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Pathogenic fungi associated with storage rot of *Colocasia esculenta* and evaluation of bioformulations against the pathogen

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ABSTRACT: Taro (*Colocasia esculenta*) is a major tropical tuber crop widely cultivated in India. Post-harvest loss of *C. esculenta* root and tubers had emerged as one of the major constraints in the production of taro and might have detrimental effects on food security. Our study on storage rot of *C. esculenta* tubers revealed that *Fusarium oxysporum* was mostly responsible for the post-harvest loss of the crop. Pathogenicity test confirmed its role as the causal agent of storage rot in taro. The pathogen was further confirmed based on molecular identification by amplifying ITS region of the rDNA using ITS1 and ITS4. Based on BLST analysis it showed maximum homology (95-99%) with *F. oxysporum*. Efficacy of a few microbial antagonists were tested against the pathogen *in vitro* which revealed that the treatment with combination of *Trichoderma viride* + *Pseudomonas fluorescens* + *Bacillus thuringiensis* showed highest inhibition of *F. oxysporum* (94.05%) followed by treatment of *T. viride* alone (65.17%).

Keywords: Bioagents, dual culture, storage rot, taro

INTRODUCTION

Taro (*Colocasia esculenta*) is a major tuber crop belonging to the family Araceae. Taro tubers are rich sources of starch (70–80 g/100 g dry taro), fiber (0.8%), and ash (1.2%), riboflavin, thiamine, vitamin C, iron, phosphorus, zinc, potassium, copper, and manganese (Quach *et al*., 2003). It is considered as the fifth most consumed root vegetable in the world (Rao *et al*., 2010). Taro crop is subjected to significant losses from several diseases. Phytophthora blight (*Phytophthora colocasiae*) and Pythium root and corm rot (*Pythium* spp.) are the most devastating fungal diseases of taro. Post-harvest loss of root and tubers has emerged as a serious problem to farmers. Presence of high moisture content in corm often results in rots that are a major reason of postharvest losses, especially when poorly handled. Corm rots are also e associated with microorganisms, including *Fusarium, Sclerotinia, Erwinia, Botryodiplodia*, and *Ceratocystis* (Paul and Chen, 2015). These microorganisms are mostly associated with field infection through wounds. After washing the roots, corms should be dried so that healing of wounds can occur. Various fungi especially have been reported to cause losses in its fields. Several soil borne microorganisms such as *Fusarium oxysporum*, *Fusarium solani*, *Sclerotium rolfsii*. *Rhizoctonia* sp. etc. may be responsible for the storage rot of *C. esculenta* (Carmichael *et al*., 2008). There are several reports on the efficacy of different fungicides (Aggarwal and Mehrotra, 1987; Ooka, 1990). However, large scale chemical use is not a desirable option from both economic and environmental perspective (Brooks, 2005). Tuber treatment, soil application and foliar application with rhizobial cultures reduced the disease severity and increased the yield compared to the untreated control (Sriram and Misra, 2007). Yam tubers inoculated with *T. viride* either totally suppressed or showed only a low percentage of rot (Okigbo and Ikediugwu, 2000). Limited work has been done so far on this aspect. With this background, the present study was undertaken on *in vitro* evaluation of biocontrol agents in the management of storage rot of *Colocasia esculenta*.

MATERIALS AND METHODS

Infected plant parts showing typical symptoms of the disease were collected from Horticultural orchard, Assam Agricultural University, Jorhat and brought to the Mycology laboratory of Department of plant pathology for further analysis.

Isolation and identification of the pathogen and preparation of pure culture

The tissues showing diseased symptoms were thoroughly washed in sterile water. The infected tissues along with healthy tissue were cut into small pieces and transferred to sterile petridishes containing 70 per cent alcohol, 1 per cent sodium hypochlorite and sterile water. The samples were transferred to PDA plates and incubated for 5-7 days at 28 ± 1°C. The desired fungi were purified using the hyphal tips technique on Potato Dextrose Agar medium and then subcultured on PDA slants for future use. The isolated fungus was identified

based on colony morphology, microscopic observation and molecular characteristics. A sterile inoculating needle was used to take a portion of mycelial colony and placed on a microscopic slide. The isolates were identified by the help of the available literature on Dematiaceous hyphomycetes (1971), Illustrated Genera of Imperfect Fungi (1972), The Bitunicate Ascomycetes and their Anamorphs (1984).

