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Isolation and characterization of the causal organism of wilt in guava (*Psidium guajava* L.)

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Abstract
Guava (*Psidium guajava* L.) is an important fruit crop cultivated throughout India. Cultivation of guava is affected due to the outbreak of nematode fungal disease complex. *Fusarium solani* is a widely distributed soil inhibiting fungus that causes wilt disease in guava. In the present investigation, six isolates of wilt pathogen, *Fusarium* species were isolated from wilt infected guava roots from Karamadai, Coimbatore district. Pathogenicity studies conducted on guava seedlings (Var L- 49) revealed that, two out of the six isolates of *F. solani* were found to be pathogenic. Molecular characterization of two isolates (F1 and F3) were carried out by amplification of Internal Transcribed Spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4. The amplified sequences were submitted in NCBI (F1- MH590324 and F3-MH590695) and phylogenetic relationship of closely related organism of *Fusarium* spp. were carried out.

Keywords: Guava, wilt, *Fusarium solani*, DNA and PCR

Introduction
Guava (*Psidium guajava* L.) a species of the Myrtaceae family, also called as poor man's fruit or apple of tropics is cultivated in more than 60 countries [23]. It is widely grown in tropical and subtropical regions of the world and well adapted to different environmental conditions. The cultivated area of Guava in India is 261 MHa with an annual production of 3961MT and productivity of 13.9MT/Ha (NHB Data Base 2017-18). Similarly, the area and production of guava in Tamil Nadu is around 9.78 Mha and 77.41MT respectively. In Tamil Nadu, guava is largely grown in districts *viz.*, Dindigul, Theni, Thiruvallur, Madurai, Kanyakumari, Virudhunagar, Thiruvannamalai and Coimbatore.

Moreover, cultivation of Guava faces several agronomic and horticultural problems such as susceptibility to numerous pathogens, mainly guava wilt caused by *Fusarium oxysporium* f. sp. *psidii* [5] and other diseases. Among these, plant pathogen especially fungi play a crucial role**.** Totally 177 pathogens (167 fungi, 3 bacteria, 3 algae, 3 nematodes and one epiphyte) are reported from different plant parts of guava. Among these diseases, wilt is the most destructive disease in guava [17].

Several pathogens have been reported as the causal organism of wilt in guava but *Fusarium oxysporum* f. sp. *psidii* and *F. solani* were found to be the important pathogens associated with the wilt disease of guava in India as per [10, 15]. However, *F. chlamydosporium* was isolated from wilt affected guava roots [11].

First report of *Fusarium* wilt in India was by Das Gupta and Rai in 1947 from the orchards of Lucknow. Later in 1990s, this destructive wilt was reported in 11 districts of Uttar Pradesh [13]. Wilt is one of the major threats to guava cultivation and it was caused by many pathogens but the most predominant fungus is *Fusarium solani* [2, 18]. Reported the occurrence of *F. solani* infections in guava plants starting from one month old seedlings to four years old plants. [7] regarded guava wilt as a national problem, causing 30 per cent yield loss in India and disease occurred in nurseries and orchards. [10, 28] reported *Fusarium* incidence from Allahabad, Agra, Farukhabad, Lucknow, Punjab, Ranchi and Rewa in India and wilt incidence was recorded from 75% to 90%, while severity ranged between 30% and 55% on infected plants.

Fusarium is a cosmopolitan soil borne fungus that colonizes the vascular system of the host and thereby blocks the movement of water and nutrients to the upper part of the plant, which in turn causes yellowing, wilting and finally death of the host plant [15].

Guava wilt have also been reported from South Africa [26], Pakistan [2], Bangladesh [12] and Canberra, Australia [14, 16]. Noted the yellowing of the leaves with inter-venial chlorosis, general drooping of the leaves, complete wilting of plants with almost dried leaves and small dried black fruits hanging on the branch from the wilt infected guava tree.

Recently, nematode and fungal wilt disease complex in guava has become a serious problem in India, particularly in the states of Tamil Nadu, Andhra Pradesh, and Karnataka. [22] Observed decline in guava orchards of Ayakudi and surrounding places of Ayakudi Dindigul district, Tamil Nadu. Presence of *M. enterolobii* and *Fusarium* sp. was confirmed by these researchers where 3-4 year old trees showed sudden yellowing, wilting symptoms, early shedding of leaves, reduction in fruit size and complete death of trees, first report from the country.

