Research Paper :

Morphological and molecular identification of *Fusarium* isolated from cumin wilt

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SUMMARY

Cumin (*Cuminum cyminum*) is one of the oldest and economically most important spices after black pepper. Production of this plant is limited due to several biotic stresses of which wilt disease are the most serious. Cumin wilt disease is caused primarily by *Fusarium oxysporum* but other *Fusarium* species have been implicated. This is the first report of wilt of *C.cyminum* caused by *F. equiseti* in India. Observation was made both at morphological and molecular levels. Isolated pathogen was confirmed by sequence analysis, ITS marker and species-specific PCR assays.

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Cumin (*Cuminum cyminum*) is one of the spices grown in India. It is commonly used as spice in our daily life. Recent studies have indicated its pharmaceutical and medicinal importance (Aruna and Sivaramakrishnan, 1996). Cumin is produced in the warm regions of the world. There has been increased demand of cumin while its production is limited and decreased (Abu-Nahoul and Ismail, 1995).

Significant loses in cumin yield can be attributed to the adverse effects caused by biotic stresses of which Fusarium wilt disease is the most serious one (Omar *et al.*, 1997).Cumin plants are attacked by *Fusarium* species responsible for wilt diseases .Wilt of cumin generally infects the crop during the last week of December when the crop is about a month old. The disease generally appears in patches and is characterised by wilting of affected plants. After the appearance of wilting, the whole plant dries up. A preliminary survey was carried out and the loss from the disease was estimated to vary between 5 to 25 per cent in North Gujarat, and 5 to 60 per cent in Rajasthan, the average in the latter case being 20 per cent. It is very difficult to control this disease through modern means of chemical based measures.

MATERIALS AND METHODS

Fungal isolates:

In January 2009 and 2010, 324 samples (wilted plants) were collected from different areas of Gujarat (India). Total of 108 cumin fields in 25 locations of 7 districts were sampled during the season (Table 1). The fields were located in the main cumin growing area in Gujarat with different climates. Each field was arbitrarily divided into five circular plots approximately 100 m in diameter and two to four samples were randomly taken from each plot. Samples were pooled in each field and two infected plant from each field were selected and used for *Fusarium* species isolation. A total of 216 *Fusarium* isolates were recovered from 108 samples collected from different geographic regions.

Identification of *Fusarium* isolate:

Infected stem sections were surface-sterilized for 3 min. with a 0.01% sodium hypochlorite solution, rinsed twice in sterile

distilled water and dried in a laminar flow cabinet. Two growth media, Potato dextrose agar (PDA) and Rose Bengal agar, were used for fungal isolations. The plates were incubated at 25ºC in the dark for 5-7days. *Fusarium* isolates were sub-cultured on PDA, using a single spore technique (Leslie and Summerell, 2006). Cultural characters were assessed by eye and by microscopic examination. Colony morphology was observed from PDA plates. Morphological identifications of isolates were made using the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006).

Pure culture of *Fusarium* isolate:

Mixed cultures or cultures contaminated with bacteria and other fungi are a problem for all types of identification. Care should be taken to obtain a pure culture (Fig. 1), particularly one derived from a single conidium or ascospore to ensure that the culture represents a clone. Methods for obtaining pure cultures are outlined by Summerell *et al*. (2003) and (Choi *et al*., 1999).

Fig. 1 : Pure culture of *Fusarium equiseti* on PDA

DNA extraction:

Fusarium isolate was grown on PDA plates for 7 days and mycelium was harvested. Total DNA was extracted from ground mycelium of isolate (~100 mg wet weight) using a Genei Plant DNA extraction Kit (Genei, India) according to the manufacturer's instructions.

Blast :

The approach for identifying an isolate of *Fusarium* using the FUSARIUM-ID database, involves obtaining a pure culture of an isolate, extracting genomic DNA, amplification of the TEF gene region, and sequencing. BLAST is then used to identify the closest matches between the unknown sequence and those contained in the FUSARIUM-ID sequence database.

Pathogenic test:

For pathogenicity tests (Koch's postulate), sterilized soil was inoculated with 7-days old conidial suspension (5x10 conidia of *F. equiseti* per ml of water) in 10 pots, followed by planting of one month old healthy cumin plants in each pot. Five pots with uninoculated plants served as control. All pots were placed in a moist chamber at 25±2°C. Every inoculated plant showed symptoms within 10-12 days and, on re-isolation, yielded the original fungus. The control plants remained healthy.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Morphological identification :

Isolations from the roots and stems of diseased plants yielded fungus is characterised by the development of abundant white aerial mycelium which turns pinkish by keeping in daylight on Potato dextrose agar (PDA) and Rose Bengal medium (Fig. 2). The colonies produced macro- and micro-conidia within 3-4 days at 25±2°C. Microconidia are single-celled, hyaline, non-septate and ovoid. Macroconidia are 2-3 septate, straight or slightly curved at apex. The size of macro-conidia was in the range of 28.0-30.5x3.5-5.25µm (Fig. 3) and the micro-conidia were in the range of 9.5-12.5x3.5-5.25µm. The pathogen culture was identified on the basis of colony and spore morphology as *Fusarium (*Summerbell *et al.*, 2003).

