Plant Archives Vol. 24, No. 2, 2024 pp. 1205-1208 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org

DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2024.v24.no.2.170

ISOLATION AND CHARACTERIZATION OF *SCLEROTINIA SCLEROTIORUM* ISOLATES INFECTING BRINJAL AND TOMATO

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(Date of Receiving-15-03-2024; Date of Acceptance-02-06-2024)

ABSTRACT

Sclerotinia sclerotiorum is among the world's most non-specific successful and omnivorous fungal plant pathogen responsible for causing wide range of diseases among different vegetable crops with a host range of more than 400 plant species. The growth of fungus is favoured by high humidity and cool weather conditions. In the present study, an attempt was made to isolate *Sclerotinia sclerotiorum* from infected brinjal and tomato plants in different regions of Uttar Pradesh, India. A total of seven isolates of *Sclerotinia sclerotiorum* were isolated and the isolates were characterized morphologically and molecularly by the amplification of the internal transcribed spacer region (ITS) using primers, followed by sequencing. All the seven isolates were confirmed the identity of fungal isolates as *Sclerotinia sclerotiorum*.

Key words : *Sclerotinia sclerotiorum*, Brinjal, Tomato, ITS, Sequencing.

Introduction

Brinjal (*Solanum melongena*) and Tomato (*Solanum lycopersicum*) are solanaceous crops belonging to the family Solanaceae or the nightshade family which have flowering plants that ranges from annual and perennial herbs to vines, lianas, epiphytes, shrubs, trees and includes a number of agricultural crops, medicinal plants, spices, weeds and ornamentals. The Solanaceae family are known for having a diverse range of alkaloids. Around the world, the production of vegetables is threatened by many biotic and abiotic stresses. Among the biotic stresses, insect pest and plant pathogens are known to pose their adverse effects on vegetable production. Solanaceous crops are attacked by number of fungal, bacterial, viral and phytoplasmal diseases like Alternaria leaf spot, Sclerotinia blight, Early blight, Late blight, Damping off *etc.* Among the various diseases the sclerotinia blight [*Sclerotinia sclerotiorum* (Lib.) De Bary] is an important disease which causes loss in quality and quantity of fruits of Solanaceous crops. *Sclerotinia sclerotiorum* is a ubiquitous plant pathogen belonging to the family *Sclerotiniaceae* and phylum *Ascomycota*. Plants susceptible to this pathogen encompasses 75 families, 278 genera and 408 species (Ziqin *et al.*, 2008). *Sclerotinia sclerotiorum* is a cosmopolitan and is one of the most devastating pathogen. A number of diseases are caused by this well known fungus and more than 60 names have been used to refer the diseases caused by this fungal (Le Tourneau, 1979; Purdy, 1979) pathogen including cottony rot, watery soft rot, stem rot, drop, crown rot, blossom blight and most commonly known white mold (Chet, Henis, 1975; Adams and Ayers, 1979; Willets and Bullock, 1992). The *Sclerotinia* infection associated with vegetables produces a white, cottony and dense mycelial mat on the surface of host and soil. Shiny water droplets were seen frequently in culture plates around the mycelia tufts later on which were converted into hard black coloured sclerotia (Boland and Hall, 1994). The disease is common in cool wet winter and spring seasons on dwarf and climbing beans, peanuts, occasionally on chilly plants and solanaceous crops. Apothecia are commonly associated with the occurrence

of diseased plants. A high level of variability among isolates of *Sclerotinia sclerotiorum* obtained by using Mycelia Compatibility Group (MCG) and RAPD markers (Junior *et al*., 2011). The genetic analysis of the isolates of *Sclerotinia sclerotiorum* have shown a medium variability in the 18s and 28s rDNA regions and thus were homologous (Kohli *et al.*, 1995).

Materials and Methods

Sample collection

Sclerotinia blight symptoms showing diseased samples of *Sclerotinia sclerotiorum* were collected from different division of Uttar Pradesh. Infected brinjal and tomato samples were collected in zip bags and transported to Plant Molecular Biology & Genetic Engineering Laboratory, A. N. D. University of Agriculture & Technology, Kumarganj, Ayodhya for further isolation of *Sclerotinia sclerotiorum*.

