Original Research Article

Characterization of Slow wilt (*Fusarium oxysporum*) and **Basal wilt (***Sclerotium rolfsii*) **pathogens from different Pepper Ecosystems of Tamil Nadu**

Abstract

Black pepper (*Piper nigrum* L.) is grown extensively across many parts of Tamil Nadu. Pepper production is greatly affected by the fungal wilt complex diseases. In this study the prevalence of two wilt complex diseases*viz*., slow wilt and basal wilt in the major pepper growing regions of TamilNadu was studied and the pathogens responsible for slow wilt (*Fusarium oxysporum*) and basal wilt (*Sclerotium rolfsii*) were isolated and the morphological and molecular characterization of the pathogens were performed to confirm their identity. The phylogenetic tree dendrograms were generated for both *F. oxysporum* and *S. rolfsii* using the MEGA 11 software and their relative similarities with known sequences were compared to identify the genetic linkage. Detailed investigations are necessary to get more knowledge on the epidemiology and economic importance of slow wilt and basal wilt diseases in black pepper.

Key words: Pepper, Slow wilt, basal wilt, *Fusarium oxysporum*, *Sclerotium rolfsii*

1. Introduction

Black pepper (*Piper nigrum* L.), widely recognized as the “King of Spices” or black gold is a perennial climbing vine belonging to the family Piperaceae[1]. The presence of two alkaloids oleoresin and piperine contributes to the aroma and pungency of black pepper. In addition to cooking, black pepper is also used as therapeutic and preservative agent[2]. Worldwide, black pepper is grown in over 26 countries and nearly 3.2 lakhtonnes of black pepper is produced every year [3]. In India,pepper is cultivated in 2.78 lakh ha with a total production of 64000 tonnes per year. Among the states, Karnataka is the largest producer of pepper in India followed by Kerala and Tamil Nadu. In Tamil Nadu, the total area under black pepper cultivation is 6098 ha, with a total production of 2000 MT/year. India is exporting about 41,000 tonnes of black pepper earning a foreign exchange of ₹ 2.4 billion every year[4].

Themajor challenges to profitable black pepper cultivation in India are thefungal wilt complexdiseases. They include quick wilt incited by *Phytophthora capsici*, slow wiltcaused by*Fusariumoxysporum*and basal wiltdue to *Sclerotium rolfsii* (Teliomorph: *Athelia rolfsii*). These diseases contribute to nearly 30% production losses in pepper cultivation [5].

Slow wilt is a debilitating disease of black pepper which was firstreported by Van der Vecht in 1932 as "geelziekte" from Bangka, Indonesia. The affected plants, in general, will often survive for several years. The affected plants exhibit necrosis of roots, yellowing and flaccidity of lower leaves with upward progression, followed by defoliation, die-back and canopy reduction. The disease progresses gradually and the pepper vines deteriorate slowly over a period of 3-4 years before complete death of plants, giving the characteristic name slow wilt or slow decline.Scientific evidences show that the slow wilt infestation weakens the pepper plants bringing down the life span of infected plants from 20 years to 6-8 years and reducing the per vine berry yield from 3.0 kg to 1.5 kg [6].

Similarly, the basal wilt of pepper caused by*S. rolfsii* is highly prevalent in the nurseries and poorly maintained plantations. It wasfirst reported by S. Choudhury from Assam in 1943. The symptoms include chlorosis of leaves and development of grayishwhite mycelia and numerous mustard-like sclerotial bodies resulting in rotting and wilting of the pepper seedlings.

Several works have been confined to the pepper quick wilt disease, while information on slow wilt and basal wilt is limited. The yield losses in pepper is not the outcome quick wilt incidence alone; slow and basal wilt diseases also contribute significantly to yield reduction in Tamil Nadu and wherever pepper is cultivated. Hence, to ascertain the prevalence and severity of less studied wilt diseases, a survey was conducted in major pepper growing area of Tamil Nadu, India, causative fungal pathogens were isolated and characterized. This article reports the morphological variability and genetic relatedness of *F. oxysporum* and *S. rolfsii*in pepper ecosystems of Namakkal, Salem, Dindigul, Coimbatore and The Nilgiris districts of Tamil Nadu.

