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RESEARCH

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First report of wilt disease in cashew (*Anacardium occidentale* L.) caused by *Fusarium decemcellulare* in Kerala, India

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Abstract

Background The incidence of wilt disease of cashew (*Anacardium occidentale* L.) caused by *Fusarium* sp. has recently emerged in two-year-old cashew trees in an experimental field at the Regional Agricultural Research Station, Pilicode, in the Kasaragod district of Kerala, India. The disease was characterized initially by chlorosis of foliage and shoots, followed by necrosis and wilting, with internal symptoms including vascular necrosis, eventually resulting in complete plant mortality.

Methods A fungal pathogen was isolated repeatedly from the infected vascular tissues onto potato dextrose agar medium. Symptomology, cultural, morphological and molecular studies were performed to characterize the pathogen causing the disease.

Results In culture, the colony of fungus was white, later turning pink. The fungus produced both macro and micro conidia in culture. Molecular characterization revealed the identity of the isolates as *Fusarium decemcellulare* Brick. Pathogenicity of the isolate was confirmed in cashew seedlings, thereby demonstrating Koch's postulates.

Conclusion To our knowledge, this is the first record of wilt disease of cashew caused by *F. decemcellulare* in India. The culture of the fungus has been deposited with the National Fungal Culture Collection of India at Agharkar Research Institute, Pune, India with accession number NFCCI 4801, and sequence of the fungus was deposited at GenBank of NCBI with accession number OP942472.

Keywords *Fusarium decemcellulare*, Cashew, Kerala, Wilt

Background

Cashew (*Anacardium occidentale* L.) is cultivated in 32 countries worldwide and India is the third largest producer, second largest consumer and biggest processor of cashew with the largest area cultivated with the crop. India has an area of 1.89 million cultivated with cashew with an estimated annual production of approximately 0.74 million tonnes of raw nuts (FAO Stat 2018-19).Cashew was once planted as a soil binder along the Western Coast of India and later christened as a 'gold mine of wasteland,' owing to its nutritional and economic value (Singh 2018; Bhatt and Venattakumar, 2006). Indeed, cashew is a major foreign exchange earning crop in India, primarily due to its nutritional value. Approximately two million small and marginal

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farmers in India are dependent on cashew, employing approximately 0.1 million people in processing factories, of which 90 per cent are women (Directorate of Cashew Research (DCR), 2015).

However, cashew trees are challenged by several biotic and abiotic stresses resulting in yield loss (Vidya et al. 2015). Biotic stresses including pests and diseases affect both quality and quantity of cashew nuts. Worldwide, there are ten major diseases reported which affect the growth and productivity of cashew (Cardosa et al. 2013). Anthracnose (*Colletotrichum gloeosporioides* Penz. & Sacc.), powdery mildew (*Oidium anacardii* F. Noack), fruit rot (*C. gloeosporioides*), gummosis of the fruit and trunk (*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl) are the most common diseases causing considerable damage to the crop (Cardosa et al. 2004; Cysneet al. 2010; FAO 2004; Freire, et al. 2002; Gama et al. 2011; Hadidi et al. 2016; Moteriro et al. 2015; Moreira et al. 2013). In addition, some countries in Africa have reported other diseases, including powdery mildew, leaf and nut black rust, and bacterial leaf spot diseases (Majune et al. 2018).

Observations of cashew germplasm show that local, open-pollinated varieties are more tolerant to disease (Cardosa et al. 1999), but recently (personal observations, 2020) a wilt disease outbreak occurred in a two-year-old germplasm collection at the All India Co-ordinated Network Project (AICRP) on Cashew in Kerala, India. Wilt diseases of cashew have not previously been reported from any area in India. The outbreak is of concern due to the potential damage as emerging wilt disease could inflict on the cashew industry in India. Hence a detailed study was undertaken to isolate, characterize, and identify the pathogen and describe the symptomatology of the disease.

