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Research Paper

Study on the Biology of the *Sclerotinia Sclerotiorum* Pathogen, Causing White Rot of Pepper

Natalia Karadzhova*
Maritsa Vegetable Crops Research Institute (MVCRI), 32 Brezovsko shosse Blvd., 4003 - Plovdiv, Bulgaria

ABSTRACT:*In this paper, the cultural characters of Sclerotinia sclerotiorum fungus on different culture media and the ability to form sclerotia under different culture conditions were studied. The culture medium was found to influence both colony morphology and mycelial growth rate and sclerotial formation in Sclerotinia sclerotiorum. The growth rate and mycelial mass of Sclerotinia sclerotiorum increases on media with a high amount of carbohydrates. Incubation of the fungus under successive alternating dark/light regimes, other conditions being equal, stimulates sclerotia formation. The mass of sclerotia increases on organic media and media with a large amount of carbohydrates. The viability of Sclerotinia sclerotiorum sclerotia is maintained for several months, with the germination capacity of large sclerotia being significantly higher than that of small sclerotia.The results of research on the biology of the fungus Sclerotinia sclerotiorum will serve to create models for predicting and selecting measures to control pepper white rot, as well as for propagation and storage of the pathogen for research purposes.*
KEYWORDS: *Sclerotinia sclerotiorum, Mycelial Growth, Sclerotial Formation, Sclerotia Viability*

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I. INTRODUCTION

*Sclerotinia sclerotiorum*fungus (Lib) de Bary is a poorly specialized phytopathogen that infects a wide range of plants causing stem rot [1,2, 3, 4] and has a wide geographic distribution [5]. Control of infection is quite difficult as the fungus is renewed by sclerotia stored on plant debris and in the soil for long periods. Currently, there are no fungicides registered for the control of this disease on pepper. Along with the development of chemical and agronomic control measures, an important aspect is the search for resistant or tolerant plant forms. The level of resistance of pepper cultivars to *Sclerotinia sclerotiorum* is unknown and potential sources of resistance to the pathogen in *Capsicum annuum* and other Capsicum spp. have not been identified [6, 7, 8, 9]. In this regard, despite the considerable number of works devoted to this pathogen species, further studies on its biology are needed to understand the physiology of the fungus and its relationship with the host plant [10, 11, 12].

The aim of the present work is to study the morphological and cultural characteristics of *Sclerotinia sclerotiorum* on different culture media and the ability to form sclerotia under different culture conditions.

II. MATERIALS AND METHODS

Isolation and Diagnosis of the Pathogen

For the work, a*Sclerotinia sclerotiorum* isolate (Scsc1) from pepper originating from a greenhouse in the village of Malo Konare, the region of Plovdiv was used, withtypical symptoms of white rot - wilting of the plant, rotting on the surface of the stem, accompanied by a white sterile plaque, formation of black, different-sized sclerotia inside the stem. Isolation was carried out using the following methodology: small fragments of the affected stem are washed under running tap water for one hour. They are then sterilized for 15 seconds in a 0.1% solution of silver nitrate [AgNO3], washed with sterile water, dried with sterile filter paper, and placed in a Petri dish on potato-glucose agar (PGA) with 2% glucose. Streptomycin sulphate 60-80 mg/l is added to inhibit bacterial growth.

The isolate was identified by microscopy as the *Sclerotinia sclerotiorum*fungus (Lib.) de Bary. In the diagnosis of the pathogen, the determinants of Pidoplichko[13] and Stancheva [14] were used.

Influence of Energy Sources on Mycelial Growth

The study on the influence of energy sources on mycelial growth was conducted by varying the glucose content of potato glucose agar (PGA)–glucose-free, with 1% glucose and with 2% glucose under different incubation regimes - natural light (room temperature, +19-21°C), without light (with temperature of +23°C and +25°C) and alternating regimes (10 days - darkness, with temperature of +20°C, then 10 days of light at +19°C - 21°C). The experiment was laid out in 4 treatments, 5 Petri dishes in each treatment.