Pathogenicity test

Healthy tubers of *C. esculenta* were washed with distilled water. Then the corms were surface disinfected with 0.1% mercuric chloride solution for 2-3 minutes. Cylindrical cores were removed from the tubers and 4 mm agar discs containing the pathogen were inserted into the holes which were properly sealed. The tubers containing the uninoculated potato dextrose agar blocks served as control (Khatoon *et al*., 2016). The treated tubers were packed into sterile polythene bags and inoculated at 28±2°C for 10 days. The tubers were continuously examined to determine the extent of rotting.

In vitro* assessment of the microbial bioagents against *Fusarium oxysporum

The widely accepted bioagents *viz*., *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Bacillus thuringiensis* were collected from the Biocontrol Laboratory of Department of Plant Pathology, AAU, Jorhat. These were transferred to fresh medium and preserved sat 4°C for further use. Antimicrobial activity of the microbial bioagents was carried out using a modified dual culture assay method (Dhingra and Sinclair, 1995) using PDA media. The diameter of the inhibition zones was measured after 24 hrs and 48 hrs of incubation. For each treatment, a 5mm disc containing the mycelium of the pathogenic fungus was placed at one side of the petriplate and was allowed to develop for 2 days due to its slow growth. Another 5 mm disc of the bioagent was inoculated at the opposite end and was incubated at 26°C. The colony diameter was recorded every 24 h. The control consisted of mycelium of the pathogen. The radial mycelial growth of *F. oxysporum* was recorded and converted into per cent mycelial inhibition using the following equation (Edington *et al*., 1971) - Mycelial Inhibition % = $[(C_2 - C_1)/C_2] \times 100$

Where,
C_1 = radial mycelial growth of *F. oxysporum* in the presence of antagonist and
C_2 = radial mycelial growth of *F. oxysporum* in control.

Characterization of the pathogen

Colony characters like colour and growth of the colony and morphological characters like size and shape of the conidia, presence of septa and chlamydospores were recorded.

Molecular diagnosis of the pathogen

Isolation of the fungal genomic DNA was done following the modified method of Al-Samarrai and Schmid (2000). The extracted DNA was quantified and its purity was assessed using Nanodrop 1000 (Thermo Scientific). The absorbance of genomic DNA was measured at 260 nm to determine the concentration of genomic DNA in solution. The absorbance was also taken at 280 nm to observe the extent of protein contamination in the extracted DNA. PCR amplification of the 18S rRNA gene region was done by using ITS primers (ITS1 and ITS4) and the purified PCR products were sent to Agri Genome Labs Private Limited, Kerala and Bioserve Biotechnologies Private Limited, Hyderabad. The sequences were aligned with Clustal W using default parameters and a neighbor-joining tree was constructed using MEGA 7 software. Statistical analysis of the data was calculated using Analysis of Variance (ANOVA) which was analyzed using CRD and Fisher's method of analysis of variance.

RESULTS AND DISCUSSION

Identification of the pathogen

The pathogen was identified based on morphological and cultural characteristics first and confirmed later on by molecular characterization. In the culture, mycelium initially appeared white but the colour changed later. Their macroconidia appeared fusiform, slightly curved, three septate, basal cells pedicellate measuring 23-54 x 3-4.5 µm whereas microconidia appeared non-septate, cylindrical measuring 5-12 x 2.3-3.5 µm. The size of chlamydospores ranged from 5-13 µm. From colony characteristics and conidial characteristic the pathogen was identified as *Fusarium* spp. However, 18S r RNA

Table 1. Results of the pathogenicity test conducted by inoculating *F. oxysporum* into colocasia tubers

	Total no. plants inoculated	No. of plants infected
F. oxysporum	6	6
Control (uninoculated)	6	0