2. Materials and Methods

2.1. Survey

During 2023,a roving survey was conducted in randomly selected villagesof Kolli Hills (Namakkal district), Thadiyankudisai (Dindigul district), Yercaud (Salem district), Kallar and Valparai (Coimbatore district) and Gudalur (The Nilgiris district) of Tamil Nadu, India to identify the prevalence and incidence of slow decline and basal wilt diseases in pepper plantations(Table 1). As the wilt disease complex is systemic in nature, the severity of the wilt diseases was expressed in terms of Percent Disease Incidence (PDI), which was calculated using the following formula according to Wheeler [7].

$$\text{Percent Disease Incidence} = \frac{\text{Number of pepper vines infected}}{\text{Total number of pepper vines observed}} \times 100\%$$

2.1.1. Sample Collection

Plant tissues (infected plant parts) were collected from symptomatic vines from different pepper growing regions of Tamil Nadu. Incase of slow wilt (*F. oxysporum*) disease,the root tissues and collar region of the pepper vines presenting yellowing and wilting symptoms were collected. Similarly for basal wilt disease (*S. rolfsii*), infected stem and collar regionsshowing grayish white mycelial outgrowth were collected.

2.2. Isolation of the Pathogens

The infected plant samples were cut into small pieces of 3-5mm size using a sterile scalpel. Then the excised tissues were surface sterilized by dipping them in 1.0% sodium hypochlorite solution for 1- 2 min, followed by dipping in 70% ethanol for 30 s. The tissues were then washed with sterile distilled water for 2-3 times to remove the traces of surface sterilizing agents. After that, the tissues were kept on sterile blotting papers to remove the excess moisture. Finally the tissues were placed on sterile Petri plates containingpotato dextrose agar (PDA) media amended with streptomycin sulphate, an antibiotic compound capable of inhibiting bacterial contamination. The Petri plates were then incubated at 26±2°Cfor *F. oxysporum*isolation and 25±1°Cfor *S. rolfsii*isolation. The whitish mycelial growth appeared after 48 h on PDA plates.Actively growing mycelial discs were made using a sterile cork borer and sub-cultured on to PDA medium, as

described by Shahidan and Kwan[8]. Finally the pure cultures of *F. oxysporum*and *S. rolfsii* were obtained using the single hyphal tip method.

2.3. Morphological Characterization

The purifiedcultures were examined for the mycelial characters, colony morphology, production of resting structures (sclerotia)and pigmentation produced by the pathogens. The conidial morphology and conidiophore characteristics were examined under the compound microscope.

2.4. Molecular Characterization

The fungal genomic DNA was extracted using the CTAB method [9]. The fungal mycelia obtained from PDA medium were ground with CTAB extraction buffer and incubated at 65 °C for 30 min for digestion. After 30 min, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added to remove the protein fractions and other impurities. After centrifugation at 12,000 rpm for 10 min, the aqueous phase was carefully collected in to a new Eppendorf tube and then the DNA was precipitated by adding equal volume of ice-cold isopropanol [9]. The precipitated DNA was washed twice with70% ethanol, followed by air-drying. Finally the DNA pellet was dissolved in 1X Tris-HCL buffer (0.5M Tris-base, pH7.6)and stored at 4 °C for further applications.

2.4.1. DNA Amplification

The genomic DNA extracted from the pure cultures of the isolated fungal pathogens (*F. oxysporum*and *S. rolfsii*) wasused for molecular analyses. The internally transcribed spacer (ITS) region of ribosomal DNA were amplified using the fungi specific universal primers ITS-1 (5'-TCC GTAGGTGAACCTGCGG-3′) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3′)through polymerase chain reaction (PCR) in a thermocycler (QIAGEN QIAmplifier 96, New Delhi, India). All the PCR reactions were performed in 20 µLvolume containing 2 µL of 10X PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCI, 20 mM $MgCl_2$], 2 µL of dNTP mixture (2.5 mM each), 2 units of Taq DNA polymerase (5 U/µL), 10 pmol each of forward and reverse primers and 1µL (100 ng) of template DNA. The temperature profile used for the DNA amplification consists of initial denaturation at 95°C for 5 min, denaturation at 95 °C for 1 min, annealing at 58°C for 1 min, extension at 72 °C for 1 min and a final extension at 72°C for 10 min [10]. The PCR products along with 100 bp marker were resolved on 1% agarose gel by electrophoresis using 1X TBE (0.13 M Tris,

