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Virulence Assay and Morpho-molecular Characterization of *Sclerotinia sclerotiorum*-The Cabbage Head Rot Pathogen

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ABSTRACT

Background: The cultivation of cabbage is impacted heavily due to cabbage head rot causing plant pathogen, *Sclerotinia sclerotiorum*. The present study was conducted to know the prevalence of cabbage head rot pathogen in Tamil Nadu and to assess its virulence.

Methods: In this study, the survey was conducted on major cabbage-growing regions of Tamil Nadu. Pathogen associated with cabbage head rot was isolated and Koch's postulates were conducted. Further the isolated pathogen was characterized morphologically and molecularly through ITS and species-specific primers.

Results: Among the surveyed areas, Nanjanadu village in Nilgiris district showed the highest percent disease incidence of 45.23% compared to other regions. A total of ten *S. sclerotiorum* isolates (TNAU-SS-1, TNAU-SS-2, TNAU-SS-3, TNAU-SS-4, TNAU-SS-5, TNAU-SS-6, TNAU-SS-7, TNAU-SS-8, TNAU-SS-9, TNAU-SS-10) were obtained during isolation. Based on the virulence assay, TNAU-SS-5 (MZ379266) isolate showed a quicker lesion size of 3.77 cm^2 in comparison to the control. The molecular conformation of pathogen was carried out using ITS 1 and ITS 4 primers targeting 18S-28S rRNA gene fragments. Furthermore, the isolates were confirmed with specific primers and identified as *S. sclerotiorum*.

Key words: Cabbage, Head rot, Sclerotia, *Sclerotinia sclerotiorum,* Virulence assay.

INTRODUCTION

Cabbage (*Brassica oleracea var. capitata)* is among the world's prominent winter vegetable crops. It is grown extensively in the temperate and tropical regions of the world. It has received attention in recent times due to high content of minerals, vitamin C and sugar affirmed with a turnover of over 70 million metric tons. Despite serving as a rich source of food and revenue to the whole world, the cultivation of cabbage is severely hindered by the ascomycete's fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, causing an economically important plant disease called head rot on cabbage. It has an expansive host range that attacks over 400 varieties and causes huge financial losses globally per annum (Kabbage *et al*., 2013). Moreover, it is one of the most lethal and prevalent necrotrophic pathogens, with the ability to form sclerotia, which act as resting structure and help in survival for a longer time in agricultural soil.

Presently, disease control strategies include chemical control and cultivar resistance, but both of them lead to pathogen resistance development. The pathogen is highly challenging to manage because of its survivability in the resting structure called sclerotia which has a great significance in disease epidemiology (Gupta *et al.,* 2016). The germination of these sclerotia, which typically occurs at the soil surface, can result in the production of massive amounts of highly infectious spores, which disseminate the pathogen *via* air currents and cause infections on the leaves. Furthermore, the use of fungicides in the soil may disrupt microbial communities and suppress beneficial soil microflora. Because of the great significance in disease epidemiology, this current study was mainly focused to study the distribution of *S. sclerotiorum* causing cabbage head rot prevailing in different ecosystem of Tamil Nadu and to assess its virulence for the head rot infection.

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MATERIALS AND METHODS

Survey and collection of cabbage head rot pathogen in Tamil Nadu

The sclerotial bodies suspecting of *Sclerotinia sclerotiorum* were collected from the infected head portion of Cabbage plants from various cabbage-growing regions of Tamil Nadu, India *viz.* Thenkari, Madampatti, Sennanur, Kuppanur and Krishnapurampudhur in Coimbatore district, Mulligoor, Coonoor, Nanjanadu in Nilgiris district and Anniyalam, Denkanikottai in Krishnagiri district (Table 1). The pathogenic sclerotia and infected samples were carefully brought to the laboratory for further examination.

Isolation and identification of cabbage head pathogen

The symptom appeared as small, white spots on the stalk or at the base of the petioles. Later the surface lesions look greyish white and the stalk become completely girdled. The pith tissue inside the surface of infected stem gets destructed and black sclerotia were observed**.** Those sclerotia were collected and they were indeed surface sterilized for 3 minutes by immersing in a 10% chlorine disinfectant (0.6 per cent sodium hypochlorite), followed by three rinses with sterile distilled water, before culturing on potato dextrose agar (PDA). The sterile plates were incubated in the dark at 27°C for 3 to 4 days until the mycelium surrounded 70% of the plate. The absolute culture of the pathogen was acquired by the single hyphal tip method (Saharan and Mehta, 2008).