sequencing was done to identify the species level. The genomic DNA was subjected to PCR-amplification of the 18S rRNA gene region using ITS1 and ITS4 primers. Agarose gel (1.2%) electrophoresis showed a clean amplification of ~500bp fragments for pathogenic fungus. PCR products were outsourced for sequencing and sequenced data were compared using National Centre for Biotechnology Information BLAST® (Basic Local Alignment Search Tool) programme. The phylogenetic analysis revealed that pathogenic fungus showed highest homology with *Fusarium oxysporum* (Fig. 1). Post-harvest loss of *Colocasia esculenta* root and tubers has emerged as a devastating problem to farmers and might have detrimental effects on food security (Olurinola *et al*., 1992). Colonization of the tubers by different pathogens led to reduction in market value (Microha *et al*., 2003). This study on storage decay of *Colocasia* *esculenta* tubers revealed that *Fusarium oxysporum* was responsible for the post-harvest loss of the crop. Several microorganisms such as *Fusarium oxysporum*, *Fusarium solani* might be responsible for the root rot of *C. esculenta* (Ooka, 1994). The symptoms caused by *Rhizopus, Mucor* and *Fusarium* on rotted *C. esculenta* corms were severe leading to complete maceration of the tissues (Agu *et al*., 2016). Fusarium rot is characterized by a light to dark brown or black rot of the potato tubers. The pathogen penetrates the tuber through wound causing rotting out at the center. Extensive rotting results in shrinkage of the tubers, usually leaving a dark sunken area outside the tuber. Sambutoxin, a new mycotoxin produced by *Fusarium oysporum* caused rotting of potato tubers (Kim and Lee, 1994). *Fusarium* colonizes the host as biotrophic fungi, produce toxins and cellulolytic enzymes during the necrotrophic stage, as they target host secondary metabolic pathways for establishment and uptake of host nutrients (Perincherry *et al*., 2019). Examples of toxins produced by *F. oxysporum* include beauvericin, fusaric acid, moniliformin, naphthazarins and sambutoxin (Li *et al*., 2013). The results of pathogenicity test revealed that the pathogen caused extensive rotting of the tissues. Based on 18S rRNA sequence analysis, the pathogen showed maximum homology with *Fusarium oxysporum*. *Fusarium oxysporum* was pathogenic to *C. esculenta* causing corm rot of Taro in Java (Widodo and supramana, 2011). In this study, *F. oxysporum* showed a velvety texture with pink mycelium, its mycelium was formed by septate hyphae and the conidiophores present clusters of macroconidia where chlamydospores were observed. The colonization of pathogens in tubers was due to the ability of the pathogen to utilize the nutrient of taro as a substrate for growth and development. These pathogens cause extensive loss of these tubers in terms of quantity and also reduce its economic and nutritive value.

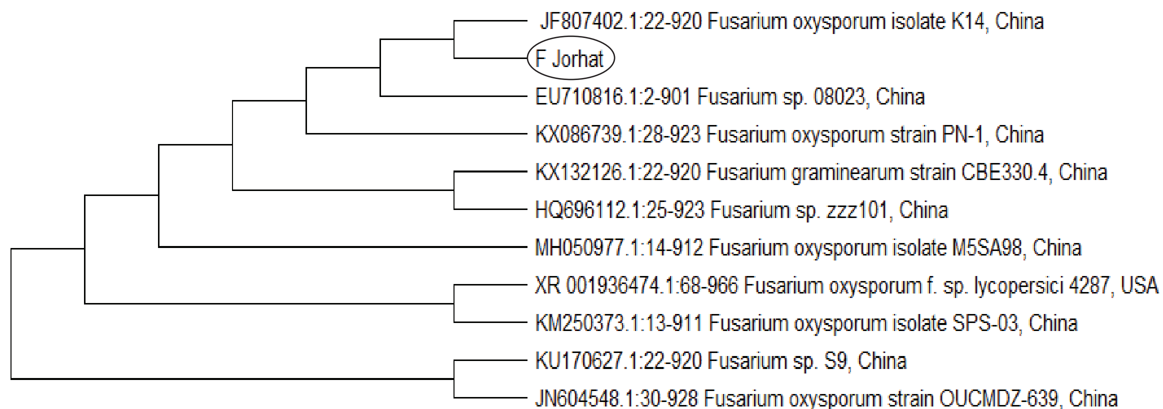

Fig. 1. Phylogenetic tree of *Fusarium oxysporum*

In vitro evaluation of antagonists

The antagonistic potential of microbial bioagents was tested against *Fusarium oxysporum* adopting dual culture method using PDA as basal media. The result of the dual culture antagonism assay (Dhingra and Sinclair, 1995) using PDA as basal media revealed that, the microbial bioagents could inhibit the growth of *F. oxysporum*. The inhibition zones produced by the microbial bioagents are described in **Table 2**. Bioformulation of *T. viride* + *P. fluorescens* + *B. thuringiensis* showed highest inhibition of *F. oxysporum* (90.8%) which was statistically at par with 0.1% Carbendazim (93.07%) which was followed by *T. viride* (81.60%) and *P. fluorescens* (61.32%)

John *et al*. (2010) reported *T. viride* as an effective biocontrol agent against *Fusarium oxysporum* f. sp. *adzuki*. Kumari *et al*., 2014 observed the efficacy of *T. viride* against the pathogen *Fusarium oxysporum* f. sp.

