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Molecular Characterization of *Fusarium oxysporum* G30 Isolated from Vegetable Wastes in Imphal, Northeast India

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Abstract

A new strain of *Fusarium oxysporum* (Schl.) emend. Synder & Hansen, viz. *Fusarium oxysporum* G30, is reported from seven different decomposing vegetable wastes and a mixture of the same in equal proportions, in Imphal, Northeast India. This strain was isolated by surface sterilization technique, followed by re-culturing till pure culture was obtained. The pure culture was characterised morphologically based on the colony features: colour, texture, colony diameter; conidial features: macro and microconidial shape, size, septation, presence of chlamydospores and foot-shaped basal cells. The strain was also identified at molecular level by partial sequencing using universal primers, ITS4 and ITS5. The ITS data reveals that isolated culture (*F. oxysporum* G30) match the existing isolates in Gen Bank (Gen Bank accession No. GQ497156.1) by 99% and it has got a code segment particular to this strain. This strain showed ubiquitous nature with mean percent frequency of occurrence ranging from 39.5 to 68% at various stages of decomposition of the selected vegetable wastes.

Keywords: complex, decomposition, frequency of occurrence, *Fusarium oxysporum* species complex, microbial growth, new strain, vegetable waste management

Introduction

Fusarium oxysporum (Schl.) emend. Synder & Hansen is an ubiquitous saprophyte found predominantly in the environment. This species has been reported to be responsible for a number of diseases in plants. *F. oxysporum* has been assigned as the most economically important species of the genus because of the numerous host range and the level of loss caused as a result of their infection in plants. Vegetable wastes which contribute 50-60 t/day of municipal solid wastes in Imphal (Anonymous, 2009) are known to be good supporters of microbial growth, especially of fungi, during the decay stages. Recent studies (Sharma and Pandey, 2012) reveal that this species occurs in maximum population in decaying vegetable wastes. Identifying this particular strain at molecular level will be useful for formulating effective biological treatment of vegetable wastes which have already become a problem for dumping in city areas. Such beneficial application of this fungus has been given less attention so far. Therefore, in the present study, the most common *F. oxysporum* strain, having the highest frequency of occurrence in decaying vegetable wastes, have been isolated and characterized at molecular level.

Materials and methods

Study materials

Selected vegetable wastes, namely, peels of *Euryale ferox* Salisb. (Nymphaceae), *Phaseolus* sp. (Fabaceae), *Solanum tuberosum* Linn. (Solanaceae), *Sechium edule* Sw. (Cucurbitaceae), petioles of *Brassica oleracea* Linn. *botrytis* (Brassicaceae), pseudostem of *Musa paradisiaca* Linn. (Musaceae), sheaths of young edible shoots of *Bambusa arundinaceae* Willd. (Poaceae) and a mixture of the vegetable wastes in equal proportions were used as the study materials.

Decomposition of vegetable wastes

Decomposition studies of vegetable wastes were carried out both in litter bags by following nylon mesh bag technique (Bocock *et al.*, 1960), as well as in experimental pots. Five gm air-dried vegetable wastes in each nylon net bag (10 x 15 cm; 1 mm mesh size) separately for each of the eight samples were randomly placed on soil surface in the experimental site located in the Department of Life Sciences, Manipur University. For each decomposing vegetable wastes at least three bags were recovered at monthly interval for a period of seven months (November-June) and brought to the laboratory in sterile polythene bags where the samples of each bag were brushed carefully to remove the adhering soil particles for the isolation of fungi. Similarly, 5 gm air-dried sample were also allowed to decompose in plastic pots placed in tin roofed experimental shade adjacent to the experimental plot. Sterilized water was sprayed at 4 days interval (1 ml/pot). As in the above case, contents of three pots for each sample were recovered at monthly interval for the isolation of fungi.

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Isolation of fungi

The technique of surface sterilization using 15% H_2O_2 and 70% Ethanol (Kinkel and Andrews, 1988) was employed for the isolation of fungi inhabiting the decomposing of vegetable wastes. Bits of samples (3 x 3 mm^2) were cut using sterilized scissors, immersed in 70% ethanol for 1 minute. These were then transferred to 15% H_2O_2 for 1 minute and again to 70% ethanol for 1 minute, followed by washing with changes of sterilized distilled H_2O for 5 minutes and air-dried in-between folds of sterilized blotting paper for 30 minutes. Five surface sterilized samples bits were then placed equidistant in each Petri dish containing 20 ml PDA medium supplemented with streptomycin (100 mg/l). The plates were incubated at 25 ± 1 °C for 7 days and fungal colonies developing on the bits were purified to identify and isolate *F. oxysporum* present. Three replicates were maintained in each case.

