Isolation, Purification, Identification and Pathogenicity of *Sclerotium rolfsii* (Sacc.) Caused by Collar Rot of Chickpea

ABSTRACT

Sclerotium rolfsii (Sacc.) is a detrimental soil-borne fungus responsible for collar rot in chickpeas. We conducted an experiment including the isolation, purification, identification, and pathogenicity assessment of fungi from infected plant materials. The plants exhibit classic signs such as complete leaf abscission, browning, desiccation, and often remain attached to the dead stem. The mycelium of the pathogen proliferates over the affected tissue and adjacent soil, creating a white mat of mycelial threads interspersed with characteristic sclerotia, ranging from brown to dark brown and resembling mustard seeds in size. Collar rot samples were collected and sent to the laboratory for isolation. The fungus was isolated and cultivated on Potato Dextrose Agar (PDA) media. The pathogenicity tests were validated by adhering to Koch's postulates and used three injection techniques. The seed and soil inoculation approach demonstrated significant efficacy, resulting in the greatest disease incidence (71.05%) and reduced seed germination (74.90%).

Keywords: *S. rolfsii;* isolation; identification; pathogenicity; collar rot; chickpea.

1. INTRODUCTION

Chickpea is the predominant pulse crop, widely farmed in India, constituting around 75 per cent of total pulse production [1].This crop is a vital component of the farmers' cropping systems nationwide, since it integrates well into crop rotation and mixed cropping practices. It has multifunctional applications and the capacity to thrive in low-fertility environments and diverse soil and climatic conditions. There are two primary varieties: desi, characterized by tiny, brown seeds, which occupies 90 per cent of the whole area, and kabuli, with larger, creamy seeds, which is cultivated in around 10 per cent of the total area under the crop. It is a substantial source of protein (20 to 25%) and enhances soil fertility via biological nitrogen fixation [2].

Worldwide, 172 different diseases (including 67 fungus, 22 viruses, 3 bacteria, 80 nematodes, and mycoplasma) target chickpea crops. [3]. Out of all of them, just few can really damage crops. Dry root rot (*Rhizoctonia bataticola*), wilt (*Fusarium oxysporum* f. sp. *ciceri*), collar rot (*Sclerotium rolfsii*), Ascochyta blight (*Ascochyta rabiei*), and wet root rot (*Rhizoctonia solani*) are among the most serious diseases.

The damaged portion of the potato plant and tuber had features with extensive mycelial development that was white in color, as shown by the fungus *Sclerotium rolfsii*. On the mycelial mat, there were several sclerotial initials. Sclerotia, which were white at first, eventually became a chocolate brown color. On the outside of the afflicted area, sclerotia that ranged in size from half a millimeter to two and a half millimeters were present. Sclerotia that have not germinated become pliable and readily broken. Cortical cell maceration was prevalent [4].Generally, the disease is severe in loamy soil regions and more common in paddy-chickpea or soybean-chickpea based cropping system, when the soil is quite damp and the weather is very humid while the seeds are first sprouting. Some seedlings become sick and eventually die. In most cases, the seedling will lay flat on its back and exhibit signs of decay around the collar area and below [5]. All of the leaves drop off, become brown and dry, and often cling to the dead stem when the illness has progressed far enough. Over infected tissue and soil, the pathogen's mycelial threads multiply to create a white mat adorned with the characteristic brown to dark brown sclerotia, which are the size of mustard seeds [6].

Significant losses in production of up to 45 per cent are produced by collar rot in chickpea agriculture, which is caused by *S. rolfsii* [7]. Among soil-borne diseases, *Sclerotium rolfsii*-caused collar rot is now among the most destructive. Chickpea seedling mortality rates range from 55 to 95 per cent due to Sclerotium rolfsii. The severity of the illness determines the annual yield loss, which may range from 10 to 30 per cent [8].