7.6, 45 mM Boric acid and 2.5 mM EDTA)as the electrode buffer. The fractionated DNA fragments were stained with Ethidium bromide and visualized using DNA gel documentation system (Gel UVITEC, UK).

2.4.2. DNA Sequencing and Analysis

The amplified DNA products of both *F. oxysporum*and *S. rolfsii*were sequencedwith the commercial sequencing services offered by Biokart India Pvt. Ltd, Bangalore, Indiaandthe sequence details were submitted in the National Centre for Biotechnology Information (NCBI) DNA database (GenBank®, National Library of Medicine, USA) under the accession Nos. OR360870 (*F. oxysporum*)and OR167040(*S. rolfsii*).TheDNA sequences were then aligned with known gene sequences in the GenBank® database using BLAST network services for comparison. The ClustalW tool was used to analyze the ITS sequence data and Phylogenetic trees were generated using the Neighborhood Joining (NJ) technique with a bootstrap value of 1000 replicated algorithmic searches using the MEGA 11 software[11].

3. Results and Discussion

3.1. Survey

The datacollected during the survey to assess the incidence of slow wilt (*F. oxysporum*) and basal wilt (*S. rolfsii*) in major pepper growing regions of Tamil Nadu indicated prevalence of slow wilt incidence from 10.12% to 42.83% while, basal wilt incidence ranged from 3.54% to 22.74% in different districts of Tamil Nadu (Table 1). The highest incidence of slow wilt was found in Thaiyankudisai (42.83%) followed by Kolli hills (40.00%) while, Kallar showed the highest incidence (22.74%) of basal wilt (Table 1). In general, higher wilt incidence was noticed during the monsoon period (data not shown).

Duarte *et al*.,[12] reported that the pathogen responsible for pepper wilting in Brazil was due to *Fusarium oxysporum*. Edward *et al*.,[13] isolated *F. oxysporum* from the infected pepper vines from Sarawak. In contrary, according to studies by Shahnazi*et al*., [10], *Fusarium solani*was identified as the causal agent of wilting on pepper vines in Malaysia.

3.2. PathogenIsolation

From the diseased pepper plants collected during the survey, five distinct isolates each of *F. oxysporum* and *S. rolfsii* were recovered on PDA medium, according to Dutta *et al*.[14]. The *F. oxysporum*isolates were designated as FMKH 1, FMKH 2, FMK 3, FMYC 4, FMTK 5 (Table 2) and*S. rolfsii*isolates were denoted as KSR 1, KHSR 2, KHSR 3, YCSR 4 and PRSR 5 (Table 3).

3.2.1.Morphological characterization

The morphological characters of each isolate of *F. oxysporum*were different from one another. Colourof the aerial mycelium varied from white to dull white while the pigmentation varied from yellowish orange to brown (Table 2; Fig 1). Similarly, the micro conidia, macro conidia and chlamydospore (vegetative spore) characteristics also differed between each isolate (Fig. 2A-C). The length of the micro conidia measured around 0.186 mm while, the macro conidia and chlamydospores measured 0.663 nm and 0.141 mm respectively. Two out of five isolates showed terminal chlamydospores while, other two isolates showed intercalary chlamydospores and one isolate (FMKR 3) did not put forth the vegetative spore (Table 2; Fig. 2E). Similar kind of results was obtained by Shahiden and Kwan [8], wherein 22 species of *Fusarium* isolates were isolated from pepper ecosystem and observed orange yellow to pink pigmentation and varying pattern of conidial morphology. Shango *et al*., [15] described different *F. oxysporum*isolates according to their mycelial and conidial characteristics.