Table 2. Suppressive effect different antagonist and their combination against *F. oysporum*

Treatment	Inhibition (%)
T. viride	81.60 (64.60)
P. fluorescens	62.32 (52..12)
B. thuringiensis	53.82 (47.38)
T. viride + P. fluorescens + B. thuringiensis	90.8 (72.34)
0.1% Carbendazim	93.07 (74.66)
F. oysporum (alone)	0.00 (0.57)
S.Ed (±)	2.25
CD(P=0.05)	4.67
CV (%)	4.17

cubense under *in vitro* conditions and found that *T. viride* could effectively inhibit the pathogen. Saravanan *et al*., 2004 reported that talc formulation of *Pseudomonas fluorescens* had higher inhibitory action against *Fusarium oxysporum* f. sp. *cubense*. Akram *et al*. (2013) reported that tomato plants inoculated with *Bacillus thuringiensis* had significant reduction of Fusarium wilt caused by *Fusarium oxysporum*.

Microbial bioagents like *Trichoderm*a spp., fluorescent Pseudomonads, *Bacillu*s spp are known as potential antagonists against a wide range of pathogens. In our study the combination of all the three antagonists were found to be superior over single bioagent which may be attributed to the cumulative effect of all the antagonists with varied mechanis of action. Khan *et al.* (2018) reported significant inhibition of *F. oxysporum f.sp lactucae* isolated from hydroponically grown lettuce by combination of *T. viride, P. fluorescens, M. anisopleae and B. thuringienis.*

Biocontrol agents employ an array of mechanisms against target pathogens. Antagonistic potential of *P. fluorescens* and *T. viride* alone or in combination has been widely reported against many major plant pathogens (Bora *et al.,* 2016). *Trichoderma* employs an array of mechanisms for suppression of plant pathogens that includes hyperparasitism, competition for nutrients and antibiosis. The genome analysis of *T. virens* and *T. atroviride* revealed an expanded arsenal of genes encoding cell wall degrading enzymes of which chitinases, β-1,3-glucanases and proteases are of significant importance (Singh *et al.,* 2014). The genus also produces a variety of antibiotics, such as gliotoxin, viridin, gliovirin, koninginins, pyrones, viridol, and peptaibols against many fungal phytopathogens (Harman *et al*., 2004). *Trichoderma* outcompetes pathogenic flora for the nutrients and hence competition is another effective mechanism of biocontrol in *Trichoderma* (Bora *et al.,* 2013). The bacterial bioagent *P. fluorescens* inhibits phytopthogens through production of antibiotics, siderophore and antifungal metabolites and lytic enzymes (chitinase and glucanase) that inhibit the mycelial growth of pathogenic fungi including *Fusarium* spp. (Saravanakumar *et al*. 2007, Sharma *et al.,* 2020). *B. thuringiensis* popularly known as bioinsecticide also has ability to control plant pathogenic fungi such as *F. oxysporum*, *F. sambucinum* and *F. graminearum* (Baysal *et al*., 2013). Chitarra *et al*. (2003) reported that *B. thuringiensis* can secrete chitinases and chitin-binding proteins; together these proteins control fungi by binding to cell wall chitin and disrupting the cell polarity, leading to fungal growth inhibition, and thus conferring an advantage as a competitor in its niche. Hence synergistic effect of *P. fluorescens, T. viride* and *B. thruingiensis* could show maximum inihibition as compared to the single formulations.

The present study revealed that *Fusarium oxysporum* has been found to cause storage rot of *Colocasia esculenta* tubers. All the biocontrol agents evaluated in this study showed great potential in inhibiting tuber rot pathogen. Regardless of the variation in their efficacy, it is evident that the BCAs showed promise in the control of post-harvest tuber rot of taro as a suitable alternative to chemical fungicides. However, more studies are required to develop module for field application and tuber treatment for ecofriendly and sustainable management of storage rot of *C. esculenta*.

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