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Isolation, Purification, Identification and Pathogenicity of *Sclerotinia sclerotiorum* (Lib.) de Bary Caused by Sclerotinia Rot of Chickpea

M. K. Sheshma [a++*], D. R. Kumhar [a#], S. L. Godara [a†], A. K. Meena [a‡] and Prahlad [a++]

[a] *Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner-334 006, Rajasthan, India.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Barry is a soil habitat plant pathogen, able of infecting more than five hundred host plant spp., all over the world and doing a crucial role in reduce the yield of economically important crops. Sclerotinia rot also known as stem rot, incited by *S. sclerotiorum* is a certical disease of chickpea. We done an experiment in which, a number of fungal isolation, purification, identification and pathogenicity from the infected plant materals. The plants appear typical symptoms like drooping of petioles and leaflets and in last stage scattered sclerotial bodies.

[++] *Ph.D. Scholar;*
[#] *Professor and Head;*
[†] *Ex-Professor and Head;*
[‡] *Assistant Professor;*
**Corresponding author: E-mail: mksheshma92@gmail.com;*

The infected isolates observed on the affected tissues after re-inoculation and generate mycelial growth and sclerotia both plants and culture plates. On re-isolation it was base that the fungus was identical to the authentic isolate. It was found that the infection was much supreme in inoculated plants as compare to control.

Keywords: Chickpea; identification; pathogenicity; sclerotinia rot; Sclerotinia sclerotiorum.

1. INTRODUCTION

Gram (*Cicer arietinum* L.) is a main grain legume in the Indian subcontinent, West Asia, Northen and Eastern Africa and Central and South America. It is known by other names like chickpea, spanish pea, chestnut bean (English), and chana (Hindi) etc. It is used as daal, besan, crushed or whole gram, roasted or cooked, green foliage and grain as vegetables. Germinated grains are suggested to cure scurvy. Oxalic and malic acids acquiring from green leaves are prescribed for intestinal sickness. Soaked grains and husk are fed to horses and animal as concentrate and roughage respectively. Being a legume crop, it enhances soil fertility by fixing atmospheric N_2 in the form of nodules, which remains in the soil after harvest time.

On worldwide basis, chickpea is the third main important grain legume after common bean and pea (Anwar et al. 2009). Asia covers 89.7 per cent in Oceania, 2.9 per cent in America and 0.4 per cent in Europe [1]. "India ranks first in terms of chickpea yield and consumption in the world. About 65 per cent of the worldwide with 68 per cent of worldwide yield is provide by India" [2]. "The significant chickpea producing countries are India, Pakistan, Ethiopia, Burma, Turkey, Mexico and Australia" [2].

"It accounts for 70 per cent cultivated *Rabi* pulses in India. The main chickpea growing states in India are Madhya Pradesh (41%) followed by Maharashtra (16%), Rajasthan (15%), Karnataka (6%), Andhra Pradesh (5%), Uttar Pradesh (5%) and other continuing states & UTs of India (12%). The total region under chickpea farming in India is about 10.56 million ha with yearly production of 11.23 million tones. The average productivity of chickpea is 1063 kg/ha" [3].

"In Rajasthan, the main chickpea growing districts are Bikaner, Churu, Jhunjhunu, Hanumangarh, Sri Ganganager, Jaipur, Sikar and Ajmer. The total region and production of chickpea in Rajasthan is 1.57 million ha and 1.67 million tones, respectively, having productivity of 1062 q/ha" [4].

Several factors which adversely affect chickpea productivity, like as fungal, viral and bacterial diseases poses a tough challenge to the farmer. Almost fifty diseases of chickpea have so far been found from many regions of the world. Among these diseases, few having economic importance *viz.,* stem rot (*Sclerotinia sclerotiorum*), fusarium wilt (*Fusarium oxysporum f. sp. ciceri*), dry root rot (*Rhizoctonia bataticola*), black root rot (*Fusarium solani*), collar rot (*Sclerotium rolfsii*) and wet root rot (*Rhizoctonia solani*). Among the leaf diseases, Ascochyta blight is observed as most important. Other leaf diseases are Botrytis grey mould (*Botrytis cinerea*), Colletotrichum blight (*Colletotrichum dematium*) and Alternaria blight (*Alternaria alternata*).

Sclerotinia rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is a main disease of chickpea and always remain in chickpea growing region of Rajasthan. It was initially found at ARS Ummedganj, Kota in the year 1993-94 and thereafter, it has been starting forever in mild to severe condition. The pathogen infects stem, collar region and foliage leading to mortality of the plants. The fungus identified by the formation of hard black sclerotia, which on germination produce cup shaped brown colored apothecia.

S. sclerotiorum (Lib.) de Bary is a necrotrophic pathogen with cosmopolitan distribution and wide host range [5] and included to the family Sclerotiniaceae [6] class Ascomycetes. It produces sclerotia and apothecia but lacks conidial stage.