Cultural and morphological characterization of *S. sclerotiorum* isolates

A total of 10 *S. sclerotiorum* isolates collected during the survey were raised on a PDA medium. A 9 mm disc of the fungus was cut from a 7-day-old culture plate with a sterilized cork borer and placed in the center of each sterile Petri dish comprising 15 mL of sterilized and solidified PDA medium. The plates were incubated (20 ± 2°C) for 7 days. The cultural and morphological includes colony growth, margin, topography and zonation of *S. sclerotiorum* were studied.

Pathogenicity test

A 5 mm culture disc of *S. sclerotiorum* was placed on the detached pinpricked cabbage head and kept on a 150 mm diameter petri dish then covered with a bell jar. Pinpricked healthy cabbage heads without fungal disc acted as the control. Incubation was performed on both inoculated and uninoculated heads (20±2°C) for 7 days. The formation of a water-soaked lesion on a cabbage head was wisely examined and measured at frequent intervals.

***In vitro* disease conformation assay on detached leaves**

Fungal conformation tests on detached leaves (using 10 isolates of *S. sclerotiorum*) were carried out in accordance with a typical procedure with slight adjustment (Emani *et al.,* 2003). In a nutshell, healthy leaves from 5-6-week-old glasshouse grown-up plants were taken and placed on damp paper towels in a Petri dish. Alternatively, *S. sclerotiorum* was grown for one week on PDA and a 9 mm disc of agar plug containing the pathogen was removed using a cork borer. The leaves were wounded at multiple points (6-7 times) for the virulence pathogen assay and agar plugs containing *S. sclerotiorum* were positioned on the surface of the leaves. To test the effects of the needle's wounding, a mock leaf was also maintained. The Petri-dishes were preserved and stored in the dark at 25°C. The trypan blue staining and survivability of ten *S. sclerotiorum* isolates were conducted according to a standard protocol with minimal changes as explained by Kabbage *et al*. (2013). On the 5th day post-inoculation (dpi), the sclerotinia-infected leaves and non-infected cabbage leaves were stained with 0.05% Trypan blue for 30 min at 27°C before washing twice with 1x phosphate buffer saline. Plasmolysis was stimulated by submerging the cabbage leaves in 1M sucrose solution for 1 hour and the leaves were photographed. The percentage of lesion zone was estimated using the millimeter graph paper method.

Molecular characterization of *S. sclerotiorum* using ITS primers

The head rot fungus was cultured in Potato dextrose broth at 20±2°C for 7 days. The mycelial mat was obtained, lyophilized and dehydrated using liquid nitrogen. The genomic DNA was isolated *via* CTAB method. The PCR amplification of 18S rRNA was performed with ITS 1(5′-TCCGATGGTGAACCTGCGG-3′) and ITS 4 primers (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.,* 1990). The PCR cyclic conditions were as follows: an initial template DNA step at 95°C for 10 min followed by denaturation at 94°C for 30s then the annealing step at 60°C for 1 min. The extension step was performed at 72°C for 1 min and the final extension step at 72°C for 10 min, followed by 35 cycles

Table 1: Distribution of cabbage head rot disease in different ecosystems of Tamil Nadu.

Isolates	Name of the village	District	Latitude	Longitude	Percent disease incidence (PDI)*
TNAU-SS1	Thenkari	Coimbatore	10°57′39.4″N	76°50′23.7″E	32.26^{d} (34.59)
TNAU-SS2	Madampatti	Coimbatore	10°58′11.4″N	76°51′35.4″E	33.25^{d} (35.21)
TNAU-SS3	Mulligoor	Nilgiris	11°16′46.2″N	76°37′09.4″E	40.52^{b} (39.53)
TNAU-SS4	Coonoor	Nilgiris	11°24′41.0″N	76°42′40.2″E	42.13^{b} (40.47)
TNAU-SS5	Nanjanadu	Nilgiris	11°24′56.4″N	76°42′23.1″E	45.23^{a} (42.26)
TNAU-SS6	Sennanur	Coimbatore	10°57′02.0″N	76°50′44.1″E	36.75^{c} (37.31)
TNAU-SS7	Kuppanur	Coimbatore	10°56′57.1″N	76°51′45.0″E	28.25^{e} (32.09)
TNAU-SS8	Anniyalam	Krishnagiri	12°32′56.3″N	77°44′39.7″E.	31.54^{d} (34.16)
TNAU-SS9	Denkanikottai	Krishnagiri	12°31′38.6″N	77°47′19.5″E	25.33^{f} (30.20)
TNAU-SS10	Krishnapurampudhur	Coimbatore	10°57′36.1″N	76°50′38.2″E	23.24^{f} (28.81)

*Values are means of three replications.

