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Full Length Research Article

Morpho-Molecular Characterization of *Fusarium proliferatum* Causing Garlic Bulb Rot in Southern Karnataka Region, India

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Abstract

The current work sought to characterize the morphological and molecular characters of the garlic bulb-rotting fungus *Fusarium proliferatum* as it is one the major disease in the garlic growing regions of Karnataka as the pathogen known to produce mycotoxin in affected garlic bulbs. This poses a substantial threat to the food safety of *Allium sativum* cultivars that are grown in various agro-climatic zones of Karnataka. Based on morphological and cultural studies, the causal organism of bulb rot disease on garlic is identified as *Fusarium proliferatum* (Matsush.) Nirenberg. The internal transcribed spacer (ITS) region of nuclear rDNA form the pathogen was amplified using the ITS-1 and ITS-4 primer to validate and supplement phenotypic and morphological identification. This finding serves as preliminary information about the garlic bulb rot disease in the surveyed region and can be a baseline for developing disease control plans for this economically significant pathogen.

***Key words*:** Bulb Rot, *Fusarium proliferatum*, Internal transcribed spacer, Mycotoxin, *Allium sativum*

The Indian subcontinent is well-known for its wide variety of spices and is one of the major exporters of spices to other countries around the world. Garlic is one of the many spices that grow luxuriantly in Indian subcontinent in different agro-climatic zones. Use of garlic has been mentioned in the ancient Egypt literature as a disinfectant to treat gangrene, and has been demonstrated that garlic has a huge potentiality in treating high blood pressure and prevent atherosclerosis [1-2]. Three hundred different varieties if garlics are known to grown in different agro-climatic zones all over the world. India is one of the most important garlic producing country with annual production of 500,000 million metric tons which accounts for five percent of the total 2.42 million tons of garlic produced globally. India ranks third behind China (sixty six percent) as the world's top garlic producer [3].

The major garlic producing states in India are Madhya Pradesh, Gujarat, Orissa, Rajasthan, Uttar Pradesh, Maharashtra, Karnataka, Bihar, Tamil Nadu, Punjab, Haryana, and Andhra Pradesh [4]. Garlic crop is being affected by number of fungi and most notably *Fusarium* sp. There are several reports that, *Fusarium* species are known to produce mycotoxins in the form of fumonisins and fusaric acid during storage and garlic being the edible product, bulb rot of garlic is a serious concern as it is being caused by *F. proliferatum* and is of considerable health concerns in humans [5-8]. The disease has been reported from different countries causing severe loss to the garlic growers as it is known to develop fungicide resistance [6], [9-11]. Garlic bulb rot is a serious issue as the causal organism is known to produce mycotoxins, mainly fumonisins which are considered to be responsible for various ailments in humans as well as animals if contaminated bulbs and other food products are consumed [12-14].

Garlic bulb rot is a serious matter of concern as the typical symptoms may not appear in the fields but after the harvest when the bulbs are stored, they may start to show typical bulb rot symptoms. In filed, generally damaged plants may exhibit diminished emergence, yellowing or browning of leaves that begins at the tips and progressively spreads to the base. The cloves or the rot chambers may have a white, light pink or reddish fungal growth on them [11]. In the present study identification and isolation of the fungi from the diseased samples collected from the farmers' field of southern Karnataka region was carried out and molecular characterization was also carried out to confirm the pathogen using molecular tools.

MATERIALS AND METHODS

Sampling sites and collection of diseased samples

During the growing seasons of 2018 to 2020, samples of infected garlic were taken from fifty-one garlic farms in six different agro-climatic areas of southern Karnataka. Each collection field was divided in five plots and samples were collected at random from each plot. Two infected bulbs from each field were chosen for the isolation of the causative agent from each field and pooled together.

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Isolation and identification of Fusarium species

The pathogen was isolated from the affected bulb. The infected garlic bulbs were surface-sterilized with 1% sodium hypochlorite solution for four minutes; infected portion was diced and rinsed two to three times with sterile double-distilled water. The edges of the lesions' infected tissue were then sliced into small pieces and aseptically placed in Petri plate containing potato dextrose agar (PDA). Streptomycin (200 mg/L) was used as antibiotic to prevent bacterial contamination. The Petri plates were incubated for seven days at 25±2°C with 12 hours photoperiod of alternate light and darkness [15].

