Isolation, purification, identification and pathogenicity of *Sclerotinia sclerotiorum* (Lib.) de Bary caused Sclerotinia rot of chickpea

ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Barry is a soil-borne plant pathogen, capable of infecting more than five hundred host plant species worldwide and plays a crucial role in reducing the yield of economically important crops. Sclerotinia rot also known as stem rot, caused by *Sclerotinia sclerotiorum* is a serious disease of chickpea. We conducted an experiment in which, a series of fungal isolation, purification, identification and pathogenicity from the infected plant parts. The plant show typical symptoms like drooping of petioles and leaflets and in advance stage scattered sclerotial bodies. The infected isolates may be seen on the affected tissues after re-inoculation and produced mycelia growth and sclerotia both plants and culture plates. On re-isolation it was found that the fungus was identical to the original isolate. It was observed that the infection was much higher in inoculated plants as compared to control.

Keywords: Chickpea, Identification, Pathogenicity, Sclerotinia rot, Sclerotinia sclerotiorum

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important grain legume in the Indian subcontinent, West Asia, Northen and Eastern Africa and Central and South America. It is known by other names like gram, spanish pea, chestnut bean (English), and chana (Hindi) etc. It is consumed as daal, besan, crushed or whole gram, roasted or cooked, green foliage and grain as vegetables. Germinated grains are recommended to cure scurvy. Oxalic and malic acids collected from green leaves are prescribed for intestinal disorders. Soaked grains and husk are fed to horses and cattle as concentrate and roughage respectively. Being a legume crop, it improves soil fertility by fixing atmospheric nitrogen in the form of nodules, which remains in the soil after harvesting.

On global basis, chickpea is the third most 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 (Gaur *et al.,* 2010). India ranks first in terms of chickpea production and consumption in the world. About 65 per cent of the global area with 68 per cent of global production is contributed by India (Reddy and Mishra, 2010). The major chickpea producing countries are India, Pakistan, Ethiopia, Burma, Turkey, Mexico and Australia.

It accounts for 70 per cent cultivated *Rabi* pulses in India. The major 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 remaining states & UTs of India (12%). The total area under chickpea cultivation in India is about 10.56 million ha with annual production of 11.23 million tones. The average productivity of chickpea is 1063 kg/ha. (Anon., 2017-18a).

In Rajasthan, the major chickpea growing districts are Bikaner, Churu, Jhunjhunu, Hanumangarh, Sri Ganganager, Jaipur, Sikar and Ajmer. The total area and production of chickpea in Rajasthan is 1.57 million ha and 1.67 million tones, respectively, having productivity of 1062 q/ha (Anon., 2017-18b).

Several factors which adversely affect gram productivity, such as like fungal, bacterial and viral diseases poses a tough challenge to the farmer. About fifty diseases of chickpea have so far been reported from different parts of the world. Among these diseases, some having economic importance *viz.*, white 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 considered as most important. Other leaf diseases are Botrytis grey mould (*Botrytis cinerea*), Colletotrichum blight (*Colletotrichum dematium*) and Alternaria blight (*Alternaria alternata*).

In Rajasthan, root rots and stunt virus diseases are commonly prevalent in most of the districts in mild to severe form in the areas where light to heavy soils prevails. A considerable damage due to Ascochyta blight (*Ascochyta rabiei*), black root rot (*Fusarium solani*) and root knot nematode (*Meloidogyne incognita)* has also been observed in some of the districts.

Stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is an important disease of chickpea and regularly occurs in chickpea growing areas of Rajasthan. It was initially observed at ARS Ummedganj, Kota in the year 1993-94 and thereafter, it has been appearing continuously in mild to severe form. The pathogen infects collar region, stem and foliage leading to mortality of the plants. The fungus characterized by the formation of hard blackish sclerotia, which on germination produce cup shaped brown colored apothecia.

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic pathogen with cosmopolitan distribution and wide host range (Purdy, 1979) and belongs to the family Sclerotiniaceae (Whetzel, 1945) class Ascomycetes. It produces sclerotia and apothecia but lacks conidial stage.