Growth intensity was judged by the diameter of the colony on day 3, when the fungus was most active.

Additionally, comparative experiments were conducted to monitor the mycelial growth intensity and sclerotial formation of the fungus on three types of agarized nutrient substrates: synthetic Czapek-DoxAgar (SCDA), semi-synthetic soy-glucose agar (SGA) and natural nutrient substrates - barley grains (BG), agarized carrot, barley and wheatgrain broth (CBWB) and carrot blocks (CB).

Influence of Energy Sources on Sclerotia Formation

The formation of sclerotia and their development were studied onsemi-synthetic potato-glucose agar (PGA) with 1% glucose; (PGA) with 2% glucose; agarized semi-synthetic soy-glucose agar (SGA) with the following composition: per 1 liter of water (g) - KH2PO4 -2, (NH4)2SO4-1, MgSO4-1, glucose-20, soybean meal-10.

On two organic media: 1. autoclaved and then moistened barley grains decomposed in Petri dishes with a layer of approximately 1 cm; 2. agarized decoction of carrots and barley and wheat grains (50 g of each ingredient per 1 liter of water).

Beakers containing 20 ml of medium were inoculated with a 5-mm-diameter block cut from the edge of a 5-day-old fungal colony grown in (PGA) with 2% glucose[15]. The full duration of incubation of Petri dishes with the fungus was 21 days from the time the culture medium was inoculated with the fungus [16]. Each variant contained 6 Petri dishes with the pathogen.

Assessment of Fungal Biomass Accumulation on Liquid Culture Media

The assessment of fungal biomass accumulation was studied on the different liquidculture media:

- glucose-free potato culture media (PLM);
- potato culture media with contents of 1% glucose (PLM) 1%;
- potato culture media with contents of 2% glucose (PLM) 2%;
- standard Czapek-Dox culture media with contents of 3% of glucose (CzDLM);
- soy-glucose culture media (SGLM).

Fermentation was carried out in an Inkubations-Schüttelschrank BS-4 B. Braun shaker (120 rpm) at +21°C for 72 hours, in 250 ml flasks of liquid culture medium, in three replicates for each variant (5 culture media). Biomass accumulation was monitored per variant in g/100 ml. For this purpose, a centrifugation program was used with a speed of 5000 rpm, a temperature of +3° C and an exposure time of 10 minutes, allowing the separation of the liquid culture medium from the biomass (mycelium).

To study the viability of the sclerotia, they were removed from the surface of the culture medium on which the fungus was cultured, measured, dried for 6 days at room temperature and weighed. This exposure was chosen experimentally by weighing the sclerotia daily for 10 days. On the 6^{th} day, the water content of the sclerotia was minimized and stabilized.

Storage of the sclerotia was performed in paper bags under ambient conditions.

Study of the Viability of the Sclerotia of the Fungus

Sclerotia were sown for germination on (PGA) with 2% glucose according to the following scheme: immediately after drying, after 1, 2, 3, 4 and 6 months of storage. For this purpose, the sclerotia were divided into small (0.2-0.3 cm), medium (>0.3-0.4 cm) and large (>0.4-0.6 cm) fractions, and each fraction was examined separately.

Sclerotia were washed in running water for two hours to soften the sclerotial envelope, surface sterilized for 1 minute with 0.1% silver nitrate solution, then rinsed twice in sterile water, dried with sterile filter paper, and placed on blocks cut from agar culture medium (6 blocks per beaker). Sclerocyte plates were incubated under ambient conditions for 7 days [17]. On the 8^{th} day, the number of germinated sclerotia was counted.

III. RESULTS AND DISCUSSION

Cultural Characteristics of *Sclerotiniasclerotiorum* Isolate (Scsc1).

