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Studies on Endophytes Associated with Medicinally Important *Saraca asoca* (Roxb.) Willd and their Antagonistic Activity against *Phytophthora colocasiae*

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Abstract

Taro (*Colocasia esculenta* (L.) Schott), a clonally propagated aroid grown largely in humid tropical areas of the world, is an important staple or subsistence crop for millions of people in developing countries. The oomycete *Phytophthora colocasiae* that cause taro leaf blight is the most devastating disease. The main purpose of the study was to evaluate the antagonistic activity of endophytic bacteria and fungi associated with medicinally important Ashoka tree **(***Saraca asoca* (Roxb.) Willd) against the pathogen *P. colocasiae*. The study involves the following phases such as isolation of endophytic bacteria and fungi, evaluation of antagonism against the pathogen using dual culture method and identification of potent endophytes. Among the 65 bacterial isolates tested, five *viz*., *Bacillus pumilis*, *Bacillus safensis, Bacillus altitudinis, Bacillus amyloliquefaciens* and *Comamonas testosterone* and 23 fungal isolates , two, *viz*., *Nigrospora oryzae* and *Diaporthe* sp. showed significantly high antagonistic effect (70-84%). This study shows that these endophytes can be utilized for developing a promising biocontrol agent against *P. colocasiae* in taro field.

Key words: Taro, *Phytophthora colocasiae*, endophytes, biocontrol, antagonism

Introduction

Taro (*Colocasia esculenta* (L.) Schott) is a major root crop of importance to hundred millions of people in tropical to sub-tropical and temperate regions of the world. It belongs to a monocot family Araceae (aroids), which is global in its distribution. Africa ranks first in area and production of taro, followed by Asia and Oceania. The world production of taro is estimated at 11.8 million tons per annum (Vishnu et al., 2012) produced from about 2 million hectares with average yield of 6 t ha^{-1} (Singh et al., 2012). Most of the global production comes from developing countries characterized by small holder production systems relying on minimum external resource input (Singh et al, 2012). FAO (2016) published global data on the taro production indicating that West Africa (Nigeria, Cameroon and Ghana) is by far the largest taro producing region. In India, two taro types viz., C.esculenta var.esculenta and C. esculenta var. antiquorum are commonly cultivated throughout the country but its exact acreage and production are not known. Taro is grown mainly for its edible leaves and corms, although all parts of the plant including corms, cormels, leaves, flowers, rhizome and stalk are edible (Lakhanpaul et al., 2003). The corms, leaves and petioles are considered as a rich source of carbohydrates, proteins, minerals and vitamins (Lakhanpaul et al., 2003).

Worldwide, it is believed that crop diseases reduce agricultural productivity by more than 10%, equivalent to half a billion tonnes of food every year (Hunter and Iosefa, 1993). Although taro is susceptible to attack by at least twenty three pathogens, only a few cause serious reduction in growth and production. Leaf blight of taro was first reported in Java about a century ago, but has since spread to various parts of Asia and the Pacific. Leaf blight caused by *Phytophthora colocasiae* (Marian Raciborski, 1900) and Pythium root and corm rot, incited by *Phytophthora* spp. are economically important disease of taro worldwide including India causing crop losses upto 30-50% and results in reduced harvest (Misra and Chowdhury, 1997). Since commercial cultivars do not have sufficient resistance to leaf blights, cultural practices and fungicide applied at 15 days intervals form the basis for leaf blight management programme.

Ashoka is the most ancient tree of India, generally known as a "ashok briksh", botanically known as a *Saraca asoca* (Roxb.), De. wild or *Saraca indica* belonging family Caesalpinaceae. These are the wonderful tree that claims to cure several diseases according to ayurvedic medicine. All parts of this plant are considered pharmacologically important and has especially been used to manage various disorders. *Saraca asoca* has been reported to contain phytoconstituents like flavonoids, steroids, glycosides, saponins, tannins, carbohydrates, proteins along with lot of pharmacological activities. Endophytes are microorganisms that are present in tissues of different plants part (leaf, stem and root) without causing any damage to plants and also develop a communal relationship with the host plant.