The disease has been reported from different parts of India and causing considerable losses. Its occurrence was first reported from Himachal Pradesh with considerable yield losses (Grewal and Pal, 1986). The extent of 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). (Vaid *et al.,* 2005). The recent observations suggested that the incidence and severity of stem rot of chickpea is increasing in the northern part of India. This increase has been aggravated by the cultivation of chickpea crop during winter months 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 India.

The stem rot of chickpea can occur either at seedling stage or at flowering and pod filling stage depending upon the environment conditions. At seedling stage, the disease causes collar rot symptoms 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.

Affected 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. In spring many water-soaked spots first appear 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

Collection, isolation and purification of the pathogen.

Diseased samples will be collected 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. Media (PDA) were sterilized by autoclaving at 1.045 kg cm^2 pressure for 20 minutes. Stems 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 1-1½ minute after three consecutive washing with sterilized distilled water, the pieces were transfer to autoclaved Potato Dextrose Agar medium in petriplates incubated at 25+1ºC and plates are placed into BOD incubator for 7 days. The fungal colonies emanating from bits were examined on 7 days of incubation.

PURIFICATION OF PATHOGEN

Pure culture of the fungus was obtained by hyphal tip method (Rangaswami and Mahadevan, 1999) on plain agar medium. For this, hypal tips were obtain from culture slants after 96 hours of incubation and were suspended in sterilized distilled water. The dilution of suspension was adjusted such that in one loopful, 5-10 spores could be counted under the low power objective of the microscope. One 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 further studies.

PATHOGENICITY TEST

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

MYCELIAL DISC METHOD

Mycelial disc of 5 mm diameter were cut from the margin of three days old culture grown on PDA and placed at stem region of injured and uninjured 30 days old healthy chickpea plants. Plants were artificially injured using carborundum powder. The inoculated plants were covered with polythene bags for a week. The uninoculated plants in injured and uninjured as control were also covered with polythene bags. Chickpea plants were examined periodically for the appearance of disease symptoms and the final data were recorded 2 weeks after inoculation.

INOCULATION WITH SCLEROTIA

Sclerotia were harvested from 15 days old culture, washed with distilled water and placed near the stem base at soil level by making injury using caborundum powder and without injury. These sclerotia were covered with sterile wet cotton swab and inoculated plants were covered with polythene bags for a week. Chickpea plants in pots were examined regularly for disease development and data recorded 2 weeks after inoculation.

IDENTIFICATION OF THE PATHOGEN

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

3. RESULTS AND DISCUSSION

Collection, isolation and purification of *Sclerotinia sclerotiorum*.

S. sclerotiorum infected plants of chickpea were collected from Institutional farm, College of Agriculture, Bikaner where disease was prevalent and collected. Samples were brought to the laboratory for isolation and further studies. The fungus was isolated on PDA from infected stems of chickpea plants under aseptic conditions. The fungus emerging form stem bits placed on PDA was observed to have profuse white cottony growth later turn brown to black sclerotia on PDA. Black hard sclerotia were formed after 10-15 days at the periphery of the colony. The culture was purified by hyphal tip technique. El– Araby (2009) observed symptoms infect collar region, stem and foliage leading to mortality 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, food and implements and cultural operation. The sclerotia and ascospores may also become air borne and cause further spread of the pathogen (Rangaswami and Mahadevan 2008).

PATHOGENICITY TEST

The infection of plants occurred in both methods of 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 appeared four to six days after inoculation as small, grayish, water-soaked lesions, which rapidly enlarged to stem length and developed into patches of soft rotting tissues (Plate 2). Stem covered with whitish mycelial mats and black colour of sclerotia in pith of varying sizes were observed on these patches when the diseased stem was split. Defoliation of leaves and death of branches of plants were noticed with advancement of the disease. Pathogen produced similar symptoms as observed in naturally infected crop, on both uninjured and injured plants but infection was higher in case of injured plants.

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. In this way, isolation, inoculation and re-isolation of the same fungus proved the pathogenicity of the pathogen. Pathogenicity of the fungus on chickpea has also been reported by Chen *et. al.,* (2006) and several other hosts by Chambers and Hardie (1961) and Newton and Sequeria (1972).

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 numerous whitest coloured 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. It was also observed that old sclerotia survived for longer time. All the above morphological 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.

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

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(A) Healthy Plant (B) Diseased Plant

(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