Though different organisms were reported as the cause, the predominant fungal species isolated in all these cases were *Fusarium* sp. In this scenario, the current study focuses on isolation and characterization of *Fusarium* sp. found in Guava grown in Tamil Nadu, as proper identification of causal organism plays a vital role in efficient management of the disease-nematode complex.

Materials and Methods

Survey and collection of guava wilt pathogen

Epizootics of fungal wilt were found naturally in Karamadai, Coimbatore district of Tamil Nadu, India. A targeted survey was thus carried out for the occurrence of guava wilt in Karamadai. The fresh roots were collected from wilt infected guava trees of 2-5 years old (L-49) showing typical symptoms of wilt (Plate 1).

A

B

Plate 1: symptoms of fusarium wilt in guava: a) intial stage symptom and b) final stage symptom

Isolation of wilt pathogen

Collected root samples were washed under running tap water for 5 min and cut into < 2 cm small thin pieces. The bits were then washed with sterile distilled water for 5 min followed by surface sterilization with 0.1% $HgCl_2$ solution for 2 min. The surface sterilized root bits were plated on half strength Potato Dextrose Agar (PDA) medium in sterile Petri plates. Petri plates were incubated at 28±2 ºC for three to five days as suggested [4]. Pure culture was obtained from single hyphal tip technique. The isolates were maintained on PDA slants at 4ºC at the Department of Nematology and Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Morphology characterization of *Fusarium* spp.

Morphological characterization of the *Fusarium* isolates have been carried out by culturing them on PDA medium at 28 ± 2ºC for seven days. The morphological characters, mycelial colour, radial growth, pigment of mycelium and characters of macro and micro conidia and chlamydospores respectively were observed [1] using Labomed compound microscope at 400X.

Pathogenicity study

In order to prove the pathogenicity of *Fusarium* isolates through Koch's postulates by stem hole technique on two month old guava seedlings (var L- 49) a study was conducted at glasshouse Department of Nematology. Fully grown *Fusarium* mycelium on tooth pick was injected to collar regions and tender roots of guava seedlings. Percentages of wilt incidence and severity were recorded at one to two months after planting. Re-isolation was carried out from infected plants showing disease symptoms as per the methodology described [19].

Molecular characterization of *Fusarium* spp.

Genomic DNA was extracted from fresh mycelium of virulent *Fusarium* isolates using CTAB method described by [6]. Mycelium of *Fusarium* spp. were lysed with CTAB ((0.7 M NaCl, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol, 1% CTAB) buffer for 5 minutes. This was followed by centrifugation at 12,000 rpm for 15 min at 4º C. The supernatant was separated and added with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) followed by centrifugation at 12,000 rpm for 15 min at 4º C. Aqueous layer was transferred into new centrifuge tube. After that DNA was precipitated by adding 0.6 volume of ice cold isopropanol, mixed well and incubated at -20ºC overnight. After incubation, trace amount of isopropanol was removed by centrifugation at 12,000 rpm for 5 min at 4º C. After that, 2 volumes of ethanol was added and the pellet was recovered by centrifugation at 12,000 rpm for 15 min at 4º C and dried, dissolved in 50 µl of nuclease free water and used as a template for PCR amplification or stored at -20 ºC until use [6].

PCR amplification of ITS regions of *Fusarium* spp.

The Internal Transcribed Spacer (ITS) region of *Fusarium* spp. was amplified with primers ITS1 (5'TCCGTTGGTGAACCAGCGG-3') and ITS4 (5' - TCCTCCGCTTATTGATATGC-3') [30]. The reaction mixture was performed for 25 µl and PCR reaction mixture contained 1 µl total genomic DNA (50 ng), 0.5 µM primers (forward and reverse) , 0.5 mM of each dNTPs, 2.5 µl 1× PCR buffer, 2.5 mM MgCl2, and 1.25µl Taq DNA polymerase (Sigma, USA). The PCR cycles began with an initial denaturation at 5 min for 94°C, followed by 30 cycles of denaturation at 1 min for 94°C, primer annealing at55°C for 45sec and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were visualized in ethidium bromide stained 1.2 % agarose gels under a UV transilluminator (Alpha imager EC 1200) [25].