Methods

Symptoms and assessment of disease incidence in the field

The disease was observed in a two-year-old germplasm collection of the All India Co-ordinated Network Project (AICRP) on Cashew in an experimental field of the Regional Agricultural Research Station, Pilicode, Kerala, India (15 m above mean sea level (AMSL)). The infected plants of the local germplasm collection (locally known as *'Tattukunnu*) were collected from the Kudiyanmala area of Eruvassy, (FAOSTAT 2019) a village in the Kannur district of Kerala situated in the northern foot hills of the Western Ghats) presented wilting that progressed to the death of the whole seedling within four to five weeks. Symptoms were described from diseased trees in the field. The per cent disease incidence (PDI) in the cashew field was calculated by the formula:

$$\text{PDI} = (\text{number of infected plants/total number of plants observed}) \times 100$$

Isolation of the fungus

Three two-year old cashew plants showing the typical wilting symptoms were uprooted and used for isolation of the pathogen. Isolations were performed from the vascular tissues in the collar region of the plant following standard isolation and culture techniques. The samples were washed under running tap water. Tissues were sectioned into small pieces (approximately 1 mm × 1 mm) using a sterile scalpel. Sections of tissue were disinfected with sodium hypochlorite solution (1%) for 30–45 s. After three rinses, in sterilized distilled water, tissue sections were placed aseptically on potato dextrose agar (PDA) medium in sterile Petri dishes. All Petri dishes were incubated at room temperature (28 ± 2 °C) under ambient light conditions and observed daily for fungal growth. After three days, development of fungal colonies was observed. Periodic subculturing of the isolate was performed following hyphal tip method from the growing edges of the colony. The selected hyphal tips were transferred to PDA slants by using a cork borer and an inoculation needle. The fungal cultures were maintained at 4 °C for further studies. The colony characteristics of the isolate were studied on a PDA medium.

Morphological characterization by microscopy

Microscope slides of samples and slide cultures were prepared, mounted and observed under a microscope (Carl Zeiss Axiolab, Carl Zeiss, Jena, Germany) at 5×, 10×, 40× and 100×. The slide culture was prepared using a pair of forceps to aseptically place a sheet of filter paper in a Petri dish. A U-shaped glass rod was placed on the filter paper, and sterile water (4 ml) was added to moisten the filter paper. A microscopic slide was placed on the U-shaped glass rod. A sterile scalpel was used to remove a 5 mm square block of water agar which was transferred to the centre of the microscopic slide. Four sides of the agar square were inoculated with mycelial fragments or with a loop full of spore suspension of the fungus, and a cover slip was placed over the agar block. The slide culture was incubated at room temperature for 48 to 72 h under ambient conditions, as already described. After incubation, the cover slip was removed carefully from the agar block and placed over a new microscope slide with a drop of lactophenol cotton blue stain and the fungal growth observed under the microscope. Measurements and photomicrographs of hyphae, conidiophores and conidia were captured using a digital camera and ZEN software (Carl Zeiss).

DNA extraction

Genomic DNA was extracted using a NucleoSpin® Plant II Kit (Macherey Nagel, Duren, German).

Approximately100 mg of fungal mycelium was scraped out from a PDA plate by using a fine spatula and afterwards homogenized using a pestle and mortar in liquid nitrogen. The powdered tissues were transferred to a microcentrifuge tube. Lysis buffer (400 µlPL1) was added and the mixture vortexed for 1 min. RNase (10 µl of RNase A solution) was added and inverted to mix. The homogenate was incubated at 65 °C for 10 min. and the lysate was transferred to a Nucleospin filter and centrifuged at 11000 × for 2 min. The flow-through liquid was collected, and the filter was discarded. Buffer PC (450 µl) was added to the liquid and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for 1 min. and the flow-through liquid was discarded. Wash buffer PW1 (400 µl) was added to the column, which was centrifuged at 11000 × for 1 min. and flow through liquid was discarded. Wash buffer PW2 (700 µl) was added and the tube was centrifuged at 11000 × and the flow through discarded. Finally, 200 µl of PW2 was added and centrifuged at 11000 × for 2 min. The column was transferred to a new 1.7 ml tube and 50 µl of elution buffer PE was added and incubated at 65 °C for 5 min. The column was centrifuged at 11000 × for 1 min. to elute the DNA. The eluted DNA was stored at 4 °C. The quality of the DNA was checked byagarose gel electrophoresis. The sample was loaded on a 0.8% agarose gel prepared in 0.5X Tris-Borate-EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed in0.5X TBE buffer at 75 V. The gel was visualized in a UV transilluminator (GeNei, Bangalore, India) and the image was captured using a gel documentation system (Bio-Rad, Hercules, USA).

