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Symptomatology, isolation and pathogenicity test of the collar rot of Chickpea (*Cicer arietinum* L.) Incitant by *Sclerotium rolfsii* (Sacc.)

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Abstract
The present study was undertaken in order to find out the prevalence and incidence of the Collar rot in Chickpea in Kota district of *Hadoti* region of Rajasthan caused by *Sclerotium rolfsii*, a roving survey done nearby places of Agriculture Research Station (ARS) Ummedganj, Kota district during *Rabi* season of 2018-19 in different places *viz.*, ARS-Ummedganj, Sangod, Sultanpur, Itawa, Galana, Dhakarkhedi and Char-chouma. Collar rot is often seen in the seedling stage, particularly under high moist condition. Maximum incidence was recorded in the field of Agriculture Research Station, Ummedganj & Mechanized Agriculture Farm, Ummedganj (19.82%), followed in decreasing order in the field of Galana (14.38%) Sultanpur (12.05%), Sangod (11.20%), Itawa (10.40%), Dhakerkhedi (9.32%) and Char-Chouma (6.69%). Thus, the incidence of collar rot in chickpea was in the range of 6.69% - 19.82%, which was quite high to causing seedling mortality and cause considerable losses to the crop. For the laboratory experimental studies, standard potato dextrose agar (PDA) medium was used for isolation and culturing *S. rolfsii*. Standard tissue isolation technique was followed for the isolation of the fungus. Later, the bit of hyphal tip of fungal growth was transferred to PDA slants for obtained pure culture. The pathogen was inoculated through Soil inoculation, Seed inoculation and Seedling inoculation for testing pathogenicity.

Keywords: Pathogenicity, soil inoculation, seed inoculation, seedling inoculation, sclerotium rolfsii, chickpea

Introduction
Chickpea (*Cicer arietinum* L.) belongs to genus *Cicer* tribe Cicereae family Fabaceae and sub family Papilionaceae (Bentham and Hooker, 1972) [6]. It is diploid in nature with chromosome number 2n=16. It is also called by various names such as gram, Bengal gram, Garbanzo, garbanzo bean, Egyptian pea, *Chana* and *chhole* in various places. It originated from South West Asia (Turkey) and is cultivated from ancient times both in Asia and Europe. Chickpea, the most important pulse crop widely grown in India, accounts for nearly 75 percent of the total pulse production in the world (Keote *et al*., 2019) [10]. It is grown as a *Rabi* season crop, under irrigated and rainfed conditions having a very important position in the arid and semi-arid farming system.

Chickpea is an important grain legume providing an enormous source of minerals, fibers, and proteins both for humans and animals (Varol *et al*., 2020) [24]. Chickpea seeds contain high mineral content has been reported for phosphorus (340mg/100g), calcium (190 mg/100g), magnesium (140mg/100g), iron (7mg/100g) and zinc (3 mg/100g) and on an average 23% protein, 64% total carbohydrates (47% starch, 6% soluble sugar), 5% fat, 6% crude fiber and 3% ash (Anonymous, 2015) [3]. Its protein is rich in lysine and has low Sulphur containing amino acids therefore, it is a protein-rich supplement and resource of cheap protein compared to animal protein to all cereal-based diets, mainly for vegetarians, so it is also known as the poor "man's meat". Its lipid ratio is high in unsaturated fatty acids, so it is extensively appreciated as healthy food. On the other hand, chickpea plays an important role in the improvement of soil fertility by fixing atmospheric nitrogen. It meets 80 per cent of nitrogen necessity from symbiotic nitrogen fixation, and can fix up to 140 Kg N/ha from air, and adds a large amount of residual nitrogen for subsequent crops. It also adds plenty of organic matter which improves soil health and fertility. Because of its deep taproot system can resist in drought conditions by extracting water from deeper layers in the soil profile (Kashiwagi *et al.*, 2005; Krishnamurthy *et al*., 2003) [9, 11].

