significant symptoms (Amusa *et al.*, 1996). The terms 'scorch', 'Apollo', 'anthracnose blotch', 'leaf and stem blight', and 'yam dieback' have been utilized to depict foliar diseases on yam. Winch *et al.,* (1984) reported that during earlier stages of infection, symptoms appear as pinpoint brown lesions (<2 mm diameter) on young leaves often surrounded by chlorotic halo which later turned reddish or dark brown to black spots with thick dark margin (3-20×3-52 mm) on the adaxial surface of leaf and finally these spots coalesced to form larger spots leading to leaf blight. The withered leaves and stem dieback gave the plant a scorched appearance hence the name 'scorch' disease.

The disease incidence was high during the post–rainy months (October – November) in *D.alata*. The rainy season has been shown to favour infection of *Colletotrichum* species, as rain flashes spread their spores (conidia) and hence maximum disease incidence was observed during winter season. Abang *et al.,* (2003) pointed out that high temperature is unfavourable for anthracnose disease development in *Dioscorea. Colletotrichum gloeosporioides* has a requirement of 26 °C to 32 °C and relative humidity of 100% for germination and growth of spores and pathogen establishment

in the host. Young foliage is more prone to anthracnose. Symptoms vary with respect to the age of the leaf, the amount of rain and the variety of yam.

2.7 THE PATHOGEN, *Colletotrichum gloeosporioides*.

Colletotrichum gloeosporioides causing anthracnose belongs to the order melanconiales and is a ubiquitous pathogen which infects monocotyledons to higher dicothyledons. *C. gloeosporioides* is a plant pathogen which is more common and widely distributed worldwide and infects about 470 different host genera. The fungus is less in temperate and more found in tropical and subtropical regions (CAB international, 2005). Some strains of *C. gloeosporioides* were identified as endophytes since they are isolated from symptomless plant parts (Cannon and Simmons, 2002; Lu *et al.,* 2004; Photita *et al.*, 2004, 2005). The pathogen also causes post-harvest problems (Prusky and Plumbley,1992). Penzig proposed *C. gloeosporioides* for the initial period as *Vermicularia gloeosporioides* in 1882. The pathogen *C. gloeosporioides* was first reported at Brazil in 1937 on *S. humilis* and in India, it was first reported by Butler in 1918 on coffee.

Variable environmental conditions of Indian subcontinent, which favors the growth and development of *Colletotrichum* spp. to infect different hosts. During the last ten years, *Colletorichum gloeosporioides*, *C. capsici, C. falcatum, C. truncatum, C. sansevieriae, C. acutatum* and *C. coccodes* are some important species reported to be associated with anthracnose disease in India in various crops.

2.7.1 Biology of *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides Penz. is an asexual facultative parasite. Fungus includes two states, *C. gloeosporioides* which is anamorph or asexual state whereas, *Glomerella cingulate* which is sexual or teleomorph state. The *Glomerella cingulate* infects broad range of host species and during asexual (mitotic) phase of their life cycle they produce acervuli within the host tissue. The teleomorph state is able to

cause disease. The fungus produces hyaline, one- celled, ovoid to oblong, slightly curved or dumbbell shaped conidia having 10-15 µm in length and 5-7 µm in width. The acervuli, are subepidermal, with setae, and short, erect conidiophores that are produced in infected tissue, (Burger, 1921). For spreading the anthracnose disease uniformly, the fungus prefers warm humid environment and effectively. Through the weakened tissues of plants fungus primarily invade and produces various specialized structures during infection process. During the interaction between host and pathogen the specialized structures viz. conidia, acervulli, setae and appressoria are formed. Conidia can be spread over short distances through rain splash. Formation of appressoria, a specialized infection structure helps in the penetration into the host. are produced During the infection process, acervli were produced, of which short crowded conidophores are formed which can be observed on the surface of diseased plants. The conidia discharge through an opening at the top of acervuli. The whole infection process, including the formation of conidia, acervuli, setae and appressoria, and results into tissue necrosis.

For the better growth it needs 25-28 °C temperature, pH 5.8-6.5 and become inactive in dry season. Potato dextrose agar, lima bean agar, malt extract agar and oat meal agar are the various medium employed for the growth and sporulation of *C. gloeosporioides*.

2.7.2 Taxonomic description

Colletotrichum gloeosporioides (Penz.) Penz. &Sacc. is an asexual facultative parasite belonging to the division Ascomycota.

Kingdom	: Fungi
Phylum	: Ascomycota
Class	: Sordariomycetes
Order	: Incertaesedis
Family	: Glomerellaceae

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Genus	: Colletotrichum
Species	: *C. gloeosporioides*
Subspecies	: *C. gloeosporioides*
Scientific Name	: *Colletotrichum gloeosporioides* (Penz.) Penz. &Sacc.
Teleomorph	: *Glomerellacingulata* (Stoneman) Spauld. & H. Schrenk

2.7.3 *Colletotrichum gloeosporioides* as pathogenic fungi

Colletotrichum gloeosporioides is one among the most significant plant pathogens in India, which can cause anthracnose in a wide range of hosts including cereals and grasses, legumes, fruits, vegetables, perennial crops and trees. The disease is characterized by spots on leaves, stems, fruits or flowers. The spots found on the host coalescence to form larger spots, and later leading to wilting or withering, and dying of infected plant. The pathogen requires warm and humid conditions to infect different plant hosts, including gymnosperm, angiosperms, ornamental and fruit plants, vegetables, crops or even grasses. As the primary inoculum is disseminated by wind or rain, the pathogen is cosmopolitan in distribution. Slight infection forms very small brown colored spots on young leaves. Later spots form larger and approach full size as of leaves, and sometime they produce pale yellow margins. Occasionally the leaf spots composed to form large irregular blotches, the centers of which may produce a'shothole' effect. Leaves with infection usually fall off. Studies of *C. gloeosporioides* anthracnose on its epidemiology, diversity, biology and control are also assessed time to time in India.

2.7.4 Management of Anthracnose

To grow resistant varieties, use of chemicals and bio-pesticide are the disease management strategies adopted at present.

Resistance differs in different varieties, some are resistant at all stage of the growth and some only when mature and have fully grown. Disease pressure will be greater

on the tolerant ones when planted together and also there is a chance of destroying susceptible varieties. Tubers should be collected from plants showing high resistance can be used for propagation. ICAR-CTCRI has released greater yam varieties, Sree Karthika and Sree Keerthi which showed high tolerance with the disease scale of 1-2, the maximum disease intensity observed in Sree Karthika was 35% and Sree Keerthi was 33% (ICAR-CTCRI, 2017). The anthracnose-resistant cultivars (e.g. TDA 291, TDA 297) bred and released by the international Institute of tropical Agriculture (IITA) are also been advocated (IITA, 1993).

In chemical control, Benomyl (no longer recommended), chlorothalonil, copper, dithiocarbamates (e.g., mancozeb) are the fungicides that have been recommended in Pacific and Caribbean islands, which can only delay the start of an epidemic and are not effective during long rainy periods (Onyeka *et al.*, 2006). Chemical control is difficult; cost intensive and continuous use of fungicides may lead to the development of fungicide-resistant strains. Foliar sprays can be applied before the symptoms develop in the crop. Imtiaj *et al.* (2005) tested different fungicides against *C. gloeosporioides* causing anthracnose of mango and found that 0.5% and 1.0% Bordeaux mixture Bavistin (0.1 % and 0.2 %) were most effective.

Biological control of diseases comprises use of microbial antagonists which has been considered as an eco-friendly method and an alternative to the use of chemical pesticides. Actinomycetes like *Streptomyces sp.* has the ability to control various fungal pathogens of different plant hosts (Samac *et al.*, 2003). Soares *et al.*, 2006 isolated Streptomyces from Brazilian soil were tested for their antagonist activity against the yam pathogens *Curvularia eragrostides* and *C. gloeosporioides in vitro.* Though, there were no reports regarding antagonistic activity of *Streptomyces species* in controllingvyam anthracnose under greenhouse or field condition. The foliar spraying and soil application of fungal biocontrol agent like *Trichoderma asperellum*, along with foliar spraying of carbendazim (0.05%) drastically reduced the disease intensity (66%) of anthracnose disease in greater yam (ICAR-CTCRI,

2016). The combination of fungicides and biocontrol agents were reported to be effective in managing the disease (Jeeva *et al.*, 2016). Yet no other effective biointensive methods have been developed to manage the anthracnose disease in greater yam.

2.8 ENDOPHYTES

The word endophyte literally signifies "in the plant" (endon Gr. = inside, phyton = plant). The use of this term is extensive as its exacting definition and range of potential hosts and occupants, e.g. bacteria fungi, and insects in plants, yet in addition for algae inside algae (Peters, 1991). Endophytes can colonize any part of the plant. In spite of the fact that there are differing uses for the word endophyte, "endophytes" are most generally characterized as those life forms whose infections are unnoticeable, the infected host tissues are at least transiently symptomless, and the microbial colonization can be shown to be internal (Stone *et al.*, 2000). Endophytes were said for first time by Bary in nineteenth century (Azevedo, 1998) and have been characterized from numerous ways. Wilson (1995) characterized endophytes as the parasites that live inside and stay asymptomatic for at least part of their life-cycle. He additionally portrayed the symptomless occupation of endophyte in plant tissue is advantageous and mutualistic in nature. But, they can also be powerful saprophytes or aggressive pathogens. Depending on their area of colonization in the plant species, microbes residing in a single plant species are categorized as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those adjacent or on plant tissue) or endophytes (microbes present inside plant tissues in leaves, roots or stems) (Andreote, 2014).Besides, because of broad investigations of these groups of microorganisms, the endophytic communities have been separated into various subgroups, for example, 'obligate' or 'facultative,' (Rosenblueth and Martínez-Romero, 2006). Endophytes that rely upon the metabolism of plants for survival, being spread among plants by the action of various sorts of vectors or by vertical transmission, are named as obligate endophytes. Though, the facultative endophytes

are those that live outside the host body during a specific phase of their life cycle and are mostly related with plants from its neighboring soil environment and atmosphere. Certainly, various reports have demonstrated that endophytic microorganisms can have the ability to control plant pathogens (Krishnamurthy and Gnanamanickam, 1997), bugs (Azevedo *et al.*, 2000) and nematodes (Hallmann *et al.,* 1998).

There are theories that, a huge number of endophytes valuable to humankind are as of now leaving yet unexplored because of constrained research consideration in this related field. Nonetheless, with ecological pollution, deforestation, natural surroundings fracture and biodiversity misfortunes, a large number of these endophytes may be for all time lost before their value is explored (Kandalepas *et al.*, 2015).

2.8.1 Bacterial endophytes

Bacterial endophytes inhabit same as that of phytopathogens making them suitable for biocontrol agents (Berg *et al.*, 2005). Bacterial endophytes are characterized as bacteria that colonize plant tissue without causing apparent symptoms in or produce any evident damage to the host. A suitable meaning of endophytic behavior stated by few authors considering that any bacterium can said to be an endophyte, if that it should be separated from plant tissue after surface-disinfection or extracted from inside the plant, and that it doesn't obviously harm the plant (Hallman *et al.*, 1997). In some cases, they can promote seedling development, improve plant growth and endorse plant establishment under antagonistic conditions (Chanway, 1997). Bacterial endophytes have been appeared to anticipate infection advancement through endophyte-intervened de novo synthesis of novel compounds and antifungal metabolites. The bacterial endophytes promote plant growth either through direct or indirect mechanisms. Coordinate advancement of plant development happens whether a bacterium encourages the procurement of fundamental nutrients or minerals or by regulates the level of hormones inside a plant. Bacterial endophytes

may have preference over microorganisms occupying the rhizosphere, meanwhile living inside plant's tissues speaks a chance to dependably in contact with the cells and in this way, promptly apply a direct useful impact. Bacterial endophytes utilize distinctive mechanisms to pick up passage in the tissues of plant, especially in roots aside from effectively settled seed-endophytes (Truyens *et al.,* 2015), which is the most widely recognized method of endophytic organisms penetrate into plant tissues which is through essential or parallel breaks in root, differing wounds in tissues happening because of plant development (Sorensen *et al.*, 2015).

2.8.2 Fungal endophytes

Endophytic fungi are ecological fungi, generally belonging to ascomycetes and anamorphic fungi. Numerous endophytic fungal strains could be seen in each plant. These endophytes have the ability to produce biologically vigorous metabolites that enable the endophyte interaction with plant (Strobel, 2003) and found that these were useful for different natural products which have application in different areas like medicine, agriculture, and industry (Selim *et al.,* 2012). And also they are able to synthesis various enzymes; like pectinases, cellulases, lipases, proteinases etc. These enzymes have vital role in biological and hydrolysis processes which are important pathways against infection (Sunitha *et al.,* 2013). Though, some endophytic fungal isolates have examined for their biological applications including their ability for antimicrobial activity; thus, it seems that screening the antimicrobial activity of fungal endophytes is valuable to discover novel antimicrobial producers.

2.9 MECHANISMS INVOLVED IN DISEASE MANAGEMENT

2.9.1 Antagonistic activity

Endophytic biocontrol agents localize in plant tissues similar to plant pathogens, and play a vital role against these pathogens. The intimate association of endophytes with plants offers an exclusive opportunity for their potential application in plant

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protection and biological control. Antibacterial or antifungal activity of endophytes is an advantage to use as biological control agents in fields, where others fail because they were unable to colonize the internal tissues of the host plant (Mathiyazhagan *et al.*, 2004; Kavitha *et al.,* 2005).

2.9. 2 Antibiotics production

Antibiotics encompass a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms. Numerous bacterial strains that produce antibiotics *in vitro* have been isolated from different soils and host plants (Thomashow and Weller, 1996). Antibiosis has been widely studied as one of the most important biocontrol mechanisms inhibiting plant pathogens. To date a number of diseases suppressive antibiotic compounds have been characterized which include N containing heterocycles such as phenazines (Thomashow *et al.*, 1990), pyrrole type antibiotics pyo-compounds and indole derivatives. A small number of antibiotics like compounds that do not contain nitrogen have also been identified (Kanda *et al.*, 1975), one of these metabolites 2, 4 - diacetylphloroglucinol (DAPG), is a major factor for the control of a range of plant pathogens (Defago, 1993). Several studies have demonstrated that many of the antibiotics produced by bacterial biocontrol agents have a broad-spectrum activity. Ongena and Jacques, 2011 reported that *Bacillus spp*. produce various secondary metabolites like cyclic lipopeptides(c-LPs) which having antifungal activity. An antibiotic Zwitter mycin A produced by *Bacillus cereus* and *Bacillus thuringiensis* which have an impact on the growth and antagonistic activity against pathogens in particular *Phytophthora* and *Pythium* species (Silo-Suh *et al.*, 1998).

2.9.3 Induced systemic resistance (ISR)

Induced resistance has been recognized as an attractive tool for plant disease management in modern agriculture. During the last two decades, studies on chemically and biologically elicited induced resistance as a biological trigger have

revealed previously unknown features of the plant defense response including defense priming. Plants establish multiple layers of defense responses, including physical barriers such as the cuticle and cell wall, as well as chemical defenses such as secretion of antimicrobial or anti-insect compounds (Pieterse *et al.,* 2009). The term ISR describes "activation of the host plant's physical or chemical defenses by an inducing agent. Further studies revealed that the endophytes can be used as microbial inoculants to control plant pathogens and promote plant growth (Kloepper and Ryu, 2006).

In cotton, Rajendran *et al.,* 2006 reported that endophytic *Bacillus* strains caused higher levels of chitinase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol, besides reduction of bacterial blight and damping off incidence. Ryu *et al.,* 2004 reported that volatile compounds like 2, 3-butanediol and various lipopeptides compounds produced by *Bacillus* spp. were the elicitors of ISR.

2.10 IDENTIFICATION OF ENDOPHYTES

2.10.1. Bacteria

The 16S rRNA gene nucleotide sequences do provide bacterium species-specific signature and hence, 16S rRNA gene sequence-based bacterial identification is considered as a precise method of bacterial identification (Clarridge, 2004; Khan and Doty, 2008; Liaqat and Eltem, 2016). The genes coding for 16S ribosomal RNA is referred to as 16S rRNA gene and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene. It is recommended that 16S rRNA gene can be used as a reliable molecular clock because 16S rRNA sequences from distantly related bacterial lineages are shown to have similar functionalities. Since these genes are encoding for important functions, the sequences are conservative across nearly all bacterial species while the features of the structural parts are largely variant and specific to one or more classes. The invention of the polymerase chain reaction (PCR) technique help the usage of the variant regions of the 16S rRNA

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genes (rDNAs) species identification. 16S rDNAs primers (15–20 nucleotides) are located in the conservative regions that flank a target region used for phylogenetic analysis (Lane *et al.*,1985).

Two of the most commonly used primers for bacterial 16S rRNA genes are 27f (spanning positions 8 to 27 in Escherichia coli rRNA coordinates) and 1492r (commonly spanning positions 1492 to 1507, though longer versions are sometimes used), which could amplify nearly the entire length of the gene. Often 8F is used rather than 27F (Sun *et al.*, 2008). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria.