"The disease has been found from many region of India and causing considerable losses. Its occurrence was first reported from Himachal Pradesh with considerable yield losses" [7]. "The extent of yield damage ranged from 21.3% to 46.7% at pod bearing stage of the crop" (Sharma, 1995). "In Jammu and Kashmir, the disease incidence varied from 18.7% (Udhampur) to 32.2% (Jammu)" [8]. The current

observations recommend that the incidence and severity of sclerotinia rot of chickpea is developing in the northern part of India. This increase has been aggravated by the cultivation of chickpea during winter seasons when conditions favour plant growth and also, development of the disease. The cultivation of chickpea under high input with irrigation increases the incidence of stem rot in northern part of India.

"The stem rot of chickpea can occur either at seedling stage or at flowering and pod filling stage depending upon the environmental conditions. At seedling stage, the disease causes collar rot at the base of stem and reduces the substantial number of plant stand. At the pod bearing stage, infection starts at upper stem or on senescent flowers by the air borne ascospores. Infected stems become pale in colour and the symptoms spread both upward and downward along the stems. Under heavy canopy and humid conditions, white puff mycelial growth becomes conspicuous" [8].

Infected plants first wilted and rapidly die, often without turning yellow. Later, as the plant dries out, the leaves turn straw color. On the surface of the root, just below ground level, small black fungal bodies called sclerotia, which are irregular in size and shape, can sometimes be seen mingled with white cottony fungal mycelium growth. In spring seasons many water-soaked lesions firstly appearing on the stems and leaves. Early symptoms of stem infection appear as white mycelial growth. Affected tissues develop a slimy soft rot from which droplets of brown liquid may exude. Infected tissues then dry out and may become covered with a web of white mycelium growth.

2. MATERIALS AND METHODS

2.1 Collection, Isolation and Purification of the Pathogen

Soil or plant tissues were observed from Institutional farm, College of Agriculture, Bikaner and brought to the laboratory for further studies. Prior isolation and other laboratory experiments, all the glasswares were cleaned with Potassium dichromate sulphuric acid, solution washed with sterilized water, sterilized in hot air oven at 180 ˚C for two hours. PDA were sterilized by autoclaving at 1.045 kg cm^2 pressure for 20 minutes. Stem rot of chickpea plants were first washed under the tap water and then cut into small piece along with healthy portion. These pieces were surface sterilized by dipping in 0.1 per cent Sodium hypochlorite solution for 1 minute after three consecutive washing with sterilized water, the pieces were transfer to autoclaved Potato Dextrose Agar medium in petriplates incubated at 25+1ºC and petriplates are placed into BOD incubator for 7 days. The fungal colonies emanating from bits were examined on 7 days of incubation.

2.2 Purification of Pathogen

Pure culture of the fungus was obtained by hyphal tip method [9] on plain agar medium. "For this, hypal tips were acquire from culture slants after 96 hours of incubation and were suspended in distilled water. The dilution of animation was conform such that in one loopful, 5-10 ascospores could be included under the low power objective of the microscope. 1 ml of above suspension was spread in Petriplates containing 20 ml sterilized plain agar medium. After 12-24 hours of inoculation, the germinating spores were located under the microscope and marked with the help of dummy objective and then transferred to PDA slant and kept in BOD for further growth. The culture was maintained by periodical transfer on PDA slants for additionally academic studies" [9].

2.3 Pathogenicity Test

"To know the pathogenic presence of the isolated pathogen under present study, experiments were managed in glass house in *Rabi* 2016-17 and 2017-18. Chickpea plants were elevate from surface sterilized seeds sown in earthen pots containing sterilized soil. Pathogenicity tests were managed by employing sclerotial inoculation mthod and mycelial disc method. For this purpose, the seeds of chickpea were surface sterilized with $HgCl_2$ solution (0.1%) and sown in 30 cm diameter earthen pots filled with autoclaved sterilized soil. 10 seeds of chickpea plants were sown in each earthen pot. The pots were continually watered and maintained for inoculation with four replications. The following two methods were employed with injury and without injury for pathogenicity test" [10].

2.4 Mycelial Disc Method

Mycelial disc of 5 mm diameter of *Sclerotinia sclerotiorum* was cut from the margin of three days old culture grown on PDA media and placed at stem area of damaged and uninjured

30 days old healthy chickpea plants. Plants were unnatural injured using carborundum powder. The inoculated plants were wrapped with polythene bags for a week. The uninoculated plants in damaged and uninjured as manage were also wrapped with polythene bags. Chickpea plants were examined continuously for the presence of disease symptoms and the final data were recorded 2 weeks after inoculation.

2.5 Inoculation with Sclerotia

"Sclerotia were collected from 15 days old culture of *Sclerotinia sclerotiorum*, washed with distilled water and placed near the stem area at soil level by making injury using caborundum powder and without injury. These sclerotia were coated with sterile wet cotton swab and inoculated plants were wrapped with polythene bags for a week. Chickpea plants in earthen pots were examined continuously for disease improvement and data recorded 2 weeks after inoculation" [10].

2.6 Identification of the Pathogen

S. sclerotiorum causing chickpea sclerotinia rot was characterized on pour cultural attributes of mycelium growth presence and sclerotia development till the period of 15 days. The pour cultural identified *viz.,* Sclerotium formation, their shape, colour, size and mycelium growth of fungus were identified under low power magnification (10X) microscope. The culture of the pathogen was also sent to ITCC, Division of Plant Pathology, IARI, New Delhi for further evidence or recognition of fungus. The fungus was identified as *S. sclerotiorum*.