Figures in the parentheses represent arcsine transformations.

Means in a column followed by same superscript letters are not significantly different according to the DMRT at P≤0.05.

and hold at 10°C. Water set as negative control instead of DNA. The PCR products were analyzed with 1% agarose gel electrophoresis recorded for amplification of estimated base pairs. For identification, the PCR products were eluted using the QIA quick gel extraction kit and sequenced using the primer ITS1 and ITS4. The partial sequence of the 5.8S gene and the flanking internal transcribed spacer (ITS4 and ITS1) of the isolated strains were submitted to GenBank and the accession numbers of sequences were obtained.

Molecular conformation of *S. sclerotiorum* using specific Primers

SSFWD (5′GCT GCT CTTCGGGGCCTT GTATGC 3′) and SSREV (5′TGACATGGACTCAATACC AAGCTG 3′) primer pairs were used, which had previously been developed for detecting *S. sclerotiorum* (Freeman *et al.,* 2002). The PCR reactions were carried out exactly as described in the ITS 1 and 4 primer pairs. The SSFWD/SSREV primers augmented a region within the ITS1/ITS4 amplified region. The PCR products were examined using 1% agarose gel electrophoresis and documented for amplification of approximate base pairs

Sequencing and phylogenetic analyses

The amplified PCR product was sequenced by Sanger's dideoxy sequencing method and the sequences were revised and aligned. The ten isolates were compared to other submitted isolates on the National Centre for Biotechnology Information (NCBI) site (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE TYPE=Blast Search) to study the variation. A phylogenetic tree was also built using the 5.8S rRNA gene sequences of ten *S. sclerotiorum* isolates. Mega 7.0 software was utilized to create a maximum neighbor-joining tree to study the phylogenetic relationship among the 10 isolates with 1000 boot-strap replication. The nucleotide sequences were aligned using Clustal W for multiple sequence alignment and a sequence identity matrix was created using bio Edit software (Version 7.0.4.1).

RESULTS AND DISCUSSION

Isolation of cabbage head rot pathogen.

A survey was conducted in June 2020, in a different ecosystem of Tamil Nadu. Among them, Nanjanadu village in Nilgiris district showed the highest percent disease incidence of 45.23% than other areas. A fungal pathogen was constantly isolated from the sclerotial bodies associated with head rot symptoms during the survey in Tamil Nadu, India (Table 1). The fungi possess hyaline, septate-branched cottony fluffy mycelium. A total of ten isolates of the fungus were isolated from various survey areas and labeled as TNAU-SS-1, TNAU-SS-2, TNAU-SS-3, TNAU-SS-4, TNAU-SS-5, TNAU-SS-6, TNAU-SS-7, TNAU-SS-8, TNAU-SS-9, TNAU-SS-10 respectively.

Morphological characterization of *S. sclerotiorum*

The *S. sclerotiorum* generally produces dense cottony mycelium on potato dextrose agar medium, the fungal mycelium covered the whole Petri plate in five days. From eight days after inoculation, immense black, dark, irregular sclerotial bodies emerged in a circular pattern along with the corners of the Petri plate. A microscopic examination of fungal mycelium confirmed that the hypha was hyaline, septate and branched. The aggregated nutrients in the sclerotia form a dew drop-like visual appearance in the Petri plates. Based on the morphological characteristics listed above, the fungus was identified as *S. sclerotiorum* (Fig 1).

Pathogenicity of *S. sclerotiorum*

Koch's postulates were confirmed to clearly recognize the pathogen associated with cabbage head rot by infecting the isolated fungus on wounded healthy cabbage. The results showed that the typical head rot symptom appeared on the inoculated cabbage head five days after inoculation. Fungus inoculated from cabbage displayed symptoms such as water-soaked lesions, resulting in the formation of white cottony fluffy mycelium and sclerotia over the rotted portion (Fig 2).