The colony morphology on pure culture plates was recorded and compared with previously published phenotypic, cultural, and other characteristics descriptions of the causal agent of bulb rot disease and also by consulting mycological monographs [16]. Characteristics features such as hyphae, reproductive structures (micro-conidia and macro-conidia), chlamydospores, and phialides were observed by sub-culturing the on PDA media and observing under compound microscope (Olympus CX 40 Binocular Microscope with Camera). Wet-mount technique was followed and lacto-phenol cotton blue stain was used to observe under microscope.

Pathogenicity test

Pathogenicity test was conducted by isolating and inoculating the leaves of garlic plants with conidial suspension of an isolate obtained from Gundlupet taluk of Chamarajanagar district in Karnataka. Inoculum was prepared by flooding with water in the Petri dish of the isolate with abundant conidial spores growing on PDA. One drop of the suspension (approximately 10µL) was pipetted onto the leaves of garlic plants. For each 15-cm pot with potting mix there were three 40-day-old plants. Sterile distilled water was pipetted onto leaves of control plants. Inoculated and control garlic plants were maintained in the polyhouse, initially surrounded by moist cloths and covered with plastic bags for 48 hours. The bags and cloths were removed and after 3 days. Inoculated plants exhibited symptoms followed by wilting of plants. Garlic bulbs were uprooted and observed for typical symptoms. The fungus was re-isolated from infected garlic bulb of inoculated plants and identified as *F. proliferatum*. Control plants developed no disease symptoms.

Molecular characterization of Fusarium proliferatm

Genomic DNA extraction

A 14 days culture of *Fusarium proliferatum* cultured in potato dextrose broth (Hi Media) was used in the present study. Mycelia from the cultured broth was filtered using sterile muslin cloth, washed in sterilized distilled water, lyophilized, and kept at -80°C until DNA extraction [17]. Mycelia were crushed using a pestle and mortar in liquid nitrogen, transferred to a 2.0 ml eppendorf micro centrifuge tube with four times the volume of pre-warmed 2 CTAB buffer (2% CTAB, 100mM Tris-HCl pH 9.5, 20mM EDTA pH 8.0, 1.4 M NaCl, and 1% mercaptoethanol, 1% Polyvinylpyrrolidine) mixture and incubated at 65°C in water bath for 1hr with occasional vortex. The mixture was cooled to room temperature and centrifuged at 10,000 rpm at 4ºC for 5 min.

Amplification and sequencing

Reliability of morphological identification was confirmed with the molecular characterization using universal primers for fungal identification. Primers set such as ITS1 (forward; 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse; 5'-TCCTCCGCTTATTGATATGC-3') [18] were used for amplification of fungal internal transcribed spacer regions of nuclear ribosomal DNA (rDNA). The PCR mixture (50 µl, total volume) contained 5µl 10×PCR buffer containing 15 mM $MgCl_2$, 149.5 µl of 2 mM of each deoxynucleoside triphosphates (dNTPs), 2 µl of each primer (5 pmole/µl), 4 µl template DNA (50-100ng), 2 µl (1U/µl) Taq polymerase and MilliQ water (30 µl). PCR reaction conditions were maintained at 94ºC (5 min) for initial denaturation and 35 cycles of a denaturation step at 94ºC (40 sec), an annealing step at 54°C (60 sec), and an extension step at 72ºC (60 sec) [19]. After the 35th cycle, a final 10-min extension step at 72ºC was performed. The PCR products were electrophoresed in 0.8% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) and the amplicons were visualized under a gel documentation system (Bio Rad). The gel section with desired band was carefully excised under UV light and subjected to extraction using a gel band purification kit (Qiagen) and sequenced bidirectionally in an ABI 3730 sequencer (Applied Biosystems, United States) using the ITS1 and ITS4 primers [20].

Sequences were subjected to online BLAST search (http://www.ncbi.nlm.nih.gov) to assign putative identity, designation of operational taxonomic units based on sequence similarity measures and phylogenetic inference. Sequences were aligned with other similar sequences retrieved from Gen Bank using Clustal Omega [21]. Phylogenetic analyses of internal transcribed spacer (ITS) sequences were constructed using the maximum likelihood (ML) method in MEGA Version 5.2 (software package) combined with bootstrap analysis with 1000 replications [22].