2. MATERIAL AND METHODS

2.1. Collection, isolation and purification of fungus

Collar rot affected plants of chickpea were collected from surveyed areas of Rajasthan *viz*., Jaipur, Kota, Bundi, Sawaimadhopur, Tonk and Alwar districts. In order to isolate the causative fungus, samples of diseased plants were used. During the field survey, samples of chickpeas infected with collar rot were isolated. To remove dirt, the afflicted sections were cleaned with tap water. A 1% sodium hypochlorite solution was used to sterilize the surface for 1 minute after small portions of about 0.5 cm in length were removed from the stem and roots. This was followed by three washes with distilled water that had been sterilized. After sterilizing the surface, the pieces were placed aseptically on PDA slants in a Laminar Air Flow Cabinet. They were then placed in a B.O.D. incubator and kept at a temperature of 25±1°C for 7 days. The *Sclerotium rolfsii* pure culture was prepared using the single hyphal tip isolation method (Rangaswami and Mahadevan, [9]). By turning the Petri plates clockwise and anticlockwise, a suspension containing 5-6 pieces of hypha per 40X microscopic field was equally distributed over 2% plain agar in one milliliter of suspension. Petri plates were placed in a B.O.D. incubator and incubated at 25±1°C for 24 hours after the surplus suspension was decanted. Under a low power microscope objective (40X), a single piece of hypha was marked out and then sliced with the aid of a dummy objective. Using an inoculating needle, individual pieces of hypha were carefully placed on PDA slants in an aseptic manner. The slants that were inoculated were maintained in a B.O.D. incubator at a temperature of 25±1°C. The physical and colony characteristics of 10 samples led to the preliminary

identification of the purified cultures as Sclerotium rolfsii. In order to create an isolate from each tehsil, the field with the greatest illness incidence was chosen. Ten separate samples were collected from different tehsils in six different districts and assigned the following codes: CSR1, CSR2, CSR3, CSR4, CSR5, CSR6, CSR7, CSR8, CSR9, and CSR10. In order to keep the pure cultures alive for future experiments, they were transferred to PDA slants at regular intervals.

2.2. Identification of the fungus

Colonies of cottony white color are produced by the pathogen *Sclerotium rolfsii* on PDA. After 7–14 days of incubation, the colonies showed signs of mycelial development ranging from a dull white to a pure white color and the beginning of sclerotia production. The sclerotia looked like mustard seeds and were brown in color. The pathogen was determined to be *S. rolfsii* based on these characteristics.

2.3. Multiplication of inoculum

Inoculum (mycelia of *Sclerotium rolfsii*) was multiplied in wheat grains for inoculations and other experiments in the current investigation. Overnight, the wheat grains were immersed in regular water. All the surplus water was drained out. Each 250 ml conical flask was filled with 30 ml of water and 150 kg of soaked wheat grains. The flasks were then sealed with cotton and sterilized in an autoclave set at 1.1 kg/cm² pressure for 30 minutes. Aseptically, the pathogen/isolates' mycelial discs (5 mm) that were seven days old were injected into the flask substrate. The substrate was then incubated at 25 ± 1°C for 20 days.

2.4. Pathogenicity test

In order to demonstrate its pathogenicity, the method of seed, soil, and soil cum soil inoculation was used to multiply isolated and purified *Sclerotium rolfsii* on sterilized wheat grains. The field soil was subjected to autoclaving for 30 minutes on three separate days at a pressure of 1.1 kg/cm². To the clay pots (30 cm diameter) with 25 g of inoculums each pot, the grains of wheat were multiplied. Before adding the soil inoculum combination, the pots were treated with a 2% formalin solution to make them surface sterilized. Each pot included three replicates, and fifteen seemingly healthy chickpea seeds of a susceptible variety (L550) were surface sterilized with a 1% sodium hypochlorite solution. The control group received the pots that did not contain inoculums. Disease symptoms were noted for a period of up to 25 days. According to (Radha Krishanan and Sen [10]), the pathogen was re-isolated and compared with the original culture after plants exhibiting signs of collar rot were harvested.

3. RESULT AND DISCUSSION

3.1. Collection, isolation, purification

Collar rot hotspots including Jaipur, Tonk, Kota, Bundi, Swaimadhopur, and Alwar were all included in the study. Infected chickpea plants were retrieved from each of these districts. After meticulously placing infected plant samples in paper bags and accurately labeling them, they were sent to the laboratory. Sampling for diversity among *S. rolfsii*, the causative agent of collar rot, was the primary objective. Collar

rot affected chickpea plants were identified in the field based on key symptoms like presence of whitish mycelium on soil surface near stem with young blackish sclerotial bodies at collar region and drying of leaves and wilting whole plant. Subsequently, the fungus was extracted from chickpea root tissues; it spreads white mycelium development on the collar area, produces fungal sclerotia, and displays the telltale signs of collar rot (Fig. 1). Following a one-minute surface sterilization with 1% sodium hypochlorite, the samples were chopped into tiny pieces and washed three times with distilled water that had been sterilized. The fragments were set on sterile Potato Dextrose Agar (PDA) in petri plates and left to incubate at 25±1°C for duration of seven days. Afterwards, the hyphal tip cut procedure was used to purify the fungus (Fig. 2).