Molecular identification:

The culture was deposited under accession number NFCCI 2157 (National Fungal Culture Collection of India) at Mycology and Plant Pathology Group Agharkar Research Institute, Pune. It showed 99% sequence similarity with genus *Fusarium* Link (1809) species *F. equiseti* (Corda) Sacc. (1886) (NCBI Accession HM130559.1) by run NCBI-BLAST (Geiser *et al*., 2004 Summerbell *et al*., 2003) ITS 1 and 2 marker was also used to confirm *Fusarium* species at a local

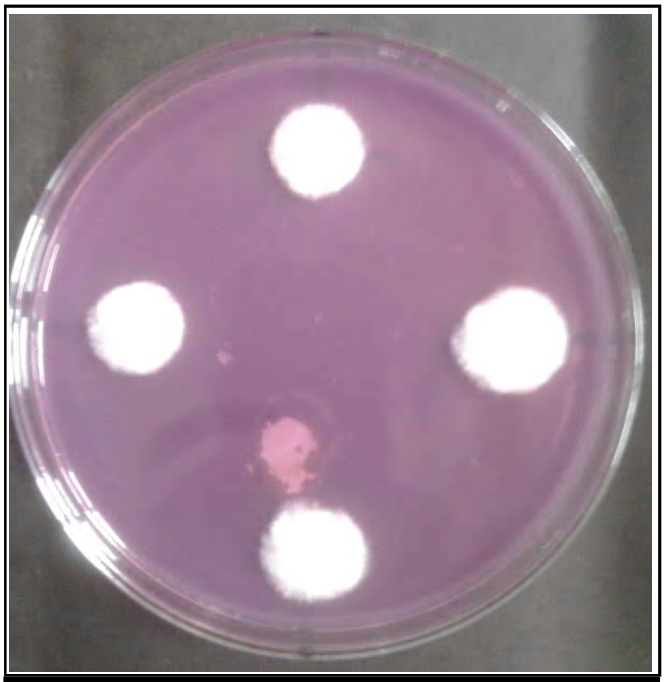

Fig. 2 : Rapid growth and profuse aerial white mycelium colonies of *Fusarium equiseti* on Rose Bengal medium

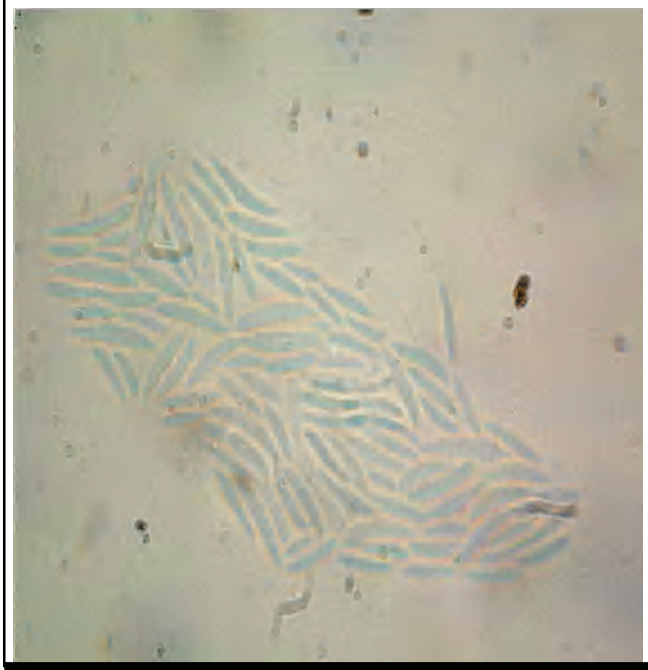

Fig. 3 : Macro conidia of *Fusarium equiseti*

Table 1 : Collections of samples from different lications

Sr. No.	District	Location	Field	Variety	No. of sample
1	Mehsana	Kadi	4	GC-4	12
		Khavad	5	GC-4	15
		Devinapur	5	GC-4	15
		Mitha	4	GC-4	12
		Dharmpur	6	GC-4	18
		Vamaj	3	GC-4	15
2	Patan	Pimpal	4	GC-4	12
		Bauchraji	4	GC-4	12
		Sami	3	GC-4	09
		Chansama	4	GC-4	12
3	Banas kantha	Desa	4	GC-4	12
		Palanpur	5	GC-4	15
		Vadgam	4	GC-4	12
4	Sabar Kantha	Megharaj	4	GC-4	12
		Bayad	5	GC-4	15
		Modasa	6	GC-4	18
		Malapur	5	GC-4	15
5	Gandhinagar	Dehgam	4	GC-4	12
		Khanpur	4	GC-4	12
6	Ahmedabad	Sanand	5	GC-4	15
		Bhavanpur	5	GC-4	15
		Tiker	4	GC-4	12
7	Surendranagar	Limadi	3	GC-4	09
		Lakhtar	4	GC-4	12
		Naliya	4	GC-4	12
Total	7	25	108		324

BLAST(Basic Local Alignment Search Tool) server, in FUSARIUM-ID v. 1.0, which can be accessed at http:// fusarium.cbio.psu.edu. Query sequence was aligned with the best score of 466/467 with 99.78% similarity. Total 10/75 *Fusarium* isolates were *F. equiseti* based on morphological characters.

DNA sequence-based identifications and species-specific PCR assays are usually needed to accurately identify species. Thus, we used species-specific PCR, ITS 1 and 2 markers and sequence analysis to confirm our morphological identification and to identify pathogen isolate.

In all 324 samples were collected from 108 cumin fields in 25 locations of 7 distrcts of Gujarat, India during season 2009 and 2011 (Table 1).

Earlier investigators have found out *Fusarium oxysporum* as causal organism of wilt in cumin. This study was based on both morphological and molecular levels that confirmed *Fusarium equiseti* is also pathogenic agent for cumin wilt. Its first study and report another species of *Fusarium* is responsible to cause wilt of cumin in India.

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