Biological agents have been considered as an alternative approach for controlling various plant diseases (Nega, 2014: Tjamos et al., 2010). Endophytes have received a great attention because of their intimate and non-detrimental association with plants, release an array of bioactive compounds that play an important role in the biological control of various pathogens. They are microorganisms that live inside the plant tissues, in most cases the microbial relationship with the host plant is symbiotic or mutualistic association with no obvious disease symptoms or morphological changes on their host (Schulz and Boyle, 2006). Because endophytes live in a steady environment inside the plant, they have more antagonistic potentiality than microorganisms isolated from rhizosphere, plant surface, or soil (Dowler and Waver, 1974; Andrews, 1992). Biological control agents are relatively cheaper, less laborious and environmentally friendly. According to Okigbo (2000), biological control has proved to be durable on its effect and has the advantage of not requiring repeated periodic application as in case of chemical fungicide.

Materials and Methods

Isolation of endophytes

For isolation of endophytes, healthy plant tissues including matured leaves, stem, young flowers and matured flowers were carefully collected from Ashoka tree (*Saraca asoca)* grown at ICAR-CTCRI during January 2017 and carried over to lab in sterile plastic sampling bags. They were processed within one hour after collection. The plant tissues were washed in running tap water and those with superficial injury that was visible to the naked eye were excluded and excised into 1-2 cm segments. The disinfection and isolation were performed according to Araujo et al., 2002. Surface sterilization was done by sequential immersion in 70% ethanol for 2 min, 4 % sodium hypochlorite for 2-3 min and finally rinsed three times in sterile distilled water and placed into nutrient agar and potato dextrose agar. The plates were incubated at 28-30ºC for 24 hrs. Surface sterility test was performed for each of the sample to ensure the elimination of surface microorganism by streaking the water of final wash in the same media. The colonies representing different morphologies were picked at random and purified by restreaking on the same medium plates for 2-3 times. Pure bacteria were sub cultured on NA, PDA slants and stored at 4ºC.

Retrieval of test pathogen and pathogenicity assay

Five isolates of *P. colocasiae* were obtained from the microbial repository of Division of Crop protection, ICAR-CTCRI, which were isolated previously from different diseased crop plants from different areas. All the five isolates of the test pathogen *P. colocasiae* were cultured on carrot agar plate and checked their virulence. A modified floating disc method was used for the virulence assay. Plants without visible symptoms of any disease were selected and three fully expanded healthy leaf disc (succeptible variety of taro, Sree kiran) were

assigned randomly. Each leaf was washed in tap water, towel-dried and placed with their abaxial side upwards on petri dishes. Excised leaf sample were floated in sterile distilled water were inoculated with mycelial disc of *P. colocasiae* and leaf fragment with only agar plug served as control. The experimental set up was incubated at 25°C in dark and observed for the symptoms. Subsequently, the lesion diameter was recorded after 4 days. Re isolation according to Koch's postulate was made from all resulting lesions. The assay was repeated twice (Nath et al., 2015). Test pathogen exhibited more virulence was used for *in vitro* screening.

Invitro screening of endophytes against *P. colocasiae*

Bacterial and fungal isolates were tested for antagonism against *Phytophthora colocasiae in vitro* with minor modification of procedure and media (potato carrot agar: carrot juice 250 g l^{-1}, potato 250 g l^{-1}, dextrose 20 g l^{-1} ,agar 20 g l^{-1}). The endophytic bacterial strains were inoculated in rectilinear streaks at opposite ends of the medium and similarly endophytic fungal plugs were used. A four mm in diameter *Phytophthora* plug was placed in the center of the petri dish. Only pathogenic fungi were kept as control. The petri dishes were incubated at 28ºC and the inhibition of mycelia growth was observed after five or seven days. The percentage of the mycelia growth was estimated by the formula $I = (C-T)/C \times 100$, where I is the percent inhibition of mycelial growth over the control, C is the mycelial growth of fungal pathogen in control and T is the mycelial growth of fungus in endophytes inoculated plate. The experiment was carried out in three independent replicates (Zhao et al., 2010).

Identification of endophytes

The selected endophytes with best inhibitory effect on *P. colocasiae* were identified by morphological characterization and 16S rRNA gene sequencing. In morphological characterization, macroscopic and microscopic features of the selected isolates were studied (Prescott et al., 1996)

Genomic DNA extraction

High molecular weight DNA of the selected strains were isolated from 24 h old cultures, grown in selected broth (Nutrient and Potato dextrose broth) at 28-30ºC using Genomic DNA purification kit (Fermentas, EU) according to manufacturer's instructions. The nucleic acid obtained was dissolved in TE buffer (100µl; pH-8.0) and stored at -20ºC for further use. The DNA concentration and integrity were checked by electrophoresis on 1% agarose gel. The DNA concentration was adjusted to 20 ng/ µl for amplification by the polymerase chain reaction.