Fig 1: Different *S. sclerotiorum* isolates of the cabbage head rot from Tamil Nadu, India.

Fig 2: Pathogenicity stages of *S. sclerotiorum* isolates in Tamil Nadu.
A-F: Stages of infection in cabbage head; a- initiation of small lesions; c and d – entire head has been covered by mycelium; e and f – rotting of entire head and formation of sclerotial bodies.

Virulence assay with *S. sclerotinia* isolates of cabbage

The first sign of mycelia infection is the presence of water-soaked lesions at the infection site, which then expand to bleached fluffy patches of white mycelium. While evaluating the virulence assay among the ten isolates, we observed that all ten isolates could effectively infect the cabbage leaves and created evident symptoms. Following the detached leaf assay, the lesion area percentage was calculated using the "Millimeter graph paper method" and the inoculated leaves were stained with Trypan Blue to estimate the level of cell death at the inoculation spot in these tissues and it revealed that higher level of lesion size was formed by the TNAU-SS-5 isolate, with a lesion size of 3.77 cm^2 in comparison to the control which showed no differences (Table 2). This suggests that TNAU-SS-5 was the highly virulent isolate responsible for the cell death caused by *S. sclerotiorum*. It was noticed that the TNAU-SS-5 isolate had a high degree of less lesion area when compared to the control plants and other isolates. Furthermore, cell death occurs in all ten isolates, although with varying impacts. As evidenced by the fact that the TNAU-SS-5 with high virulence activity was chosen for further experimentation in cabbage based on its pathogenicity assessment in glasshouse plants.

Molecular characterization of *S. sclerotiorum* using ITS 1 and ITS 4 primers

The genomic DNA isolated from *S. sclerotiorum* isolates was subjected to a PCR amplification using primer sets corresponding to the 5.8S rRNA region. The DNA from all ten isolates was magnified using a PCR amplification size of approximately 580 bp. The rDNA homology sequence alignment BLAST indicated that the nucleotide sequence identity was more than 98 percent with the existing isolates of *S. sclerotiorum* available in the NCBI database. Nucleotide sequence from ITS region of all the *S. sclerotiorum* isolates were submitted to NCBI and designated their accession numbers (Table 2). A phylogenetic tree was also constructed using the Neighbour-joining method, which revealed the formation of a single cluster, indicating a close relationship between all ten Sclerotinia isolates.

Molecular characterization of *S. sclerotiorum* using specific primers

The identity of *S. sclerotiorum* was molecularly validated using currently accessible primers. The specific primers were constructed using alignment of rDNA ITS sequences (Freeman *et al.,* 2002). SSFWD and SSREV were the specific primers used in this study. The PCR product obtained from all the ten isolates was amplified with an amplicon size of approximately 280 base pairs as predicted.

Phylogenetic analysis of *S. sclerotiorum* isolates

The phylogenetic tree was constructed based on the nucleotide sequence of different *Sclerotinia* isolates using MEGA7.0 software. The percent identity ranged from 50 to 95 percent. The results revealed that the two clads were generated, in which all the 10 isolates including the virulent isolate TNAU-SS-5 (MZ379266.1) formed separate major clad 1 which phylogenetically varied from clad 2. Clad 1 includes five major sub-clad. Under major sub-clad 1, the 10 isolates, namely TNAU-SS-1, TNAU-SS-2, TNAU-SS-3, TNAU-SS-4, TNAU-SS-5, TNAU-SS-6, TNAU-SS-7, TNAU-SS-8, TNAU-SS-9 and TNAU-SS-10 were clustered together as many minor sub-clads.The sub cluster 1 was phylogenetically 51 per cent similar with the other Sclerotinia isolates available in NCBI. In major sub clad 2, isolate PMB SS8 was 69 per cent similar with the isolate MTR SS3. *Rhizoctonia solani* isolate OA3S1-51 (MG214604.1) and *Alternaria alternata* strain YJY-3 (OL958426.1) kept as outgroup. The phylogenetic analysis revealed that the virulent isolate TNAU -SS-5 was phylogenetically similar with all other isolates of *S. sclerotiorum* (Fig 3).

Table 2: Lesion size and accession number of different *S. sclerotiorum* isolates.