Morphological characterization

The isolated strain (*Fusarium oxysporum* G30) was morphologically characterized on the basis of colony features: colour, texture, colony diameter; conidial features: macro and microconidial shape, size, septation, presence of chlamydospores and foot-shaped basal cells by plating the pure culture of the fungus on three different media: (i) Potato Dextrose Agar (PDA) [Potato (peeled) 200 g; Dextrose 20 g; Agar 20 g; Distilled H_2O 1L]; (ii) Czapek's Dox + Yeast extract agar (CYA) [Sucrose 30 g; $NaNO_3$ 2 g; K_2HPO_4 1 g; $MgSO_4 + H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4 + 7H_2O$ 0.01 g; Agar 15 g; Distilled H_2O 1 L] (Onions *et al*., 1981) and (iii) Lignocellulose Agar (LCA) [Glucose 1 g; KH_2PO_4 1 g; $MgSO_4.7H_2O$ 0.2 g; KCl 0.2 g; $NaNO_3$ 2 g; Yeast Extract 0.2 g; Agar 13 g; Distilled H_2O 1 L] (Miura and Kudo, 1970). The pH of the test media was maintained at 5.5 being optimal for the growth and sporulation for majority of fungi. The Petri dishes were then incubated for 7 days at 25 ± 1 °C in BOD incubator and the morphological characterization of the fungus was done by consulting standard literatures *viz*., Leslie and Summerell (2006) and Watanabe (2002) and confirmation of the culture identity was done at NFCCI (National Fungal Culture Collection of India), ARI, Pune, India (Accn. No. NFCCI 2065).

Molecular characterization

Molecular characterization of the culture isolate was performed at NFCCI, ARI, Pune by partial sequencing (ITS).

Genomic DNA was isolated in pure form from the culture. Nearly 500 dp rDNA fragments were successfully amplified using universal primers-ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') and ITS5 (5'GGA AGT AAA AGT CGT AAC AAG G3'). The sequencing PCR was set up with ABI-BigDye Terminator v3.1 Cycle Sequencing Kit (Part No. 4337455). PCR reaction was performed in a 25 µL volume containing 10 X PCR buffer, 200 mM dNTP, 1U Taq polymerase, 10 pM of each primers and 10 ng genomic DNA. While the amplification cycle consisted of an initial denaturation step of 95 °C for 5 mins, followed by 30 cycles of (a) denaturation (95 °C for 1 min), (b) annealing (56 °C for 1 min) and (c) extension (72 °C for 1 min) further followed by a final extension step of 10 mins at 72 °C. The sequence data was aligned with publically available sequences and analyzed to reach identity (Table 1).

Table 1. List of reference cultures of *Fusarium oxysporum* in Gen Bank

Accession	Description	Max. ident.
GQ497156.1	*Fusarium oxysporum* strain KKK 19	99%
FJ158124.1	*Fusarium oxysporum* f. sp. gladioli isolate UAS007	99%
FJ158128.1	*Fusarium* sp. UAS011	99%
EU849584.1	*Fusarium oxysporum* f. sp. vasinfectum strain Anyang City	99%
EU750682.1	*Fusarium* sp. 14018	99%

Data analysis

Percent frequency of occurrence was calculated by the formula:

Frequency of occurrence (%) = (Number of sample bits on which fungal species occurred/total number of bits observed) × 100.