3. RESULTS AND DISCUSSION

3.1 Collection, Isolation and Purification of *Sclerotinia sclerotiorum*

"S. sclerotiorum injured plants of chickpea were assemble from Institutional farm, College of Agriculture, Bikaner where disease symptoms was prevalent and collected. Disease samples were brought to the laboratory for isolation and further studies. The fungus was isolated on PDA media from injured stems of chickpea plants under hygiene conditions. The fungus emerging form stem bits kept on PDA was observed to have prolife white cottony growths which later turn brown to black sclerotia on PDA. Black hard sclerotia were formed after 10-15 days of incubation at the periphery of the mycelia. The culture was purified by hyphal tip technique" [11,12] observed "typical symptoms infect collar region, stem and foliage leading to death of the plants. The fungus characterized by the formation of hard blackish sclerotia, which on germination produce cup shaped brown colored apothecia". "The fungus is mainly a soil dweller and spreads from plant to plant through irrigation water, farm implements and cultural operation. The ascospores may also become air borne and act as secondary inoculum to cause further spread of the pathogen" [13].

3.2 Pathogenicity Test

"The pathogenicity of the pathogen was provably both methods like inoculation using mycelial disc and sclerotia. In both cases, typical symptoms of sclerotinia rot disease appeared on the plants (Plate 1 & Table 1). A higher percentage of infection was recorded when the inoculum was directly placed on the stem of the plant. Inoculation with mycelial disc was found to be severe, it caused 80% infection in injured and 50% infection in un-injured plants. While the sclerotia used as inoculum were found to be less infective. Symptoms of disease formed four to six days after inoculation as small, grayish, water-soaked lesions, which rapidly coalesces to stem length and developed into patches of soft rotting tissues (Plate 2). Stem covered with whitish mycelial mats and black colours of sclerotia in pith of varying sizes were observed on these patches when the diseased stem was split opened. Defoliation of leaves and death of branches of plants were noticed with advancement of the infection. Similar symptoms was also observed in naturally infected crop, on both uninjured and injured plants but infection was higher in case of injured plants" [10].

"This fact suggested that the injury predisposes the plants to fungal attack. Re-isolation from lesions developed on artificially inoculated plants, yielded the same fungus, which was previously isolated from the naturally infected chickpea plants and which proven the pathogenicity test of the same fungus" [10]. "Pathogenicity of the fungus on chickpea has also been reported by Chen et al. [14] and several other hosts" by Chambers and Hardie [15] and Newton and Sequeria [16,17].

3.3 Identification of the Pathogen

Identification of the pathogen was done on the basis of white cottony mycelia growth appeared on sterilized PDA media. Formation of immature

Table 1. Pathogenicity test of *Sclerotinia sclerotiorum* on chickpea plants

Treatments	Seeds sown	Germinated seeds	Infected plants	Infection (%)
Inculation with mycelial disc				
1.With injury	40	40	31	80.00
2.Without injury	40	40	18	50.00
Inculation with sclerotia				
1.With injury	40	40	19	60.00
2.Without injury	40	40	17	40.00
Control (without inoculum)				
1.With injury	40	40	0	0.00
2.Without injury	40	40	0	0.00

(A) Healthy Plant (GNG-1581) (B) Diseased Plant (GNG-1581)

(C) Pure Culture (D) Sclerotial stage of *S. sclerotiorum*

Plate 1. Pathogenicity test of *Sclerotinia sclerotiorum* with chickpea plant

(A) Mycelial disc method (B) Inoculation with sclerotia

Plate 2. Different methods of Pathogenicity test of *Sclerotinia sclerotiorum* with chickpea plant

numerous whitish sclerotia in Petri plate after 4 days incubation also used for identification of the fungus (Plate 1 d). The sclerotia of fungus were initially round and looked like irregular chickpea seed, later it became brown to dark black. On maturity sclerotia were very hard and plate became dry after utilization of nutrients from the media. It was also observed that old sclerotia survived for longer time. All the above morphological and microscopic characters of sclerotia, apothecia, asci and ascospores of fungus led to identification of *Sclerotinia sclerotiorum.* It was further confirmed from the Indian type culture collection (ITCC), Division of Plant Pathology, IARI, New Delhi as *Sclerotinia sclerotiorum* (Lib.) de Bary.

4. CONCLUSION

This study represents that the pathogen was isolated from infected chickpea plants and multiplied on sorghum grains. Pathogenicity was proved using variety GNG-1581, after 25-30 days of sowing, typically water soaked lesions appeared on collar region. The pathogen was found to produce characteristic symptoms such as fluffy white mycelial mats on the surface of infected tissues of stem, leaves and development of many black sclerotia of different shape and size. Black sclerotia were also developed inside the infected stems. The pith was found with abundant black and irregular sclerotia similar to those formed in culture. Higher per cent disease incidence was found in mycelial disc and sclerotia inoculation technique.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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