PCR analysis

The internal transcribed spacer (ITS) region of the rDNA partial gene was amplified using ITS4 and ITS5 primers (White et al. 1990). PCR amplification reactions were performed in a 20 µl reaction volume containing 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2 mM of each of the dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5 M Betaine, and 5 pM of forward and reverse primers. The PCR amplification was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Waltham, MA, USA). ThePCR thermal cycler was programmed as follows: initial denaturation was performed at 98 °C for 30 s followed by denaturation at 98 °C for 5 s, annealing at 60 °C for 10 s,extension at 72 °C for 15 s, and a final extension step at 72 °C for 60 s. The denaturation, annealing and extension steps were repeated 40 ×. The PCR products were checked for size and quality in a 1.2% agarose gel as described above. A 2-log DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as the molecular size standard.

Sequencing and sequence analysis

An ExoSAP-ITkit (GE Healthcare, Chicago, IL, USA) was used to clean up the PCR products prior to sequencing. The sequencing reaction was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Waltham, MA, USA), using a BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing PCR temperature profile consisted of a 1st cycle at 96 °C for 2 min followed by 30 cycles at 96 °C for 30 s, 50 °C for 40 s and 60 °C for 4 min for all the primers. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the sequences were performed using Geneious Pro v5.1 (Drummond et al. 2010).To determine the identity of the fungus, sequence analysis was conducted using the BLASTn programme of the National Centre for Biotechnology Information [NCBI, Bethesda, MD (http://ncbi.nlm.nhm.gov/blast)].

Phylogenetic analysis

A phylogenetic analysis was performed using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei 1993). A phylogenetic tree was constructed with 100 bootstrap replications. The tree with the highest log likelihood (− 1857.36) was selected. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 software (Tamura et al. 2021). This analysis involved 16 nucleotide sequences including the ITS sequence of the isolate and top 15 sequences producing significant alignments with thesequence of the isolate in a BLASTn analysis of NCBI.

Pathogenicity tests

Cashew seedlings were grown in sterilized soil in polybags (10 × 15 cm). The soil was collected from an experimental field of the Regional Agricultural Research Station, Pilicode, Kerala, Indiawhere cashew plants were growing. The collected soil was sterilized by autoclaving at 121 °C at 6895 Pa (approximately 15 psi) for10 min. The sterile soil was used to fill the polybags, and was

supplemented with nutrient fertilizers (organic manure and chemical fertilizers containing N, P and K) at the recommended rate (KAU, Crops 2016) and irrigated on an as-needed basis using a watering can. Cashew seeds were sown in the soil, from which polybags with healthy seedling were selected. The pathogen was cultured in potato dextrose broth (PDB) in a shaking incubator under ambient conditions as described previously. After 7 days of incubation, the mycelial mass was collected by filtering the PDB through a Whatman no. 1 filter paper. A conidial suspension of the fungus was obtained by pouring 100 ml of sterile distilled water over the separated mycelial mat and vortexing for 5 min. The spore count was adjusted to 10^6 spores mL^{-1} using a hemocytometer.A15 ml aliquot of the conidial suspension was challenge inoculated into the root zone of five seedlings at the 5–7 leaf stage by soil drenching. An additional three plants were treated with sterile water to serve as controls. The seedlings were maintained under a shade net in an isolated location and observations were taken daily to assess symptom development. As with the diseased plants in the field, symptoms of wilt were described under the shade net conditions.