Results and discussions

In both decomposition studies a particular isolate of *Fusarium oxysporum* G30 was obtained from all the study samples throughout the study period with mean percent frequency of occurrence (F.O. %) ranging from 39.5% (*M. paradisiaca*) to 68.0% (*B. arundinaceae*) in litter bag decomposition and 44.0% (*B. arundinaceae*) to 63.5% (mixed sample) in pot decomposition (Fig. 1). This result is another evidence to show the ubiquitous nature of *F. oxysporum*. Earlier, ubiquitous nature of *F. oxysporum* was reported by Sharma *et al*. (2011) while working on the diversity of microfungi associated with surface soil and decaying leaf litter of *Quercus serrata*. It has also been reported that *F. oxysporum* is the most widely dispersed *Fusarium* species and can be recovered from most soils - arctic, tropical or desert and cultivated or not (Leslie and Summerell, 2006). In fact, the near ubiquity of this fungus in soil worldwide has led to its inclusion in what has been termed the global mycoflora (Gordon and Martyn, 1997).

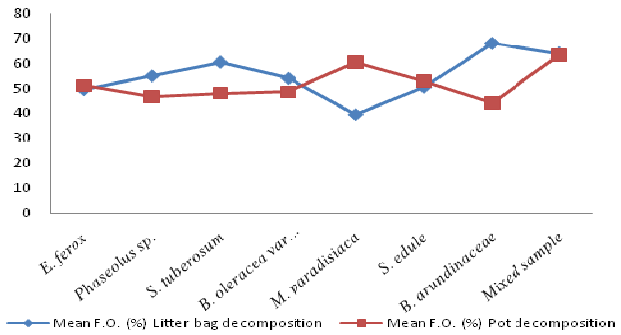

Fig. 1. Mean F.O. (%) of *Fusarium oxysporum* G30 in decomposing vegetable wastes

Morphological characterization

Culture media differentially influence the growth, colony characters and sporulation of fungi (Sharma and Pandey, 2010) hence use of more than one culture media has been recommended for morphological characterization of this fungi also. Seven days old colonies (Fig. 2a) of *F. oxysporum* G30 on PDA measured 52.0 ± 3.1 mm in diameter, floccose, magenta pink with concentric zones of dark and light reddish colouration; reverse magenta pink turning violet with age. Colonies on CYA measured 85.7 ± 0.3 mm in diam., white,

lacking zonations; reverse colourless, while colonies on LCA exhibited 79.7 ± 0.3 mm diam. in growth, light cottony in texture, hyaline; reverse colourless after seven days of incubation. Microconidia and macroconidia were produced in all three culture media. Microconidia 1 to 2 celled, hyaline, fusiform to kidney shaped, produced singly from the tips of phialides, 0-Septate 4-14 × 2-4 µm, 1-Septate 9- 33 × 2-5 µm. Macroconidia slightly curved usually with three to five septa, rarely more; no foot-shaped basal cells observed, 3-Septate 20 54 × 2 - 5 µm, 5-Septate 30 – 65 × 3 - 6 µm. Chlamydospores present, abundant in older cultures, terminal and intercalary, single in majority of the cases measuring 10 - 15 µm in diameter (Fig. 2b).

Molecular characterization

Morphological characterization alone is not enough as genetic information is not provided for identification of a species since the process is emphasized on differences of the morphological features which could readily be altered by environmental and cultural factors (Edel *et al*., 1995). In fact, fungal taxonomy is in a state of reflux because of the recent researches based on molecular approaches. Despite of the advancements and their considerable value, molecular approaches to fungal identification still remains

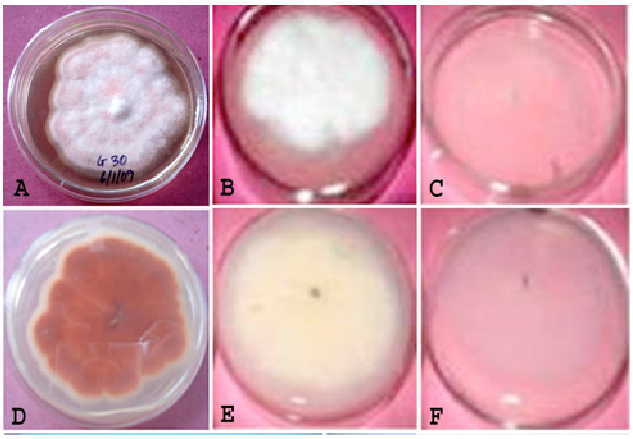

Fig. 2a. A. *Fusarium oxysporum* G30 on PDA media; B. *Fusarium oxysporum* G30 on CYA media; C. *Fusarium oxysporum* G30 on LCA media; D. *Fusarium oxysporum* G30 on PDA media (Reverse); E. *Fusarium oxysporum* G30 on CYA media (Reverse); F. *Fusarium oxysporum* G30 on LCA media (Reverse).