At the beginning of its development, the mycelium of the fungus is visually poorly expressed, spider-like. After a time, abundant dichotomous branching of the primary hypha begins [18, 19] and a distinct, at

first white dense, later greying mycelium appears. The reverse of the colony on (PGA) is white, on soy-glucose agar it is dark yellowish. After maturation of the sclerotia, a putrid odour appears. It is weaker on (PGA), more pronounced on natural substrates. After the sclerotia have fully matured, the smell weakens and is absent in the old crop.

Influence of Energy Sources on Mycelial Growth of *Sclerotiniasclerotiorum*

The mycelial growth rate of (Scsc1) depends on the composition of the culture medium (Table 1).

The colony diameter of the fungus 3 days after incubation on high glucose medium was larger compared to the other treatments. Mycelial growth under natural light and +19-21°C conditions was 4.6 mm on 1% glucose medium and 5.6 mm/24 h on 2% glucose medium. In our experiment, increasing the incubation temperature of (Scsc1) from +23°C to 25°C in light-free conditions did not significantly affect the mycelial growth rate. The lower incubation temperatures also showed a trend towards faster colony growth on medium with higher glucose content, about 6.5 mm on medium with 1% glucose and 7.5 mm/24 h on medium with 2% glucose.

Table 1. Colony growth of Sclerotinia sclerotiorum on (PGA) with different glucose content under different temperature and light regimes.

Content of glucose in (PGA), g/l	Colony growth, mm/24 hours on the 3rd day		
	Natural light	Darkness	
	+19 - 21ºC	+23ºC	+25ºC
0	2.85±0.1	4.36±0.1	4.36±0.1
10	4.60±0.2	6.45±0.1	6.66±0.1
20	5.61±0.1	7.17±0.2	7.90±0.2

The culture medium influences both colony morphology and the rate of mycelial growth and sclerotia formation in *Sclerotinia sclerotiorum*. The mycelial growth rate of the fungus is highest on semi-synthetic media containing natural components. On the third day of sowing, the colony diameter of (Scsc1) was 44-46 mm on (SGA) and (CBWB) agar, 20 mm on 1% and 2% (PGA) and 32.5 mm on sterile barley grains. In these variants, the fungal mycelium occupied the entire surface of the culture medium on the fifth day of counting, and in the 1% and 2% (PGA) variants– on the 6th day (Table 2).

Table 2. Growth of the colony diameter of *Sclerotinia sclerotiorum* on cultural media with different composition and glucose content in a thermostat without light at a temperature of 23ºC.

Medium/glucose content,g/l	Colony diameter (mm) on 3, 5 and 6 days			Colony growth, mm/24 hours
	3-day	5-day	6-day	на 3-day
(PGA)- 0	13,68	41,67	56,67	4,36
(PGA)- 10	19,58	80,41	90,00	6,45
(PGA)- 20	21,50	82,50	90,00	7,17
(SGA) - 20	43,75	90,00	90,00	14,58
(CBWB) glucose-free	46,25	90,00	90,00	15,42
(BG)glucose-free	32,50	90,00	90,00	10,83

Abbreviation: (PGA) – potato-glucose agar, (SGA) – soy-glucose agar, (CBWB) – agarized carrot, barley and wheat grain broth, (BG) – barley grain.

Dynamics of Mycelial Growth and Sclerotia Formation

The morphology of the fungal colony cover changes dynamically by day and has its own characteristics on different culture media.

On day 3 of sowing on barley grains, mycelium was abundant and dense, but weak and tender on (PGA) 1% and (PGA) 2%. On day 5 on (SGA), abundant, dense mycelium was formed with a tendency to thicken and form sclerotia; on (CBWB), mycelium was tender and loose, without the beginnings of sclerotia. In the variant with barley grains the mycelium is diffusely distributed over the entire surface of the food substrate, and in the middle of the Petri dish there is a well-developed dense mycelium on which large black sclerotia form. On day 6, small to medium-sized sclerotia form on (SGA) and on (CBWB), mycelial thickening was observed at the periphery of the colony. On day 7, mass mycelial thickening was observed on (PGA) 1% and the