PCR amplification

PCR amplification of 16S rRNA was carried out using 8F (AGA GTT TGA TCC TGG CTC AG) and 1492 R (CGG CTA CCT TGT TAC GAC TT) for bacteria. ITS1-ITS4 region of rDNA was performed using primer ITS1 (TCC GTA GGT GAA CCT GCG G) as described by Rhoden et al., 2012 and primer ITS4 (TCC TCC GCT TAT TGA TAT GC) for fungi as described by White et al., 1990. PCR was carried out in 25 µl reaction volumes, containing 18.5 µl ultra-pure water, 2.5 µl of Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 0.5 µl dNTP, 0.5 µl Taq DNA polymerase (MerkGeNi, India), 0.5 µl forward primer, 0.5 µl reverse primer and 2 µl of template DNA. Amplification was performed in an Agilent sure cycler 8800 (Agilent Technologies, USA). Thermocycling conditions of 16S rRNA consisted of initial one denaturation step of 95ºC for 2 min followed by 35 amplification cycles of cooling denaturation 95ºC for 30 sec, annealing at 55.5ºC for 30 sec, elongation at 72ºC for 1 min and final elongation at 72ºC for 8 min. On the other hand, for ITS amplification the thermal cycler was programmed as 2 min at 94ºC, 35 cycles of 30 s at 94 ºC , 1 min at 57.1 ºC and 1 min 30 s at 72 ºC, and finally 8 min at 72 ºC. PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by Alphaimager HP. Purified PCR products were sequenced, the sequence data obtained was refined and was BLAST analyzed at NCBI website http://www.ncbi.nlm.nih.gov to determine the similarity of the sequence with already reported gene sequences in the Gen Bank. The nucleotide sequence data obtained was deposited to the Gen Bank and the accession numbers were obtained.

Statistical analysis

The significance of the interaction effects of the antagonistic bacteria and fungi was determined by ANOVA (SSCNARS analysis). The antagonistic effects were compared using TUKEY s Honest Significant Difference ($P \leq 0.05$).

Results and Discussion

Isolation of endophytes

In morphological characterization the endophytic bacterial isolates exhibited the diverse colony shapes, colors, margins and texture including round to irregular colonies, off white, pink and yellow colonies with regular or wavy margins. A total 65 endophytic bacteria were isolated, of which 34 isolate from leaves, 16 isolates from stems, 7 isolates from mature flowers and 8 isolates from young flowers. On the other hand 23 different endophytic fungi were randomly selected according to their morphological characteristics such as colony coloration, pigment formation and growth of mycelial colonies on potato dextrose agar (Fig.1. and Fig.2.). Among this 10 isolates were from leaves, 6 isolates from stem, 3 isolates from matured flower and 4 isolates from young flowers. The isolation efficiency of bacteria from leaves was higher than from other parts.

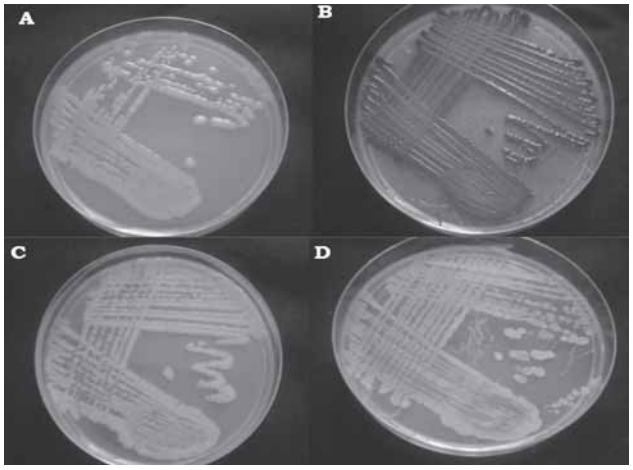

Fig. 1. Morphologically different endophytic bacteria isolated from *Saraca asoca*