Isolation of *Sclerotinia sclerotiorum* and morphological characterization

Samples (stems, leaves and sclerotia) collected from the infected samples were used for isolation of the pathogen on PDA medium, a small portion of diseased tissue along with a portion of adjacent healthy tissue and sclerotia were cut into small pieces (3 to 5 mm in length) and then surface sterilized with 0.1% $HgCl_2$ for 30 seconds. The pieces were then rinsed thrice with sterilized distilled water. Sterilized and rinsed pieces were inoculated aseptically on sterilized Petriplates containing PDA medium. Potato Dextrose Agar (PDA) medium was prepared and sterilized using method described by Johanston and Booth (1983). The inoculated Petriplates were incubated at 25°C for five to six days. All the isolates were analyzed morphologically by the observation of radial growth, colony colour, sclerotia formation, number and size of sclerotia.

Isolation of DNA from the Isolates of *Sclerotinia sclerotiorum*

After successful isolation of *Sclerotinia sclerotiorum* from all the samples, total DNA extracted using protocol described by Sambrook *et al*. (1989). Bands corresponding to genomic DNA were observed by performing electrophoresis in 1% agarose gel.

Molecular characterization of Isolates by PCR amplification of ITS Region

The ITS region of the isolates was amplified with ITS based forward/reverse primers, ITS(F) 5'-TCCGTAGGTGAACCTGCGG-3', ITS(R) 5'-TCCTCCGCTTATTGATATGC -3'. Grabicoski *et al.* (2015) used genus specific forward/reverse primers ITS4SS and ITS5SS for the ITS region amplification of isolates. PCR was carried out in automated thermal cycler (Applied Biosystems) with 20 µL of total reaction mixture containing: 1 µL of genomic DNA (~50 ng), 10 pM of upstream (forward) primer, 10 pM of downstream (reverse) primer, 2 µL of 10× PCR buffer, 2 µL of 1.5 mM each dNTP mix and 1 unit of Taq DNA polymerase. Cycling conditions for PCR were: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. One cycle of final elongation was performed at 72°C for 7 min. The PCR product was analyzed using 1% agarose gel electrophoresis.

Purification of PCR amplified DNA product and DNA sequencing

All the seven characterized samples were selected for further identification by sequencing of the amplified ITS region. PCR amplified DNA products of all the samples were purified from gel using gel purification kit (DNA gel/PCR purification miniprep kit). Purified PCR product was then sent for sequencing to Biokart India Pvt. Ltd., Bangalore, India. BLAST was performed for sequence analysis using BLASTn suite with nucleotide collection (nr/nt) as database and for search Megablast program was used.

Phylogenetic analysis

The phylogenetic tree was constructed using maximum likelihood method based on the Tamura-Nei model. The analysis was performed with ITS region sequences of all the isolates.

Results and Discussion

Isolation of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum was successfully isolated from infected samples (stems, leaves) of brinjal and tomato plants and by using sterilized sclerotium. A total of five isolates were collected from brinjal and two from tomato. The identity of all the isolates was confirmed by cultural and morphological characters of the fungus. All the seven fungal isolates covered the entire petri plate surface of 85-90 mm diameter after 4 days of incubation. Isolates SS3 and SS4 were fast growing (radial growth of 90.00 mm in 96 hours) among the 7 isolates while isolate SS6 was slowest growing (radial growth of 86.33 mm in 96 hours). All the isolates had fluffy regular type of growth and had white colour of colony in SS6 and SS7 isolates, creamy white colour colony in SS1, SS3, SS4 and SS5 isolates and dull white colony inSS2 isolate. Sclerotia formation in the isolates were found black and dull black colour. The largest size of sclerotia (5.66 mm