In case of*Sclerotium rolfsii*, the mycelial characteristics varied from white to hyaline with cottony growth (Fig.1B)and took 6-8 d for the formation of golden brown to brownish black sclerotia(Table 2; Fig. 2F-G). The length of immature sclerotium measured around 0.048 mm (Fig.2G). Prajapati *et al*.[16] isolated *S. rolfsii* from chilli and observed similar kind of morphological characteristics.

3.3. Molecular Characterization

To avoid the misconceptions through morphological confirmation of the pathogens, molecular characterization is warranted. Hence, to check the genetic relatedness and identity of the fungal wilt complex pathogens, PCR analysis was performed with all the isolates of *F. oxysporum*and *S. rolfsii* using the universal primers ITS-1 and ITS-4, as described elsewhere. The products were fractionated on agarose gel through electrophoresis.

3.3.1.DNA Sequencing and Analysis

The fungus specific ITS primers, amplified a 550 bp DNA fragment both in*F. oxysporum* and *S. rolfsii* (Fig. 3&4). The amplified DNA fragments were purified and sequenced. The sequences obtained (query sequences) were analysed using the BLAST software and matched with NCBI GenBank® database for confirmation.The BLAST search showed nearly 97-98%homology with previously reported sequences of *F. oxysporum* and *S. rolfsii*, thus confirming its identity. The matching accession numbers are presented in Fig. 5. After the confirmation, the sequence details were deposited under the accession numbers OR360870 for *F. oxysporum* isolate FMKH 1 and OR167040 for *S. rolfsii*isolate KSR 1 respectively. Elanchezhiyan *et al*. [17] confirmed the identity of *Fusarium oxysporumf.sp. lycopersic*i by PCR amplification of 18S-28S rRNA region using fungal universal primers ITS 1 and ITS4.Similarly, Mahadevakumar*et al*., [11] employed the same molecular tools to confirm the identity of *S. rolfsii*isolated from *Cucurbita maxima* with online DNA database. Shahidan and Kwan [8] obtained a single amplicon of 550 bp size with the universal fungus specific primers and constructed a Neighbourhood phylogenetic tree with the related sequences of *F. oxysporum* causing slow decline in black pepper.

5.5.3. Phylogenetic Analysis

To further the knowledge on the genetic relatedness of the isolated pathogens, the isolated query DNA sequences were aligned with known sequences in the GenBank@ database by the ClustalW software and the phylogenetic dendrograms were generated using the MEGA 11 software (Fig. 5).The analysis showed that thesequences of *F. oxysporum* isolate FMKH 1 and *S. rolfsii* isolate KSR 1 shared 97-99% similarities with the accession numbers MW513788.1 and KJ546416.1 respectively in the NCBI GenBank@ database, both forming a single cluster (Fig. 5).

4. Conclusion

In Tamil Nadu, most of the published reports on the prevalence of wilt complex diseases in the black pepper vines are restricted to quick wilt caused by *P. capsici*. Our study reports for the first time, the severity of two less studied wilt diseases in different pepper growing areas of Tamil Nadu. Though considered minor when compared to quick wilt, the survey data shows that the slow wilt and basal wilt diseaseshave the potential to cause significant damageto pepper vines and reduce yield.

Hence, to mitigate thelosses from those diseases, a clear knowledge on the pathogens, their mode of spread and symptomatology are to be studied in details. The present study provides information regarding the prevalence of *Fusarium oxysporum* and *Sclerotium rolfsii* in Tamil Nadu, their abundance and distribution, the morphological and molecular traits.