Phylogenetic affiliation

The ITS sequence data sets of each were aligned using the default promoters that have been created to give the best average benchmark accuracy, as detailed in the original description of the algorithm, and the CLUSTAL programme, which is available along with MEGA software. Visual inspections were used to check alignments, and manual corrections were made as needed. The Kimura-two-parameter model was used to produce the NJ trees, and preliminary phylogenetic trees were constructed based on two very distinct techniques neighbour-joining (NJ) and maximum parsimony (MP) as implemented in MEGA 5.2. Gaps were considered missing data and all characters were run in an unequal order with equal weight. Zero-length branches collapsed, saving all the various, equally parsimony trees. 1,000 bootstrap replicates were run for each study to assess the node support of the resulting trees. The ClustalW software was used for alignments [23]. Manual corrections were made to the aligned sequences with a gap-positioning focus. To calculate pair wise % sequence divergence, DNA sequence data were analyzed. Phylograms were created using the sequence alignment data in MEGA programme (MEGA 5.2).

RESULTS AND DISCUSSION

Sampling of garlic bulbs and morphological identification of causal organism

Garlic bulbs of specific cultivars that were commercially grown in various agro-climatic localities of southern Karnataka region districts namely; Chamarajanagara, Mysore, Hassan, Chitradurga, Tumkur and Mandya (Fig 1a) were examined to detect symptoms like water-soaked lesions and whitish or pinkish dark spots on the surface of the bulb. The bulbs exhibiting symptoms (Fig 1b-c) were collected for identification and isolating of the causal organism. Ninety

garlic bulbs collected from various fields samples were cultured on potato dextrose agar media, incubating at 25±2°C and observed for cultural characteristics. Out of ninety *Fusarium* cultures, sixty-six developed whitish colony (Fig 2) with aerial mycelium on PDA that later turned pale orange in colour. Based on culture, morphological, and reproductive structures, the causative organism was identified as *Fusarium proliferatum* (Matsush.) Nirenberg. Macro conidia, which were composed of three to five long cone-shaped cells, are asexual reproductive structure produced on specialized hyphae known as conidiophores (Fig 3a) which were thick-walled measuring length of 609.42µm, and width of 886.82µm. Micro-conidia had one or two cells formed in a false chain measuring length of 259.56µm and width of 68.60µm (Fig 3b). Conidiophores were either branched or un-branched (Fig 3b). Chlamydospores were also observed (Fig 4a) found. False heads in small numbers (Fig 4b) and mycelium coiling (Fig 4c) in some areas were also noted having single, double, and multiple phialides (Fig 5a-c). Both spore forms are produced in enormous quantities and the one that rapidly germinate on hosts with suitable substrates developed into colonies that continue to produce spores. Based on cultural, morphological and microscopic observation, the isolate displayed same colony characteristics similar to *F. proliferatum* confirming the causal agent as *F. proliferatum*. Our results corroborate with previous research reports from Germany, India, Argentina, North Carolina (USA), Egypt, Spain, Italy, China and France stating that *F. proliferatum* is the worldwide causal agent of garlic bulb rot which was identified based on phenotypic, cultural, morphological, and molecular characters form different parts of the world [4], [8], [15], [19], [24-27].

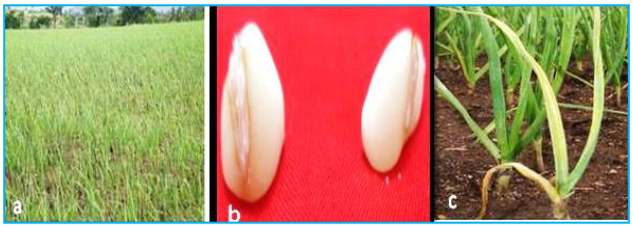

Fig 1 (a) Garlic field of southern Karnataka region, (b) Infected bulb collected from garlic growing southern Karnataka regions, (c) Infected plant