Our results are parallel to the findings of Punja and Rahe [11] who isolated fungus by standard tissue isolation method and identified as *S. rolfsii* based on mycelial and sclerotial characteristics. They observed characters like small tan to dark brown spherical mustard like sclerotia and Kokub*et al*. [12] also reported similar characters. Awasthi *et al*. [13]; Kamthe*et al*. [14] further verified Koch's postulate and cultured fungus on Potato Dextrose Agar (PDA) media. In addition to keeping track of morphological and cultural details, we measured things like dry mycelial weight, mycelial diameter, the quantity of sclerotia generated, the pattern they took on, and when they first appeared. All over the Petri dishes, tiny sclerotia with a reddish brown color were discovered.

3.2. Identification

The isolated fungus was primarily identified by its observable physical and cultural traits. Colonies of cottony white color are produced by the pathogen *Sclerotium rolfsii* on PDA. Colonies began to take shape after 7–14 days of incubation and displayed characteristics such as heavily branched, fluffy, fan-shaped mycelial development that covered the whole Petri dish. The color of the colonies ranged from dull white to pure white. A sclerotia looks like a mustard seed and is black or dark brown in color. The pathogen was determined to be *S. rolfsii* based on these characteristics.

White mycelium strands of *S. rolfsii* on root system

Collar rot symptoms on aerial parts of plant in field

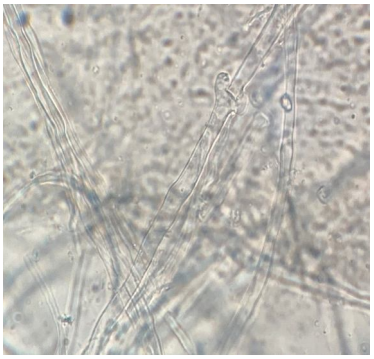
Fig.1. Typical symptoms of collar rot of chickpea

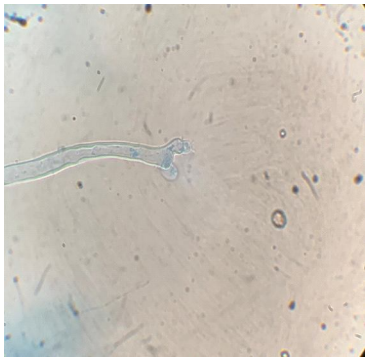
Diseased root

Pure culture

Mature sclerotia

Pure culture slants





Mycelia at 40x

Fig.2. Characteristics of *Sclerotium rolfsii*

3.3. Pathogenicity test

Under greenhouse circumstances, the pathogenicity test of *S. rolfsii* was carried out using the soil inoculation technique. Each *S. rolfsii* isolate was tested for its pathogenicity on the sensitive chickpea variety, L 550. Soil and wheat grains were used to multiply the pathogen in containers. No inoculum was added to the control group. The collar area was the first site of infection. The diseased plants' leaves became a pale green, then yellowed and drooped. Roots completely encircled the collar area as white mycelium expanded at the advanced infection stage. The stem's base had rotted and become brown. Over time, the plant withered away. Infected tissues gave rise to the Sclerotial bodies. The result of pathogenicity of pathogen revealed highest disease incidence (71.05%) under seed cum soil inoculation technique followed by soil inoculation technique (65.35%) and seed inoculation technique (58.69%) (Table 1 and Fig. 3). Likewise, minimum seed germination (15 days after sowing) (74.90%) was observed with seed cum soil inoculation followed by soil inoculation (77.87%), seed inoculation (79.94%) and maximum seed germination was in un-inoculated control (89.84%).

The present findings are in agreement with the result of Muthukumar and Venkatesh [15] and Prasad *et al*. [16] who used soil inoculation as a means of demonstrating pathogenicity. Also, in a study conducted by Singh *et al*. [17], it was shown that germination rates varied depending on the inoculation method. In infected soil, germination was only 70 per cent, in seed treated with pathogen it was 35 per cent, in seedling stage it was 86 per cent, and in un-inoculated soil it was 90 per cent. There was no mortality in the control group, but pre-emergence mortality rates of 30 per cent in soil infected with *S. rolfsii* and 65 per cent in seeds inoculated with the pathogen were significantly higher.