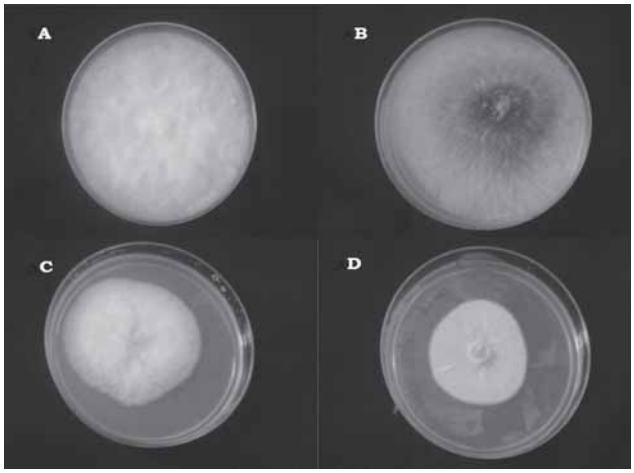

Fig. 2. Morphologically different endophytic fungi isolated from *Saraca asoca*

Endophytes are residing in different tissues of living plants are relatively unstudied and are being considered as potential source of novel natural products to be used in medicine, agriculture, and industry. The surface disinfection of plant tissues is the most important step in the isolation process and aims to eliminate the external community of microorganisms, maintaining a viable internal community of plant samples according to the procedure outlined by Arajuo et al., 2002. The endophytes were isolated from different inner parts of *Saraca asoca*. The sterilized distilled water which had been used to wash the sterilized plant tissues was cultured on NA and PDA plates. If there were no colonies observed within 2-3 days, it could be assumed that the disinfection procedure was done properly and the isolates were considered as endophytic bacteria and fungi.

Pathogenicity assay

The susceptible variety of taro leaf was inoculated with the pathogen and incubated at 25ºC. All the five isolates were found to be virulent and reproduced typical leaf blight symptoms. The isolates initiated lesion development after 4 days of inoculation and the inoculated sites showed water soaked lesions at the beginning which turned brown upon progression of the disease. Test pathogen exhibited more virulence was used for *in vitro* screening (Fig. 3).

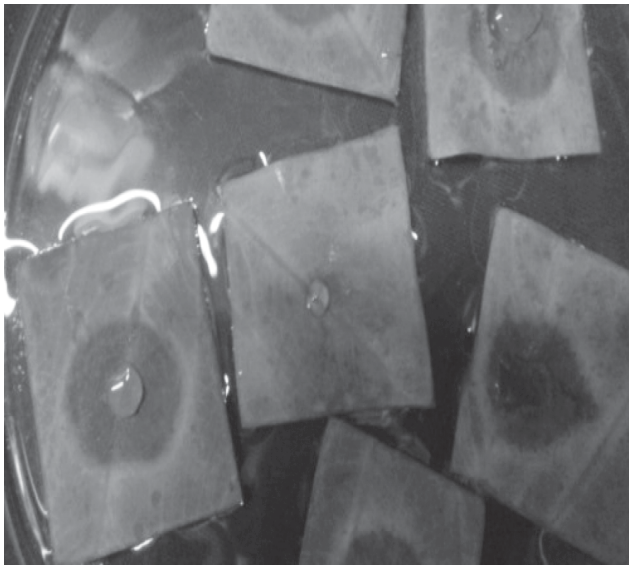

Fig. 3**.** Virulence assay of *P. colocasiae*

In vitro screening of endophytes against *P. colocasiae*

Results showed that all the 65 endophytic bacteria and 23 endophytic fungi tested in this study exhibited antagonistic activities against *P. colocasiae* in varying degrees. Mycelial growth of the pathogen was considerably hindered by all the test antagonists under this study. In a control petridish (without endophytes) the pathogen grew at a faster rate and covered the whole petridish within 8 days where as the pathogen showed comparatively a slower growth in the petri dish with dual culture. There was a significant difference in percentage inhibition of mycelia growth of pathogen by all the test anagonists (Table 1. and Table 2.). The inhibitory rates of 10 bacterial isolates were more than 70%, 14 isolates were more than 60%, 41 isolates were less than 60%. Among the isolate, SA29 isolated from *Saraca asoca* leaf gave the strongest inhibitory effect at 84% (Fig.4.). In case of fungal endophytes six isolates showed more than 70% mycelial inhibition, one isolate showed more than 60% inhibition and other 16 isolates were less than 60%. Among the fungal isolate SAF2 showed strong inhibitory effect of 77.33% (Fig.5).

The evaluation of the endophytic bacteria and fungi against plant pathogens did not require direct contact because the antagonists most likely produced diffusible antibacterial and antifungal metabolites into the medium (Geetha and Vikineswary, 2002). Microscopic examination of the dual culture assay showed an alternation of the mycelium of the pathogen where it was in contact with antagonist. The most common mode of action observed was antibiosis which appeared in the co-inoculated dishes as an inhibition zone (Fig. 4.).