first sclerotia began to form on (PGA) 2%. In the (SGA) and (CBWB) variants, a process of abundant sclerotia formation from the periphery towards the centre of the colony begins. On the barley grains, the fungus massively formed large black sclerotia. Mass formation of sclerotia of different size fractions was observed on all culture media on the 17th day of incubation. Depending on the culture medium, the size of sclerotia varied strongly: on (PGA) 1% small and medium, less frequently large fraction of sclerotia was formed; on culture media (PGA) 2%, (SGA) and (CBWB) mass formation of large sclerotia was recorded. The largest sclerotia formed on barley grains, occupying the entire surface and interior of the substrate.

Accumulation of Fungal Biomass in a Liquid Culture Medium:

An indicator of the dependence of fungal growth on the amount of carbohydrate was the accumulation of mycelial biomass from a 3-day culture in liquid medium (Table 3). In the absence of glucose in the culture medium (PLM), the mycelial mass was less compared to (PGLM) with 1% and (PGLM) with 2% glucose content. In order to select the culture medium that was most favorable for mycelial mass accumulation, Czapek-Dox liquid medium(CzDLM)and soy-glucose liquid medium (SGLM) were tested. When the fungus was grown on standard (CzDLM), little mycelial growth was observed - 1.241 g/100 mm, whereas when the fungus was cultured on (SGLM), the biomass accumulation was significant - 6.15 g per 100 ml of cultural medium, indicating that this agar is the most favourable for the growth of *Sclerotinia sclerotiorum*.

Table 3. Evaluation of biomass accumulation of Sclerotinia sclerotiorum in liquid cultural media during incubation for 72 hours and 23°C.

No.	Liquid cultural medium	Biomass in 100 ml medium,g
1	Glucose-free potatomedium	1,060
2	Potato medium with 1% of glucose	1,161
3	Potato medium with 2% of glucose	1,191
4	Czapek-Dox medium with 3% of glucose	1,241
5	Soy-glucose mediumwith 2% of glucose	6,150

Centrifugation at 5000 revolutions, temperature +3ºC degrees, exposure 10 minutes

Formation of Sclerotia on Different Cultural Medium

It is generally accepted that the size and shape of sclerotia vary depending on the growing conditions and the culture medium[18].

In our experiment, the formation of sclerotia on solid culture media was recorded in the following sequence: on barley grains - on the 5th, (SGA) - on the 6th, 2% (PGA) and (CBWA) - on the7th, and on the 10th day on 1% (PGA)(Figure 1).

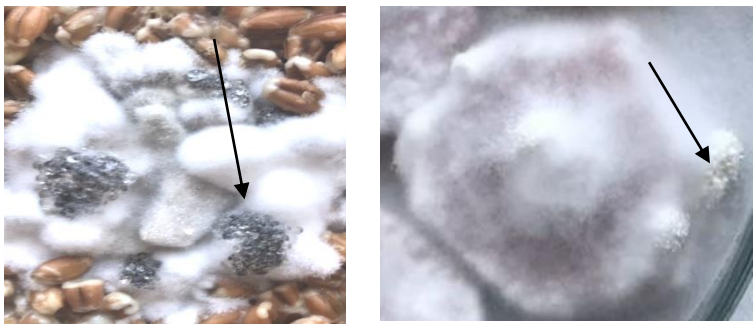

Figure 1. Sclerotia formationof *Sclerotinia sclerotiorum* onorganic media: 1 - barley grains, 2 - carrot discs.