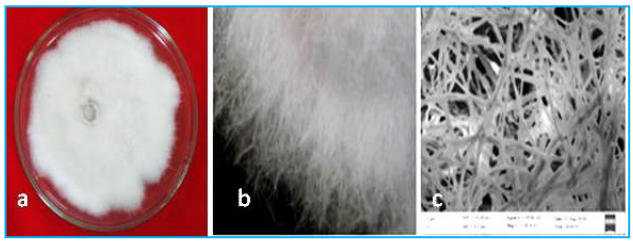

Fig 2 (a) colony morphology of 7 days old *F. proliferatum,* (b) Sterio microscopic features of *F. proliferatum,* (c) SEM image of *Fusarium proliferatum*

Pathogenicity test

Pathogenicity test was conducted to prove the Koch's postulate. The results revealed that, garlic plants inoculate with conidial suspension of an isolate obtained from Gundlupet taluk of Chamarajanagar district exhibited typical symptoms of bulb rot and wilting of plants on 60 days old garlic plants. The garlic bulbs which were showing typical symptoms of garlic bulb rot were used to re-isolate the pathogen and identified as *Fusarium proliferatum*. Control plants did not develop any disease symptoms.

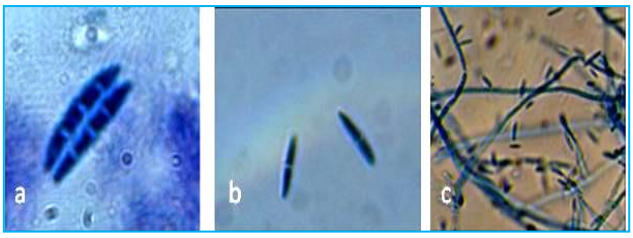

Fig 3 (a) Macro conidia (under 100x magnification), (b) Micro conidia (under 100x magnification, (c) Conidiophores (under 40x magnification)

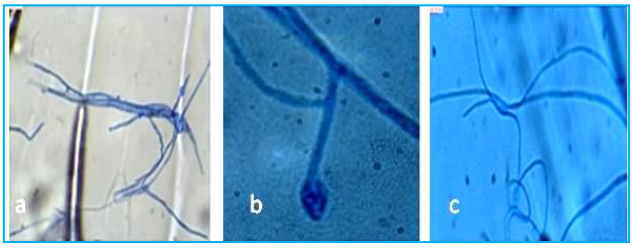

Fig 4 (a) Chlamydospore (under 40x magnification), (b) Fales head (under 100x magnification, (c) Coiling of hyphae (under 100x magnification)

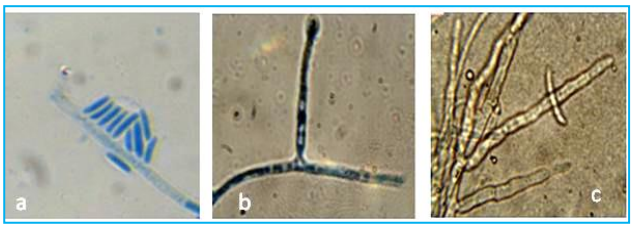

Fig 5 (a) Mono phialide bearing macro conidia (under 100x magnification), (b) Double phialide (under100x magnification), (c) Multi phialide (under 100x magnification)