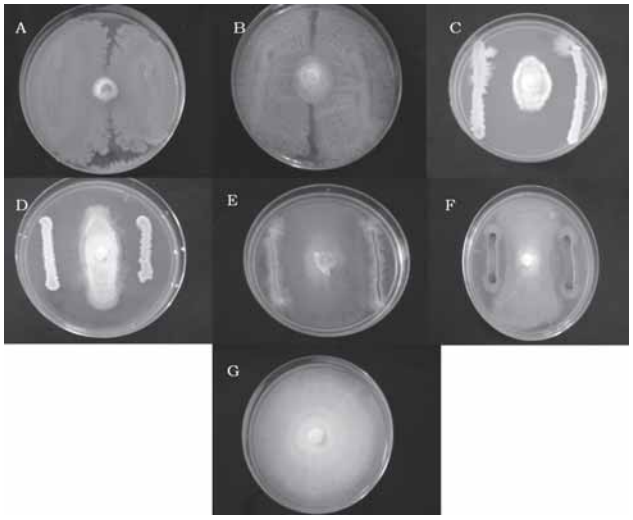

Fig.4. Antagonistic effect of the endophytic bacterial isolates on *P.colocasie* in dual culture assay \

Table 1. In vitro inhibition of *P. colocasiae* by different bacterial endophytes

Isolate code	Mycelial growth Inhibition (cm) ± standard deviation	Percent of inhibition *
SA1	2.1 ± 0.3	71.98^{CD}
SA2	4.96 ± 0.34	33.77^{Q}
SA3	2 ± 0.26	73.3^{C}
SA4	4.76 ± 0.45	36.44^{P}
SA5	6 ± 0.46	20^{T}
SA6	5.23 ± 0.50	30.22^{R}
SA7	2 ± 0.25	73.3^{C}
SA8	3.2 ± 0.45	57.3^{L}
SA9	3 ± 0.28	60 ^{JK}
SA10	6.03 ± 0.45	19.55^{T}
SA11	4.5 ± 0.50	40^{O}
SA12	5.46 ± 0.30	27.11^{S}
SA13	3 ± 0.32	60^{JK}
SA14	2.9 ± 0.45	61.33^{IJ}
SA15	2.73 ± 0.34	63.53^{HI}
SA16	2.7 ± 0.35	64^{H}
SA17	3.9 ± 0.40	48^{MN}
SA18	4 ± 0.35	46.6^{MN}
SA19	2.73 ± 0.36	63.5^{HI}
SA20	2 ± 0.26	73.3^{C}
SA21	1.6 ± 0.24	78.6^{B}
SA22	3 ± 0.50	59.9^{JK}
SA23	6 ± 0.5	20^{T}
SA24	5.5 ± 0.25	26.6^{S}
SA25	2.33 ± 0.30	68.8^{EF}
SA26	2.26 ± 0.35	69.75^{DE}
SA27	5 ± 0.28	33.3^{Q}
SA28	5 ± 0.30	33.3^{Q}
SA29	1.5 ± 0.20	84^{A}
SA30	3 ± 0.36	60^{JK}
SA31	2.1 ± 0.32	72^{CD}
SA32	2.96 ± 0.35	60.44^{JK}
SA33	3 ± 0.42	60^{JK}
SA34	5 ± 0.30	33.3^{Q}
SA35	2.2 ± 0.42	70.66^{DE}
SA36	3.03 ± 0.42	59.5^{JKL}
SA37	2.9 ± 0.35	61.3^{IJ}
SA38	2.93 ± 0.42	60.86^{JK}

Table 1. contd.