India ranks first in conditions of chickpea production and consumption in the world. In India, area under chickpea was 10.57 million hectares with a production of 11.15 million tonnes and

productivity of 1055 kg/ha, whereas in Rajasthan it grown on area 1.38 million hectares with a production of 1.47 million tonnes and productivity of 1065 kg/ha during *Rabi* 2017-18 (Anonymus, 2019) [4]. The highest production has been received from Madhya Pradesh (41%) followed by Maharashtra (16%), Rajasthan (13%), Karnataka (8%), Andhra Pradesh (6%), Uttar Pradesh (6%) and other remaining states. India is the major chickpea growing country of the world and in India the major producing states are Madhya Pradesh, Rajasthan, Maharashtra, Uttar Pradesh, Karnataka and Andhra Pradesh together contributing to 90 per cent of the area and 91 per cent of the production in the country (Singh, 2010) [22].

Despite the high total production and more nutritive value, productivity of chickpea was low due to many biotic and abiotic constraints. Among the biotic constraints Chickpea crop is attacked by 172 pathogens (67 fungi, 22 viruses, 3 bacteria, 80 nematodes and mycoplasma) from entire the world (Nene *et al.*, 1996) [15]. Among all, only a few of them have the potential to destroy crops. Some of the severe diseases in order of their importance are wilt (*Fusarium oxysporum* f. sp. *ciceri*), dry root rot (*Rhizoctonia bataticola*), collar rot (*Sclerotium rolfsii*), wet root rot (*Rhizoctonia solani*) and Ascochyta blight (*Ascochyta rabiei*). Among the soil borne diseases such as Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceri*), dry root rot (*Rhizoctonia bataticola*) and collar rot (*Sclerotium rolfsii*) are the major limiting factors in chickpea production. Chickpea diseases may cause yield losses up to 100% depending on time of infection. Dry root rot and collar rot are emerging as a major threat to chickpea production due to drastic climate change (Pande *et al.* 2010) [16].

Among the biotic causes collar rot caused by *Sclerotium rolfsii* Sacc. is an important disease in areas where seedling is exposed to high temperature and high moisture in the soil, affected seedlings turn yellow, which can be easily pulled out. The fungus placed in the form of genus *Sclerotium rolfsii* by Saccardo (1913) [19] as it forms differentiated sclerotia and sterile mycelia. Lower portion of stem of herbaceous plants decay with development of white mat of mycelium at the lesion site. This often spreads out on to the near-by soil surface. Shortly after the mycelial mat develops, small (0.5-1mm), white round, fuzzy mycelial bodies begin to appear. These further developed into mustard grain sized light to dark brown structures known as sclerotia, serve as over wintering bodies and may be seen in the mycelium, on diseased tissues above or below ground, on soil surface, or in soil crevices. The characteristic white mycelial mats and sclerotia also develop at the infection sites near the crown under favorable conditions. Foliage wilting and die-back develop as a consequence of rolling of the lower trunk or crown tissue. *S. rolfsii* is a ubiquitous and aerobic pathogen. The drying of plants in chickpea field is observed due to wilt and root/collar rot complex. Out of these diseases, collar rot caused by *Sclerotium rolfsii* Sacc. is becoming more serious at a seedling stage especially in the area where paddy or soybean-based cropping system is followed.

Collar rot of chickpea caused by *Sclerotium rolfsii* is an important soil borne and fast spreading fungal pathogen, which causes considerable damage to the plant stand. Seedling mortality in chickpea due to *S. rolfsii* has been reported to vary from 54.7 to 95.00 per cent (Shrivastava *et al.*, 1984) [20]. Under field conditions, *S. rolfsii* has been reported to cause 22 to 50 per cent reduction in yield of chickpea. The pathogen caused significant reduction in plant population having wide host range (Aycock, 1966; Punja, 1988) [5, 17]. Ghosh *et al*. (2013) surveyed four chickpea growing states of India *i.e.*, Andhra Pradesh, Karnataka, Madhya Pradesh and Chhattisgarh and reported that losses from collar rot disease ranged from 7.1 to 10.5%. This disease is more problematic for chickpea farmer in the *Hadoti* region of Rajasthan.