2.10.2 Fungi

The intervening internal transcribed spacer (ITS) region has been utilized by different researchers for the identification of fungi (White *et al.* 1990; Gul *et al.*, 2017; Naik *et al.*, 2017). The Internal Transcribed Spacer (ITS) regions are highly mutable sequences which gained unlimited significance in differentiating fungal species through PCR. The earliest PCR primers to gain wide acceptance for work with fungal Internal Transcribe Sequences (ITS) were "ITS1" and "ITS4" which amplify the highly variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the Small SubUnit-coding sequence (SSU) and the Large SubUnit-coding sequence (LSU) of the ribosomal operon. Intergenic transcribed spacer regions (ITS1 and ITS2) are located between 18S and 5.8S and between the 5.8S and 28S ribosomal DNA (rDNA) which is non-coding region, cannot be translated into rRNA. These primers amplify a extensive range of fungal strains and help to examine DNA from specific fungus (Manganyi *et al.,* 2018).

2.11 PRIMER DESIGNING

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

Primer sequences need to be chosen that it should not bind to any other regions of DNA. Specificity of primer is the major concern while designing the primers. Most common method used is BLAST search. Here, all the possible regions to which a primer may bind can be predicted. The nucleotide sequence as well as the primer sequence can be BLAST searched. The free online NCBI Primer-BLAST tool integrates primer design and BLAST search into a single application. Many other online tools are also freely available for primer design, including some tools which focus on specific applications of PCR. The most popular tools used widely for primer designing are Primer3Plus and PrimerQuest.

MATERIALS AND METHODS

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3. MATERIALS AND METHODS

3.1 LOCATION

The study was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2017-2018. The detailed description of materials and methods used for carrying out various experiments is described in this chapter.

3.2 SAMPLE COLLECTION

Leaves, stem and roots of greater yam varieties/accessions, Sree Karthika, Sree Keerthi, Da 251 and Da 265, taro (var. Muktakeshi), coleus (var. Sree Latha) and arrowroot were collected for the isolation of bacterial and fungal endophytes. Leaves from anthracnose infected greater yam (var. Orissa Elite) plants were taken for isolation of the pathogen *Colletotrichum gloeosporioides.* Samples for the study were collected from fields of ICAR- CTCRI.

3.3 ISOLATION OF *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides was isolated from the anthracnose infected greater yam plants (var. Orissa Elite) of ICAR- CTCRI farm. Symptomatic leaves from infected plants showing spots were taken and washed under running tap water. Then the infected regions from leaves were excised and cut into small pieces of about 5 mm^2 size. These leaf portions were placed in Petri dish and surface sterilized 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 $mgml^{-1}$) ampicillin (Appendix I) under aseptic conditions and incubated at 25±2 °C for 5-7 days. After 7 days a loop full of fungal culture were taken on a glass slide and

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examined under light microscopy at 40X magnification (Nikon Eclipse E200) for the presence of conidia and conidiophore. After confirmation the cultures were purified by single spore isolation technique and maintained on PDA slants for further studies.

3.3.1 Testing the pathogenicity of *Colletotrichum gloeosporioides* on detached leaves

Pathogenicity of isolated *C. gloeosporioides* from various fields was tested on detached matured leaves of greater yam susceptible variety Orissa Elite and more virulent *C. gloeosporioides* was selected for antagonist activity. For pathogenicity test15 µl of spore suspension (5×10^5 spores ml^{-1}) was sprayed on the leaf surface and incubated at 26^0C in BOD incubator (ANALAB INSTRUMENTS INDIA, SID - 10) for 8 days (Hong and Hwang, 1998). In order to maintain humidity for the development of disease, sterile distilled water was sprinkled or the filter paper was moistened and placed inside. Symptoms appeared were observed at regular intervals.

3.3.2 Molecular Identification of the Pathogen

3.3.2.1 DNA isolation

Genomic DNA was extracted from the mycelial mat of *C. gloeosporioides* isolates by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee (1996). For this, the pathogen was grown in 50 ml Potato Dextrose Broth and the mycelia were harvested by filtration through sterile filter paper. 80mg of mycelium was ground to fine powder with liquid nitrogen using sterile mortar and pestle and transferred into 2 ml centrifuge tube. 2 ml of extraction buffer (Appendix II) was added to the tissue powder and vortexed it for 30 sec followed by addition of 5 µl Proteinase K (20 mg ml^{-1}), gently mixed it and incubated at 65 °C for 45 min. Then 5 µl RNase (20 mg ml^{-1}) was added to the crude extract and incubated at 37 °C for 20 min. The tubes were centrifuged at 10,000 rpm (Kubota High Speed Refrigerated Centrifuge 500, Japan) for 10 min at 27 °C. The supernatant was transferred to a fresh 2 ml tube and added equal volume of phenol-chloroform-isoamylalcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by

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centrifugation at 12,000 rpm for 15 min. The supernatant was transferred to a clean tube and mixed with equal volume of chloroform-isoamylalcohol (24:1). The upper aqueous phase was transferred after centrifugation at 12,000 rpm for 15 min at 27 °C. DNA was precipitated by adding 0.8 volume of ice cold isopropanol to the upper layer and mixed by inverting it 20-30 times. The tubes were centrifuged at 12,000 rpm for 15 min at 27 °C to pellet out the DNA. The pellet obtained was washed twice with 70 % ethanol and centrifuged at 10,000 rpm for 5 min at 27 °C. The pellet was resuspended in 1 X TE buffer (Appendix II) and stored at -20 °C

3.3.2.2 Agarose gel electrophoresis

The DNA obtained were separated in 1 % (w/v) agarose gel in 1X TAE buffer (Appendix II) containing 0.5 μgml^{-1} ethidium bromide. An aliquot of DNA (3 µl) mixed with the loading dye (Appendix II) was loaded in each wells of the gel. The electrophoresis was carried out 80 Vcm^{-1} (BIO RAD Power Pac HV, USA) for 20 min. The gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA).

3.3.2.3 Nanodrop spectrophotometric analysis

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

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3.3.3 PCR amplification using species specific primers

The Primer pairs CgsF1 (GGCGGGTAGGGTCTCCGTGAC) / CgsR1 (TTTGAGGGCCT ACATCAGCT) (Raj *et al.*, 2012) were used for PCR to confirm the pathogen.

PCR reaction mix:

Component	25µl reaction	Final concentration in PCR
10X KAPPA Taq buffer (with 25 mM $MgCl_2$)	2.5 µl	1X
dNTP mixture (10 mM)	0.5 µl	0.2 mM each
Forward Primer (10 µM	1 µl	0.4 µM
Reverse Primer (10 µM)	1 µl	0.4 µM
KAPA Taq DNA Polymerase (5U/µl)	0.2 µl	1 U
Template DNA	1µl	100 ng
PCR -grade water	18.8 µl	Upto 25 µl

Total volume: 25 µl

The PCR profile was denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 62 °C for 40 sec, and 72 °C for 40 sec, and then a final extension at 72 °C for 5 min. Amplified products were resolved on a 1.5% agarose gel and image was visualized by ethidium bromide staining through Gel Doc System (Alpha Innotech Corporation, San Leandro, CA, USA).

3.4 ISOLATION OF BACTERIAL AND FUNGAL ENDOPHYTES

The leaves, stems and roots of greater yam, taro, coleus and arrowroot were brought to the laboratory. 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 in

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the following steps: washing in distilled water followed by sodium hypochlorite 3 % (v/v) (3 min), ethanol 70 % (1 min) and two washings with autoclaved distilled water (Anjum and Chandra, 2015). Each isolation procedure was done in triplicate for each cultivar. Bacterial endophytes were isolated by placing the samples in Nutrient agar (Appendix I) incubated at 37 °C for 24 h and fungal endophytes were isolated in Potato Dextrose Agar containing ampicillin (100 mg ml^{-1}) (Appendix I), 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 in respective media were recorded. Codes were given based on the name of the crop, variety, fungus/bacteria, the part used and a number.

Dioscorea alata (Da), var. Sree Karthika (Sk), Sree Keerthi (Ski), Da 251 (251), Da 262 (262), *Colocasia esculenta* (Cs), *Plectranthus rotundifolius* (Sr), and *Maranta arundinacea* (Ma).

3.5 *IN VITRO* SCREENING OF ENDOPHYTES AGAINST *Colletotrichum gloeosporioides*

The antifungal activity of isolated endophytes was tested by dual culture technique (Cherif and Benhamou, 1990) using PDA medium. A mycelial disc (9 mm diameter.) of the *C. gloeosporioides* was placed at one end of the Petri dish and the bacterial antagonists were streaked on both sides, one cm away from the periphery of the Petri dish just opposite to the mycelial disc of the pathogen and fungal endophytes were tested by placing a mycelia disc similar to pathogen disc on the opposite side of pathogen. 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. Experiments were conducted with three sets of replication plates.

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

Growth inhibition (%) = [(dc - dt)]/dc] × 100, where dc and dt represent the fungal growth diameter in control and treated sample.

3.6 MOLECULAR IDENTIFICATION

PCR has been performed to identify the potential isolates selected from the above study.

3.6.1 Isolation of DNA

3.6.1.1 Bacterial DNA isolation

The DNA from the potent bacterial endophytes were isolated by SDS method as described by Wilson, 2001. Antagonist bacterial endophytes were grown in 1.5 ml nutrient broth at 28 °C. Total DNA was extracted from cultures grown for 16 h in nutrient broth were centrifuged 12,000 rpm for 10 min into a pellet and resuspend the pellet in 567 µl TE buffer (Appendix I) by repeated pipetting. To give a final concentration of 100 µg ml^{-1} proteinase K in 0.5 % SDS, 30 µl of 10 % SDS and 3 µl of 20 mg ml^{-1} proteinase K was added. Mix thoroughly and 5 µl of RNase (20 mg ml^{-1}) was added and incubated 1 h at 37 °C. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was added, mixed thoroughly, and centrifuged at 12,000 rpm for 15 min at 27 °C in a microcentrifuge (Kubota High speed Refrigerated centrifuge, 6500, Japan). Aqueous, viscous supernatant was collected to a fresh microcentrifuge tube, leaving the interface behind and added an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) mixed thoroughly, and centrifuged at 12,000 rpm for 15 min at 27 °C. Transferred the supernatant to a fresh tube and 0.6 vol isopropanol was added to precipitate the nucleic acids. Invert the tube back and forth until a stringy white DNA precipitate becomes clearly visible and centrifuged to pellet out the DNA at 10,000 rpm for 10 min. The pellet was

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transferred to a fresh tube and washed the DNA with 70 % ethanol, spinned 10,000 rpm 5 min at room temperature. Carefully removed the supernatant and briefly dried the pellet in a Bio incubator at 37 ° C for 45 min. The pellet was re dissolved in 30 µl TE buffer (Appendix II). The DNA obtained was checked in 1% (w/v) agarose gel in 1X TAE buffer (Appendix II) and stored at -20^{0}C.

3.6.1.2 Fungal DNA isolation

Genomic DNA was extracted from the mycelial mat of antagonist fungal endophytes by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described in Sl. No. 3.3.2.1.

3.6.2 Agarose gel electrophoresis

A small amount of the isolated DNA from both fungus and bacteria was run on 0.8 % agarose gel was prepared in 1X TAE buffer (Appendix II) as described in 3.3.2.2.

3.6.3. Nanodrop spectrophotometric analysis

The absorbance of DNA samples from bacteria and fungus were recorded to determine the quantity and quality of DNA isolated as described in 3.3.2.3.

3.6.4 PCR amplification using universal primers

3.6.4.1 PCR amplification of bacterial DNA using 16S rRNA targeting primers

To identify strains of bacteria, 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).

PCR mixture is same as described in 3.3.3

The samples were amplified on DNA thermalcycler (Eppendorf Master Cycler Gradient, German) using the PCR conditions initial 95 °C for 2 min followed by 35

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cycles denaturation 90 °C for 20 sec, annealing temperature at 55.5 °C for 45 sec 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).

3.6.4.2PCR amplification using RecA for bacterial DNA

RecA primers (Guo *et al.,* 2015)

Forward primer: 5' TGAGTGATCGTCAGGCAGCCT 3'
Reverse primer: 5' TTCTTCATAAGAATACCACGAACCGC 3'

In order to standardize the annealing temperature of primers, gradient PCR was performed. The reaction mix was optimized as follows:

Component	25µl reaction	Final concentration in PCR
2X PCR Master Mix (EmeraldAmp GT PCR TAKARA BIO INC)	12.5µl	1X
Forward Primer (10 µM)	1 µl	0.4 µM
Reverse Primer (10 µM)	1 µl	0.4 µM
Template DNA	1µl	100 ng
PCR -grade water	18.8 µl	Upto 25 µl

Total volume: 25 µl

After the preparation of the reaction mix, the tubes were vortexed and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 92 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 sec, annealing at temperature gradient from 55-66 °C for 30 sec and extension at 72 °C for 1 min. The final extension was carried out

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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).

3.6.4.3 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 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3') primers (White *et al.*, 1990).

PCR mixture as same as described in 3.3.3

The samples were amplified in Agilent Technologies sure Cycler 8800 (USA) using the PCR conditions initial 94 °C for 3 min followed by 35 cycles denaturation 94 °C for 30 sec, annealing temperature at 53 °C for 30 sec and extension at 72 °C for 1 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).

3.6.5 Purification of PCR product

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

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

3.6.6. DNA sequencing

The isolated DNA were sequenced at the Agri genome, India using Sangers method (Capillary sequencer <3130xl>A)

The sequence obtained was first edited with Geneious Sequence Alignment Editor Programme version 11.1.4. The edited sequence was analyzed using NCBI BLAST program and identified the organism.

3.7 SPECIES SPECIFIC PRIMER DESIGNING

Primers were designed based on the obtained DNA sequence information. Primer 3 version 0.4.0 was used to design specific primers. The obtained primer pairs from Primer 3 software were subjected to NCBI BLAST analysis and primer pairs that bind specifically to the desired organism was selected.

The designed primers were subjected to *in-silico* analysis for determining specificity and other characteristics. FastPCR programme was used to study the length of primers as well as the PCR product, self-complementarity and possibilities for primer dimer formation, GC content, melting temperatures and feasible annealing temperatures. The primer sequences thus validated by *in-silico* methods were sent to PrimerX, Xcleris Lab ltd, Ahmedabad for synthesis.

3.7.1 Gradient PCR for standardizing annealing temperature

The newly designed primers were used to amplify the identified potential endophytes. This primer pair was designed based on the common region of the 3 potential bacterial endophytes identified so it can amplify all the identified ones.

(Forward primer) sequence: 5' ACC TTG ACG GTA CCT AAC CA 3'
(Reverse primer) sequence: 5' GTC CAT TGT AGC ACG TGT GT 3'

The synthesized primers were diluted to a final concentration of 10 µM with sterile water to obtain the working solution. In order to standardize the annealing temperature of primers, gradient PCR was performed. The reaction mix was optimized as follows:

Component	25µl reaction	Final concentration in PCR
2X PCR Master Mix (Thermo Scientific, USA)	12.5µl	1X
Forward Primer (10 µM)	1 µl	0.4 µM
Reverse Primer (10 µM)	1 µl	0.4 µM
Template DNA	1µl	100 ng
PCR -grade water	18.8 µl	Upto 25 µl

Total volume: 25 µl

After the preparation of the reaction mix, the tubes were vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 92 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 sec, annealing at temperature gradient from 49.9 –59 °C for 30 sec and extension at 72 °C for 1 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

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

3.8 TISSUE CULTURE OF *D.alata* VARIETY Orissa Elite

Nodes from the tissue cultured greater yam (var. Orissa Elite) susceptible to anthracnose variety maintained at ICAR-CTCRI were used to raise plantlets for the study.

3.8.1 Media preparation

Half Murashige and Skoog (MS) media (HIMEDIA PT021X1L) was used to establish nodal culture of plants. Each pack contains 4 g of MS powder which was dissolved in distilled water and added with sucrose (30 g) and PVP (0.1 %). The pH was adjusted to 5.7 with 1N NaOH/1N HCl and the volume was made to 2 L. The medium was poured into test tubes (15 ml media in 150 x 25 mm tubes) and sterilized at 121 ^{o}C and 15 psi pressure for 20 min (Appendix III). After sterilization the culture tubes were stored in culture room until use.

3.8.2 Explant preparation and inoculation

All the inoculation operations were carried out under aseptic conditions inside a laminar air flow chamber, which is alcohol wiped and UV sterilized for 20 min before use. The tissue cultured plantlet was taken from the inoculated test tube using a sterile forceps and leaves were removed using a sterile scissors. The nodes were dissected out from the plant into a sterile filter paper in a sterile Petri dish. Every single node from the Petri dish as held inside the sterile filter paper wicks placed in the test tubes containing ½ MS medium. (Saranya *et al.,* 2013). The inoculated tubes were maintained in tissue culture room at 25±1^{0}C under 16/8h photoperiod of around 2000 lux light intensity provided by white fluorescent tubes with 60% relative humidity.

3.8.3 Hardening of plants

After 2 months of subculturing on half MS media, the plants were taken out of the culture tube and hardened before transferring to soil. The plantlets were taken out of the culture tubes with the help of forceps avoiding any mechanical damages. A thorough washing in tap water was done to remove the adhering medium to avoid infections in the future and the plants are carefully placed in plastic cups containing sterile coirpith. The hardened plants are thoroughly watered and kept in shade under a humidity range of 60-70 % which were utilized for colonization study.