Isolates	Lesion size(cm)	Accession numbers
TNAU-SS1	1.00^{g} (0.99)	MW926795.1
TNAU-SS2	1.73^{d} (1.31)	MZ398265.1
TNAU-SS3	1.33^{e} ^{f}(1.15)	MZ379262.1
TNAU-SS4	1.47^{e} (1.21)	MZ379265.1
TNAU-SS5	3.77^{a} (1.94)	MZ379266.1
TNAU-SS6	2.47^{c} (1.57)	MZ401153.1
TNAU-SS7	1.27^{f} (1.12)	MZ379264.1
TNAU-SS8	2.23^{c} (1.52)	MZ382865.1
TNAU-SS9	3.23^{b} (1.79)	MZ382864.1
TNAU-SS10	1.40^{ef} (1.18)	ON025544.1

*Values are means of three replications.

Figures in the parentheses represent square root transformations. Means in a column followed by same superscript letters are not significantly different according to the DMRT at P≤0.05.

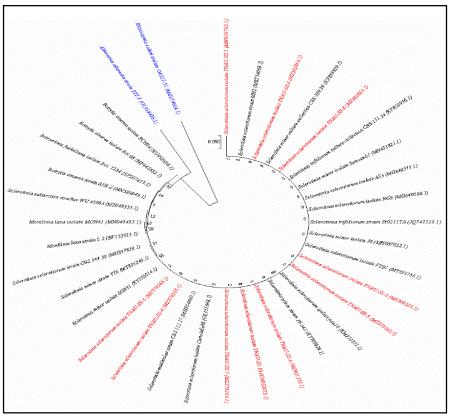

Fig 3: Phylogenetic analysis of *S. sclerotiorum*.

The current study primarily focused on isolation, morphological and molecular characterization of cabbage head rot pathogen in Tamil Nadu. The pathogen *S. sclerotiorum* was found in head rot infected samples of cabbage collected during the survey, produced cottony aerial mycelium that was hyaline and well developed. Sclerotia were produced at the colony's growing margins or center after 5 days of incubation, forming concentric rings which generally produced black, irregular sclerotial bodies. The fungus was identified as *S. sclerotiorum* (Lib.) de Bary and the characters were compared with the keys obtained by previous researchers (Goswami *et al.,* 2012). The fungal pathogen isolated from cabbage head rot infected cabbage heads was cultured on a PDA medium, a mycelial disc was inoculated and pathogenicity was confirmed on a pin-pricked head in the current study. Similarly, Krishnamoorthy *et al*. (2016) confirmed *S. sclerotiorum* pathogenicity on cabbage by inoculating a fungal disc on the cabbage head.

The detached leaf assay is one of the most frequently used methods for assessing disease symptoms. This assay has previously been used successfully to determine the antifungal effectiveness of diverse compounds against *S. sclerotiorum*. Similarly, we used this technique to score the disease symptoms of *S. sclerotiorum* in cabbage and found that the pathogen TNAU-SS-5 was highly virulent compared to other isolates against these foliar pathogens. The identification of the fungus *S. sclerotiorum* was further confirmed by molecular characterization using ITS and species specific-primers. The amplified products of ITS and species-specific primers were partially sequenced and identified as *S. sclerotiorum* by comparing sequences available in NCBI database. Similarly, Gupta *et al.*, (2016) and Kumar *et al.*, (2015) confirmed the fungus *S. sclerotiorum* by molecular characterization using ITS and species specific primers using ITS and specific primers.

In the light of phylogenetic investigation, the 10 isolates, namely TNAU-SS-1, TNAU-SS-2, TNAU-SS-3, TNAU-SS-4, TNAU-SS-5, TNAU-SS-6, TNAU-SS-7, TNAU-SS-8, TNAU-SS-9 and TNAU-SS-10 were clustered together as many minor sub-clads. The sub-cluster 1 was phylogenetically 51 per cent similar to the other Sclerotinia isolates available in NCBI. The phylogenetic analysis revealed that the virulent isolate TNAU –SS-5 was phylogenetically similar to all other isolates of *S. sclerotiorum*.

Disclosure of conflicting interests

The authors declare that there is no conflict of interest to disclose.

CONCLUSION

The results of this study showed that prevalence of different strains of *S. sclerotiorum* infecting the cabbage plant in Tamil Nadu. These findings were further confirmed using PCR with ITS and specific primers targeting 18S-28S rRNA gene fragment of *S. sclerotiorum.* Based upon this study the most virulent isolate TNAU-SS-5 has been identified and that has been further used for future experimental analysis.

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