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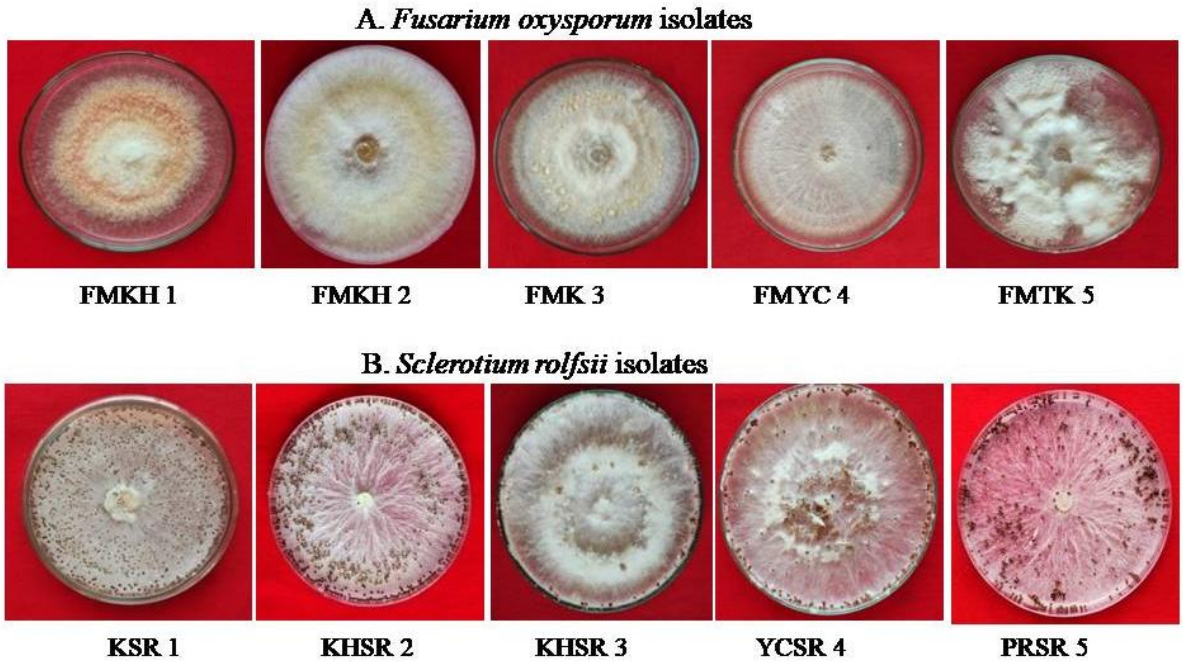

Fig. 1. Morphological characteristics of *F. oxysporum* (A) and *S. rolfsii* (B) isolates