3.9. CHECKING COLONIZATION OF POTENTIAL ENDOPHYTES ON ROOTS, STEM AND LEAVES OF TISSUE CULTURED *D.alata*

3.9.1 Inoculation of endophytes on tissue cultured plantlets

The two-month-old plantlets on paper cup containing sterile coirpith were inoculated with each of the bacterial suspensions using two methods: pruned root dip method (Musson et al. 1995) and spraying method (Bressan and Borges, 2004). In root dipping method, the roots from tissue culture plants were washed in sterile distilled water and the root tips (3 mm) were trimmed using a sterilized scalpel to facilitate the uptake of the inoculum. The plantlets were then placed in sterile 50 ml conical flask at 25 °C for 3 h with only their roots in contact with the inoculum suspension of each isolate (at 10^8 cfu ml^{-1}) and planted on the corresponding paper cups. As control, plantlets with severed root tips were treated with sterile distilled water. In spraying method, the plantlets in paper cups were sprayed with the bacterial suspension (10^8 cfu ml^{-1}). The inoculated plantlets were covered with polythene bags and maintained under tissue culture conditions. The covers were removed after seven days.

3.9.2. Isolation from endophytes from inoculated tissue culture plantlets

The inoculated potential endophytes were re isolated from the tissue cultured plants undergone both root dipping and spraying treatment after eight days of inoculation.

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The standard procedure was followed for the isolation. The leaves, stem and roots of plant from both treatments was surface sterilized (Anjum *et al.*, 2015) and placed in nutrient agar (Appendix I) and incubated at 37 °C for 24 h. The endophytes were sub cultured after purification and identified.

3.9.3. DNA isolation of isolated potential endophytes

The DNA of re isolated endophytes from leaves, stem and roots of tissue culture plantlets were isolated by SDS method (Wilson, 2001) as described in Sl. No. 3.6.1.1

3.9.4. PCR amplification using species specific primers

The isolated DNA were amplified using the designed species-specific primer

The reaction mix was optimized as follows:

Component	25µl reaction	Final concentration in PCR
2X PCR Master Mix (Thermo Scientific, USA)	12.5µl	1X
Forward Primer (10 µM	1 µl	0.4 µM
Reverse Primer (10 µM)	1 µl	0.4 µM
Template DNA	1µl	100 ng
PCR -grade water	18.8 µl	Upto 25 µl

After the preparation of the reaction mix, the tubes were gently vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with an initial denaturation at 92°C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 sec, annealing temperature at 58 °C for 30 sec and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 8 min. The PCR amplified products along

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with Gene Ruler 1 kb plus DNA ladder (Thermo Scientific, USA) were separated on agarose gel (1.5%). The gel was viewed using Alpha Imager (Alpha Innotech, USA).

3.10 EFFECT ON ENDOPHYTES ON GREATER YAM PLANTS IN GROW BAGS AGAINST ANTHRACNOSE DISEASE- POT TRIAL STUDIES

For studying the effect of potential endophytes against anthracnose disease, two pot trials were conducted one is placed inside glass house with artificial inoculation of pathogen and the other one is open condition with natural infection. The experiments were laid out with ten treatments. Five replications for each treatment were maintained.

3.10.1 Details of different treatments applied

T1- Tuber treatment of *Bacillus cereus*

T2- Soil treatment with dolomite as a carrier of *Bacillus cereus*

T3- Spraying of *Bacillus cereus*

T4- Combinatorial of tuber treatment, soil treatment and spraying of *Bacillus cereus*

T5-Tuber treatment of *Bacillus subtilis*

T6- Soil treatment with dolomite as a carrier of *Bacillus subtilis*

T7- Spraying of *Bacillus subtilis*

T8- Combinatorial of tuber treatment, soil treatment and spraying of *Bacillus subtilis*

T9-Control

T10- Fungicide control

For tuber treatment the bacterial suspension was prepared by inoculating 1 ml of 12 h grown culture in nutrient broth into 100 ml broth and incubated at 37 °C for 24 h in shaking incubator. The bacterial cells were pellet out by centrifuging at 7500 rpm for

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20 min and the pellet were suspended in sterile distilled water and make up to the dilution 10^8 cfu ml^{-1}. The tubers for planting were dipped in bacterial suspension for 1 h prior to planting.

The soil treatment was done with the help of dolomite carrier. A 50 ml of 24 h grown bacterial culture were centrifuged at 7500 rpm for 20 min at 26 °C. The cells harvested were diluted with 10 ml sterile distilled water and mixed with 100 mg autoclaved dolomite and incubated at 37 °C for overnight and spread on the planted pots.

In spraying method, the bacterial culture was pellet out by centrifuging at 7500 rpm for 20 min and diluted with sterile distilled water up to the dilution of 10^8 cfu ml^{-1}. The spray was given after 20 days of planting when the leaves were arising (each spray volume consisted of 60 ml per plant). The bacterial suspension was sprayed with an interval of 10 days.

Control plants were planted without any of the treatments and in fungicide control, the fungicide Starbenz (1 mlL^{-1}) were applied to the plants once in a month and 10 days interval.

3.10.2. Pot trial studies in glass house

The greater yam plants were raised by planting aerial tubers in small pots applied with treatments (T1, T2, T3, T4, T5, T6, T7 and T8) and control (T9 and T10). After 1month of growth artificially pathogen *C. gloeosporioides* were inoculated by spraying method. The spore suspension of pathogen (10^5 ml^{-1}) were uniformly sprayed on the plants and the covered with polythene bags and water was sprayed on the plants two times a day intermittently to maintain the humidity and moisture.

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3.10.3 Pot trial studies in environmental conditions

The plants were raised by planting ~ 250 g of greater yam tubers in grow bags which were applied with all the treatments as described in 3.10. Four spraying of bacterial endophyte suspension were done at an interval of ten days.

3.10.4 Efficacy of application of endophytes on growth parameters

The efficacy of application of endophytes on growth parameters of the treatment plants was recorded after three months of planting. The length of vine (cm) and number of leaves were recorded in all the plants of each treatment in both glasshouse and natural condition.

3.10.5 Efficacy of endophytes on disease intensity

The disease development was scored in all the treatments based on the following scale (Sharada and Shamarao, 2015) after four months of planting.

Disease intensity based on 0-5 scale,

0 - Nil

1 - 1-10 %

2 - >10 to 25%

3 - >25 to 50%

4 - >50 to 75%

5 - >75%

3.11. STATISTICAL ANALYSIS

The data were statistically analyzed using the The SAS System. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) and the means were compared using Duncan's Multiple Range Test.

RESULTS

4. RESULTS

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The results of the study entitled "Identification and evaluation of endophytes from tropical tuber crops against *Colletotrichum gloeosporioides,* causing anthracnose in greater yam (*Dioscorea alata* Linn.)" carried out at the Division of Crop Protection, ICAR- CTCRI, Sreekariyam, Thiruvananthapuram during 2017- 2018 are presented in this chapter.

4.1 SAMPLE COLLECTION

Leaves, stem and roots of greater yam (var./accessions Sree Karthika, Sree Keerthi, Da 251 and Da 265), taro (var. Muktakeshi), coleus (var.Sree Latha) and arrowroot were collected for the isolation of bacterial and fungal endophytes. Leaves from anthracnose infected greater yam (var. Orissa Elite) plants were taken for isolation of the pathogen *Colletotrichum gloeosporioides.* The yam field (Plate 1) and the disease symptoms observed in field is shown in Plate 2. All the samples for the study were collected from fields of ICAR- CTCRI.

4.2 ISOLATION OF *Colletotrichum gloeosporioides*

The infected leaves of greater yam which showed typical anthracnose symptoms as brown to black lesions, larger than one cm were collected and used for isolation of the pathogen. *C. gloeosporioides* (Plate 3). The isolate was identified based on the morphological descriptions of *Colletotrichum* species outlined by Mordue (1971) and Sutton (1992). Mycelial growth was observed after two days incubation in Potato Dextrose Agar. The isolate was subcultured on same media and the spore development was found within 8-10 days of culturing. Plate 4 shows the culture and conidia of isolated *Colletotrichum gloeosporioides* under light microscopy at 40X magnification. Subculturing of fungi was done at 10 days interval.

Plate 1. Field view of greater yam

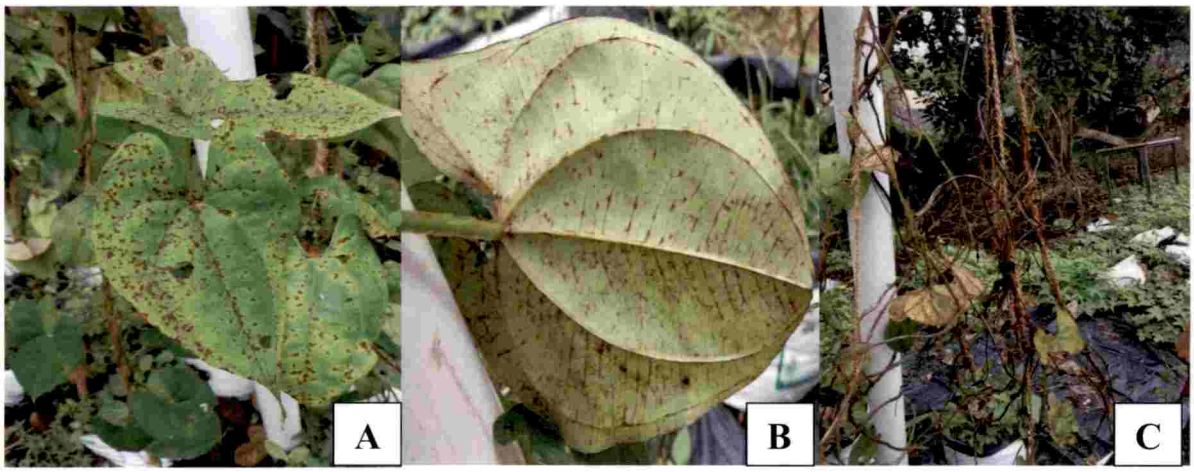

Plate2. Symptoms of anthracnose infection in greater yam. (A) Brown spots on leaf (B) Necrosis of leaf on ventral side (C) Die-back symptom

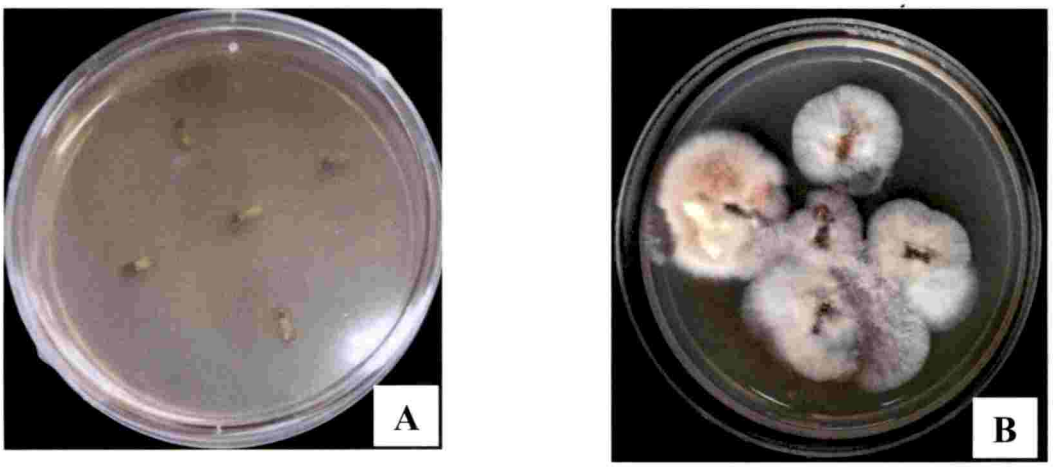

Plate 3. Isolation of *C.gloeosporioides*. (A) Disease infected leaf placed on PDA. (B) Growth of fungi from infected leaf

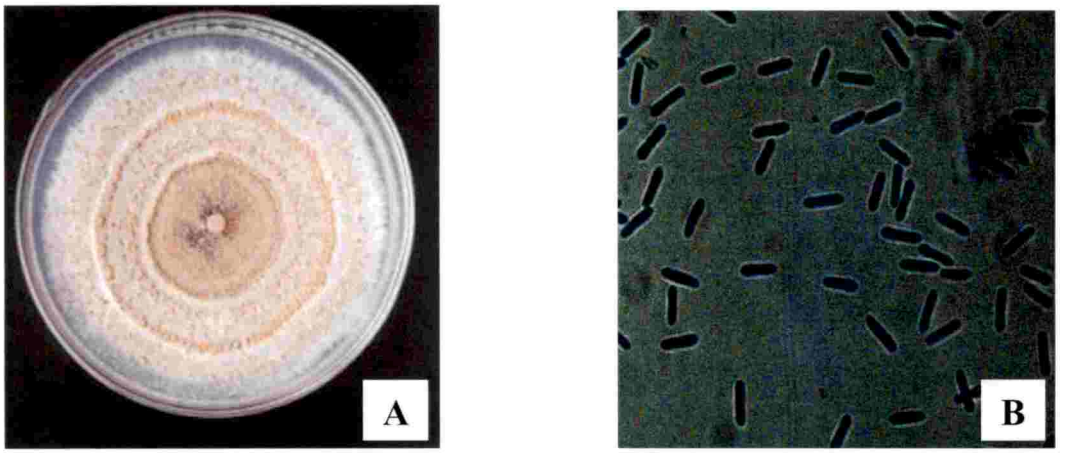

Plate 4. (A) Seven days old culture of *C.gloeosporioides* on PDA media. (B) Spores of *C. gloeosporioides* observed under microscope (40X)

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4.2.1 Testing the pathogenicity of *Colletotrichum gloeosporioides* on detached leaves

Pathogenicity of *Colletotrichum gloeosporioides* to cause infection was tested on detached young leaves of greater yam (var. Orissa Elite). After three days of inoculation symptoms of anthracnose were developed. Initially symptoms appeared as small brown spots and after 5 days smaller spots coalescence to larger spots and necrosis were found. The mature healthy leaves from greater yam were inoculated with the pathogen at a concentration of 5×10^5 spores ml^{-1} and reisolation from lesions always yielded an isolate with characteristics similar to the one used to inoculate the leaves (Plate 5). From the five isolates, most virulent one was selected.

4.2.2 Molecular identification of the pathogen

4.2.2.1 DNA isolation

CTAB method was used for the isolation of DNA from the *Colletotrichum gloeosporioides.* The extracted genomic DNA was run on agarose gel (1%) and visualized in Alpha Imager to observe the bands (Plate 6). The concentration of DNA obtained was 350 ng $\mu l^{-1}$and quality (absorbance ratio A 260/ 280) was 1.56 using a nanodrop spectrophotometer.

4.2.2.2 PCR amplification using species specific primers

PCR amplification with species specific primers (CgsF1 and CgsR1) on *C. gloeosporioides* DNA samples yielded amplicons of ~300 bp size when separated on 1.5% agarose gel (Plate 7). These results further confirmed that the isolate was *C. gloeosporioides.*

4.3 ISOLATION OF BACTERIAL AND FUNGAL ENDOPHYTES

The bacterial and fungal endophytes were isolated from leaves, stem and roots of greater yam, taro, coleus and arrowroot on Nutrient Agar and Potato Dextrose Agar

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respectively and the surface sterilization protocol of isolated strains was found to be effective in removing epiphytic organisms. Thus, the bacterial and fungal strains obtained after sterilization can be considered as true endophytic organisms since the control plates did not show any bacterial or fungal growth (Plate 8). Among 139 endophytes isolated, of which 65 and 74 were bacterial and fungal endophytes respectively. The total number of endophytes isolated from each crop is shown in Table 1. Morphologically distinct ones were selected for further studies which comprise 37 bacterial and 37 fungal endophytes. The morphological characteristics and growth rate of selected bacterial endophytes and fungal endophytes are shown in the tables Table 2 and Table 3 respectively.

4.4 *IN VITRO* SCREENING OF ENDOPHYTES AGAINST *Colletotrichum gloeosporioides*

The antagonistic effect of bacterial and fungal endophytes on *C. gloeosporioides* were calculated as inhibition of mycelial growth by dual culture method (Plate 9) and mycelial inhibition of bacterial endophytes were presented in Table 4, Fig. 1 and fungal endophytes in Table 5, Fig. 2. Among the thirty-seven bacterial endophytes screened, the isolate from arrowroot leaf (MaL1), coleus stem (SrS1) and coleus root (SrR2) exhibited minimum *C. gloeosporioides* mycelial growth of 8 mm, 1 mm and 7 mm and with the maximum antifungal activity of 88.6, 85.7 and 84.6% over control, respectively (Plate 10). They are significantly higher than other isolates.

Thirty-seven fungal endophytes were screened against *C. gloeosporioides* to test their efficacy in inhibition of mycelial growth. The mycelial growth of pathogen was minimal in dual culture with four fungal endophytes. They were endophyte from the greater yam (var. Sree Keerthi) stem (DaSiFS1), Da 251 root (Da251FR1), taro stem (Cs1FS1) and coleus stem (SrFS3) with the antifungal index of 67.1, 55.7, 49.3 and 45.3% over control, respectively (Plate 11).