Results

Symptoms and assessment of disease incidence in the field

In the field, symptoms appeared initially as chlorosis (Fig. 1a) followed by necrosis of foliage (Fig. 1b) with wilting and dieback of shoots (Fig. 1c) eventually resulting incomplete plant death (Fig. 1d). Infected plants showed internal symptoms of the disease including vascular necrosis (Fig. 1e). A sample size of 73 plants was observed for the incidence of wilt symptoms. Only three plants (4.11%) showed typical wilting symptoms (Fig. 1a–e).

Isolation and cultural characterization of the fungus

The colony of the isolate was white coloured, later turning pink. After sub-culturing, the mycelium of the colony

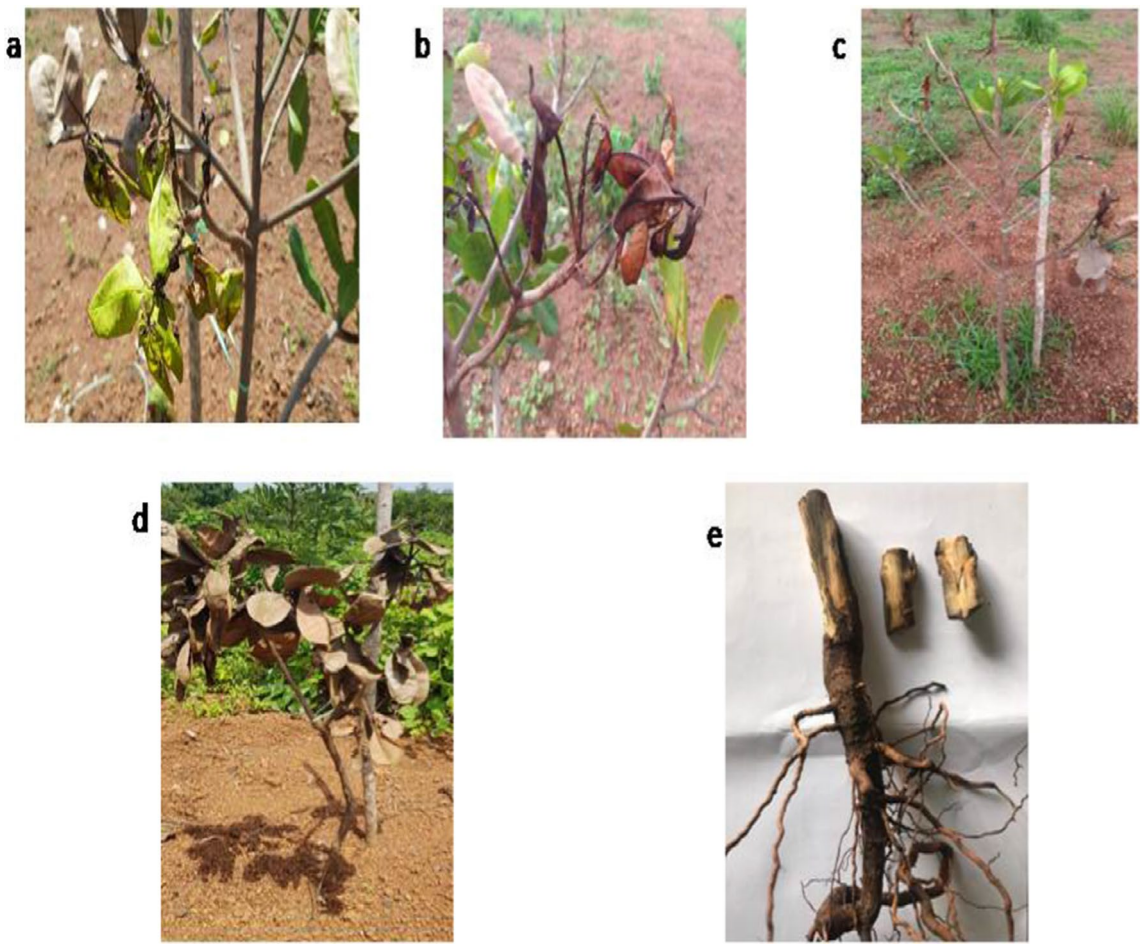

Fig. 1 Symptoms of the wilt disease of cashew caused by *Fusarium decemcellulare*. **a** Chlorosis and wilting of the foliage, **b** Necrosis and death of the foliage, **c** Dieback of shoots, **d** A completely wilted, moribund plant, and **e** Vascular discolouration due to the pathogen