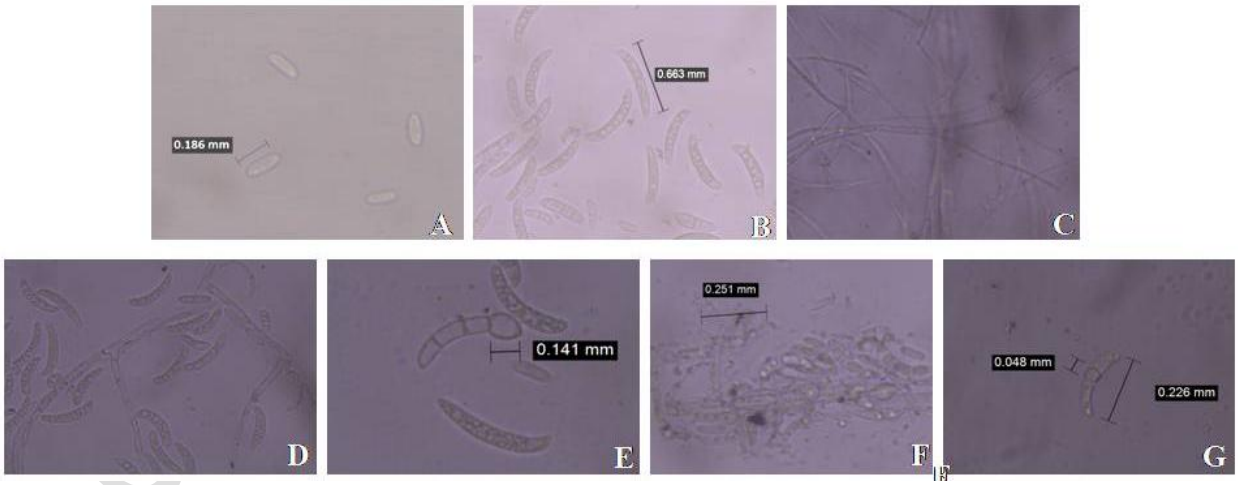

Fig. 2. Morphological characteristics slow wilt and basal wilt pathogens. A. Micro conidia, B. Macroconidia, C. Mycelium and D. Chlamydospores of *F. oxysporum*. E. Early sclerotium induction and F. Immature sclerotium of *Sclerotium rolfsii*.

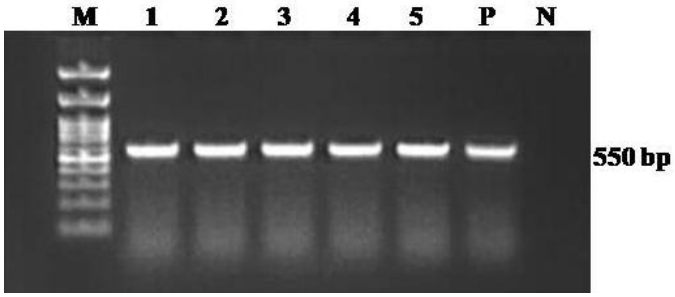

Fig. 3. PCR analysis of *Fusarium oxysporum* isolates. M. 100 bp marker, 1. FMKH 1; 2. FMKH 2; 3. FMKR 3; 4. FMYK 4; 5. FMTK 5; P. Positive control (*Fusarium oxysporum* isolate FGS1) and N. Negative control.

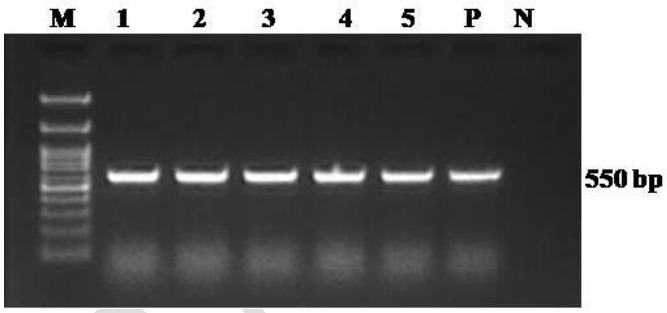

Fig. 4. PCR analysis of *Sclerotium rolfsii*isolates. M. 100 bp marker, 1. KSR 1; 2. KHSR 2; 3. KHSR 3; 4. YCSR 4; 5.PRSR 5; P. Positive control (*Atheliumrolfsii* isolate ARGS1) and N. Negative control.