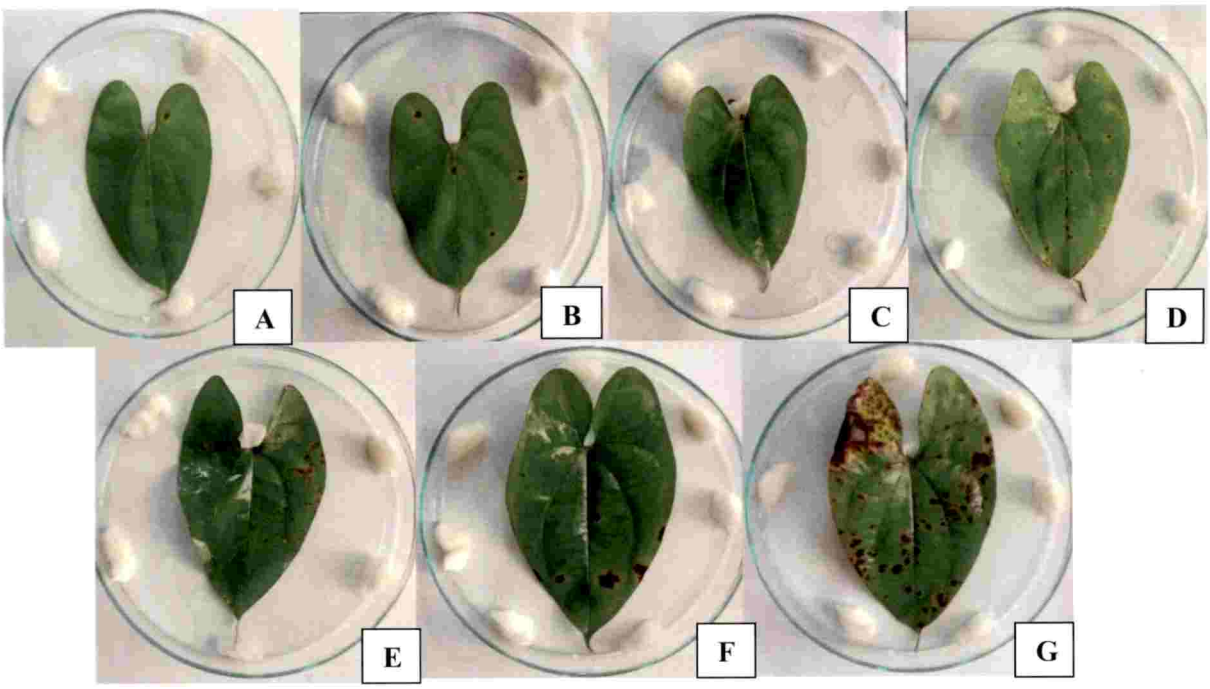

Plate 5. Development of symptoms after three days of spraying with *C. gloeosporioides*. (A) 1st day; (B, C) - 3rd day; (D, E) - 5thday; (F) - 7th day; (G) 9th day of disease development

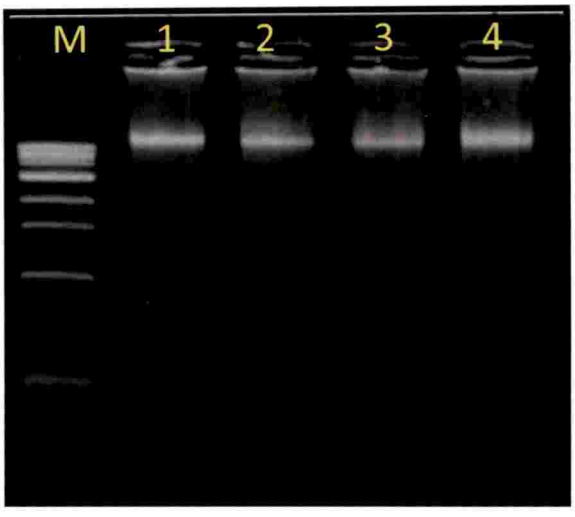

Plate 6. DNA isolated from *C. gloeosporioides*. M- ladder 1 kb, Lane 1-4 DNA from the most virulent *C. gloeosporioides* obtained

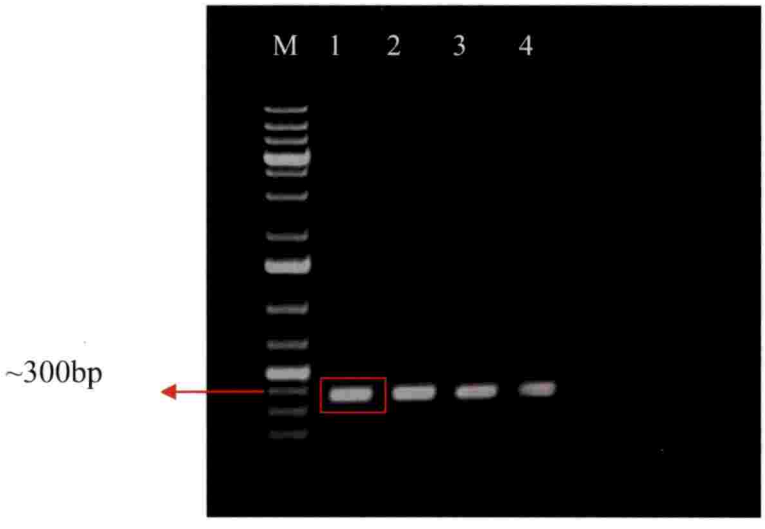

Plate 7. PCR amplification of *C. gloeosporioides* using specific primer M- 1kb plus ladder. Lane 1-4 PCR product of *C. gloeosporioides*

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Among the endophytes used in this study, the mean antifungal index was maximum in bacterial endophytes (86%) compared to fungal endophytes (54%). It was observed that results were quite consistent with findings made through conidial germination assay. The potent three bacterial endophytes (MaL1, SrS1 and SrR2) were taken for further studies.

4.5 MOLECULAR IDENTIFICATION

4.5.1 Isolation of DNA

4.5.1.1 Bacterial DNA isolation.

SDS method was performed for the isolation of DNA from the three endophytic bacterial isolates which found to be potent in antagonist activity (dual culture). The genomic DNA extracted was checked in agarose gel (1%) and visualized in Alpha imager to observe the bands (Plate 12).

The concentration (ng μl^{-1}) and quality (A260/A280) of DNA was estimated using Nanodrop spectrophotometer and results are shown in Table 6.

4.5.1.2 Fungal DNA isolation

CTAB method was performed for the isolation of DNA from the four endophytic fungal isolates which found to be inhibitory in antagonist activity (dual culture). The genomic DNA extracted was checked in agarose gel (1%) and visualized in Alpha imager to observe the bands (Plate 13).

The good concentration (ng μl^{-1}) and quality (A260/A280) of DNA was confirmed using Nanodrop spectrophotometer and results are shown in Table 6.

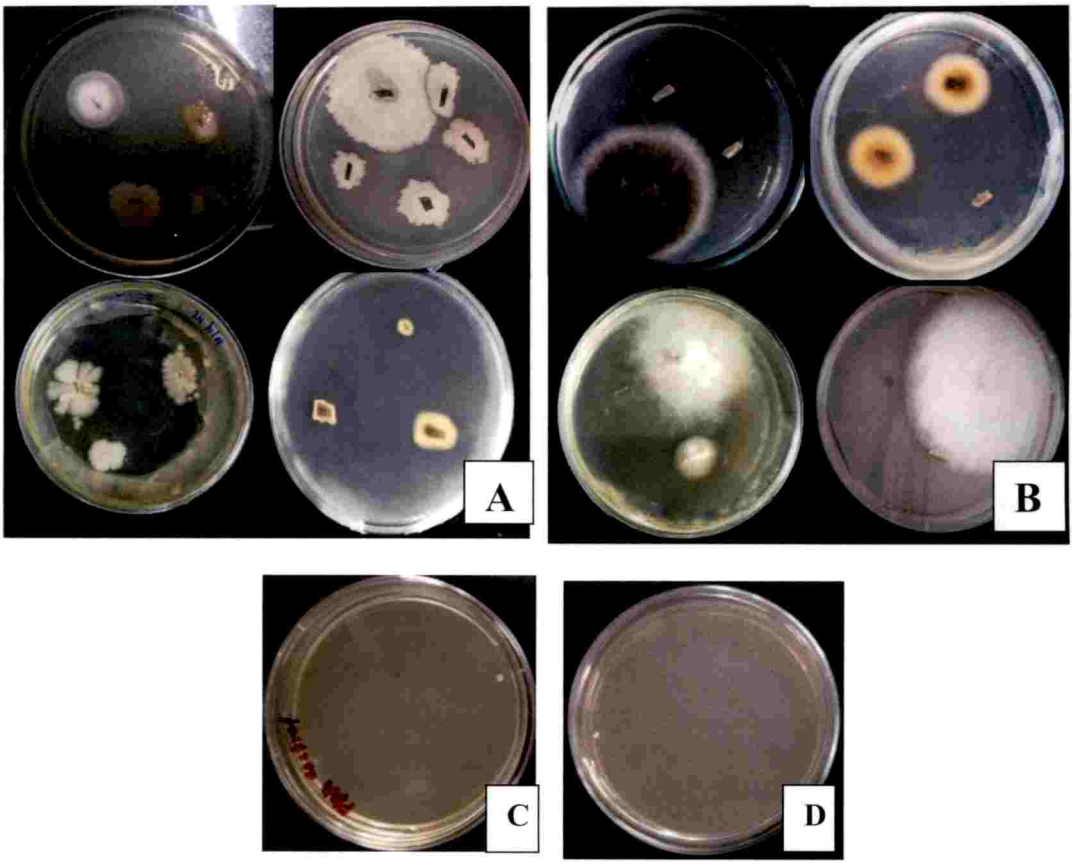

Plate 8. Endophytes isolated from different tropical tuber crops (A) Bacterial endophytes (B) Fungal endophytes (C) PDA control plate (D) NA control plate

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Table 1. Total number of bacterial and fungal endophytes isolated from different tropical tuber crops

Sample	**No. of Bacterial endophytes**			**No. of Fungal endophytes**			**Total no. of endophytes from each crop**
	Leaf	Stem	Root	Leaf	Stem	Root	
1. Greater yam *(Dioscorea alata)*							
• Sree karthika	5	3	2	4	4	1	19
• Sree keerthi	4	5	3	2	5	4	24
• Da 215	4	2	1	3	2	3	15
• Da 262	2	5	2	4	5	2	19
2. Taro (*Colocasia esculenta)* • Mukthakeshi	4	2	2	5	1	7	21
3. Coleus (*Plectranthus rotundifolius)* • Sree Latha	5	4	2	6	7	2	26
4. Arrowroot (*Maranta arundinacea)*	2	4	1	5	1	2	15

Table 2: 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	Irregular	Entire	Flat	Moderate	Opaque	Positive
25	SrS2	Circular	Entire	Flat	Punctiform	Opaque	Positive
26	SrR1	Irregular	Entire	Flat	Moderate	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	Irregular	Entire	Flat	Moderate	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

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Table 3. Morphological characteristics of fungal endophytes

Sl no.	Name of isolates	Shape	Colour	Size (in cm) on 5th day	*Growth rate	Texture
1	DaSkFL1	Round	Cream 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	Cream white	6.2	Fast	Cottony
5	DaSkFR1	Irregular	Greyish white	4	Slow	Cottony
6	DaSiFL1	Irregular	Greyish white	5	Medium	Powdery
7	DaSiFS1	Irregular	Black brown	4.5	Slow	Cottony
8	DaSiFS2	Irregular	Cream 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	Cream white	7.3	Fast	Cottony
13	Da262FS1	Round	Black 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	Cream white	5	Medium	Cottony
18	Cs1FR1	Irregular	Black brown	7.2	Fast	Powdery
19	Cs2FL1	Round	White	7.4	Fast	Cottony
20	Cs3FS1	Irregular	Black brown	6.4	Fast	Cottony
21	SrFL1	Irregular	Greyish white	7.2	Fast	Powdery
22	SrFL2	Irregular	White	5	Medium	Cottony
23	SrFS1	Irregular	Cream white	7.4	Fast	Powdery
24	SrFS2	Irregular	Greyish white	5.3	Medium	Cottony
25	SrFS3	Round	Cream white	6.4	Fast	Cottony
26	SrFS4	Round	Cream white	5	Medium	Cottony
27	SrFS5	Round	Cream white	7	Fast	Cottony
28	SrFS6	Round	Greyish white	7.4	Fast	Powdery
29	SrFR1	Round	Black 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	Cream white	4.9	Slow	Powdery
36	MaFR2	Irregular	Cream white	5	Medium	Powdery
37	MaFR3	Irregular	Cream white	5	Medium	Cottony

*Growth rate: <5 cm – Slow; 5 to 6 cm- Medium; >6 cm- Fast growing

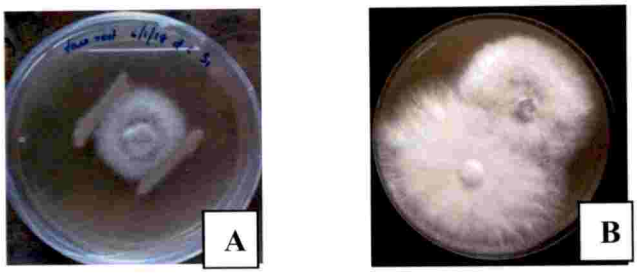

Plate 9. Antagonist activity by dual culture (A) Bacterial dual culture (B) Fungal dual culture

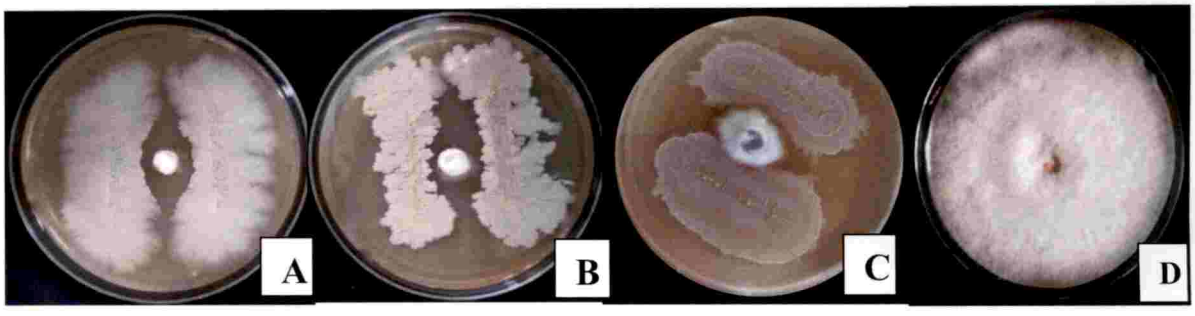

Plate 10. Potent bacterial endophytes (A) bacterial endophyte from arrowroot leaf (MaL1) (B) bacterial endophyte from coleus stem (SrS1) (C) bacterial endophyte from coleus root (SrR2) (D) control

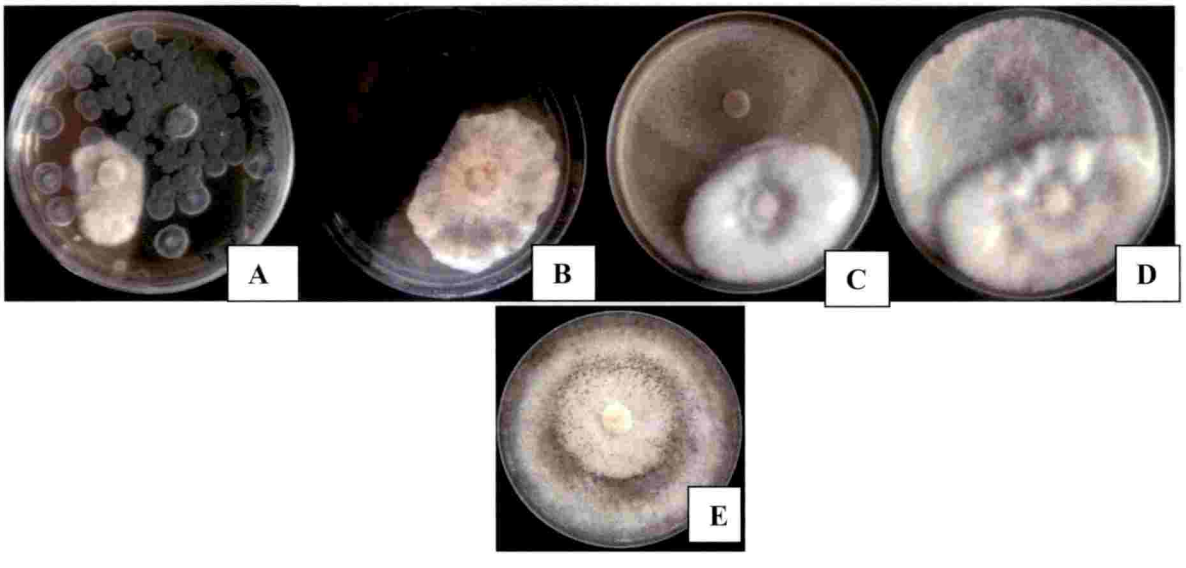

Plate 11. Fungal endophytes having more than 45% inhibition (A) Greater yam (var. Sree Keerthi) stem (DaSiFS1), (B) Taro stem (Cs1FS1), (C) Coleus stem (SrFS3), (D) Greater yam (Da 251) root (Da251R1), (E) Control

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Table 4. Mycelial inhibition of bacterial endophytes

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

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

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Table 5. Mycelial inhibition of fungal endophytes

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

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

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Fig 1 Graphical representation of antagonistic activity of bacterial endophytes on pathogen

