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New Perspective

First Report of *Fusarium proliferatum* Causing Rot of Onion Bulbs (*Allium cepa* L.) in India

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Abstract

A rot disease was observed on onion bulbs in major growing areas of Kadapa and Kurnool districts of Andhra Pradesh, India during 2010 to 2012. Based on pathogenicity, morphology and ribosomal DNA spacer sequences, the pathogen was identified as *Fusarium proliferatum* (Matsushima) Nirenberg. The fungus was isolated from onion bulbs presenting purple and reddish lesions, obtaining *F. proliferatum* consistently. The fungus produced effuse white colonies, branched hyphae, short conidiophores, slightly curved macroconidia, and single celled microconidia measuring 5.6-10.5 X 2.0-3.5 µm in diameter. Morphological identification of the fungus was confirmed using ribosomal DNA sequence data. Kotch's postulates were confirmed by performing pathogenicity test on healthy onion bulbs. This is the first report of *F. proliferatum* causing rot disease on onion bulbs in India; although it had already been reported for onion in the USA and Serbia.

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INTRODUCTION

The onion (*Allium cepa* L.), is a monocot bulbous biannual or perennial herbaceous plant of the Liliaceae, native to southwestern Asia and is the most widely cultivated species of the genus *Allium*. This genus also contains several other species variously referred to as onions and cultivated for food, such as the Japanese bunching onion (*A. fistulosum*), the Egyptian onion (*A. xproliferum*), and the Canada onion (*A. canadense*). Present species, *A. cepa* is one of the most familiar species of the group, is cultivated and used around the world. It is also known as the bulb onion or common onion, is used as a vegetable. It is widely used in the cuisine, often used as thickening agent for curries and gravies, but can also be eaten raw in salads, being rich vitamin C, B_6 and folic acid (James, 1994). It contains chemical compounds such as phenolics and flavonoids that have potential anti-inflamatory, anti-cholesterol, anti-cancer, antioxidant and antibacterial properties (Yang *et* al., 2004). Most onion cultivars are about 89% water, 4% sugar, 1% protein, 2% fibre and 0.1% fat. (Slimestad *et al*., 2007) It is estimated that around the world, over 9,000,000 acres (3,642,000 ha) of onions are grown annually. About 170 countries cultivate onions for domestic use and about 8% of the global production is traded internationally (Chengappa *et al*., 2012).

India is the second largest producer of onion in the world next to China. Onion is considered one of the most profitable crops in India, with a planted surface area of 1.04 million hectares and a domestic production in 2011-12 of 15.75 million tones (Chengappa *et al*., 2012). 50% of onion production is concentrated in the states of Maharashtra, Karnataka, Madhya Pradesh, Gujarat, Rajasthan, Andhra Pradesh, Uttar Prdesh, Bihar, Orissa, and Tamil Nadu both in rabi (winter) and kharif (rainy) seasons (Chengappa *et al*., 2012). In these regions, fungal diseases are the main cause of huge economic losses, including damping-off, purple blotch, block mould, white rot, basal rot, and onion smut.

In Andhra Pradesh, onion bulbs are grown for large domestic and regional export markets. In October of 2010, diseased onion bulbs were received from producers and exporters in Kadapa district, Andhra Pradesh, India. From 2010 to 2012, similar symptoms were observed at harvest on onion bulbs in Kadapa and Kurnool districts, Andhra

Pradesh, India. Considering the importance of the disease in onion growing regions, the present study was carried out for identification of the causal agent associated with diseased onion bulbs.

MATERIALS AND METHODS

Isolation of Pathogen

A total of 93 diseased samples were collected from all localities. Infected tissues were surface sterilized in 1% sodium hypochlorite for 2 min, rinsed three times in sterile distilled water, and plated on potato dextrose agar (PDA), and then incubated for 7 days at 25^0C under 12 hours light and dark conditions.

Morphological Identification of Pathogen

After seven days, hyphal tips from the margin of each developing colony were subculture on PDA. Micro slides of fungal culture were prepared in lactophenol-cotton blue, examined under microscope for their morphological characters and identified with the help of the standard keys provided by Domsch *et al*. (1980), Nelson *et al*. (1983), Nirenberg and O'Donnell (1998), Lesile and Summerell (2006), in their representative manuals besides consulting relevant published literature. The measurements of the spore forms and vegetative structures were taken with the help of an ocular micro meter. The identified fungus was stored on potato dextrose agar slants in the refrigerator at 4^0C prior to use.

Molecular Identification of Pathogen

For DNA extraction, a pure fungal culture, growing on PDA, was used as a source of DNA after incubation at 25^0C using the BioGene kit method. Squares of the cultured mycelia (0.5 cm^2) were cut from one week old cultures. The agar was scraped from the bottom of the pieces to exclude as much agar as possible. The pieces were ground in the presence of dry ice using a mortar and pestle. The genomic DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in sterile double distilled water and 1 µl samples of this solution were used for PCR amplification. The polymerase chain reaction (PCR) primers ITS-4 and ITS-5, developed by White *et al*. (1990) were used to amplify the internal transcribed spacer regions of ribosomal DNA, which encompass the 18S rDNA gene and both ITS-1 and ITS-2 regions.