Along the periphery of the colony, 1.0 – 1.5 cm away from the edge of the Petri dish, a ring-like area of a white mycelium was formed, which thickened and started forming germs of sclerotia on it.The germs gradually enlarged, obtain a specific shape, and, depending on the composition of the culture medium, the surface of the future sclerotium is covered with colourless droplets of moisture on days 5-6. On the following day, from the moment of the appearance of the exudate, the sclerotia begin to turn grey, then darken and the exudate gradually disappears. The sclerotial sheath thickens and sclerotia form on days 10-14 of mycelial seeding. On the 14th day of inoculation, the sclerotia have a high moisture content. On the 21stday after sowing, the sclerotia are fully mature, easily removed from the surface of the medium, in some cases raised above the

substrate, and lose 40 to 60% moisture after drying. When the fungus was incubated on (PGA) with 2% sucrose at 20%, the crude mass of 1 sclerotium averaged 10.14 mg, and after drying, 0.08 mg, 52% less.

The ripening process of small-sized sclerotia is faster and they ripen already on day 8-9 after medium inoculation.

The type of growth and sclerotia formation described above is characteristic of the isolate (Scsc1) when incubated on agar (PGA).

In the course of work, other types of sclerotia formation were also detected, e.g., in concentric circles on (SGA), in piles (barley grains, carrot discs), at large spacing (PGA1%, CBWA).

On the glucose-free potato agar, slow growth of weak myceliumwas observed, (approximately 0.5-0.7 cm), as sclerotia formed at the periphery of the colony no earlier than 10 days of incubation. When the fungus was incubated on a rich culture medium of (SGA), the mycelium reached the edge of the dish already on day 3, after which the process of sclerotia formation began. The sclerotia formed in the middle of the colony were larger in size than the sclerotia at the periphery butwere present in smaller numbers.

Shape of sclerotia of *Sclerotinia sclerotiorum*.

Sclerotia have a variety of shapes - convex above, lenticular below or with a slightly concave base, lenticular-oblong (the length slightly exceeds the width), elongated or rounded. The sclerotia are arranged more singly, or merge together and take on a convex ellipsoid shape. When grown on a hard substrate (barley grain, wheat), they fill the cavities between the grains, envelop the grains, merge, and often have an irregular shape (Figure 2).

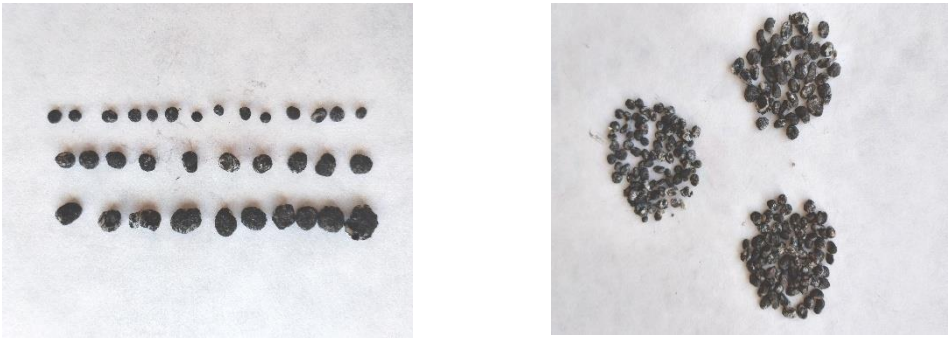

Figure 2. Different fractions of sclerotia of *Sclerotinia sclerotiorum*.

A clear dependence of sclerotium mass on the amount of carbohydrate was found. When the fungus was grown on (PGA) under all incubation regimes, the mass of sclerotium increased as the glucose content of the medium increased (Table 4).

Table 4. Influence of the food substrate and the glucose content at the 21st day from the beginning of the experiment.

Medium	Glucose content (g/l)	Number of sclerotia	Percentage content of sclerotia by fractions, (%)			Total mass of sclerotia (mg)	Mass of one sclerotium (mg)
			2-3 (mm)	3-4 (mm)	над 4 (mm)		
(PGA) 1%	10	19	10,5	42,1	47,4	424	22,32
(PGA)2%	20	16	37,5	62,5	0,0	470	26,23
(SGA)	20	19	47,4	36,5	16,1	5310	27,66
(CBWBA)	0	14	100,0	0,0	0,0	80	5,71
(CD)	0	38	31,6	15,8	52,6	1000	26,32
(BG)	0	365	53,3	35,0	11,7	10730	29,38

Development of the fungus on organic media.

