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Isolation and characterization of a new fungal species, *Fusarium equiseti* (Corda) Sacc., from *Brassica juncea* in Bharatpur

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Abstract

A field survey of emerging collar root disease incidence at farmer's fields of Indian mustard (*Brassica juncea* L.) was conducted in Bharatpur district of Rajasthan to confirm the casual organism of the disease. The pathogen from collected diseased samples was isolated, purified and supply to Agarkar Research Institute, Pune, India for identification. Analysis of the sequences of different fragments of the ribosomal genes demonstrated that isolated pathogen species belongs to the Nectriaceae family and is genetically different from other morphologically similar species of *Fusarium*. This new species is unique in having colonies on PDA at 25°C± 2°C, fast growing, rosy pink to vanacous, reverse pell leuteus to light ochraceous. As per the micromorphology, hyphae was hyaline, smooth, pigmented and 2.75-4.77µm wide. Single-spore pure culture produced chlamydospores were intercalary, fusoid, hyaline and smooth walled. Although, macroconidia were abundantly produced, fusoid, smooth walled, hyaline, foot cell present and sickle shaped, 2-septate with 14.59-45.48 x 2.88-4.10µm in size. Though, microconidia were fusoid, 1-septate and 2.5-2.67 x 2.67-13.7 µm in size. As per our studies, the fungus was morphologically identified as *Fusarium equiseti* (Corda) Sacc. (NFCCI 4564).

Introduction

Collar rot is emerging as the sporadic disease of rapeseed-mustard in Bharatpur, India. Recently, Indian mustard plants with rotting stems above ground level followed by premature death were observed at farmer's field in Bharatpur, observed upto 20% incidence. Now a days, there are other fungi responsible to cause rot problem in Indian mustard (*Brassica juncea* L.) just after first irrigation (35-45 d.a.s) including *Sclerotium rolfsii* Sacc., *Rhizoctonia solani* etc. Although, *Fusarium equiseti* (Corda) Sacc. is considered to be a weak pathogen on cereals and is sporadically establish to be associated with Fusarium head blight-infected kernels (Xue *et al.,* 2006). This species generally occurs in tropical and subtropical regions (Booth, 1978; Bosch and Mirocha, 1992), but it has also been recovered from cereals in temperate areas, including the Soviet Union, Europe, and North America (Kosiak *et al*., 2003; Stack *et al*., 1997; Tekauz *et al*., 2005; Wing *et al*., 1993; Xue *et al*., 2006). *Fusarium equiseti* is a soil inhabitant and can transmit a disease to seeds, roots, tubers, and fruit of numerous crop plants. It has been formerly concerned as a causal agent of disease on different plant species, such as cotton (*Gossypium hirsutum* L.; Chimbekujwo, 2000), cowpea (*Vigna unguiculata* (L.) Walp.; Rodrigues and Menezes 2005), lentils (*Lens culinaris* Medik.; Chaudhary and Kaur, 2002), sugar beet (*Beta vulgaris* L.; Stojsin *et al*., 2001), potato (*Solanum tuberosum* L.; Rai, 1979; Theron & Holtz, 1989) and pine (Pinusspp.; Ocamb & Juzwik, 1995).

Seed decay and reddish brown to black lesions were observed on hypocotyls and roots of kidney bean (*Phaseolus vulgaris*), bush bean (*Phaseolus lunatus*), broad bean (*Vicia faba*), chickpea (*Cicer arietinum*), and pea (*Pisum sativum*). A brownish discolouration and water-soaking symptoms developed on roots of alfalfa (*Medicago sativa*), canola (*Brassica napus*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and oat (*Avena sativa*) seedlings. Tomato (*Lycopersicon esculentum*), pepper (*Capsicum annum*), carrot (*Daucus carota*), and cucumber (*Cucumis sativus*) plants did not exhibit any visible symptoms (Goswami *et al*., 2008). The disease is distressing farmers to cultivate Indian mustard crop. Keeping in view the seriousness of the problem the present investigations were carried out.

Materials and Methods

Experiments were carried out in controlled environmental conditions at ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur, India. Though, identification of the pathogen was carried out by morphological and molecular analysis at Agharkar Research Institute, Pune, India.