Table 1. Pathogenicity test of *S. rolfsii* with chickpea by different inoculation techniques

Inoculation technique	Seed germination (%)	Per cent disease incidence (%)
Soil inoculation	77.87 (61.92)	65.35 (53.93)
Seed inoculation	79.94 (63.37)	58.69 (49.99)
Seed + Soil inoculation	74.90 (59.92)	71.05 (57.45)
Un-inoculated (control)	89.84 (67.07)	0.00 (0.00)
SEm±	0.55	0.77
CD (P=0.05)	1.82	2.54

* Average of six replications; Figures in parentheses are angular transformed value

Inoculated

Un-inoculated

Fig.3. Pathogenicity test of *S. rolfsii* with chickpea

4. CONCLUSIUON

Sclerotium rolfsii (Sacc.) is one of the most destructive pathogen of chickpea, depositing the growers in significant economic losses. The pathogen was isolated from the collected collar rot samples by using hyphal tip techniques, and the pathogenicity of the pathogen was proved by following Koch's postulates and using three methods of inoculation *i.e.* seed, soil and seed cum soil inoculation. The highest collar rot incidence (71.05%) and lower seed germination (74.90%) was recorded in the seed cum soil inoculation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Keote GA, Prakash Reddy MS, Kapgate OY, Wasnikar AR and Bhoyar SD. Effect of bio-inoculants for the management of collar rot of chickpea. *International Journal of Chemical Studies.* 2019; 7(4): 1857-1861.

2. Jukanti AK, Gaur PM, Gowda CL and Chibbar RN. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition*. 2012; 108(1): 11-26.

3. Nene YL, Sheila VK and Sharma SB. A world list of chickpea and pigeonpea pathogens. ICRISAT, Patancheru, 5th edition, 1996; 27.

4. Narasimha SR. Biological control of wilt of potato caused by *Sclerotium rolfsii* Sacc. M.Sc. (Agri.) Thesis, University of Agricultural Sciences, Dharwad, 2000.

5. Shirsole SS, Khare N, Lakpalme N and Kotasthane AS. Detection of resistant sources against collar rot of Chickpea caused by *Sclerotium rolfsii* Sacc. under field conditions. *International Journal of Current Microbiology Applied Sci*ence, 2018; 7(1): 502-505.

6. Hasna MK, Kashem MA, and Ahmed F. Use of *Trichoderma* in Biological Control of Collar Rot of Soybean and Chickpea.*International Journal of Biochemistry Research and Review.* 2020*;* 29(9): 25-31.

7. Sarkar A, Patel JS, YadavS, Sarma BK, Srivastava JS and Singh HB. Studies on rhizosphere-bacteria mediated biotic and abiotic stress tolerance in chickpea (*Cicer arietinum* L.) *Vegetos,*2014; 27: 158-169.

8. Shyam S and Tiwari RKS. Efficacy of *Trichoderma harzianum* alone and its integration with fungicides against collar rot caused by *Sclerotium rolfsii* in chickpea. *International Journal of Agriculture Sciences,*2018; 10(9): 5945-5947.

9. Rangaswami G and Mahadevan A. Diseases of crop plants in India (4th ed). New Delhi, PHI Learning Private Limited, 2004; 275-278.

10. Radhakrishnan P and Sen B. Efficacy of different methods of inoculation of *Fusarium oxysporium* and *Fusarium solani* for inducing wilt in muskmelon. *Indian Phytopathology*, 1982; 38(1):70-73.

11. Punja ZK and Rahe JE. Method for research on soil borne phytopatogenic fungi. St. Poul., APS Press, 1992; 160-170.

12. Kokub DF, Azam A, Hassan M, Ansar MJ and Khanum A. Comparative growth, morphological and molecular characterization of indigenous *Sclerotium rolfsii* strains isolated from different locations of Pakistan. *Pakistan Journal of Botany*, 2007; 89(5): 1849-1866.

13. Awasthi DP, Das P, De B, Hazari S and Nair N. Isolation and characterization of *Sclerotiumrolfsii* Sacc. causing collar rot of pigeonpea in tripura. *Innovative Farming*, 2018; 3(3): 103-105.

14. Kamthe HJ, Ghante PH, Hingole DG and Khaire PB. Chickpea (*Cicer arietinum* L.) collar rot disease: Prevalence, symptomatology, isolation, identification and pathogenicity. *The Pharma Innovation Journal,* 2023; 12(8): 01-08

15. Muthukumar A and Venkatesh A. Occurrence, virulence, inoculum density and plant age of *Sclerotium rolfsii* Sacc. causing collar rot of Peppermint. *Journal of Plant Pathology and Microbiology,*2013; 4(10): 1-4.

16. Prasad SD, Basha ST and Reddy NPE. Molecular variability among the isolates of *Sclerotium rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. *EurAsian Journal of Biosciences*, 2010; 4: 80-87.

17. Singh K, Meena CB, Gautam C and Jewaliya B. Symptomatology, isolation and pathogenicity test of the collar rot of Chickpea (*Cicer arietinum* L.) Incitant by *Sclerotium rolfsii* (Sacc.). *The Pharma Innovation Journal*. 2022; 11(3): 23-29.