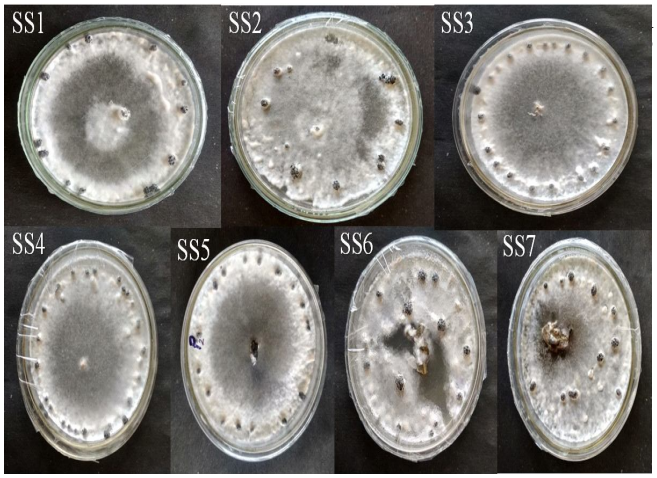

Plate 1 : PDA plates showing growth of different isolates of *Sclerotinia sclerotiorum* after 10 days of incubation at 25^{0}C.

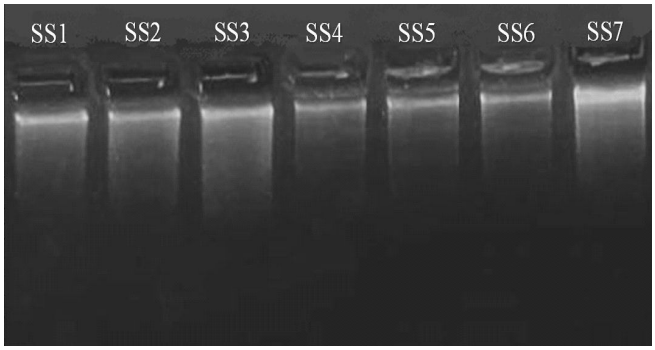

Plate 2 : Agarose gel showing DNA from the selected seven isolates of *Sclerotinia sclerotiorum*.

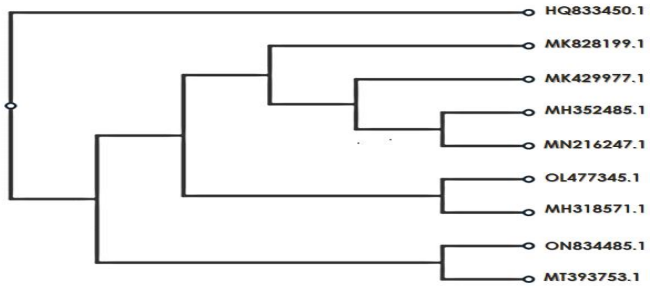

Fig. 1 : Phylogenetic tree showing evolutionary relationship of *Sclerotinia sclerotiorum* isolates based on Tamura-Nei model.

in width and 5.00 mm in length) were found in isolate SS6 while the smallest size of sclerotia (2.00 mm both in width and length)and highest number of sclerotia (22) were found in isolate SS4 whereas minimum number of sclerotia (10) were formed in SS2 isolate (Plate 1).

Molecular characterization of Isolates

Total DNA was extracted from all the seven isolates of *Sclerotinia sclerotiorum*. Sharp bands corresponding to the DNA of all seven isolates were observed on agarose gel (Plate 2). Isolated genomic DNA was used in PCR reactions with ITS specific primers for the *Sclerotinia sclerotiorum* was obtained successfully in the PCR reaction with all the isolates which confirmed the identity of all the isolates as *Sclerotinia sclerotiorum*.

DNA Sequencing and data analysis

Purified PCR product was sequenced and a sequence of 544 bp, 541 bp, 541 bp, 541 bp, 484 bp, 557 bp and 545 bp corresponding to SS1, SS2, SS3, SS4, SS5, SS6 and SS7 isolates were respectively obtained. The sequence was then analyzed using BLASTn tool, which revealed that ITS region of isolates SS1, SS2, SS3, SS4, SS5, SS6, and SS7 were 99-100% identical to the ITS region of *Sclerotinia sclerotiorum* (Accession No. MH318571.1, HQ833450.1, MN216247.1, MN216247.1, MT393753.1, MH352485.1 and ON834485.1, respectively).

Phylogenetic analysis

The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model. Evolutionary analysis were conducted in Mega 6. Four clads were formed in the phylogenetic analysis (Fig. 1), which were showing similarity with each other.

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