Plant material

Total 10-seeds in each cemented pots (30x30x25cm) were sown on 5 November 2018 using cultivar NRCHB 101 of mustard under controlled condition. Recommended dose of fertilizers applied at sowing time and soil moisture was adjusted to 50% WHC and amount of water lost was restored after each 24 hrs. There were four replicates of each treatment and the pots were randomized on a screen house bench. Data on pathogen colonization were recorded after 10-days of inoculation by counting % infected and healthy plants in each treatment.

Isolation of fungi

After washing the tissues thoroughly in sterile water, the causal fungus was isolated from plant tissues exhibiting clear symptoms. The infected tissues along with adjacent small unaffected tissue were cut into small pieces (2–5 mm squares) and by using flame-sterilized forceps, they were transferred to sterile petridishes containing 0.1% NaOCl solution used for 5 min surface sterilization of plant tissues. The plant parts were transferred to Potato Dextrose Agar (PDA) plates containing Streptomycin @ 0.2 g L^{-1} and incubated for 7-days for the complete growth of fungi. The resulted fungi were purified using the hyphal tips technique on PDA medium and then subculture of isolated fungus on slant medium for future studies.

Preparation of inoculums

Inoculum of fungus was mass multiplied usin corn seed medium. Wet Corn seeds were autoclaved for 20 min at 15 psi twice before incolation of the fungi into 250 ml conical flasks. A 5 mm diameter inoculum disc from an actively growing culture of *F. equiseti* (isolated from Indian mustard) on PDA was transferred into each flask. The flasks were incubated at 25^0C temperature for ten days for production and maturation of macrocondia. The inoculum was then mixed on a sterilized soil used for sowing in pots.

Pathogenecity of the pathogen

Study on inoculation methods in cemented pots was carried out in cage house at ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur, India. Four different methods and inoculum sources were used in this study. Methods applied were: 1) soaking seeds in mycelial fragment suspension, 2) sowing seed in soil mixed with culture, 3), inoculating basal stems of 75 day-old plant (germinated on sterilized, moistened cotton rapped with parafilm), 4) sowing seeds in soil mixed with fungus growing on autoclaved corn seeds at 75 days after sowing (d.a.s.) and 5) uninoculated check were taken. Plants treated with sterile water served as control. For soil inoculation, pathogen was grown for mass multiplication on Corn seed media for ten days was incorporated in soil. Stem inoculation was done using pick tooth method. Inoculating through wounds on stem and tooth pick techniques were applied to inoculate mature plants (75 days after sowing).

Effect of different media on growth

Fungal mycelial growth rate of *F. equiseti* was recorded on seven different culture media *viz*. Potato dextrose agar, Oat meal agar, Brassica leaf extract agar, Czapek-dox agar. Mustard stubble agar, Corn meal agar and Chickpea agar in four replications from 7-days old culture with 24 hours interval.

Morphological identification of fungi

To determine the causal agent of the disease, small pieces of stems were surface-sterilized with 0.1% sodium hypochlorite, washed thrice in sterile distilled water, placed on potato dextrose agar (PDA) plates and incubated at 25°C for seven days. Fungal identification was done based on fungal culture, macromorphology, micromorphology, and microconidia at Agarkar Research Institute, Pune, India. The fungus was identified according to cultural characters described by Gilman (1957), Barnett and Hunter (1972) and Nelson *et al.* (1982).

Molecular identification of isolated pathogen

Genomic DNA was isolated in pure form, from the culture isolated from Indian mustard plant from Bharatpur district of Rajasthan, India. The ITS-rDNA partial gene was successfully amplified using primers ITS4 & ITS5. The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit. The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences and analyzed to reach identity.

Results and Discussion

Diseased Indian mustard plants were received from farmer's field, showing wilting of leaves and twigs and necrosis of stem, crown and roots were observed during 2016 to 2018, with an incidence of up to 20%. The fungus responsible for collar rot was morphologically identified as *Fusarium equiseti* (Corda) Sacc. (NFCCI 4564).