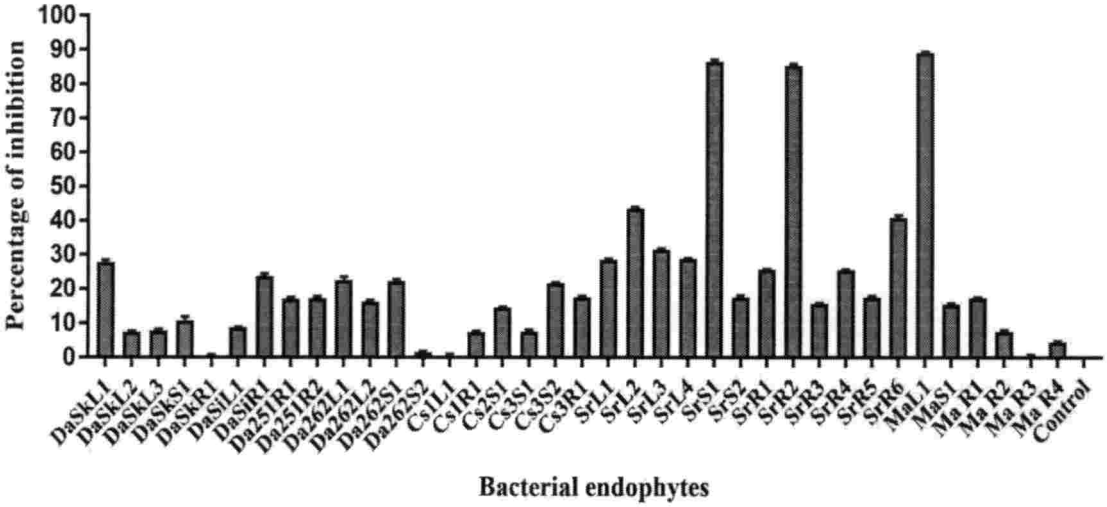

Fig 2 Graphical representation of antagonistic activity of fungal endophytes on pathogen

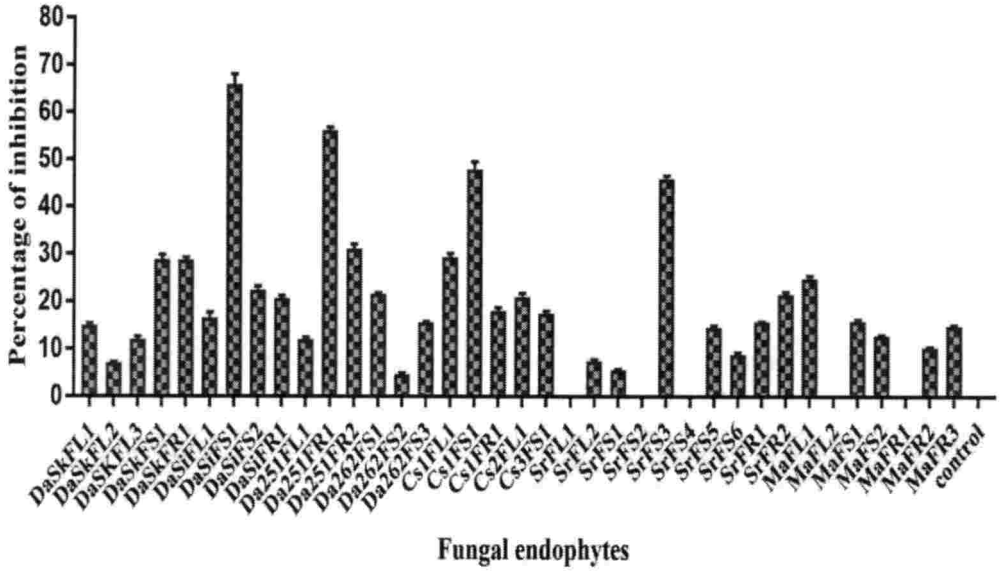

Plate 12. DNA isolated from potent bacteria. Lane 1- DNA from MaL1, Lane 2- DNA from SrS1, Lane 3- DNA from SrR2

Plate 13. DNA from fungus having above 45% inhibition. Lane 1- : DNA from DaSiFS1, Lane 2- DNA from Cs1FS1, Lane 3- DNA from SrFS3, Lane 4- DNA from Da251R1

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Table 6. Quantity and quality (absorbance ratio A 260/ 280) of isolated bacterial DNA and fungal DNA estimated using Nanodrop spectrophotometer

Sample	Concentration (ng μl^{-1})	A 260/A 280
Bacterial endophytes		
MaL1	486	1.84
SrS1	540	1.76
SrR2	886	1.88
Fungal endophytes		
DaSiFS1	336	1.89
Cs1FS1	815	1.77
SrFS3	111	1.64
Da251R1	773	1.78

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4.5.2 PCR amplification using universal primers

4.5.2.1 PCR amplification of bacterial DNA using 16S rRNA targeting primers

PCR amplification using 16S - 23S rRNA intervening sequence 8F, 1492R on bacterial DNA samples yielded amplicons of 1500 bp size when separated on 1.5% agarose gel (Plate 14).

4.5.2.2 PCR amplification using RecA for bacterial DNA

The bacterial DNA was again PCR amplified using another set of primers RecAF and RecAR in order to confirm the species. The annealing temperature was standardized by using gradient PCR temperature range from 57-65.3 °C. The optimum Tm value for better amplification of bacterial DNA was observed at 58.3 °C (Plate 15) and yielded amplicons of ~800 bp size when separated on 1.5% agarose gel (Plate 16).

4.5.2.3 PCR amplification of fungal DNA using ITS1 and ITS4 targeting primers

PCR amplification of fungal DNA using ITS1 and ITS4 targeting primers yielded a product size of ~750 bp when separated on 1.5% agarose gel and visualized in Alpha imager (Plate 17). The products were purified using Gene JET PCR Purification kit.

4.5.3 DNA sequencing

The purified PCR products were sent to the Agri Genome, India. The sequencing results were obtained as electropherogram resulting from capillary sequencing in.aib format. The forward sequences and reverse sequences were initially aligned and edited using Geneious R11version 11.1.4. The sequences after edition were run through the online BLAST (BLASTn) program of NCBI. Through the BLASTn search, the database sequences which were similar to the input nucleotide query were identified.

The BLAST analysis of sequences identified the bacterial (Fig 3, 4, and 5) and fungal endophytes (Fig 6,7,8 and 9). The bacterial endophytes MaL1, SrS1 and SrR2

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identified using 16S rRNA targeting primers were *Bacillus sp.*, *Bacillus subtilis* and *Bacillus subtilis* respectively. While the MaL1 bacteria was identified as *Bacillus cereus* when PCR amplified using RecA primers.

The fungal endophytes DaSiFS1, Da251FR1, Cs1FS1 and SrFS3 were identified as *Penicillium citrinum, Phanerochaete australis, Curvularia pseudobrachyspora and Diaporthe batatas* respectively.

The sequences were submitted in NCBI and accession numbers are shown in the Table 7

4.6 SPECIES SPECIFIC PRIMER DESIGNING

The primers were designed specifically to amplify the *Bacillus subtilis* and *Bacillus cereus* using Primer BLAST software. The specific primers were synthesized from PrimerX, XclerisLab, Ahmedabad utilizing the common region in both bacteria.

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

The synthesized primer pair is as follows:

EndoBa1_Forward primer: 5' ACCTTGACGGTACCTAACCA 3'
EndoBa1_Reverse primer: 5' GTCCATTGTAGCACGTGTGT 3'

4.6.1 Gradient PCR for standardizing annealing temperature

After the synthesis of primers, the annealing temperature was standardized by using gradient PCR. Being an important step of PCR, the annealing temperature of the primer pair was optimized by conducting gradient PCR from 49.9- 59 °C, and the optimum Tm value for better amplification was observed at 53 °C (Plate 18).

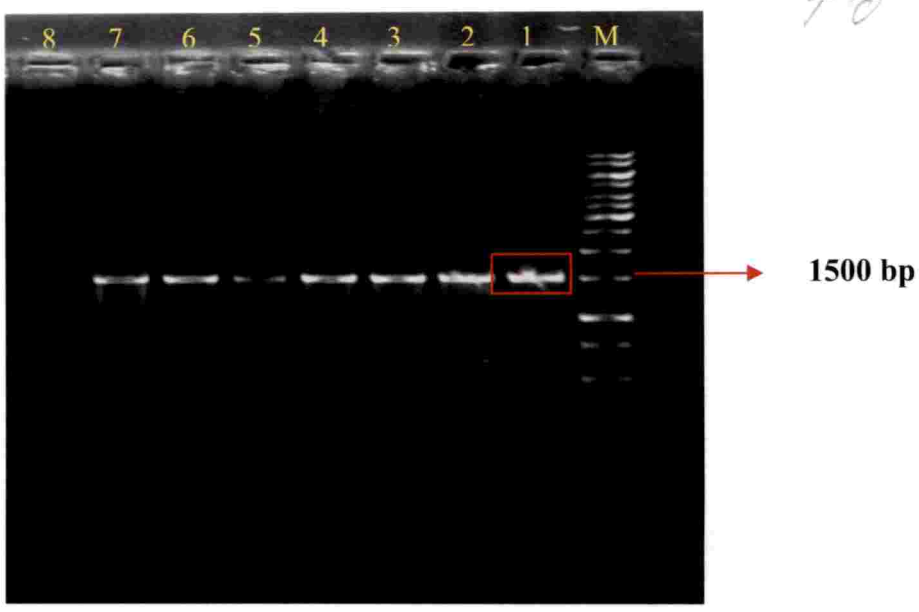

Plate 14. PCR amplification of bacterial DNA using 16SrRNA primers. M- Ladder 1kb, Lane 1,2- PCR product of MaL1, Lane 3,4 - PCR product from SrS1, Lane 5,6 - PCR product from SrR2, Lane 7- Positive control, Lane 8- Negative control

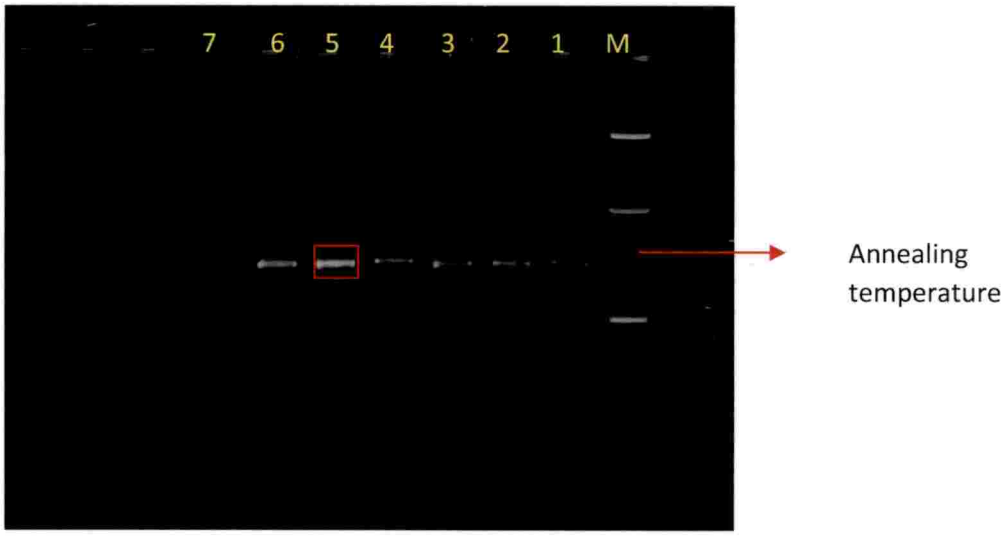

Plate 15. Gradient PCR (57-65.3°C) using RecA primers M- ladder 1 kb plus Lane 1- 65.3 °C, Lane 2- 64.1 °C, Lane 3- 62.3 °C, Lane 4- 60 °C. Lane 5- 58.3 °C ,Lane 6- 57 °C, Lane 7- control

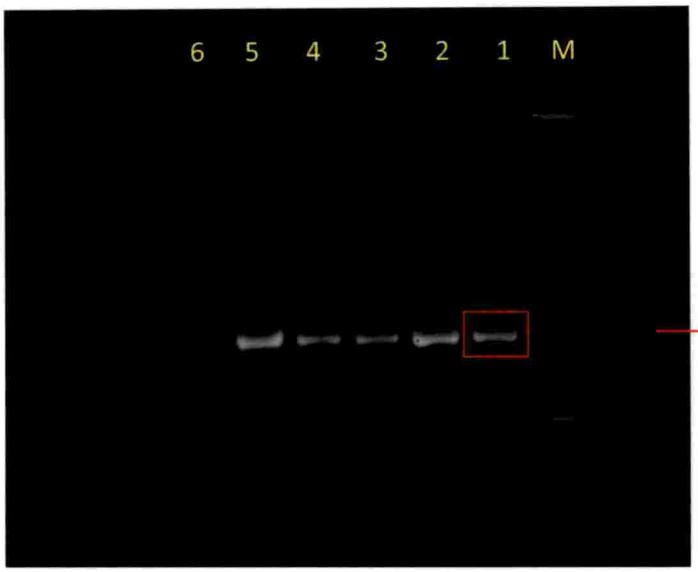

Plate 16. PCR amplification of bacterial DNA using RecA primers. M- ladder 1 kb plus Lane 1,2 - PCR product of MaL1, Lane 3- PCR product from SrS1, Lane 4 - PCR product from SrR2, Lane 5- Positive control, Lane 6- Negative control

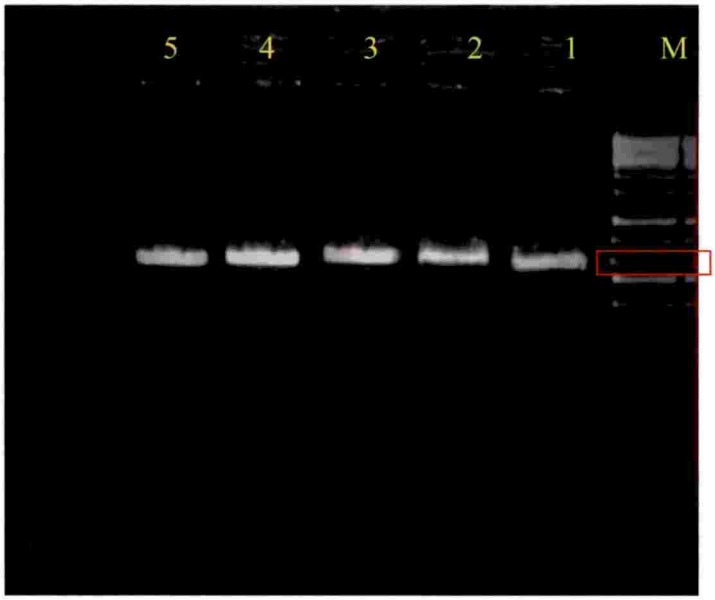

Plate 17. PCR amplification of fungal DNA using ITS 1 and ITS 4 primers. M- Ladder 1kb, Lane 1- PCR product of DaSiFS1, Lane 2- PCR product of Cs1FS1, Lane 3-PCR product of SrFS3, Lane 4-PCR product of Da251R1, Lane 5-Positive control, Lane 6-Negative control

Sequences of bacterial endophytes:

Bacillus cereus

GCTCTTAAACAAATAGAAAAACAGTTCGGCAAAGGTTCCATTATGAAACT
GGGAGAAAAGACAGATACAAGAATTTCTACTGTCCCAAGCGGCTCCCTCA
CTCTTGATACAGCACTAGGAATTGGCGGATATCCGCGCGGACGGATTATG
AAGTATACGGTCCTGAAAGCTCAGGTAAAACAACCGTGGCGCTTCATGCG
ATTGCTGAGGTTCAGCAGCAGGGCGGACAAGCTGCGTTTATCGATGCGGA
GCATGCGTTAGATCCGGTATACGCGCAAAAGCTCGGTGTCAACATCGAAG
AGCTTTTACTGTCGCAGCCTGACACAGGCGAGCAGGCGCTTGAAATTGCG
GAAGCGTTGGTTCGAAGCGGCGCAGTTGACATCGTTGTTGTCGACTCTGC
GGCTCTCGTCCCGAAAGCGGAAATTGAAGGCGACATGGGAGATTCGCAT
GGTTTACAAGCTCGCTTAATGTCTCAAGCGCTTCGTAAGCTCTCAGGGGC

Bacillus subtilis

GCTCTTAAACAAATAGAAAAACAGTTCGGCAAAGGTTCCATTATGAAACT
GGGAGAAAAGACAGATACAAGAATTTCTACTGTACCAAGCGGCTCCCTCG
CTCTTGATACAGCACTGGGAATTGGCGGATATCCTCGCGGACGGATTATT
GAAGTATACGGTCCTGAAAGCTCAGGTAAAACAACTGTGGCGCTTCATGC
GATTGCTGAAGTTCAGCAGCAGGGCGGACAAGCCGCGTTTATCGATGCGG
AGCATGCGTTAGATCCGGTATACGCGCAAAAGCTCGGTGTTAACATCGAA
GAGCTTTTACTGTCTCAGCCTGACACAGGCGAGCAGGCGCTTGAAATTGC
GGAAGCATTGGTTCGAAGCGGGGCAGTTGACATTGTCGTTGTCGACTCTG
TAGCCGCTCTCGTTCCGAAAGCGGAAATTGAAGGCGACATGGGAGATTCG
CATGTCGGTTTACAAGCACGCTTAATGTCTCAAGCGCTTCGTAAGCTTTCA
GGGGCCATTAACAAATCGAAGACAATCGC