SA39	3 ± 0.24	60^{JK}
SA40	6.06 ± 0.42	19.10^{T}
SA41	3 ± 0.36	60^{JK}
SA42	4.96 ± 0.45	33.75^{Q}
SA43	3 ± 0.28	60^{JK}
SA44	2.63 ± 0.30	64.8^{GH}
SA45	2.5 ± 0.30	66.6^{FG}
SA46	2.5 ± 0.32	66.6^{FG}
SA47	3 ± 0.40	60^{JK}
SA48	4.03 ± 0.35	46.17^{MN}
SA49	2.3 ± 0.35	69.3^{E}
SA50	3.1 ± 0.28	58.6^{KL}
SA51	3.06 ± 0.38	59.06^{JKL}
SA52	2.9 ± 0.44	61.33^{IJ}
SA53	3 ± 0.42	60^{JK}
SA54	2.1 ± 0.30	72^{CD}
SA55	4.9 ± 0.30	33.75^{Q}
SA56	5 ± 0.45	33.3^{Q}
SA57	5 ± 0.42	33.3^{Q}
SA58	2.9 ± 0.40	61.33^{IJ}
SA59	4.06 ± 0.28	45.75^{N}
SA60	3.1 ± 0.35	58.66^{KL}
SA61	3 ± 0.20	60^{JK}
SA62	2.2 ± 0.48	70.6^{DE}
SA63	3 ± 0.30	60^{JK}
SA64	3.86 ± 0.35	48.42^{M}
SA65	3.1 ± 0.25	58.6^{KL}

*Mean of three replicates

Values followed by the same letters (A-T) are not statistically significantly different (P ≤ 0.05) according to TUKEY's Honest Significant Difference.

Table 2. In vitro inhibition of *P. colocasiae* by different fungal endophytes

Isolate code	Mycelial growth Inhibition (cm) ± standard deviation	Percent of inhibition *
SAF1	2.03 ± 0.28	72.86 ^{B}
SAF2	1.7 ± 0.40	77.33 ^{A}
SAF3	3.5 ± 0.42	53.33 ^{E}
SAF4	3.03 ± 0.30	59.53 ^{D}
SAF5	5 ± 0.52	33.33 ^{G}
SAF6	1.9 ± 0.20	74.6 ^{B}
SAF7	2 ± 0.20	73.3 ^{B}
SAF8	5.36 ± 0.35	28.44 ^{H}
SAF9	6 ± 0.50	20 ^{I}
SAF10	4.9 ± 0.45	34.6 ^{G}
SAF11	4.9 ± 0.32	34.66 ^{G}
SAF12	2.3 ± 0.36	69.33 ^{C}
SAF13	3.66 ± 0.40	51.11 ^{F}
SAF14	5 ± 0.28	33.3 ^{G}
SAF15	3 ± 0.25	60 ^{D}
SAF16	2.23 ± 0.30	70.2 ^{C}
SAF17	6 ± 0.34	20 ^{I}
SAF18	6 ± 0.24	20 ^{I}
SAF19	6 ± 0.26	20 ^{I}
SAF20	5.96 ± 0.30	20.43 ^{I}
SAF21	3.6 ± 0.35	52 ^{EF}
SAF22	2 ± 0.22	73.3 ^{B}
SAF23	6 ± 0.25	20 ^{I}

*Mean of three replicates

Values followed by the same letters (A-I) are not statistically significantly different (P ≤ 0.05) according to TUKEY's Honest Significant Difference.

Mycelial growth inhibition of the target pathogen revealed that the suppression rate was highly reciprocal, with a wider inhibition zone. The isolates most effectively inhibited fungal pathogen growth in the dual culture experiment generated such a large zone of inhibition, indicating that the endophytic bacteria produce certain non-volatile antibiotics and metabolites. A microbial biocontrol agent may express different mechanisms against pathogens during their antagonistic activity.

Production of extremely diverse bioactive compounds by endophytic bacteria and their potential use as biocontrol agents has been reported to be dependent on many parameters., such as taxonomical position, physiological characters, geological conditions (Sharma et al., 2009). Endophytic bacteria might either become localized at the entry point or spread throughout the plant tissues (Liu et al., 2015). They can effectively antagonize pathogens via releasing various bioactive molecules. Various members of the genus *Bacillus* are under focus for their broad antagonistic potentiality against wide array of phytopathogenic fungi and bacteria. They release minimum 66 diversed antibiotic compounds (Roberti and Selmi, 1999).