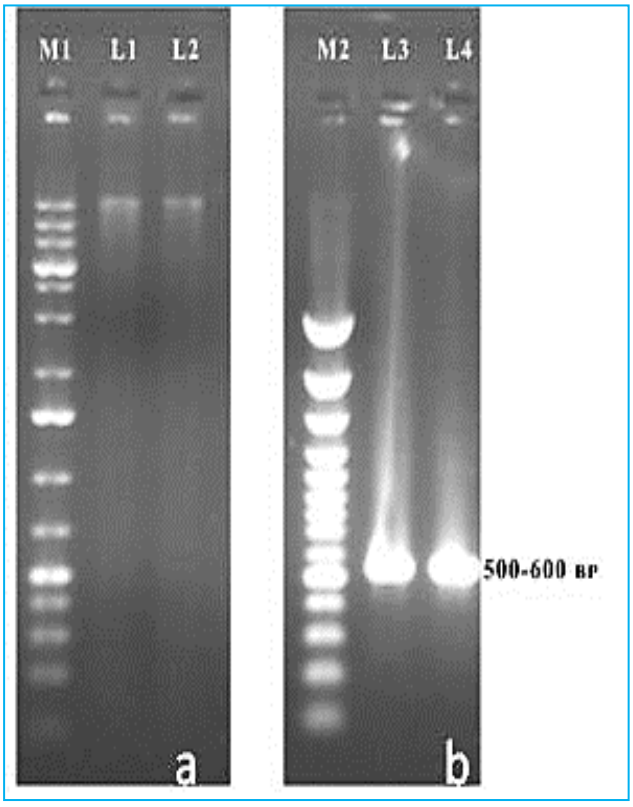

Fig 6 (a) 1% Agarose gel showing genomic DNA in Lane-1 and Lane-2, (b) 1% Agarose gel showing amplicon of ~550-600 base pair Lane-3 and Lane-4

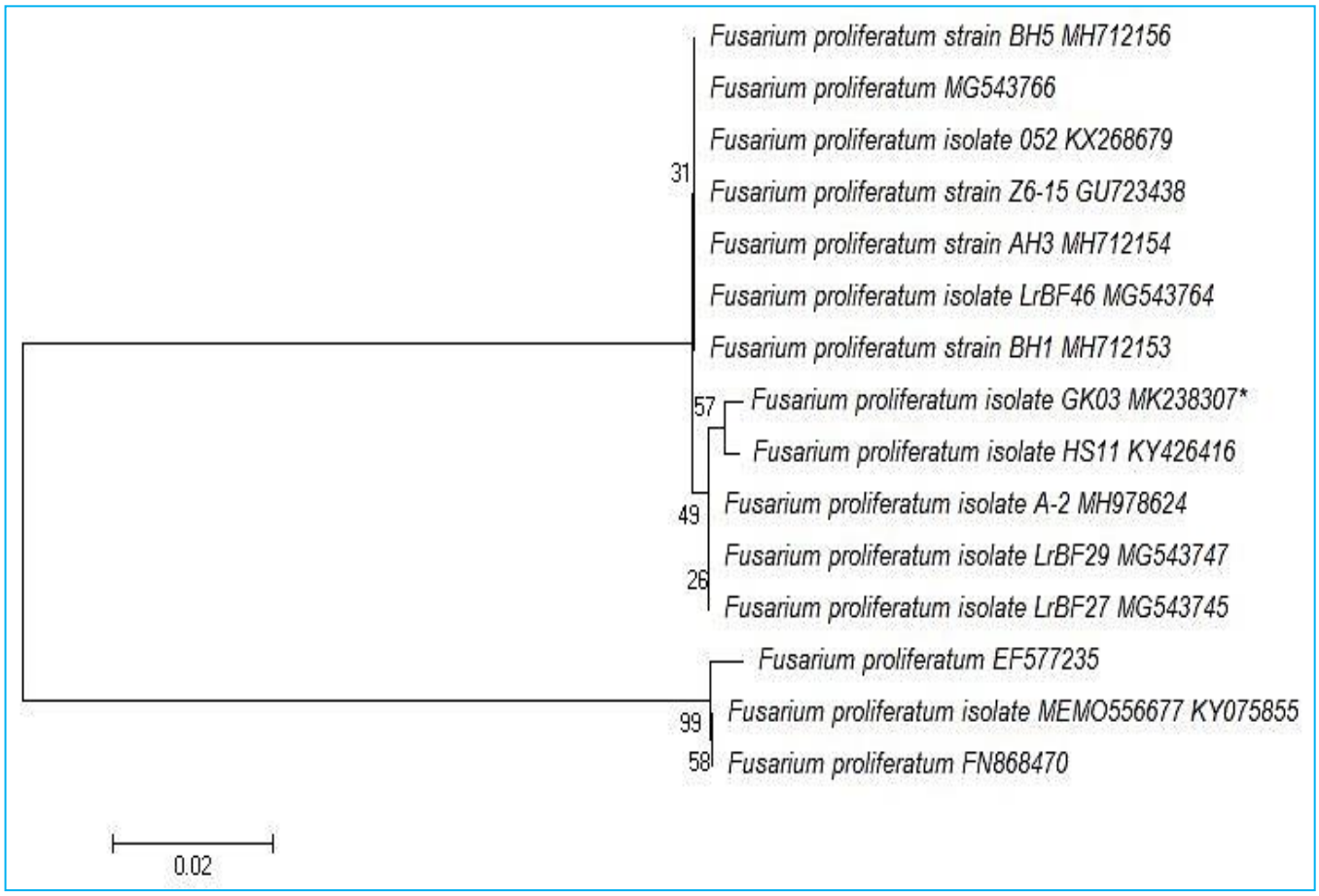

Fig 7 Phylogenetic affiliation of *Fusarium proliferatum* GK03 MK238307* using the Neighbour-joining method using MEGA software 5.2 (* Represents isolate obtained in the present study)