Bacillus subtilis

GCTCTTAAACAAATAGAAAAACAGTTCGGCAAAGGTTCCATTATGAAACT
GGGAGAAAAGACAGATACAAGAATTTCTACTGTACCAAGCGGCTCCCTCG
CTCTTGATACAGCACTGGGAATTGGCGGATATCCTCGCGGACGGATTATT
GAAGTATACGGTCCTGAAAGCTCAGGTAAAACAACTGTGGCGCTTCATGC
GATTGCTGAAGTTCAGCAGCAGGGCGGACAAGCCGCGTTTATCGATGCGG
AGCATGCGTTAGATCCGGTATACGCGCAAAAGCTCGGTGTTAACATCGAA
GAGCTTTTACTGTCTCAGCCTGACACAGGCGAGCAGGCGCTTGAAATTGC
GGAAGCATTGGTTCGAAGCGGGGCAGTTGACATTGTCGTTGTCGACTCTG
TAGCCGCTCTCGTTCCGAAAGCGGAAATTGAAGGCGACATGGGAGATTCG
CATGTCGGTTTACAAGCACGCTTAATGTCTCAAGCGCTTCGTAAGCTTTCA
GGGGCCATTAACAAATCGAAGACAATCGC

81

Sequences of fungal endophytes:

Cuvularia pseudobrachyspora

AAGGACTGAGATCCTACTGATCGAGGTCAAACGTAAAAATGTAGTCTTGA
TGGATTGCCGTCCTTTTTGCTGATTGCAAGCGCAAAAATGTGCTGCGCTGC
GAAACCAGTAGGCCGGCTGCCAATCGTTTTAAGGCGAGTCTTTGGGCGAG
GCCAAAGACAAAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAAT
GACGCTCGAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGT
TCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACGTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTGTAAATGATTTACATTTGTTATACTGACGCTGATTGCAACTGCATAAAA
AAAGGTTTATGGTGTGGTCCTGGTGGCGGGCGAACCCGCCCAGGAAACA
ACAAGTGGYAAAAAAGGGTGATATATCACCTTTTTATTGTTGTCCGCGGT
CCCCCCAGGACACCACACAAAAAACCTTTTTTTGGGCAGGCGAACGGGGG
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GGAAATCAAAAAGGGCAGGCCGGTCCGGGCGCCATTTGCCCCCCCAGCTT
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ATAAAAATGATGGGCGGCCGGCCTACTGGTTCCAAAAATTATTTTTTTTTG
CTCTGGAAGCAGGAAAAAGGCCCCCACCCATCAGATTTTTTTTATTTTGAC
GCGGACCGGGCGGCTTCCCTTTTTTTTTAGGAAACGGGGGGGGGGGGAAC
CCC

Penicillium citrinum

AACGACTGCACCTACTGATCGAGGTCACCTGAGATAATTAAAGGTTGGGG
GTCGGCTGGCGCCGGCCGGGCCTACTAGAGCGGGTGACGAAGCCCCATA
CGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCC
CGGCGGCGGGACGGGACCCAACACACAAGCCGGGCTTTGAGGCGCAGCA
ATGACGCTCGGACAGGCATGCCCTCGGGAATACCAGAGGGCGCAATGTG
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CATTTCGCTGCGTTCTTCATCGATGCGGGAACCAAGAGATCCGTTTGGTTG
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GACAGGTTCATGAAGAACGATTAGTCAGACTTTATTGCGAATCTCTGTTT
TCACAATGAAGACGGGGAAAATGCAAAAACTATGTAATTGCAGATTTGG
GGAATCTTCGTTTTTTGAACGCACTTCCCCCATTGGTATTCGGGAGCTTTG
CCTGCCAATTTCATTGTGCCCTCAGGCGGGTTTTGTGTGTGGCCCCCCCCC
CCCCGCGGGGAACAACAAAAAGGAAGCGGCGCACGCTCGGTCTGAGCGT
AGGACCTCCCACCGGAGAGAGGGCGGGCGGGCACCACCATATTATATTAT

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CTAGTTGACCCGGACAGGTAGAATCCCGCTACTAAGATACGAGAGGCGA
GAAT

Diaporthe batatas

GTGCGGGCCATCGTACCTGATCGAGTCAATTTTCAGAAGTTGGGGGTTTA
ACGGCAGGGCCCAGGGCCTCCAGACTGACTTACCCGCCTGGCGGCGCCCT
TCGGGCCCCCCGAGGGAGGCAGCCCCCCAACCACTTTTATACTCAGAGAA
AAAAGAATAAKTTCAACAGATCCTTTCTGATAAACACGATCGTGTTAATC
AGAAAGGACTGAATTTGACATTTTGTTTTCTGAGTCATTACAGTGGTTGGG
GGCTGCCCCTCGGGGGCCGAAAGCGCTCGCCGACAGGGAAGTTACAGTCT
GGGCCGGCGGCGCTACCCCACGATTACCCACGAGAATACCCGTGAACTAG
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Phanerochaete australis

AAGACTGGCCAAATCGTACCTGATTTGAGGTCAGATGTCAAGTAAGTTGT
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GCGCAGATAATTATCACACCGAAGCGATCCGTTACACTCACGCTAATACA
TTTAAGAGGAGCCGATTCAACGAGGAACCAGCACGACCTCCAAGTCCATG
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CTTCATCGATGCGAGAGCAAAGAGATCCGTTGCTGAAAGTGGTATAAAGA
TGCGTTATACGCAAGTTTACATTTCTTAAACTGAAGCGTTTGTAGTAAAAC
ATAGGGAAGGCTTCCACGCGAGCTGTTTAGACCTCGCCCCTTCTGCCGAC
CTACAAAAAGTGCACAGAGGGTTGAAGAGTGGATGAGCAGGGTGTGCAC
CATGCCCCGAGAGCCCAGCTACAACCCGTTCAGTTACTCGTAAATGATCC
TTCCGCAGGTTCACCCTACGGAAAGGCTTCGGGTATACGGTGAGTCTGGG
ATGCCCCTGGTCACACCTCACTGCTTTTGTGGGAATAACGGGGGATTTTTT
TTTTAACCTTTGAAAAACCGTTAAAATTTTAATTTAATTTTTTTGTGAAAC
CAGAGAGGAAAAGGGGAAATTTTAAACCTTTCCCCCCCCCAAGCGCGTTG
GGGGATCCCCCCCCCAAATTTTAAAAGAGGGGGGGGCCTCTGCGCCCTTTA
GGGGAAGACTCGTGAATTATTSGCCGCGGAANAAAAAAAGCCCCTCTCCT
TTCTCTTTGCTCACTCCACGAGACCCTTGAAAGAGAAGAACCGGAGAAAA

83

Fig 3. BLAST analysis of MaL1 isolate (*Bacillus cereus*)

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus cereus strain MBGJa3 chromosome	913	913	100%	0.0	100%	CP026523.1
Bacillus intestinalis strain T30, complete genome	845	845	100%	0.0	97%	CP011051.1
Bacillus subtilis subsp. spizizenii strain NRS 231, complete genome	845	845	100%	0.0	97%	CP010434.1
Bacillus subtilis subsp. spizizenii strain DK1-SA11 recombinase A (recA) gene, partial cds	845	845	100%	0.0	97%	KF779126.1
Bacillus subtilis subsp. spizizenii str. W23, complete genome	845	845	100%	0.0	97%	CP002183.1
Bacillus subtilis strain QB61 chromosome	832	832	100%	0.0	96%	CP029461.1
Bacillus subtilis strain GS 188 genome	832	832	100%	0.0	96%	CP022391.1
Bacillus subtilis strain SR1 genome	832	832	100%	0.0	96%	CP021985.1
Bacillus subtilis subsp. subtilis strain SRCM101392, complete genome	832	832	100%	0.0	96%	CP021921.1
Bacillus subtilis strain TLO3, complete genome	832	832	100%	0.0	96%	CP021169.1
Bacillus subtilis strain GQJK2, complete genome	832	832	100%	0.0	96%	CP020367.1
Bacillus subtilis strain NCIB 3610 chromosome, complete genome	832	832	100%	0.0	96%	CP020102.1

Fig 4. BLAST analysis of SrS1 (*Bacillus subtilis*)

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis strain QB61 chromosome	957	957	100%	0.0	100%	CP029461.1
Bacillus subtilis strain GS 188 genome	957	957	100%	0.0	100%	CP022391.1
Bacillus subtilis strain TLO3, complete genome	957	957	100%	0.0	100%	CP021169.1
Bacillus subtilis strain GQJK2, complete genome	957	957	100%	0.0	100%	CP020367.1
Bacillus subtilis strain NCIB 3610 chromosome, complete genome	957	957	100%	0.0	100%	CP020102.1
Bacillus subtilis subsp. subtilis str. 168, partial genome	957	957	100%	0.0	100%	CP019663.1
Bacillus subtilis subsp. subtilis str. 168 genome	957	957	100%	0.0	100%	CP019662.1
Bacillus subtilis subsp. subtilis strain QB5413 genome	957	957	100%	0.0	100%	CP017313.1
Bacillus subtilis subsp. subtilis strain QB5412 genome	957	957	100%	0.0	100%	CP017312.1
Bacillus subtilis strain BS16045, complete genome	957	957	100%	0.0	100%	CP017112.1
Bacillus subtilis subsp. subtilis strain 168G, complete genome	957	957	100%	0.0	100%	CP016852.1
Bacillus subtilis subsp. subtilis strain PS38 genome	957	957	100%	0.0	100%	CP016789.1

Fig 5. BLAST analysis of SrR2 (*Bacillus subtilis*)

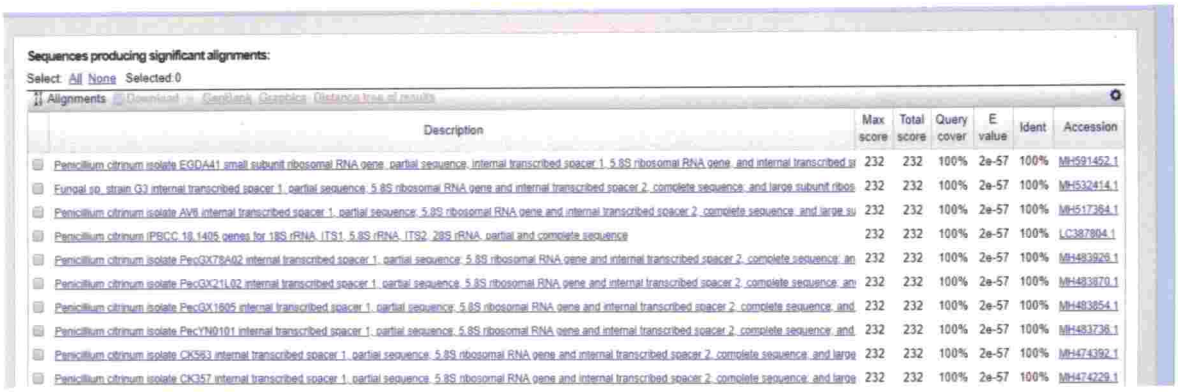
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis strain QB61 chromosome	957	957	100%	0.0	100%	CP029461.1
Bacillus subtilis strain GS 188 genome	957	957	100%	0.0	100%	CP022391.1
Bacillus subtilis strain TLO3, complete genome	957	957	100%	0.0	100%	CP021169.1
Bacillus subtilis strain GQJK2, complete genome	957	957	100%	0.0	100%	CP020367.1
Bacillus subtilis strain NCIB 3610 chromosome, complete genome	957	957	100%	0.0	100%	CP020102.1
Bacillus subtilis subsp. subtilis str. 168, partial genome	957	957	100%	0.0	100%	CP019663.1
Bacillus subtilis subsp. subtilis str. 168 genome	957	957	100%	0.0	100%	CP019662.1
Bacillus subtilis subsp. subtilis strain QB5413 genome	957	957	100%	0.0	100%	CP017313.1
Bacillus subtilis subsp. subtilis strain QB5412 genome	957	957	100%	0.0	100%	CP017312.1
Bacillus subtilis strain BS16045, complete genome	957	957	100%	0.0	100%	CP017112.1
Bacillus subtilis subsp. subtilis strain 168G, complete genome	957	957	100%	0.0	100%	CP016852.1

Fig 6. BLAST analysis of DaSiFS1 (*Penicillium citrinum*)



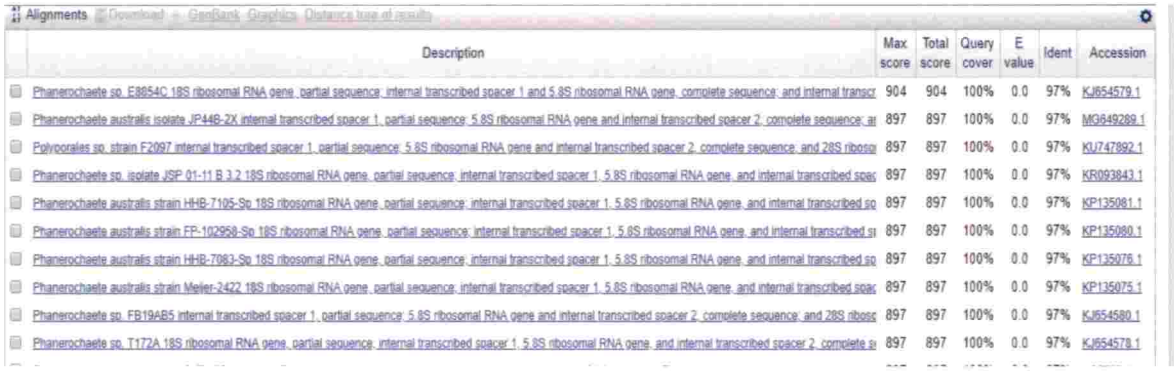
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Penicillium citrinum isolate EGDA41 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	232	232	100%	2e-57	100%	MH591452.1
Fungal sp. strain G3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribos	232	232	100%	2e-57	100%	MH532414.1
Penicillium citrinum isolate AV6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large su	232	232	100%	2e-57	100%	MH517384.1
Penicillium citrinum IPBCC 18.1405 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	232	232	100%	2e-57	100%	LC387804.1
Penicillium citrinum isolate PecGX78A02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	232	232	100%	2e-57	100%	MH483926.1
Penicillium citrinum isolate PecGX21L02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; an	232	232	100%	2e-57	100%	MH483870.1
Penicillium citrinum isolate PecGX1605 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	232	232	100%	2e-57	100%	MH483854.1
Penicillium citrinum isolate PecYN0101 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	232	232	100%	2e-57	100%	MH483736.1
Penicillium citrinum isolate CK583 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large	232	232	100%	2e-57	100%	MH474392.1
Penicillium citrinum isolate CK357 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large	232	232	100%	2e-57	100%	MH474229.1

Fig 7. BLAST analysis of Da251R1 (*Phanerochaete australis*)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Phanerochaete sp. E8854C 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcr	904	904	100%	0.0	97%	KJ654579.1
Phanerochaete australis isolate JP44B-2X internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; ar	897	897	100%	0.0	97%	MG649289.1
Polyporales sp. strain F2097 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosor	897	897	100%	0.0	97%	KU747892.1
Phanerochaete sp. isolate JSP 01-11 B 3.2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spac	897	897	100%	0.0	97%	KR093843.1
Phanerochaete australis strain HHB-7105-Sp 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	897	897	100%	0.0	97%	KP135081.1
Phanerochaete australis strain FP-102958-Sp 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	897	897	100%	0.0	97%	KP135080.1
Phanerochaete australis strain HHB-7083-Sp 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	897	897	100%	0.0	97%	KP135076.1
Phanerochaete australis strain Meijer-2422 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spac	897	897	100%	0.0	97%	KP135075.1
Phanerochaete sp. FB19AB5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosc	897	897	100%	0.0	97%	KJ654580.1
Phanerochaete sp. T172A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete se	897	897	100%	0.0	97%	KJ654578.1

Fig 8. BLAST analysis of Cs1FS1 (*Curvularia pseudobrachyspora*)

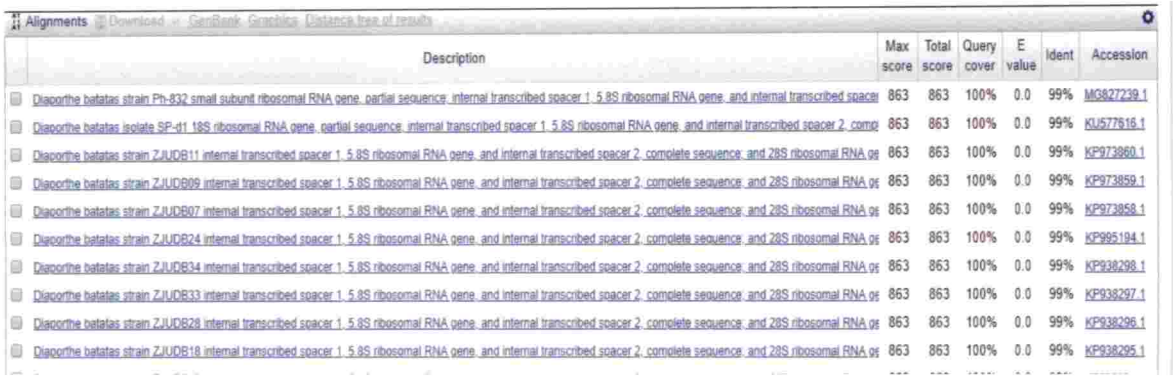
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Curvularia pseudobrachyspora strain CIMAP: Ac-112017 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and i	941	941	100%	0.0	100%	MG645008.1
Curvularia pseudobrachyspora strain CPC 28808 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	941	941	100%	0.0	100%	MF490819.1
Curvularia lunata small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete s	941	941	100%	0.0	100%	MF289553.1
Curvularia aeria strain IP 2328.96 isolate ISHAM-ITS_ID MITS1388 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	941	941	100%	0.0	100%	KP131939.1
Cochliobolus lunatus isolate MBC002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	941	941	100%	0.0	100%	JQ685447.1
Cochliobolus lunatus 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seque	941	941	100%	0.0	100%	JN542520.1
Dothideomycete sp. 7407 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete s	941	941	100%	0.0	100%	EU680517.1
Macrophomina sp. IBL 03164 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comple	941	941	100%	0.0	100%	DQ682587.1
Curvularia lunata strain IP 2328.95 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cc	941	941	100%	0.0	100%	DQ836800.1