Results and Discussion

Survey

In the present study, six isolates of *Fusarium* spp. were isolated from wilted guava roots. Typical wilt symptoms *viz.*, stunting, yellowing of leaves, defoliation, reduced fruit size due to improper nutrient uptake and premature leaf and fruit shedding were regarded as identifiers for the sample collection (Plate 1). Similar symptomology of wilt affected plants were documented [16]. They noticed yellowing of the leaves with inter-venial chlorosis, general drooping of the leaves, complete wilting of plants with almost dried leaves and small dried black fruits hanging on the branch. [10] also reported stunted growth with rare flowering as well as wilting within very short period of time.

The findings of the present study agreed with [7]. [10] reported that *F. solani* and *Macrophomina phaseoli* either individually or in combination can cause wilt in guava. Guava wilt is also reported from various countries such as South Africa [26], Brazil (Junqueira *et al*., 2001), Pakistan [2] Bangladesh [12] and Canberra, Australia [14].

Morphological characterization of *Fusarium* spp.

A total of six isolates were isolated from the roots of wilted guava plants and were morphologically identified as *Fusarium* species (Plate 2). The mycelium of the *Fusarium* isolates were white in colour with a mat like appearance on PDA except the isolate F4 which was slightly pinkish. Macro and micro conidia were observed for all the 6 isolates. Microconidia were single, oval to reniform shaped without any septation (Plate 3 and Table 1) and size ranged from 6.50 - 14.50 x 2.50 - 3.50 µm. Macroconidia were falcate to almost straight, usually 3 septate, rarely 4-5 septate, thin walled, pointed at both ends and in few cases slightly curved. The dimensions of macroconidia ranged from 20.27 - 40.50 x 5.00 - 6.75 µm (Plate 3 and Table 1). All the isolates produced thick walled, both terminal and intercalary, globose, smooth and single celled chlamydospores (Plate 3). The isolates were identified as *F. solani* with the presence of long and unbranched monophialides, cream mycelia that may vary in pigmentation as well as using the size and shape of macro and microconidia. The radial growth in diameter varied from 5.8 to 7.0 cm at 7 days after inoculation at a temperature of 28 ± 2 ˚C. The fungal morphological characters were compared with the description given by [8] and morphologically confirmed as *F. solani*. The genus *Fusarium* contains a number of soil inhibits species with worldwide distribution and known to be important plant pathogen [20] reported that *F. solani* was the causal organism for guava wilt. *F. oxysporum* f. sp. *psidii* and *F. solani* are the important fungal pathogens to cause wilt disease by [10] and [21].

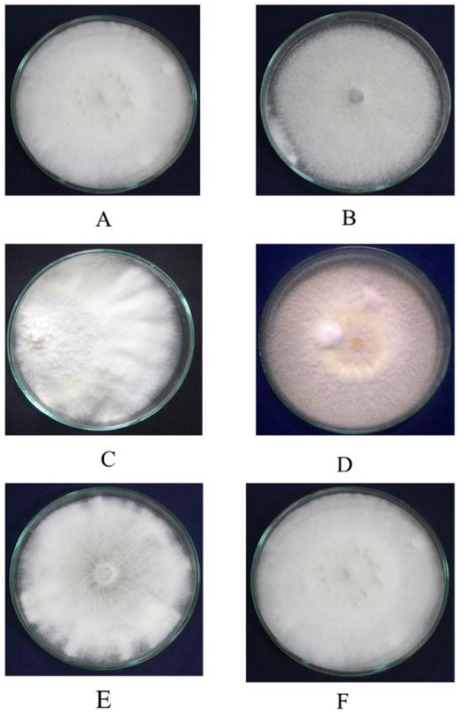

Plate 2: a-f different isolates of *f. solani.*

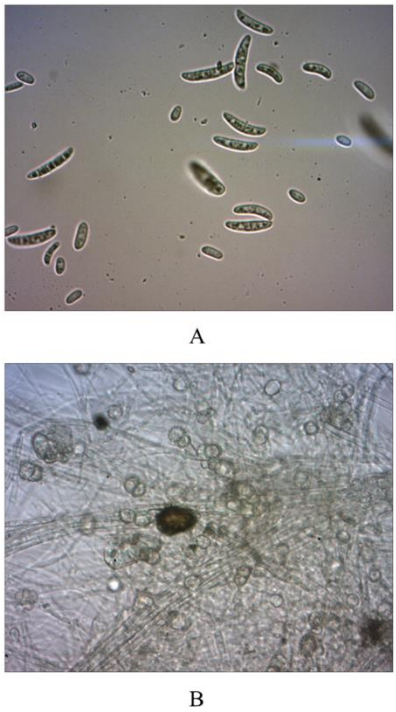

Plate 3: Conidia and chlamydospores of *f. solani*: a) macro and micro conidia and b) chlamydospores

Table 1: Macroconidia and Microconidia measurement and colony colour of 6 isolates of *Fusarium solani* isolates on PDA medium.