Analysis of PCR amplification results and phylogenetic affiliation

Identification of *Fusarium proliferatum* as the causal agent of bulb rot disease on garlic in Southern Karnataka region was validated by a combined data, based on both morphological characters and aplified sequenseces of ITS region of nulcear rDNA analyses. *F. proliferatum* is widespread throughout the world and is frequently linked to the bulb rot diseases that affect a variety of plant species in various nations [7], [10], [26], [28]. Due to the extensive phenotypic classification system, morphological identification of the *Fusarium* genus is considered to be challenging, convoluted, confusing, and time-consuming. Numerous physical characteristics are shared by all species of the genus *Fusarium*, such as macro-conidia that are nearly straight, small basal cells, and micro-conidia arranged in chains [29-30]. To overcome these problems, many phytopathogenic fungi that belong to various taxonomic groups can now be detected and identified using molecular techniques, particularly PCR-based assays. Because the ITS of the nuclear rDNA region is cosidered to be highly conserved, this region is a preferable option for phylogenetic research and is used to differentiat species within the genus *Fusarium* as DNA sequence analyses of the ITS region has the capacity to provide greater information regarding resolution at the sub-species level [31-32].This technique show good specificity and can distinguish closely related species [33-35].

The isoalted pure culture of *F. proliferatum* was subjected to molecular characterisation based on amplified internal transcribed spacer regions of nuclear rDNA fragment and molecular phylogenetic affiliation to complement and validate the morphologically identified pathogenic fungal strain. Using agarose gel electrophoresis, the genomic DNA recovered from the *F. proliferatum* was verified to be in tact (Fig 6a). Using forward (ITS-1) and reverse (ITS-4) primers, the ITS region of nuclear rDNA was amplified, with the predicted amplicon size being between 550 and 600 base pairs (Fig 6b). The PCR result was then bidirectionally sequenced.

Blast results of the ITS sequences of the fungal isolate that produced a PCR product (deposited in GenBank as accession number MK238307) confirmed that the causal agent of bulb rot disease on garlic in southern Karnataka region belongs to the *Fusarium proliferatum* (100% similarity and E-values of 0.0 for *F. proliferatum* of GenBank as accession number KY426416) which formed a homogenous subclade within *Fusarium proliferatum* group (GK03 MK238307) when subjected to Neighbour-joining tool using MEGA software 5.2 (Fig 7). Hence, isolate found in garlic bulb samples from Southern Karnataka is identified *as F. proliferatum* using both morphological traits and molecular studies of the ITS region of nuclear rDNA.

Previous phylogenetic analyses have demonstrated that *F. proliferatum* is a member of the homogeneous clade, which is quite similar in terms of morphology and biology and is capable of producing a wide variety of mycotoxins. High bootstrap values from a phylogenetic analysis based on the ITS sequences showed that different species were divided into distinct groups, demonstrating the utility of the ITS rDNA region as a molecular marker for *Fusarium* species identification as it is easy to distinguish between each isolate and hence verified that *F. proliferatum* is the causal agent of garlic bulb rot disease which corroborated earlier report [5-11], [36]. The current study describes the importance of morphological as well as molecular taxonomic studies for the successful, sensitive, and reliable detection, isolation, and identification of the fungus *F. proliferatum* that causes bulb rot in a variety of hosts including garlic [6], [37].

CONCLUSION

In the present study a detailed investigation on morphological and molecular study was carried out to confirm the causal organism of garlic bulb rot which is becoming a serious threat of economic loss to garlic growers in the southern Karnataka region where small-scale garlic farming is practiced. Our results can become a baseline in developing management strategies of the garlic bulb rot disease caused by *F. proliferatum.* To the best of our knowledge this is the first report of garlic bulb rot disease caused by *F. proliferatum* from the Southern Karnataka region, India.

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