Materials and Methods

Survey to assess disease incidence of collar rot of chickpea in Kota district of Rajasthan

The rapid roving survey of chickpea fields of nearby places of Agriculture Research Station (ARS) Ummedganj, Kota district of Rajasthan was surveyed to find out the incidence of collar rot during *Rabi* season of 2018-19 in different places *viz.*, ARS-Ummedganj, Sangod, Sultanpur, Itawa, Galana, Dhakarkhedi and Char-chouma. Thus, total of 7 localities were selected and in each locality 3 fields (minimum area 1 acre) were randomly selected. To know the disease incidence, an area of 1m x 1m was marked diagonally across the field at five spots on each field and per cent disease incidence (PDI) was calculated with the help of following formula: -

$$\text{Percent disease incidence} = \frac{\text{Number of plant exhibited collar rot}}{\text{Total number of plant observed}} \text{X100}$$

The localities wise incidence of collar rot was worked out by calculating the mean of three fields of respective locality. During the disease appearance, growth of the plant is checked, initially the leaves of infected chickpea plants turn light pale in colour, plant start drying and finally die, seedling pulled up very easily. The seedling was found to collapse and showed rotting at collar region. White mycelial growth adhered to collar region along with mustard like sclerotia were observed. During survey, personal discussions were also held with the cultivators regarding incidence and severity of the disease *viz.*, agronomic practices and field history.

Collection of diseased samples

Infected plants which showing typical collar rot symptoms were collected during month of October to December, 2018 from the chickpea fields of Agriculture Research Station, Ummedganj (Kota). Samples were brought in to Department of Plant Pathology, College of Agriculture, Ummedganj, (Kota) for isolation and further studies.

Isolation of fungus

The part of collar region showing typical symptoms of disease was cut into small pieces. Then these pieces were surface sterilized with 0.1% $HgCl_2$ solution for one minute. Such pieces were washed thoroughly in sterile distilled water three times to remove the traces of mercuric chloride solution, and then aseptically transferred to sterilized potato dextrose agar (PDA) plates. They were incubated at 25±1°C for three days for growth of fungus. Later, the bit of fungal growth was transferred to PDA slants. The pure culture of fungus was obtained by further growing culture and following hyphal tip culture under aseptic conditions. As method suggested by (Aneja, K. R., 2018) [2]. The pure cultures were maintained on PDA slants at 4±1 °C for further studies.

Identification of fungus
The pathogen *S. rolfsii* forms cottony white colonies on PDA. The colonies appeared as dull white to pure white mycelial growth and initiation of sclerotia formation after 8-9 days of incubation. Sclerotia are brown in colour and mustard seed like in shape. On the basis of these characters pathogen was identified as *S. rolfsii.* Further, pathogen was identified from ITCC (Indian Type Culture Collection) Lab, IARI, Division of Plant Pathology, New Delhi.

Maintenance of pure cultures
The fungus *S. rolfsii* was sub-cultured on PDA slants and allowed to grow at 25±1 ℃ temperature in the incubator for ten days. The culture was obtained stored in a refrigerator at 4±1 ℃ and sub cultured once in a month.

Mass multiplication
The sand sorghum medium (SSM) was used for mass multiplication (mass inoculum) of *S. rolfsii* in laboratory. The sorghum grains were soaked in water for overnight. These grains were spread on clear blotter paper for drying. Soaked grains were taken in tray and @ 25 g/kg calcium carbonate was added to grains for avoid clumping. About 150 g moistened grains and 75 g dry sand was filled in each 500 ml flasks, plugged, wrapped with paper and autoclaved for 30 min at 121.6℃ temperature & 1.05 kg/cm^2 pressure 2 times by keeping 24 hrs. gap between the two successive autoclaving. The flasks containing SSM were inoculated with pure culture of *S. rolfsii* (6-8 bit) of mycelial disc of 6 mm cut with the help of sterilized cork borer under aseptic condition and incubated at 25±1℃ for 2 weeks. Flasks were shaken on alternate days to avoid clumping of grains and to facilitate early growth of fungus on the grains. The grains turned whitish due to mycelial growth of the test pathogen. The giant culture so obtained was used for further studies.

Proving the pathogenicity by different method of inoculation
Soil inoculation
Sterilized soil was filled in cemented pots of size 45 x 30 cm^2. Fifteen days old culture grown on sorghum grains10 g mass culture of *S. rolfsii* grown on sorghum seeds was added to upper 15 cm layer of soil in pots and mixed thoroughly with soil to get sick soil. Then healthy seeds of collar rot susceptible chickpea variety (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution, washed thrice in tap water before sown in the pots containing sterile soil. Pots without *S. rolfsii* culture served as control. Moisture content in pot was maintained to field capacity by adding required amount of water periodically. Observations on germination, pre and post emergence mortality were recorded on seven days after sowing. Re-isolation was made from such affected portion of the plant tissue and compared with that of original culture (Ashan *et al.*, 2018).