Fig 9. BLAST analysis of SrFS3 *(Diaporthe batatas)*



Description	Max score	Total score	Query cover	E value	Ident	Accession
Diaporthe batatas strain Ph-832 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	863	863	100%	0.0	99%	MG827239.1
Diaporthe batatas isolate SP-d1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comp	863	863	100%	0.0	99%	KU577616.1
Diaporthe batatas strain ZJUDB11 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP973860.1
Diaporthe batatas strain ZJUDB09 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP973859.1
Diaporthe batatas strain ZJUDB07 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP973858.1
Diaporthe batatas strain ZJUDB24 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP995194.1
Diaporthe batatas strain ZJUDB34 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP938298.1
Diaporthe batatas strain ZJUDB33 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP938297.1
Diaporthe batatas strain ZJUDB28 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP938296.1
Diaporthe batatas strain ZJUDB18 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ge	863	863	100%	0.0	99%	KP938295.1

Table 7. 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%	Submitted
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%	Submitted

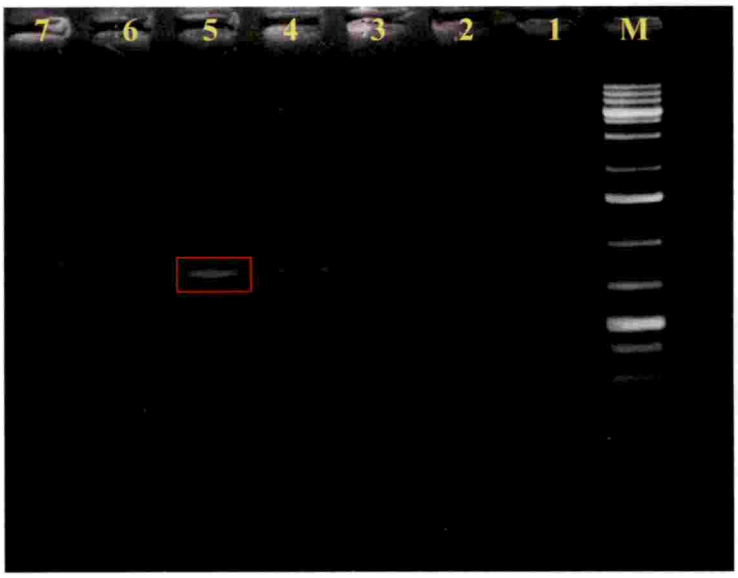


Plate 18. Gradient PCR of synthesized primer. M- ladder 1kb plus ; Lane 1- 59 °C; Lane 2- 58.3 °C; Lane 3- 57.1 °C; Lane 4- 55.3 °C; Lane 5-53 °C; Lane 6 – 51.3 °C Lane 7-49.9 °C

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4.7 TISSUE CULTURE OF *D.alata* VARIETY ORISSA ELITE

4.7.1 Explant preparation and inoculation

The tissue cultured plantlets maintained in ICAR-CTCRI were utilized as mother culture for the nodal culture of *D.alata.* Nodal culturing of *D.alata* (var. Orissa Elite) was done in half MS media. The cultured tubes were maintained in culture room at 25±1^{0}C at under 16/8 h photoperiod of 2000 lux light intensity and 60% relative humidity inside culture room. Single node was excised aseptically from a plantlet and kept in half MS media in test tubes.

Shoot induction was observed within 2 weeks of inoculation and root development was observed within 4 weeks. When the plants reached a height of ~10 cm with about 9-10 nodes in 3 months, they are sub cultured in half MS media.

4.7.2 Hardening of plants

After three months of subculturing the plants were ready for hardening. The sufficiently rooted plants were removed from culture tube and washed thoroughly to remove adhering media. They were then transplanted to paper cups containing sterile coir pith and kept inside the growth chamber. Plate 19 shows the different developmental stages of tissue culture plants – Orissa Elite which were utilized for colonization study.

4.8 CHECKING COLONIZATION OF POTENTIAL ENDOPHYTES ON ROOTS, STEM AND LEAVES OF TISSUE CULTURED *D.alata*

4.8.1 Inoculation of endophytes on tissue cultured plantlets

The potent *Bacillus cereus* and *Bacillus subtilis* were inoculated into the tissue culture plants through root dip method (Plate 20) and spraying method to confirm the colonization of two bacterial endophytes. Three replications were maintained for each treatment (Plate 21). Control plants were maintained without inoculating the bacteria.

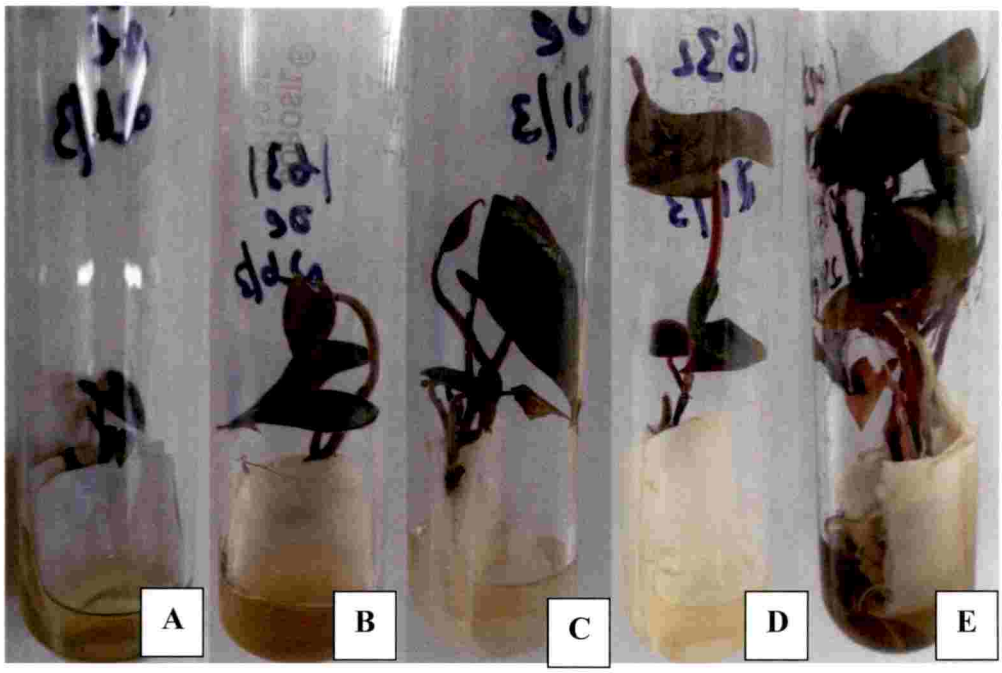

Plate 19. Different growth stages of *D.alata* : (A and B) shoot initiation after 1 week of inoculation (C and D) shoot and root development after 2 months (E) adequately rooted plants ready for hardening (F) hardened plants in paper cups containing sterile coir pith.

4.8.2 Endophytes re isolation from inoculated tissue culture plantlets

The bacteria *Bacillus cereus* and *Bacillus subtilis* were isolated from the inoculated plantlets. The leaves, stem and roots of plantlets from both methods of inoculation were used for the isolation in order to confirm the colonization of bacteria in leaves, stem and root. The isolation was done after eight days of treatment. The growth of bacteria was observed in all plant parts within 24 hr after inoculation on nutrient agar (Plate 22).

From root dipping method, a total of 13 colonies and from spraying method 18 isolates were obtained. The bacteria obtained were subcultured and identified using species specific primers.

4.8.3 DNA isolation

The DNA was isolated from the re isolated endophytes from leaves, stem and roots by SDS method. The genomic DNA extracted was checked in agarose gel (1%) and visualized in Alpha imager and observed the bands (Plate 23).

4.8.4 PCR amplification using species specific primers

The DNA of bacterial isolates from leaves, stem and roots were amplified using the designed species-specific primers yielded amplicons of 750 bp size when separated on 1.5% agarose gel (Plate 24). These results further confirmed that the isolate was *Bacillus subtilis and Bacillus cereus.* Hence the colonization of the potent endophytes *Bacillus subtilis* and *Bacillus cereus* in leaves, stem and roots were confirmed.

Plate 20. Root dip method. (A) root tips pruned plantlets dipped in bacterial suspension (B) Plantlets after root dipping

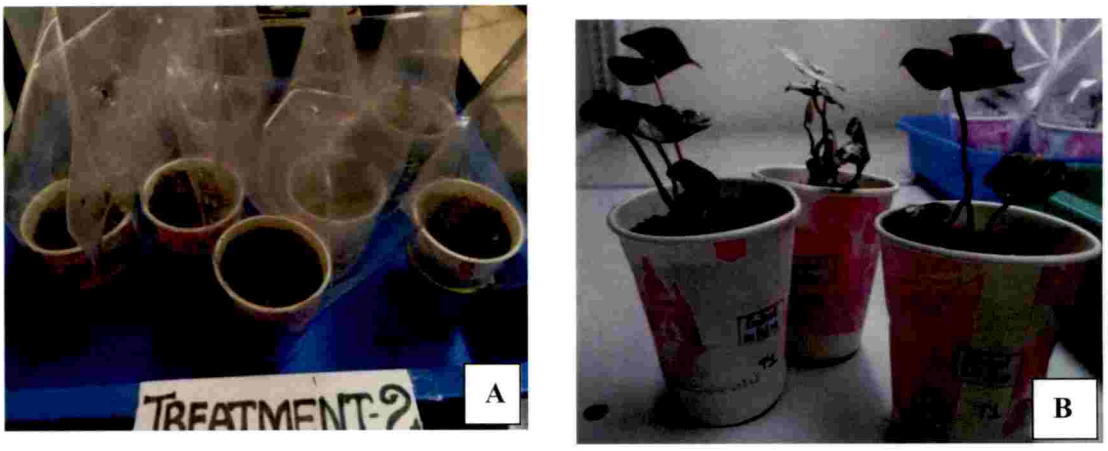

Plate 21. (A) Endophytes inoculated plants by spraying method (B) Three replications were maintained

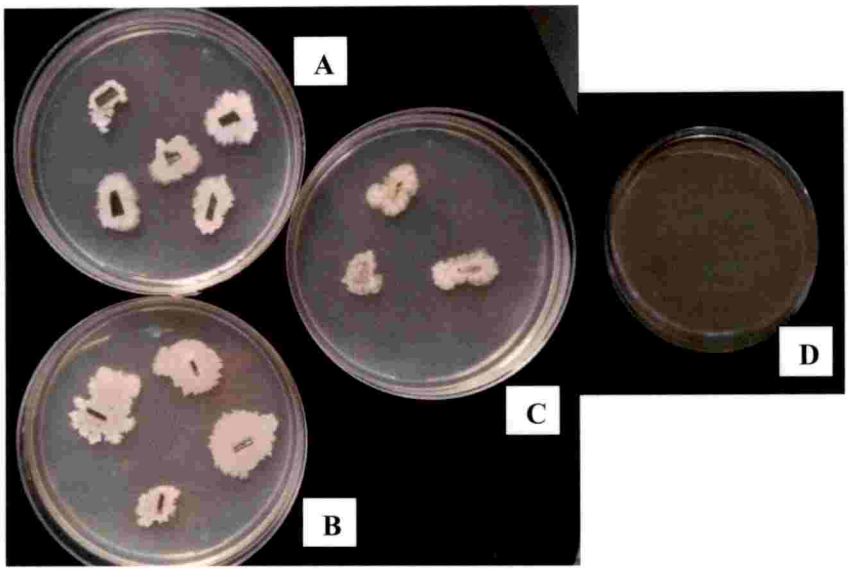

Plate 22. Endophytes isolated from inoculated plants (A) isolated from leaves (B) from stem (C) from root (D) control

Plate 23. DNA from the isolated endophytes. Lane 1,2- endophyte from leaves; Lane 3,4- from stem; Lane 5,6,7- from root

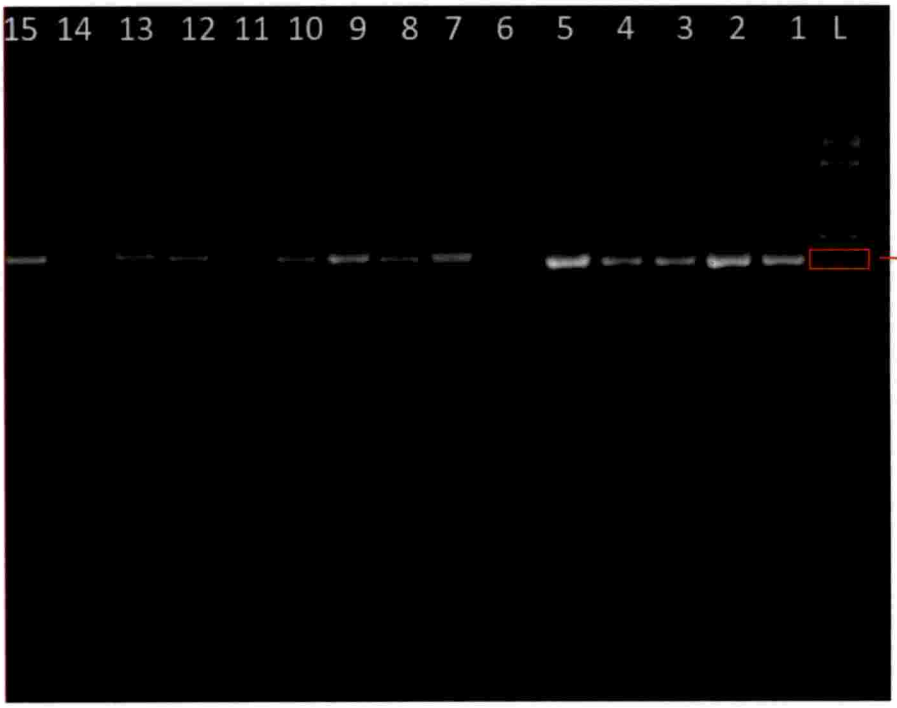

Plate 24. PCR amplification using species specific primers. M- 1kb ladder; Lane 1,2- PCR product of endophyte from leaves (T1); Lane 3,4- from stem (T1); Lane 5,6- from root (T1); Lane 7,8- from leaves (T2); Lane 9-10- from stem (T2); Lane 11-15- from root (T2)

4.9 EFFECT ON ENDOPHYTES ON GREATER YAM PLANTS IN GROW BAGS AGAINST ANTHRACNOSE DISEASE- POT TRIAL STUDIES

The plantlets were treated with all the treatments described in 3.10.1. The tubers were successfully grown with 100% seed germination and the bacterial endophytes were shown great effect in managing the disease as well as growth promotion. A total of 50 treatments were maintained containing four treatments of two bacteria and control, fungicide control. Five replications were maintained.

4.9.1 Pot trial studies in glass house

The plantlets were maintained inside the glass house (Plate 25) and disease was initiated after spraying with the pathogen (10^5 spores ml^{-1}). Endophytic spraying was done four times with ten days interval. Two spraying was given after the initiation of disease symptoms. 20 ml bacterial suspension was sprayed to each plant every time. The growth parameters like length of vine and number of leaves was recorded after two months of planting were shown in Table 8. The disease intensity was calculated and score was given which were shown in Table 9.

Bacillus cereus was found to be efficient in controlling the disease followed by *Bacillus subtilis*. The intensity of disease observed in *Bacillus cereus* treated plants was only 11.9% and the score was 2 while *Bacillus subtilis* had disease intensity 35% with a score of 3. The growth parameters were found to be increased in tuber treatment with *Bacillus cereus.*

Among all the treatments, even though the fungicide (carbendazim (0.05%) treatment (T10) did not show significant increase in length of vine and number of leaves, it showed highest reduction in disease intensity compared to control (94%) and other treatments. Among the endophyte treatments, T1, Tuber treatment with *B. cereus* showed significantly highest length of vine (91.2 cm), number of leaves (21) and lowest disease intensity (10.90 %). The disease intensity is 86.4 % decrease over absolute control. This is on par with T3 (Spraying of *B. cereus*), T4 (combination of

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tuber, soil and spraying of *B. cereus*, T5 (tuber treatment with *B. subtilis*) and T2 (soil application of *B. cereus*), which showed 84.5 to 86.3 % decrease in disease intensity over control.

4.9.1 Pot trial studies in open condition

The plants were placed in open condition with all treatments without the application of the pathogen (Plate 26). The growth parameters were recorded after 3 months of planting and shown in Table 10. The disease intensity and score of each treatment was tabulated and presented in Table 11. The endophyte *Bacillus cereus* treated plants were shown to be efficient by 11.6 % disease intensity and score of 2 which is followed by *Bacillus subtilis* with 32.5 % disease intensity and score of 3. The spraying treatment of *Bacillus cereus* shown less disease incidence when compared with other treatments but spraying was found to be not much efficient in other bacteria. Overall comparison makes tuber treatment was best treatment followed by combination of all treatment. The growth parameters found to be increased in *Bacillus cereus* treated plants as same as in glass house studies. Thus, the endophyte *Bacillus cereus* has great growth promotion activity. In open condition also generally, same trend was observed. The fungicide (carbendazim (0.05%) treatment (T10) did not show significant increase in length of vine and number of leaves. However, it showed highest reduction in disease intensity compared to control (92.8 %) and other treatments. While comparing the endophyte treatments T3 (Spraying of *B. cereus*) showed significantly highest length of vine (827.2 cm), number of leaves (76). The lowest disease intensity was observed in T3 (Spraying of *B. cereus*)(9.56%) The disease intensity is 89.3 % decrease over absolute control. This is on par with T1 (Tuber treatment with *B. cereus*) , T2 (soil application of *B. cereus*) and T4 (combination of tuber, soil and spraying of *B. cereus* which showed 83.7 to 88.8 % decrease in disease intensity over control.