was floccose and appeared white to peach in colour with small, dotted cushion structures, which were the sporodochia on the surface of the medium (Fig. 2a).

Morphological characterization by microscopy

Hyphae were branched, septate, smooth-walled, hyaline and 1.5–5.2 µm wide (standard deviation [SD] 1.260). The conidiophores were 65–70 × 1.18 to 2.2 µm (SDs 1.629, 0.392, respectively) and produced from the lateral side of the hyphae. Macroconidia were 2 to 6 septate, fusoid, straight to falcate, smooth walled with a dimension of 23.79–70 × 4.31–7.44 µm (SDs 14.464, 1.148, respectively) (Fig. 2b). Micro conidia were oval, hyaline, and smooth-walled and produced in chains each with a dimension of 4.78–7.77 × 2.88 to 2.95 µm (SDs 0.916, 0.025, respectively) (Fig. 2c). Hyphae, macroconidia and microconidia often occurred together (Fig. 2d). The measurements of the morphological structures are presented in Additional file 1: Table S1. Additional summary statistics are presented in Additional file 1: Table S2.

Molecular characterization

The BLASTn analysis of the isolate showed homology with *Fusarium decemcellulare* Brick with 100% sequence similarity. The culture of fungus has been deposited in the National Fungal Culture Collection of India at Agharkar Research Institute, Pune, India with accession number NFCCI 4801 and the ITS sequence of the fungus with a length of 511 bp was deposited at GenBank of NCBI with accession number OP942472. The sequence is also presented in Additional file 1: Fig. S1. Sequence analysis was confirmed against NCBI GenBank accession number KX78159.1. The five hits with the highest % identity from theBLASTn analysis are presented (Table 1). A phylogenetic analysis of the isolate OP942472*F. decemcellulare* and the top 15 sequence alignments from GenBank is presented in the phylogenetic tree (Fig. 3).

Pathogenicity test

The results of the pathogenicity test performed on healthy cashew seedlings (Fig. 4a) revealed that the isolate was pathogenic on the inoculated seedlings. The seedlings developed wilting symptoms 21–30 days after inoculation. Initially, the lower leaves showed wilting followed by symptom development in younger leaves. The vascular tissues developed dark discoloration, wilting extended to the petioles and shoots leading to stunting and death of the seedlings (Fig. 4b, c). The fungus was consistently re-isolated from infected plants onto PDA. In contrast, control plants did not develop any symptoms (Fig. 4d).