Seed inoculation
Healthy seeds of chickpea (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution for one minute followed by three washings with sterilized water and seeds were dressed with test fungi @ 4g/kg seeds, 10 treated seeds were sown in sterilized soil in cemented pots. Proper isolation and replication were maintained. No seed dressing with test fungus was used as control. Observations on germination, pre and post emergence mortality were recorded on seven days after sowing (Jayale, 2014) [8].

Seedling inoculation
Sterilized soil was taken in cemented pots, Healthy seeds of chickpea (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution for one minute followed by three washings with sterilized water sown in cemented pots in net house. The test fungus was inoculated on fifteen days old seedlings at collar region @ 20 g/pot. Proper control was maintained where the test fungi were not inoculated. Observation on the mortality of seedlings was recorded seven days after inoculation at regular interval (Mahato *et al.*, 2018) [13].

Observation recorded
The percentage seed germination, pre-emergence seed rot and post-emergence seedling mortality were calculated by the formulae.

a. Germination (%) = $\frac{\text{Number of seed germinated}}{\text{Total number of seed sown}}$ X 100

b. Pre-emergence seed rotting % (PESR) = $\frac{\text{Number of seed not germinated}}{\text{Total number of seed sown}}$ X 100

c. Post-emergence seedling mortality % (PESM) =$\frac{\text{Number of seedling died}}{\text{Total number of seedling}}$ X 100

Results and Discussion
Survey for assessing the collar rot incidence of chickpea
To find out prevalence of disease on farmer's field and research field a roving survey of chickpea growing areas were undertaken at different locations of nearby places of ARS, Ummedganj, Kota district of Rajasthan, to assess the incidence of collar rot on chickpea during *Rabi* 2018-19. In all the locations surveyed none of the field remained free from the disease.

Table 1: Incidence of collar rot of chickpea at research or farmer's fields in Kota district of Rajasthan

S. No.	Location surveyed	Disease incidence (%)
1.	Agriculture Research Station, Ummedganj	19.82
2.	Sangod	11.20
3.	Sultanpur	12.05
4.	Itawa	10.40
5.	Galana	14.38
6.	Dhakerkhedi	9.32
7.	Char-chouma	6.69

* Average of three fields of each locality.

The data presented in Table-1, showed that the maximum incidence was recorded in the field of Agriculture Research Station, Ummedganj & Mechanized Agriculture Farm, Ummedganj (19.82%), followed in decreasing order in the field of Galana (14.38%) Sultanpur (12.05%), Sangod (11.20%), Itawa (10.40%), Dhakerkhedi (9.32%) and Char-chouma (6.69%). Thus, the incidence of collar rot in chickpea was in the range of 6.69% - 19.82%, which was quite high to causing seedling mortality and cause considerable losses to crop. Wilt, collar rot and dry root rot of chickpea occurs in all most all the countries wherever chickpea is grown. During

survey in Jabalpur and adjoining area collar rot appeared at 15 - 45 days old crop and it ranged from 5 - 30 percent (Gupta and Mishra, 2009) [7]. It suffers severely due to seedling mortality of plants from emergence to seedling stage of 60 DAS known as collar rot, which is caused by *Sclerotium rolfsii*. The collar rot is one of the major limiting factors for successful cultivation of chick in India, causing significant economic losses every year. It is considered as economically important disease of chickpea in India (Nene, 1985) [14]. The incidence is related to higher moisture content and presence of undecomposed organic matter near soil surface. It's problem is severe in the seedling stage, except in irrigated crops where the disease can occur at any stage if temperature is not low. Chickpea followed by paddy or soybean cropping system shows more disease incidence. In the present investigation, the collar rot disease of chickpea was observed more prominent factor causing significant yield loss in the farmer field of *Hadoti* region of Rajasthan.