S. No	Isolates	Macroconidia (Length and breath µm) *	Microconidia (Length and breath µm) *	Chlamydospores	Colour	Colony diameter 7th day (cm)
1	F1	20.25 - 41.50 and 5.00 - 6.50 µm	6.50 - 14.00 and 2.50 3.50 µm	+	White	7.0
2	F2	21.50 - 40.00 and 4.50 - 6.75 µm	7.50 - 16.50 and 2.50 4.00 µm	+	White	6.0
3	F3	22.00 - 40.00 and 5.00 - 6.00 µm	7.00 - 15.00 and 2.50 - 4.50 µm	+	White	7.0
4	F4	20.27 - 40.50 and 5.00 - 6.75 µm	8.00 - 16.50 and 1.00 - 3.00 µm	+	Pink	6.50
5	F5	22.00 - 42.50 and 4.00 – 7.00 µm	6.50 - 17.00 and 3.50 - 4.50 µm	+	White	5.80
6	F6	20.50- 38.50 and 4.50 - 6.00 µm	6.00 - 15.50 and 2.50 - 4.00 µm	+	White	6.0

*Dimensions obtained the average values of ten reading

+ - Present

Pathogenicity

The pathogenicity of the isolates under study was proved in artificially inoculated plants through stem hole technique. This technique was proposed [16] for pathogenicity study in guava. The wilting symptoms were recorded at 45 days after inoculation. The establishment of infection varied within the tested isolates of *Fusarium*. Among the six isolates, two isolates (F1 and F3) were found to be highly pathogenic to guava variety L-49. The infected plants showed the characteristic wilting symptoms *viz.*, chlorosis, wilting of plant, yellowing and shedding of leaves. Similar pattern of wilt was documented [27, 16]. Reported that infected guava plants died 3-4 weeks after infection whereas few plants took 6-8 months for complete wilting. Some trees required 16 days for initiation of wilting in some cases 252 days required for wilting (M. However, in the present study, some isolates (F2, F4, F5 and F6) of *Fusarium* spp. artificially inoculated guava plants does not shown symptoms and they was characterized as not pathogenic. [9] Too observed difference in pathogenicity of *Fusarium* species. Among the 89 isolates of *Fusarium* sp., only *F. oxysporum* f. sp. *psidii* and *F. solani* were found pathogenic to guava. [16] Observed wilting in healthy guava plants inoculated with *F. oxysporum* f. sp. *psidii* and *F. solani.*

Molecular characterization of *Fusarium solani*

In order to confirmation of *Fusarium* spp. the isolates F1 and F3 (which were pathogenic to guava), were characterized with two nuclear markers through PCR. The two marker (ITS1 and ITS4) led to positive amplifications and sequencing for both isolates. The amplicon size was approximately 560 bp for *F. solani* ITS region (Plate 4). Amplified PCR products was purified and partially sequenced (Chromous Biotech PVT ltd. Bangalore, India). The sequences was deposited in BLAST and Gen accession numbers were obtained (F1 MH590324 and F3 MH590695. Both isolates possessed 100 per cent similarity of identity with the orthologous sequence of *F. solani* (accession number HQ 38439).

The total size of the ITS1 and ITS4 regions, 5.8S rDNA gene of the isolates varied from 380 to 620 bp [24]. According to the present study, distinguishing species of *Fusarium* based on morphological characteristics were not reliable. [10] Also made similar remarks suggesting molecular characterization for *F. solani* using PCR analysis for proper identification.