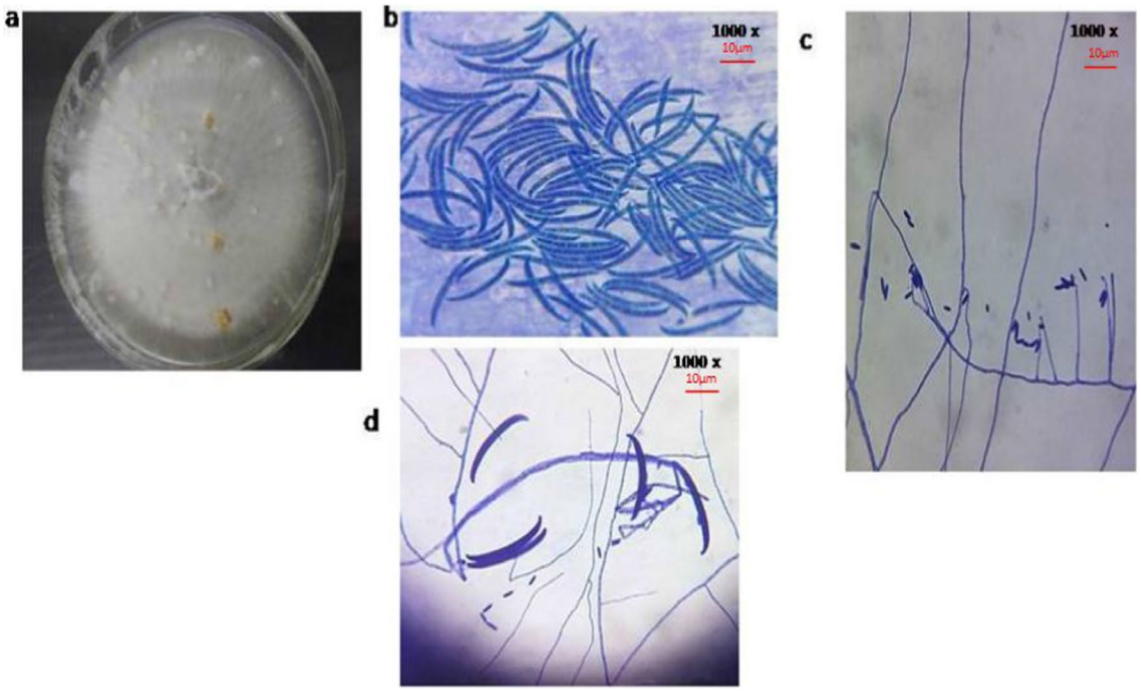

Fig.2 Cultural and morphological characteristics of the fungus causing wilt of cashew in India identified as *Fusarium decemcellulare*. **a** *F. decemcellulare* growing on potato dextrose agar, **b** Macro conidia, **c** Micro conidia, and **d** Hyphae, macroconidia and microconidia of the fungus

Table 1 The top five hits based on aBLASTn analysis of the fungal isolate from cashewidentified as *Fusarium decemcellulare* Brick. (Altschul et al. 1990)

Genbank accession no	Description	Max score	Query cover	Query coverage	E value	Identity (%)
KX78159.1	*Fusarium decemcellulare* isolate SZ060692	944	944	100%	0.0	100.00
ON931753.1	*Fusarium* sp. strain NRRL 22512	944	944	100%	0.0	100.00
ON931745.1	*Fusarium* sp. strain NRRL 53937	944	944	100%	0.0	100.00
ON931738.1	*Fusarium* sp. strain NRRL 53976	944	944	100%	0.0	100.00
ON003547.1	*Fusarium decemcellulare* culture NFCCI:5202	944	944	100%	0.0	100.00

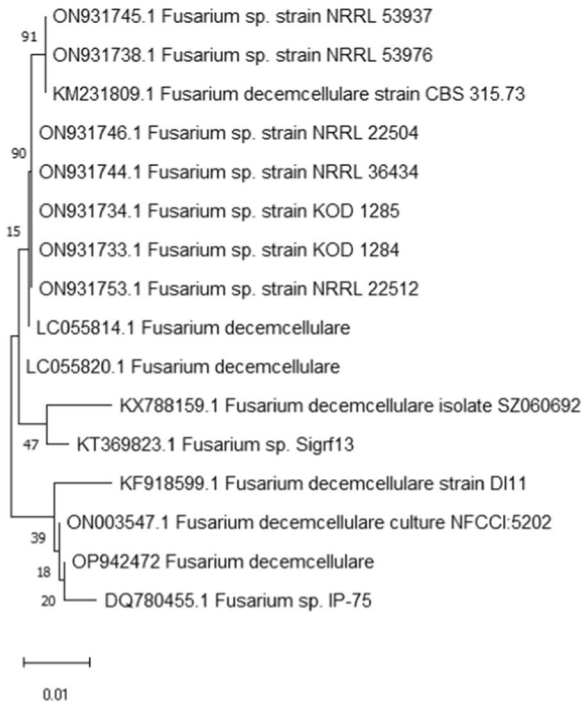

Fig. 3 Phylogenetic analysis of the isolate OP942472 of *F. decemcellulare* based on the maximum likelihood method using the internal transcribed spacer (ITS) region of the rDNA partial gene compared to the ITS sequence of other fungi (GenBank accession numbers are indicated). Numbers at the nodes represent the proportion of times that node branched the same way based on 100 bootstrap replications