Symptomatology

The Collar rot appears in patches in field condition and infected plants were observed at seedling stage up to six weeks after sowing. The affected plants turned yellow and uprooted very easily. The seedling was found to collapse and showed rotting at collar region. White mycelial growth of pathogen adhered to collar region along with mustard seed like sclerotia were observed. On the basis of visual observations at different crop growth stages revealed that the severity of the disease was more pronounced during seedling stage *i.e.*, up to 45 days after sowing and gradually decrease with age of crops. Similar symptoms were also reported by Siddaramaiah *et al.* (1978) [21]. The disease is usually appeared in patches in the field. Singh *et al.* (2011) [23] in his studies on survey, symptomatology, isolation pathogenicity test of the collar rot in chickpea at Bundelkhand region during the year, 2005-06 and 2006-07 in the districts *viz.* Hamirpur, Jhansi, Lalitpur, Mahoba and Urai reported that collar rot is seen in the seedling stage, particularly under wet soil conditions and the incidence of disease is more at higher soil moisture (30%) and temperature (30 ℃) at sowing time. *S. rolfsii* infected plants show white to mustard coloured sclerotia and thin white mycelial mat on almost all the infected and wilted plants (Kumar and Venkatesh, 2013) [12].

Collection of diseased sample and isolation of fungus

Chickpea infected plants showed typical collar rot symptoms were collected during the months of November to December 2018 from chickpea fields of ARS, Ummedganj, Kota. Samples were brought in laboratory of Department of Plant Pathology, College of Agriculture, Ummedganj, Kota for isolation and further studies. Potato dextrose agar (PDA) media were used for isolation and culturing *S. rolfsii* for laboratory experiments. Standard tissue isolation technique was followed for isolation of fungus. Later, the bit of fungal growth was transferred to PDA slants. The pure culture of fungus was obtained by further growing culture and following hyphal tip culture under aseptic conditions. The pure cultures were maintained on PDA slants at 4 °C±1 for further studies. The isolates were obtained by standard tissue isolation method and identified as *S. rolfsii* based on mycelial and sclerotial characteristics as given by (Punja and Rahe, 1992) [18].

Identification of fungus

The pathogen *S. rolfsii* form cottony white colonies in petriplate containing PDA. The colonies appeared as pure white mycelial growth and initiated production of sclerotial bodies after 9 days of incubation. Sclerotia are brown in colour and mustard seed like in shape. On the basis this character's pathogen was identified as *S. rolfsii*. Further, the identification of pathogen was confirmed from Indian Type of Culture Collection, Division of Plant Pathology, IARI, New Delhi (Ref. No. PP/3260; Date- 25/03/2019).

Mass multiplication of fungus

The sand sorghum medium (SSM) was used for mass multiplication (mass inoculum) of *S. rolfsii* in laboratory. The sorghum grains were soaked in water for overnight. These grains were spread on clear blotter paper for drying. The grains were taken in tray and @ 25 g/kg calcium carbonate was added in grains to avoid clumping. About 150 g moistened grains of sorghum and 75 g dry sand was filled in each 500 ml conical flasks, plugged, wrapped with paper and autoclaved for 30 min at 121.6 ℃ temperature & 1.05 kg/cm^2 pressure 2 times, by keeping 24 hrs. gap between 2 successive autoclaving. The grains in flasks were inoculated with pure culture of *S. rolfsii* (6-8 bit) of mycelial disc of 6 mm cut with the help of sterilized cork borer under aseptic condition and incubated at 25±1 ºC for 2 weeks. The flasks were shaken on alternate days to avoid clumping of grains and to facilitate early growth of fungus on grains. The grains turned whitish due to mycelial growth of test pathogen. The giant culture so obtained will be used for further studies.

Proving the pathogenicity by different inoculation method

Soil inoculation

Sterilized soil was taken in cemented pots of size 45 x 30 cm^2. Two weeks old culture grown on sorghum grains 10 g mass culture of *S. rolfsii* grown on sorghum seeds was added to upper 15 cm layer of soil in pots and mixed thoroughly with soil before 48 hrs. of sowing to get sick soil. Then healthy seeds of collar rot chickpea variety (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution, washed thrice in tap water before sown in the pots containing sterile soil. Pots without *S. rolfsii* culture served as control. Sterilized seeds were sown in earthen pots (containing infested soil) to test the effect of pathogen on germination per cent and pre and post emergence mortality recorded at regular interval up to 45 days after sowing. Moisture content in soil was maintained to field capacity by adding required amount of water periodically.