Plate 25. Pot trial in glass house

Plate 26. Pot trial in open condition

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Table 8. Efficiency of endophytic bacteria on growth parameters in glass house pot trial

Treatment	*Length of vine (cm)**	*Number of leaves**
T1	91.2^{A}	21^{BA}
T2	78.4^{BAC}	15^{DC}
T3	73.8^{BC}	15^{DC}
T4	74.7^{BC}	22^{A}
T5	87.0^{BA}	19^{BAC}
T6	74.0^{BC}	18^{BAC}
T7	78.4^{BAC}	13^{D}
T8	81.4^{BAC}	17^{BDC}
T9	68.4^{DC}	14^{DC}
T10	55.0^{D}	12^{D}

* Mean of five replications ** Means with the same letter are not significantly different.

Table 9. Efficency of endophytic bacteria on disease management

Treatment	*Disease intensity**	Disease score
T1	10.90^{D}	2
T2	10.98^{D}	2
T3	10.44^{D}	2
T4	12.48^{D}	2
T5	10.98^{D}	2
T6	26.38^{C}	3
T7	27.52^{C}	3
T8	33.72^{B}	3
T9	80.30^{A}	5
T10	4.36^{E}	1

* Mean of five replications ** Means with the same letter are not significantly different.

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Table 10. Efficiency of endophytic bacteria on growth parameters of pot trial open condition

Treatment	*Length of vine (cm)**	*Number of leaves**
T1	827.2^{A}	76^{A}
T2	432.8^{C}	53^{ABCD}
T3	733.8^{AB}	63^{ABC}
T4	709.5^{AB}	68^{AB}
T5	527.0^{BC}	53^{ABCD}
T6	433.6^{BC}	45^{BCD}
T7	478.4^{BC}	43^{BCD}
T8	461.4^{BC}	43^{BDC}
T9	368.4^{C}	38^{DC}
T10	275.0^{C}	32^{D}

* Mean of five replications **Means with the same letter are not significantly different.

Table 11. Efficency of endophytic bacteria on disease intensity

Treatment	*Disease intensity**	Disease score
T1	12.33^{D}	2
T2	14.34^{D}	2
T3	9.56^{D}	1
T4	10.01^{D}	1
T5	23.54^{C}	2
T6	27.35^{C}	3
T7	35.45^{B}	3
T8	41.78^{B}	3
T9	89.42^{A}	5
T10	6.45^{E}	1

* Mean of five replications ** Means with the same letter are not significantly different.

DISCUSSION

5. DISCUSSION

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Tropical root and tuber crops are important edibles of many developing countries including India during food scarcity. Apart from cassava, sweet potato, yams are the important tropical tuber crops with high production potential. Greater yam (*Dioscorea alata*) is the major yam species cultivated in India. Anthracnose/ die back disease caused by *Colletotrichum gloeosporioides* is a serious foliar epiphytotic disease of *D. alata*, which can cause extensive yield loss globally. Isolates of *Colletotrichum* has been found to cause damage in different crops such as coffee, cereals, fruits, vegetables, legumes and grass. *Dioscorea* is a plant with very high medicinal and nutritional value. Thus, its disease management is of prime concern. The existing strategies like chemical methods and development of resistant varieties have not been much effective. There are no exclusive and effective biointensive strategy developed for anthracnose management till date. The management of disease using endophytes is most promising and a durable strategy. The presence of endophytes had been studied in some tuber crops but sufficient information is lagging concerning the utilization of endophytic microbes in these life saving crops (Nayak *et al.*, 2017). In the current study, isolation of a wide range of bacterial as well as fungal endophytes and subsequently their bioactive potential has been explored and the results are discussed here.

Endophyte is any organism including fungus and bacteria which can colonise any part of the plant without producing any symptoms on the host. They are considered as beneficial organisms with growth promotion activity and induction of host resistance through antibiosis, competition etc. Endophytes especially bacterial endophytes are seen in most of the plants (McInroy and Kloepper, 1995; Sturz, 1995). The host endophyte interaction has been defined as altruism, commensalisms, symbiosis or passivity to pathogenicity. Some of the endophytic isolates are known to produce antibiotics and siderophores (Sessitsch *et al.*, 2004).

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Endophytic bacteria are able to reduce or prevent the deleterious effects of certain pathogenic organisms. Berg *et al.,* (2005) reported that endophytes from potato plants displayed antagonistic activity against several fungi and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas.* Endophytic bacteria interact more closely with the host plant and therefore, could be efficient biological control agent in sustainable crop production.

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 were shown antagonist activity against *Colletotrichum gloeosporioides* causing anthracnose in greater yam *in vitro*. Bacterial strains belonging to *Erwinia* and *Bacillus* species complex have been isolated from yam rhizomes or tubers by Zhang *et al.*, (2010). *Bacillus* endophytes were reported to colonise the internal regions of plants and found effective in the biocontrol of various plant diseases more than one caused by different soilborne pathogens and which can be used in commercially available biocontrol products (Ongena *et al.*, 2005). There are reports showing that *Bacillus subtilis* and *Bacillus 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. Still, *B. subtilis* and *B. cereus* has not been reported as a biological control agent against anthracnose in greater yam.

Molecular techniques exhibit high sensitivity and specificity for identifying microorganisms. The endophytes were identified using universal primers. 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 indications have stated that identification of *B. subtilis* and *B. cereus* cannot rely on the limited information obtained from 16S rRNA gene analysis and

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biochemical and physiological assays (Heather and Geraldine, 2011; Oleg *et* al., 2004), which is consistent with our results. 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).Some of the *Bacillus* strains share almost equal 16S rRNA gene sequences (99.2–99.6 %) which usually exemplify limited diversity for members of closely related strains (Ash *et al.*, 1991).Recombinase A (RecA) gene sequence analysis is reported to be more efficient for 16S rRNA gene sequencing in differentiating between closely related *Bacillus* species or strains (Kwon *et al.*, 2009). Milad *et al.,* 2016 reported that RecA provided twofold more discrimination than 16S rRNA gene analysis of *Bacillus subtilis* and *Bacillus cereus*. In our study *Bacillus cereus* was identified using Rec A primers.

Two potential isolates explored in this study (*B. cereus* and *B. subtilis*) from tropical tuber crops were applied to study the management of anthracnose in greater yam plants under glass house and open condition. Colonization of endophytic bacteria is a prerequisite for a successful biocontrol agent to be able to establish efficient protection (de Weert and Bloemberg 2006; Weng *et al.*, 2013). The colonization of both the endophytes was proved through recovery from greater yam tissue cultured plants inoculated with endophytes in media and also amplification using designed specific primers. The results from present study showed that *B. cereus* and *B. subtilis* provided evidence of biocontrol of *Colletotrichum gloeosporioides*, as well as of plant growth promotion in pot experiment as well as glass house experiment over control.

The present results on the effect of antagonistic bacteria against *C. gloeosporioides* were consistent with the results of some of other studies. Work on the effect of endophytes on *C. gloeosporioides* causing greater yam anthracnose is not available. However, evidence of this coincidence 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), in the treatment of several

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strains of *Streptomyces sp.* for controlling anthracnose, reduced significantly the number and size of spots on leaf seedlings and suspension of 108 and 109 $cfuml^{-1}$ of *Streptomyces sp.* caused reduction of disease by 79 % and 93 %, respectively. In the present experiments, disease severity index in inoculated greater yam plants with *Bacillus* isolates varied from 1 to 3 that showed significant difference from untreated control with disease severity index of 5. Furthermore, these results were consistent with the results of other investigators including Intra *et al.*, (2011), in which *Streptomyces cavourens is* was used against *Colletotrichum* spp.

The suggested and established mechanisms of *Bacillus spp.* for antagonistic activity comprised of metabolite production (Furuya *et al.,* 2011) and also induction of resistance by triggering different defense mechanisms. Therefore, we could conclude that the inhibitory effect may be due to the antifungal substances and also effectors which could induce resistance in host plants which needs further study.

Efficiency of bacterial endophyte on *Colletotrichum gloeosporioides* in the present study showed that the infection by the pathogen was significantly reduced by endophyte precolonization. Also, it was found that the bacterial endophytes were not only able to control anthracnose in greater yam but also promoted plant growth too. The growth stimulation is may be due to endophyte association provide nitrogen fixation (Hurek *et al.*, 2002; Iniguez *et al.*, 2004; Sevilla *et al.*, 2001) or phytohormones production or by enhancing availability of minerals (Sessitsch *et al.*, 2002; Sturz *et al.*, 2000). Moreover, these endophytes produce 2-3 butanediol and aceotin which is a newly discovered mechanism for plant growth promotion (Ryu *et al.,* 2003).

As a conclusion, the endophytic bacteria studied in this work had a great potential in managing the anthracnose in greater yam and it could be an effective bio intensive method as an alternative to chemical control and extensive development of resistant varieties. In future the molecular mechanism of arresting the disease intensity at

endophyte as well as host level may contribute to develop the suitable delivery system for managing the disease.

SUMMARY

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5. SUMMARY

The study entitled "Identification and evaluation of endophytes from tropical tuber crops against *Colletotrichum gloeosporioides* (Penz.) Sacc. causing anthracnose in greater yam (*Dioscorea alata* L.)" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during 2017-2018. The objective of this study was to isolate and identify the potential endophytes against the pathogen and confirmation of its colonization in greater yam plants.

Greater yam (var. Orissa Elite) leaves showed typical spots of anthracnose infection were used for the isolation of the pathogen *Colletotrichum gloeosporioides.* From the isolates obtained most virulent one was selected for the study through pathogenicity testing. The isolated pathogen was confirmed through PCR amplification using species specific primer. DNA was isolated from the pathogen by CTAB method and the product size obtained (~360bp) confirmed that the isolate was *C. gloeosporioides.* The endophytes for the study were isolated from leaves, stem and roots of different tropical tuber crops such as greater yam (var. Sree Karthika, Sree Keerthi, Da 251 and Da 263), taro (var. Muktakeshi), coleus (var. Sree Latha) and arrowroot. A total of 139 endophytes were isolated comprising 65 bacterial endophytes and 74 fungal endophytes. Morphologically distinct ones were selected for the further studies which included 37 bacterial and 37 fungal endophytes. These endophytes were further screened for antagonist activity against *C. gloeosporioides in vitro* by dual culture method and growth inhibition was calculated. Three bacterial endophytes MaL1, SrS1 and SrR2 with maximum antifungal index of 88.6%, 85.7% and 84.6% over control respectively and four fungal endophytes having more than 45% inhibition were obtained. However, the activity was less when compared to bacterial endophytes.

The potent endophytes were identified by PCR method. The DNA of three bacterial endophytes was isolated by SDS method and PCR amplified using 16SrRNA primers and recA primers. The amplicon size obtained was 1500 bp and ~ 800 bp respectively which were sequenced and identified as *Bacillus cereus* (MaL1), *Bacillus subtilis* (SrS1) and *Bacillus subtilis* (SrR2. Similarly, fungal DNA was isolated using CTAB method and PCR amplified using ITS1 and ITS4 primers. The products obtained at 750 bp size were sequenced and identified the organisms as *Penicillium citrinum, Curvularia pseudobrachyspora, Diaporthe batatas* and *Phanerochaete australis.* The sequences of both bacteria and fungus were submitted in NCBI. Since the fungal endophytes were found to be less potent when compared to bacterial endophytes, only the bacterial endophytes were utilized for further evaluation in managing disease and colonization efficiency.

A specific primer was designed which could amplify both the bacteria, based on the common region in the sequence of *Bacillus cereus* and *Bacillus subtilis.* The primers were synthesized and annealing temperature was found out using gradient PCR. The predicted size of the product was 750 bp. For the colonization studies, tissue culture plantlets of greater yam (var. Orissa Elite) were utilized and endophytes were inoculated by two methods, viz., root dipping and spraying. The *Bacillus cereus and Bacillus subtilis* were inoculated on the plantlets and the inoculated endophytes were isolated after eight days of inoculation. The DNA from the isolated endophytes were PCR amplified using the designed specific primer and the product obtained at ~800bp confirmed the colonization of *Bacillus cereus* and *Bacillus subtilis* in the inoculated plants.

The efficiency of endophytes on disease management was studied by pot trial method. Two pot trial method were followed, first one inside the glasshouse with artificial inoculation of the pathogen and second one in the open condition with

natural infection. In the studies, ten treatments were applied comprising tuber treatment, soil treatment, spraying and combination of all treatments of *Bacillus cereus* and *Bacillus subtilis*, control and fungicide control. For each treatment five replications were maintained. From the two experiments, it was observed that the anthracnose intensity was less in *Bacillus cereus* followed by *Bacillus subtilis* inoculated plants over control with a disease intensity of 11.6 % and 32.5 % respectively. It was found that spraying treatment of *B.cereus* showed highest reduction in disease severity followed by tuber treatment and combination treatment.

The growth parameters like length of vine and number of leaves of *Bacillus cereus* and *Bacillus subtilis* inoculated plants were recorded. After three months of planting it was found that *Bacillus cereus* had significant growth promoting activity followed by *Bacillus subtilis* when compared with control.

The 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 pathogens 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 disease in field after evaluation. Moreover, the endophytes could also be utilized for various important diseases of tropical tuber and other crops.

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APPENDICES

APPENDIX I

CULTURING OF *Colletotrichum gloeosporioides* AND ENDOPHYTES

Nutrient Agar (NA)

Suspend 2.8 grams of NA powdr (HIMEDIA) in 100 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50 °C. Mix well and pour into sterile Petri plates.

Potato Dextrose Agar (PDA)

Suspend 3.9 g of PDA powder (HIMEDIA) in 100 ml distilled water and boil to dissolve. Sterilize by autoclaving at 15 psi pressure, 121°C for 15 minutes. Add 100 µl of Ampicillin (100 mg ml^{-1}) to cooled media, mix well and pour into the sterile Petri plates.

APPENDIX II

DNA ISOLATION

CTAB Extraction Buffer

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

SDS Buffer

10 % SDS- Dissolve 1 g SDS in 10 ml of distilled water

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SDS (10 %) – 30 µl

Proteinase K (20 mg ml^{-1})- 3 µl

TAE Buffer, 50X (pH 8.0)

Tris-base - 242 g

Glacial acetic acid - 57.1 ml

0.5 M EDTA - 100 ml

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

TAE buffer, 1 X

2 ml 50 X TAE + 98 ml distilled water

Agarose Gel (0.8%)

Agarose - 1.5 g

1 X TAE Buffer - 100 ml

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

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

APPENDIX III

TISSUE CULTURE OF *D. alata*

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

MS media powder (HIMEDIA PT021X1L) – 2.4 g

Sucrose – 15g

Polyvinylpyrrolidone (PVP) – 1.0 g

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

0.1N HCl (100 ml)

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

0.1 N NaOH (100 ml)

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

ABSTRACT

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IDENTIFICATION AND EVALUATION OF ENDOPHYTES FROM TROPICAL TUBER CROPS AGAINST *Colletotrichum gloeosporioides* (PENZ.) SACC. CAUSING ANTHRACNOSE IN GREATER YAM (*Dioscorea alata* L.)"

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Abstract of the thesis

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9. ABSTRACT

Greater yam (*Dioscorea alata*) is recognized as a major tuber crop in tropical countries. One of the main constrain in greater yam production is anthracnose disease which is caused by a fungus *Colletotrichum gloeosporioides*. Several chemical fungicides, resistant varieties and biological control has been adopted for managing the disease and Still these methods are not found to be much effective in controlling the disease. Endophytes are microorganisms, both fungi and bacteria reside inside the host without causing any apparent harm to the host and they possess various potential funtions in host plant like growth promotion, disease control etc. In this study, the potential endophytes both bacteria and fungi against *Colletotrichum gloeosporioides* causing anthracnose in greater yam was screened and identified. The endophytes from different tropical tuber crops were utilized for screening against the pathogen by dual culture method. A total of 139 endophytes isolated, 37 bacterial and 37 fungal endophytes were found to be morphologically distinct and were selected for screening. From that, 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. These endophytes were identified using molecular methods as bacterial endophytes were *Bacillus cereus, Bacillus subtilis*, and *Bacillus subtilis.*, the fungal endophytes as *Penicillium citrinum, Phanerochaete australis, Curvularia pseudobrachyspora and Diaporthe batatas* repectively. The fungal endophytes were found to be less potent when compared to bacterial endophytes. Specific primers were designed based on the common regions of potent bacteria and its colonization in tissue culture plants were confirmed using these species-specific primers. The pot trial studies in glass house and in open condition proved that *Bacillus cereus* followed by *Bacillus subtilis* had potential activity in managing the anthracnose disease as well as growth promotion activity. In glass house as well as open condition, *Bacillus cereus* showed a disease intensity of 11.6 % with a score of 2. It could be a best biointensive method to manage anthracnose disease in greater yam.