Pathogenecity of the pathogen

Disease initiation was first reported in soil inoculation + seed inoculation methods after 65 days after sowing (d.a.s.). With tooth-pick inoculation technique (28.8%),

symptoms developed more severe than seed inoculation and soil inoculation methods. After inoculation of for 10-days, stem infection by *F. equiseti* was recorded highest in seed+soil inoculation method (40.0 %). Four methods developed significantly different results, plants died within 10-days but the seed+soil inoculation method was found to be the best and practicable method (Table 1).

Table 1. Per cent disease incidence in four inoculation methods

Treatments	% incidence
Seed inoculation	21.9
Soil inoculation	19.6
Stem inoculation	28.8
Seed+soil inoculation	40.0
Check	3.1
CD(p=0.01)	9.6

Morphological identification of fungi

This new species is unique in having colonies on PDA at 25°C± 2°C, fast growing, rosy pink to vanacous, reverse pell leuteus to light ochraceous. A dense white mycelium developed, that turned first beige, finally buff brown. Micromorphology showed that the hyphae were hyaline, smooth, pigmented and 2.75-4.77µm wide. Single-spore pure culture produced chlamydospores were intercalary, fusoid, hyaline and smooth walled. While, macroconidia were abundantly produced, fusoid, smooth walled, hyaline, foot cell present and sickle shaped, 2-septate with 14.59-45.48 x 2.88-4.10µm in size. Though, microconidia were fusoid, 1-septate and 2.5-2.67 x 2.67-13.7 µm in size.

Molecular identification

The tested fungal strain showed 100% sequence similarity with *Fusarium equiseti.* Sequnce analysis with NCBI accession number EU326202.1, *Fusarium equiseti* isolate XSD-80 resulted in following alignment statistics. Query length-506, Score-913 bits (1012), Expect-0.0, identities-506/506 (100%), Gaps-0/5.6 (0%), Strand- Plus/ Minus.

Effect of different media on growth

The growth of *F. equiseti* was noted faster on Mustard stubbles extract agar (86.8 mm) followed by Oat meal agar (86.6 mm), Corn meal agar (86.5 mm), Czepak dox agar (86.3 mm), and Mustard leaf extracts agar (85.9 mm) medium. Slowest growth was recorded on Potato dextrose agar (38.8 mm) followed by Chickpea agar (83.8 mm) medium (Table 2). The mycelia growth rate pattern was at par in all medium except very slow in PDA (Fig. 1). Although, host medium both leaf and stubbles extract were found suitable for abundant growth of *F. equiseti* which is first report so far.

Table 2. Mycelial growth of *Fusarium euiseti* on different media

Media	Mycelial growth (mm)
Czepak Dox Agar	86.3
Leaf Extract Agar	85.9
Poatao Dextrose Agar	38.8
Chick Pea Agar	83.8
Oats Meal Agar	86.6
Mustard Stubble Agar	86.8
Corn Meal Agar	86.5
CD (p=0.01)	3.53

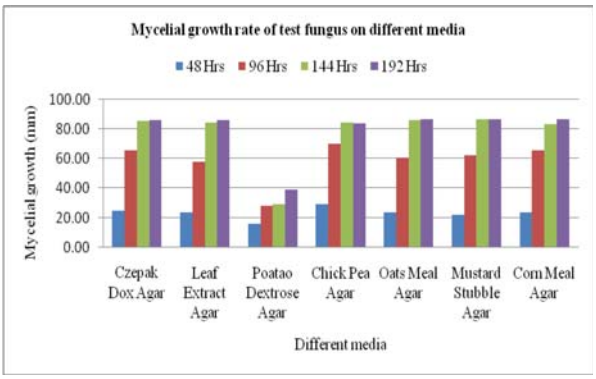

Fig. 1. Growth rate of *Fusarium equiseti* on different media

Conclusion

As per the available reports, this is the first report of *F. equiseti* causing collar rot disease on Indian mustard in Rajasthan where about 50 per cent of total Indian mustard cultivation in India. Mustard leaf extract and Mustard stubbles extract were first time found suitable media for the profuse growth of *F. equiseti.*

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