The effect of the culture medium on the mass of the sclerotia and their size was confirmed during incubation of the fungus on organic media. On carrot discs, sclerotia reached 0.9 cm (0.5-0.9 cm) and the mass of a sclerotium was 26.32 mg. These indices reach even higher values when the fungus is grown on barley grains-the size of the sclerotia is up to 1 cm (0.5-1.0) and the average weight is 29.38 mg.

The total mass of sclerotia in a Petri dish with the same amount of glucose in the culture medium was almost the same regardless of the temperature and light–about 424 mg at 10 g glucose and about 470 mg at 20 g (Table. 4). Accordingly, as the number of sclerotia increased, their mass was lower and vice versa. With alternating dark/light regimes, both the number and total mass of sclerotia decreased.

Incubation of the fungus under successive alternating dark/light regimes, other things being equal, stimulates sclerotia formation. This indicates that the process of sclerotia formation on medium with sufficient nutrients may continue until the nutrient supply in the medium is exhausted. The highest number of sclerotia and their total mass were observed under alternating lighting regimes, and the maximum mass per sclerotium was observed when grown at $+25^0$C in a thermostat. In general, the mass of sclerotia in a Petri dish increased on organic media and on media with a large amount of carbohydrates.

***Sclerotinia Sclerotiorum*Sclerotia Viability**

The viability of *Sclerotinia sclerotiorum* sclerotia is known to persist for several months [19]. In our experiments, after 5 months of storage, most of the sclerotia remained viable. The viability of sclerotia was found to be size dependent. The germination ability of large sclerotia was higher. At the end of the storage period, it decreases by approximately 14%, whereas it is almost halved in the small ones. This may be explained by the limited amount of nutrients in small sclerotia (Table 5).

Table 5. The viability of sclerotia depending on the period of storage and mass.

Sclerotia size (cm)	Percentage (%) of germinated sclerotia after storage (months)				
	1	2	3	4	5
0.2-0.3 (small)	83.0±4.6	81.0±5.2	78.0±6.1	74.0±6.3	58.1±4.0
>0.3-0.4 (medium)	86.0±3.1	74.0±4.4	83.0±3.7	80.2±3.9	81.0±3.1
>0.4-0.6 (large)	89.1±2.6	86.5±3.1	85.3±3.0	86.7±3.1	84.6±3.0

IV. DISCUSSION

The period of preservation of sclerotia in the soil is, according to some data, about 2.5 years, according to others, up to 8 years [20]. Presowing treatment of seeds with fungicides protects seedlings of crops for 40 days from diseases, pathogens of which are active already in early spring [21]. To reduce the damage of plants by *Sclerotinia sclerotiorum*, first, it is necessary to observe the correct crop rotation, and for this, in addition to information about the possibility of maintaining the ability of sclerotia to germinate in the soil, it is necessary to know what their potential viability is [22]. In our experiments, after 5 months of storage, most of the sclerotia remained viable.The results of research on the biology of the fungus *Sclerotinia sclerotiorum*will serve to create models for predicting and selecting measures to control pepper white rot, as well as for propagation and storage of the pathogen for research purposes.

V. CONCLUSIONS

As a result of the conducted studies, it was found that the growth rate and mycelial mass of *Sclerotinia sclerotiorum* increased on media with a large amount of carbohydrates and, especially, on media that included soybean meal (SGA). The same pattern applies to sclerotia formation - the higher the nutrient properties of the medium, the more sclerotia are formed and the larger their size. The best indicators for mycelial growth and sclerotial formation are a constant incubation regime at +23-25°C in a thermostat or in natural light.The viability of *Sclerotinia sclerotiorum* sclerotia is maintained for several months and the germination ability of large sclerotia is significantly higher than that of small sclerotia.

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