The reaction mixture contained 50 µl of 1U Taq DNA polymerase, 5 µl of 10×PCR buffer (10mM Tris HCl, pH 8.3, 500 mM KCl, 15mM $MgCl_2$), 160 µM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 10pmoles of each ITS-5 and ITS-4 primers, 2 µl of 5M betaine and 50 ng of genomic DNA. The final volume (25 µl) was adjusted using PCR-grade double distilled water (Fisher Scientific, Wembley, Western Australia). The PCR amplification was performed in a thermocycler (eppendorf Pvt. Ltd). Cycle parameters consisted of an initial denaturation at 94^0C, 55^0C and 72^0C for 30, 45 and 60 s, respectively, and a final extension step of 7 min at 72^0C was included. The resulting PCR products were checked on 0.8% agarose gel electrophoresis and purified with QIAquick spin column (QIAGEN) following the manufacturer's instructions.

PCR product of ITS-amplified region containing ITS-1, 18S rDNA and ITS-2 was directly sequenced using ITS-5 (forward primer) and ITS-4 (reverse primer) by using the ABI PRISMTM Bigdye Terminator Cycle Sequencing kit, Version 3.1 (Applied Biosystems Inc.) and analysed on an ABI prism 3730XL automated DNA sequencer (Applied Biosystems Inc.). The sequence data obtained from ITS-4 reverse primer was inversed using gene doc software and clubbed with sequence data of ITS-5 to obtain complete sequence of amplified ITS product. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the GenBank database using BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST).

Pathogenicity Test

To determine pathogenicity, bulbs were surface disinfected in 70% ethanol for 1 min, rinsed with sterile distilled water, and injured to a depth of 0.5 cm with a sterile 2 mm diameter probe. The wounds were filled with PDA colonized by the appropriate isolate from a 7-day-old culture. Ten bulbs for each tested isolate received sterile PDA as a control. The bulbs were incubated at 25^0C for 2 weeks. The tests were repeated once.

RESULTS AND DISCUSSION

The fungal colonies were fast growing with white aerial mycelium and violet to dark pigments in the PDA. Hyphae were septate and hyaline. Conidiophores were short, simple or branched. Macroconidia were sparse, slightly curved to almost straight, 3- to 5- septate, and 31-53 × 3.5-5.0 µm. Microconidia were abundant, single celled, oval or club-shaped, and measuring 5.6-10.5 × 2.0-3.5 µm, and in chains on monophialides and polyphialides. On the basis of morphological characteristics, the pathogen was identified as *Fusarium proliferatum* (Matsushima) Nirenberg (Nirenberg and O'Donnell, 1998; Leslie and Summerell, 2006).

The identity of the fungus was confirmed through internal transcribed spacer-polymerase chain reaction (ITS-PCR) technique, where the amplified products yielded around 547-bp. The resulting sequence was compared to the GenBank database using the NCBI BLAST search program. BLAST analysis of the 547-bp amplicons showed 99% similarity with ITS sequence of *F. proliferatum* (GenBank Accession No. FN868470.1). Sequence from this study was submitted to GenBank database (Genbank Accession No. AB675035). *F. proliferatum* (synonym *Gibberella intermedia*) is the anamorphic form of the *G. fujikuroi* complex that belongs to the Nectriaceae family (Nirenberg and O'Donnell, 1998).

After 2 weeks of artificial inoculation, rot symptoms similar to the original symptoms developed on all inoculated bulbs and *F. proliferatum* was consistently re-isolated from symptomatic tissue, fulfilling Koch's postulates. No fungi recovered from the control bulbs.

F. proliferatum has previously been reported on onion in the United States (Mohan *et al*., 1997; du Toit *et al*., 2003) and Serbia (Stankovic *et al*., 2005). To the best of our knowledge, this is the first report of *F. proliferatum* causing rot disease on onion bulbs in India. This plant pathogen has economic importance since it affects crops such as rice, corn, banana, sorghum, asparagus, pine trees, palm trees (Leslie and Summerell, 2006), and garlic (Dugan *et al*., Ravi Sankar and Prasad Babu, 2012). This species is known to produce fumonisin, beauvericin, and moniliformin toxins, among others (Stankovic *et al*., 2007),

this is of significance as it may pose toxicological risks to consumers if onion bulbs become infected.

CONCLUSION

Rot disease of onion bulbs caused by *F. proliferatum* is reported for the first time in India. *F. proliferatum* is an important pathogen producing huge losses in agriculture, due to its broad range of hosts. It is one of the main fumonisin producing species in the *Fusarium* genus and, in the near future, the mycotoxigenic hazard of *Fusarium* infections in onion should be determined. This disease poses a potential threat to the production and biodiversity of this important food crop. Urgent interventions are necessary to halt this emerging epidemic in India.

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