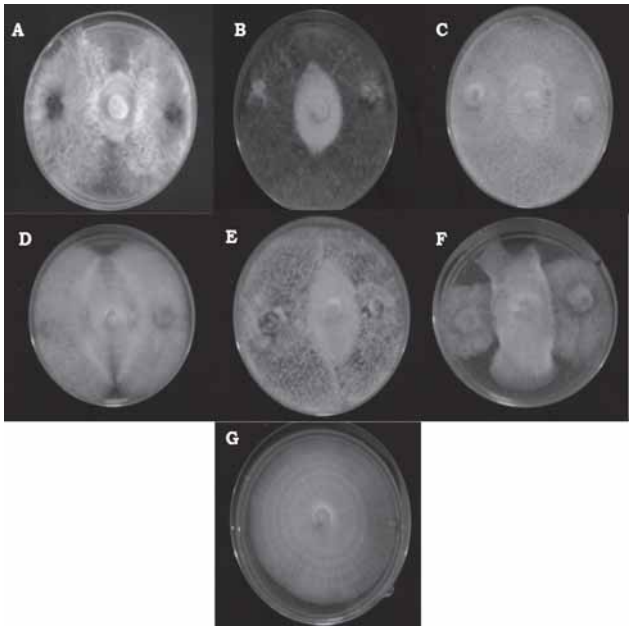

Fig. 5. Antagonistic effect of the endophytic fungal isolates SAF2, SAF6, SAF7, SAF22, SAF1, and SAF16 on *P.colocasie* in dual culture assay

Identification of endophytes

In morphological characterization endophytic bacteria and fungi exhibited diverse phenotypic characteristics. Bacterial and fungal endophytic growth was observed on NA, PDA agar after 24 hrs of incubation, colonies of different colours such as white, cream, pink and yellow were observed. Morphological characterization of the 65 bacterial isolates based on gram test revealed that 35 of the endophytic bacterial isolates were gram negative while 30 were gram positive.

Besides the phenotypic characteristics, gene sequencing was used for the molecular identification which gives more taxonomic information. DNA was isolated from more potent endophytic bacteria (SA3, SA7, SA20, SA21, SA29, SA31) and endophytic fungi (SAF1, SAF2, SAF6, SAF7,SAF16 SAF22). Quality was evaluated on 1% agarose gel, a single band of high molecular weight DNA has been observed. Fragments of gene were amplified by PCR using 8F, 1492R for bacteria, ITS1 and ITS4 for fungi from the above isolated DNA. Amplified products were resolved on 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and scanned through the Gel Doc system. A single PCR amplicon band of 1500 bp, 800 bp was observed respectively. The PCR product was purified and processed for sequencing. The obtained sequences were aligned with the corresponding sequences of Gen Bank using BLAST programme.

The bacterial isolates SA3, SA7, SA20, SA21, SA29, SA31 were identified as *Bacillus pumilis*, *Bacillus safensis*, *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Endophytic bacterium* and *Comamonas testosteroni* respectively. The fungal isolates SAF1, SAF2, SAF6, SAF7, SAF16, SAF22 were identified as *Phomopsis* sp., *Nigrospora oryzae*, *Phomopsis liquidambaris*, *Diaporthe* sp., *Colletotrichum gloeosporioides*, and fungal endophyte sp. This endophytic bacterial and fungal isolates having 97% homology, were deposited in National Center for Biotechnology Information (NCBI). The highest similarities found with different endophytes and Gen Bank accession numbers of selected isolate were summarized in Table 3.

Table 3. Isolate code, Name, Genbank accession numbers, % of similarity and Query cover of potent endophytes

Isolate code	Isolate name	Genbank accession number	% of similarity	Query cover (%)
SA3	*Bacillus pumilis*	MG551744	100	100
SA7	*Bacillus safensis*	MG551745	100	100
SA20	*Bacillus altitudinis*	MG551746	100	100
SA21	*Bacillus amyloliquefaciens*	MG551747	100	100
SA29	*Endophytic bacterium*	MG551748	100	100
SA31	*Comamonas testosteroni*	MG551749	100	100
SAF1	*Phomopsis sp.*	MG798661	99	77
SAF2	*Nigrospora oryzae*	MG735753	100	100
SAF6	*Phomopsis liquidambaris*	MG798662	99	99
SAF7	*Diaporthe sp.*	MG733134	100	100
SAF16	*Colletotrichum gloeosporioides*	MG786768	97	97
SAF22	Fungal endophyte *sp*.	MG786769	100	45

Conclusion

The current study raises numerous implications on the present management strategies for taro leaf blight. The present study mainly conducted to assess the antagonistic activity of isolated endophytic bacteria and fungi against *P.colocasiae*. These selected endophytes can release a wide array of extracellular bioactive metabolites with high capability to inhibit the pathogen thus they can be used to manage leaf blight of taro and may be recommended as good biocontrol agents. The use of biocontrol agents such as endophytes as an alternate way to manage leaf blight of taro is ideal option, apart from chemical method.

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