Seed inoculation

Healthy seeds of chickpea (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution for one minute followed by three washings with sterilized water and seeds were dressed with test fungi @ 4g/kg seeds, 10 treated seeds were sown in sterilized soil in cemented pots. Proper isolation distance and replication were maintained. No seed dressing with test fungus was used as control. Observations on germination and pre-emergence mortality were recorded on seven days after sowing. Post emergence seedling mortality was recorded at regular interval up to 45 days after sowing.

Seedling inoculation

Sterilized soil was taken in cemented pots, healthy seeds of chickpea (JG-14) were soaked overnight in water and surface sterilized with 0.1% $HgCl_2$ solution for one minute followed by three washings with sterilized water sown in cemented pots in net house. The pathogen was inoculated on fifteen days old seedlings at collar region @ 20g/pot. Proper control was maintained where pathogen was not inoculated. Observations on the mortality of seedlings was recorded seven days after inoculation at regular interval.

The data presented in Table- 4.2 revealed that the germination of seed was only 70% in infested soil, 35% in seed dressed with pathogen and 86% where pathogen inoculated in seedling stage, while highest germination *i.e.*, 90% was recorded in control (uninoculated). Pre-emergence mortality of 30% was observed in *S. rolfsii* infested soil and highest 65% in seed inoculating with pathogen as compared to no mortality in control. Similarly, the 95% post emergence mortality was recorded in the test fungus infested soil and 100% in both as well as in seed and seedling inoculation method as compared control. Re-isolation was made from such affected portion of the plant tissue and compared with that of original culture for proving Koch's postulates.

Table 2: Testing of pathogenicity by different inoculation method

Treatment	Germination %	Mortality %	
		Pre-emergence Mortality	Post emergence Mortality
S. rolfsii inoculated in soil	70.00% *	30.00%	95.00%
S. rolfsii inoculated with seed	35.00%	65.00%	100.00%
S. rolfsii inoculated in seedling	86.00%	0.00%	100.00%
Un inoculated	90.00%	0.00%	0.00%

* Average of three replications.

Summary and Conclusion

To find out the prevalence of collar rot of chickpea on farmer's field and research field a roving survey of field in chickpea growing areas were undertaken at different locations of nearby places of ARS, Ummedganj, Kota district of Rajasthan, to assess the incidence of collar rot on chickpea during *Rabi* 2018-19. In all the locations surveyed none of the field remained free from the disease. Maximum incidence was recorded in the field of Agriculture Research Station, Ummedganj & Mechanized Agriculture Farm, Ummedganj (19.82%), followed in decreasing order in the field of Galana (14.38%) Sultanpur (12.05%), Sangod (11.20%), Itawa (10.40%), Dhakerkhedi (9.32%) and Char-chouma (6.69%). Thus, the incidence of collar rot in chickpea was in the range of 6.69% - 19.82%, which was quite high to causing seedling mortality and cause considerable losses to the crop. The symptoms of collar rot in chickpea include initial yellowing of affected plant leaves and show signs of rotting at collar region. On uprooting such plants, the collar region was covered with a white cottony mycelial growth and large number of sclerotial bodies of pathogen on collar region.

Plate 1: Isolated Fungus growth and sclerotia within plate & pure culture slant

Plate 2: Symptoms sowing Chickpea seedling and pre-emergence seed rotting

Plate 3 (a): Symptoms sowing chickpea seedling (Seedling inoculated) and seed rotting (Seed inoculated)

Plate 3 (b): Pathogenicity test of *S. rolfsii* by soil inoculation

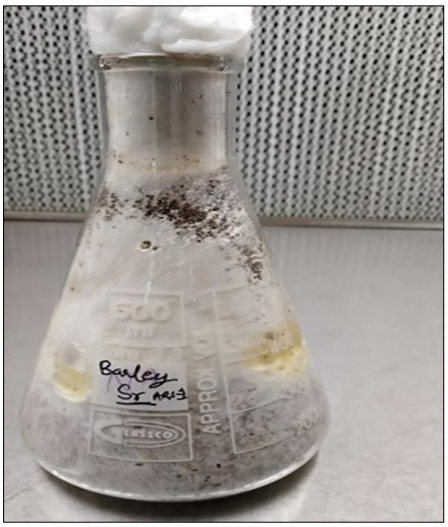

Plate 3 (c): Mass multiplication of Pathogen (*S. rolfsii*)

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