Discussion

There have been several reports of *Fusarium* species causing various diseases of tree crops, including vascular diseases. Singh and Singh (1978) reported the occurrence of *F. decemcellulare* on living galls of *Zizyphus mauritiana* in India. Ploetz et al. (1996) reported *F. decemcellulare* as a pathogen of mango in the United States.The first report of dieback of mango in China caused by *F. decemcellulare* was more recent (Qi et al. 2013). The first report of *F. decemcellulare* causing green point gall of cocoa in Cuba was made by Vincent et al. (2012). In Brazil, Matos et al. (2016) reported inflorescence over sprouting and vascular and rachis necrosis in cashew caused by *F. decemcellulare*, and in the same year, Tibuhwa and Shomari (2016) reported that *F. oxysporum* caused massive wilting of cashew trees in Magawa, in the coastal region of Tanzania. Kathoon et al. (2017) reported *F. oxysporum* in cashew causing seedling blight and root rot disease in Odisha, India, and Lee et al. (2017) reported fruit rot disease in hongro apples caused by *F. decemcellulare* in Gyeongsangbuk-do Province in Korea (Additional file 2).

The cultural and morphological characteristics of the fungus we isolated were similar to those reported by others describing *F. decemcellulare* (Singh and Singh 1978; Ploetz et al. 1996; Vicente et al. 2012; Qi et al. 2013; Matos et al. 2016; Lee et al. 2017). The sequence analysis of the ITS region confirmed the causal agent as *F. decemcellulare*. The pathogenicity studies demonstrated Koch's postulates. A review of the literature reveals that wilt disease caused by *F. decemcellulare* in cashew has not previously been reported in Kerala, India. Thus, to the best of our knowledge, this is the first report of the pathogen causing wilting of cashew in Kerala, India.

Conclusion

The occurrence of a wilt disease of cashew caused by a fungal pathogen was recently observed in two-year-old cashew trees in Kerala, India. The cultural, morphological and molecular characterization resulted in the fungus being identified as *F. decemcellulare* Brick. Pathogenicity was demonstrated by fulfilling Koch's postulates. The inoculated seedlings showed typical symptoms of the wilt disease, and *F. decemcellulare* was consistently re-isolated from infected plants. This identification of *F. decemcellulare* as the causal agent of wilt of cashew in India will be a basis for further studies of the disease etiology, epidemiology and management.

Fig.4 Results of inoculating cashew seedlings with *Fusarium decemcellulare*. **a** Healthy cashew seedlings used for the pathogenicity test, **b** and **c** Challenge inoculated plants presenting typical symptoms of cashew wilt from severe wilting to death, and **d** A healthy control plant

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-023-00142-w.

Additional file 1: Figure S1. The 511 bp fragment ITS sequence of the fungus identified as *Fusarium decemcellulare*. **Table S1.** The measurements of the morphological structures used to characterizethe fungusby microscopy. **Table S2.** Summary statistics of the measurements of the morphological structures used to characterizethe fungus by microscopy.

Additional file 2. Phylogenetic tree of *Fusarium decemcellulare*.

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Author contributions

AVMM undertook the field investigation on disease incidence. PKL carried out isolation, characterization and pathogenicity studies. Amal Premachandran carried out symptomatology studies and MV maintained the sample collection, and captured the images. All the authors contributed for manuscript preparation and read the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
A culture of fungus has been deposited in the National Fungal Culture Collection of India at Agharkar Research Institute, Pune, India with accession number NFCCI 4801. Sequence analysis with NCBI accession No. KX788159.1 *Fusarium decemcellulare* isolate SZ060692 has resulted in 100% identity alignment statistics.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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