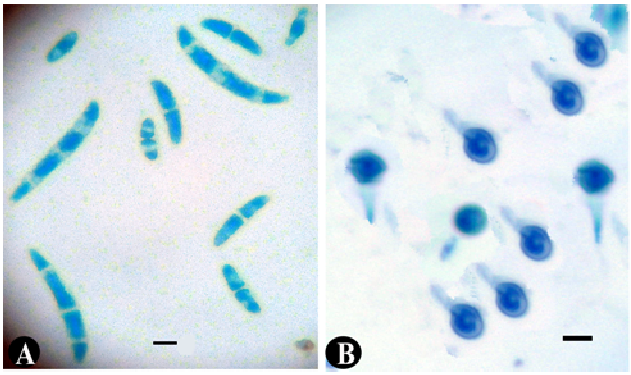

Fig. 2b. *Fusarium oxysorum* G30 (Scale = 10 µm): A. Macro- and microspores; B. Chlamydospores

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Query  1    GTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   GTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCC  95

Query  61   GCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   GCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAAT  155

Query  121  CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCG  215

Query  181  ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC  275

Query  241  CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTG  335

Query  301  GTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  GTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCC  395

Query  361  ATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  ATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCA  455

Query  421  ACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATTA  480
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Sbjct  456  ACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATTA  515

Query  481  AAGCGGAGGAA  491
            ||||||| |||
Sbjct  516  AAGCGGAAGAA  526
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Fig. 3. Sequence analysis (491 bases) with (NCBI sequence Accession GQ497156.1)/ G30 Sequences producing significant alignments

untouched in Northeastern region of India, which forms an important portion of the Indo-Burma biodiversity hotspots (Pawar *et al*., 2007). On molecular characterization this particular strain, *Fusarium oxysporum* G30, has got the following characters compared to other already defined isolates available in the Gen Bank database (Fig. 3).

The ITS data reveals that the isolated culture *F. oxysporum* G30 match the existing isolates in Gen Bank (Gen Bank accession No. GQ497156.1) by 99% and has got a code segment in the Quary 481 to 491 of the sequence which is particular to this strain (Fig. 3). *Fusarium oxysporum* is a species complex consisting of morphologically indistinguishable strains. Therefore, molecular identification will serve the gene level documentation of this strain. In India, about 320 million tonnes of agricultural wastes are generated annually (Suthur *et al*., 2005) of which vegetable wastes alone is in major proportion. These vegetable wastes are either burnt or dumped elsewhere causing environmental pollution. Biological treatment of these wastes appears to be cost effective and carry a less negative environmental impact (Paraskeva and Diamodopoulos, 2006). The filamentous fungus, *Fusarium oxysporum* has attracted a lot of scientific interest due to its ability to produce lignocellulolytic enzymes which are required for degradation of the lignin and cellulose that form the main components of such wastes. Infact, *Fusarium oxysporum* is one of the most powerful lignin degraders isolated during a screening of lignolytic microorganisms in forest soil (Rodriguez *et al*., 1996). In this connection, the present strain identified might have potential significance in biodegradation with the following properties: high F.O. percent (63.5%) in mixed vegetable in pot, ubiquitous nature of the strain for universal use and ability to grow in different media. Future detailed investigation may be carried out on assessing the

biodegradability of this fungal strain in terms of weight loss and lignocellulose contents.

Conclusions

Morphologically this particular strain of *Fusarium oxysporum* G30 is hardly distinguishable from the other known strains viz. *Fusarium oxysporum* strain KKK 19 (GQ497156.1), *Fusarium oxysporum* f. sp. *gladioli* isolate UAS007 (FJ158124.1), *Fusarium* sp. UAS011 (FJ158128.1), *Fusarium oxysporum* f. sp. *vasinfectum* strain Anyang City (EU849584.1) and *Fusarium* sp. 14018 (EU750682.1). However, molecular identification of this particular strain showed 99% sequence similarity with the above mentioned known strains. The difference lies in Quary 481 to 491 of the sequence. Thus, the present strain is a distinct strain of *Fusarium oxysporum* and reported for the first time as a new strain.

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