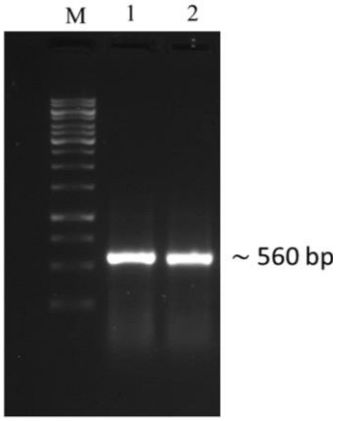

Plate 4: PCR amplifications of conserved regions of *f. solani* using ITSI and ITS4 promer. M) 1 kb ladder, lane 1&2) f. solani isolates

Phylogenetic tree

Phylogenetic reconstructions were performed with F1 and F3 isolates using the neighbor-joining method [3]. The present study, an approximately 560 bp DNA fragment of internal transcribed spacer of *F. solani* isolates were amplified and partially sequenced. Sequences were aligned with other *Fusarium* species obtained from GenBank database and phylogenetic tree was constructed. The Maximum Likelihood tree (Fig 1) was highly resolved and showed that the present study populations F1 (MH590324) and F3 (MH590695) of *F. solanii* we studied formed a clade together with sequences other *F. solani* (HQ384397) isolates retrieved from GenBank database. Separate clades were formed for other species of *Fusarium* with high bootstrap support [29].

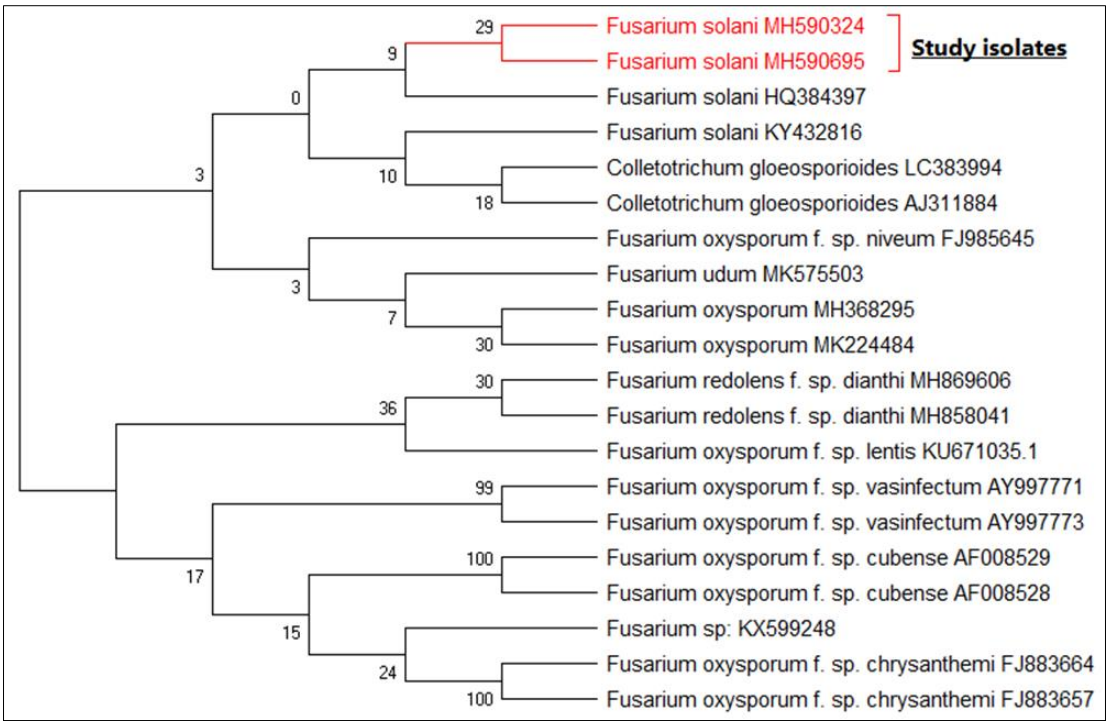

Fig 1: Maximum likelihood tree after an alignment of consensus sequences based on the ITS region of *Fusarium solani* identified in this study. Newly obtained sequences in this study are in labelled as study isolates. Analysis was done using 1000 bootstrap replicates. The bootstrap support value for each clade is indicated on the nodes

Conclusion

The survey of wilt infected guava orchards in Karamadai resulted in collection, isolation and characterization of *Fusarium solani* based on morphological and molecular techniques. Wilt itself being a dreadful disease of guava, association with nematodes results in high level of damage to the crop. In this scenario, proper identification of causal organism is mandatory for devising effective management strategies.

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