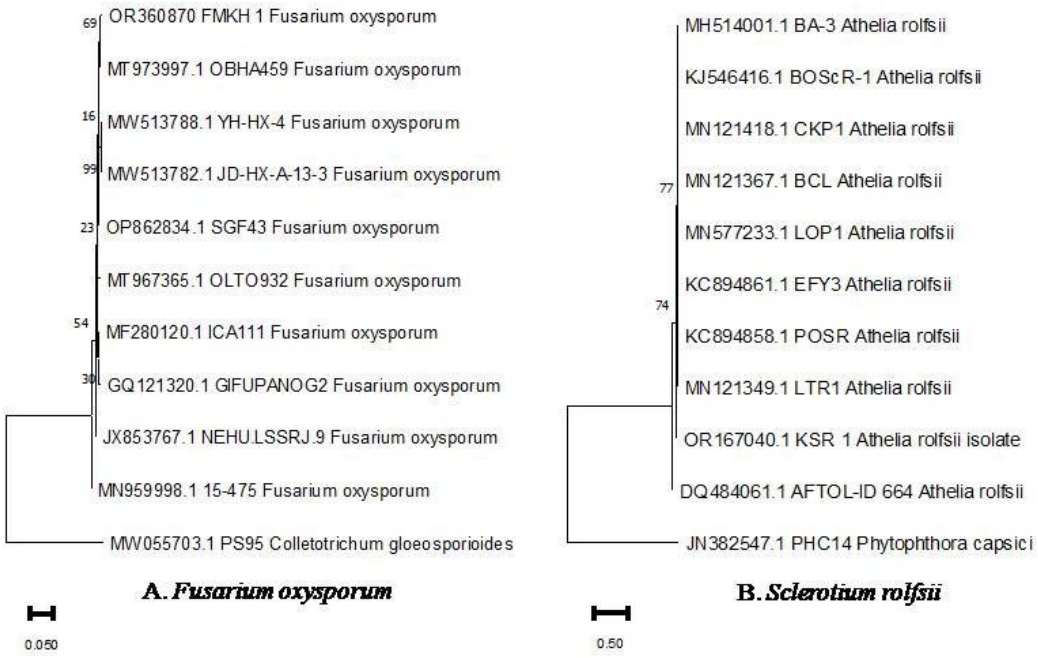

Fig. 5. Phylogenetic analysis of *F.oxyporum*isolate FMKH 1 and *S. rolfsii*isolate KSR 1.

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Table 1. Survey for slow wilt and basal wilt in major pepper growing districts of Tamil Nadu

S.No.	District	Location/ Villages	Geological Coordinates		PDI (%)	
			Latitude	Longitude	SW	BW
1.	Namakkal (Kolli Hills)	Karavalilkombai	11.30275584°	78.34885688°	40.00	9.86
2.		Valavanthinadu	11.28407583°	78.35097889°	38.59	16.85
3.	Coimbatore	Kallar	11.33605301°	76.80065064°	10.12	22.74
4.		Vaalparai	11.33624777°	76.86169548°	16.47	3.54
5.	The Nilgris	Naduhatty	11.422832°	76.833792°	13.43	4.67
6.	Dhindugal	Thaiyankudisai	11.786541°	76.328755°	42.83	15.88
7.	Salem (Yercaud)	Anthiyur	11.79417°	78.211476°	33.45	10.74
8.		Kombaikkadu	11.782627°	78.202198°	30.90	7.58

Table 2. Morphological Characteristics of *Fusarium oxyporum* isolates from Pepper ecosystem

Sl. No.	Isolates	Mycelial Pattern	Pigmentation	Microconidia	Macroconidia	Chlamydospores
1.	FMKH 1	Flaccose, thick mycelia	Orange	Abundant, Oval, aseptate	Falcate, pointed ends, 3-5 septate	Terminal
2.	FMKH 2	Soft Cottony	Yellow	Sparse, oval, septate	Falcate, pointed ends, 3-4 septate	Intercalary
3.	FMKR 3	Fluffy aggregates in centre with water droplets	Yellow	Sparse, ellipsoid, septate	Falcate to straight, blunt ends, 2-3 septate	Absent

4.	FMYK 4	Sparse thin concentric mycelia	Brown	Abundant, oval, aseptate	Falcate, pointed ends, 3-4 septate	Terminal
5.	FMTK 5	Scattered fluffy mycelia	No pigmentation	Abundant, ellipsoid, septate	Straight, blunt ends, 2-3 septate	Intercalary

Table 3. Sclerotial characters of *Sclerotium rolfsii* isolates from Pepper ecosysystem

Sl. No.	Isolates	Mycelial Characters	Sclerotial Characters			
			Colour	Size (mm)	Arrangement	Days for sclerotium formation
1.	KSR 1	Whitish cottony mycelia	Brown	0.8- 1.0	Scattered equally	6 d
2.	KHSR 2	Hyaline to white	Golden brown	1.2	Arranged regularly in outer circle	8 d
3.	KHSR 3	White with cottony aggregates at centre	Brown	1.2 -1.4	In the corner of the plates	8 d
4.	YCSR 4	Fluffy cottony white mycelia	Dark brown	1.0-1.2	In the centre and in the corner	7-8 d
5.	PRSR 5	Hyaline mycelia	Brownish black	1.4- 2.0	Scattered irregularly	7 d