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Obtaining, maintaining and characterizing the morphogenesis of a pure culture of *Septoria helianthi* Ellis & Kellerm pathogen *in vitro*

Levytska Kh[1,2]*, Lyakh V[2,3], and Soroka A[3]

[1] *Ivano-Frankivsk National Medical University, Ivano-Frankivsk, 76000, Ukraine.*
[2] *Zaporizhzhia National University, Zaporizhzhia, 69600, Ukraine.*
[3]*Institute of Oilseed Crops of NAAS, Zaporizhzhia, 70417, Ukraine.*

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Abstract

Septoria leaf spot is becoming increasingly widespread in Ukraine. However, methods of isolation and features of *Septoria helianthi* morphogenesis *in vitro* remain not studied in detail. Our study aimed to establish the characteristics of the isolation, cultivation and development of pathogen under *in vitro* conditions. We modified the method for isolating *S. helianthi*. The potato-glucose agar medium was suitable for cultivating the pathogen and the pathogen retained its pathogenicity long time under optimal thermostat conditions on a nutrient medium. It has been proven that the development of *S. helianthi* colonies *in vitro* changed under the influence of cultivation temperature.

Keywords – colony – *in vitro* – nutrient medium – pathogen – *Septoria helianthi* – sunflower

Introduction

Sunflower *Septoria* disease is caused by the *Septoria helianthi* Ellis & Kellerm fungus (Das et al. 2020). *Septoria* leaf spot is widespread throughout the world including Ukraine (Block 2005, Liu & Li 2007, Marques et al. 2011), where its prevalence sometimes increases to 100% (Retman et al. 2020). It is known that *Septoria* leaf spot can cause damage to sunflower plants together with other fungal diseases (Levitskaya et al. 2022). The disease develops at an optimal temperature of 22–25°C. The primarysource of infection is crop residues, and initial inoculation occurs with pycnospores. In the presence of favorable weather conditions for the pathogen, the *Septoria* leaf spot can threaten sunflower crops (Petrov & Arsenijevic 1996).

The harmfulness of *Septoria* leaf spot manifests itself in the suppression of plants, premature drying of leaves, which leads to a decrease in chlorophyll content and an increase in the intensity of transpiration. The disease causes the destruction of chloroplast, which leads to a decrease in the size of the assimilation surface and leads to a decrease in the rate of photosynthesis and respiration rate (Brand et al. 2020).

The nature of the pathogen cannot be determined based only on the existing symptoms of the disease. Similar symptoms can be caused by different pathogens. This complicates the diagnosis of a particular disease. That is why the isolation and identification of the *S. helianthi* pathogen is an important component for studying the biological characteristics of this pathogen and, accordingly, for protecting sunflower cultivation from *Septoria* leaf spot.

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Corresponding Author: Khrystyna Levytska – e-mail – krlevitskaya92@gmail.com

There are methods to isolate fungal pathogens in pure culture, including *Septoria* leaf spot pathogens in different crops (Belay 1982, Eyal et al. 1987). However, there are no generally accepted methods for isolating and maintaining the *S. helianthi* pathogen. According to this, it is necessary to master the method of cultivation of the pathogen and know the specifics of the development of *S. helianthi* under *in vitro* conditions.

Our work aimed to establish the characteristics of the isolation, cultivation and development of *Septoria helianthi* under *in vitro* conditions.

Materials & methods

Sample collection and preparation

The sunflower samples affected by *Septoria* leaf spot served as the material for the study. Affected sunflower leaves were collected at the stationary infectious disease nursery of the Institute of Oilseed Crops of the National Academy of Agrarian Sciences. For isolation of the pathogen *S. helianthi*, we used fresh and herbarium leaves of sunflower with symptoms of damage. The stationary infectious plot was organized in the field crop rotation of the Institute of Oilseed Crops in 2005 to evaluate the breeding material of oil crops for a complex of diseases, including *Septoria* leaf spot. Every year it is enriched with infected plant residues collected in different regions of the Ukraine.

Research on the isolation and cultivation of the *Septoria* leaf spot pathogen was carried out in laboratory conditions at the Institute of Oilseed Crops of the National Academy of Agrarian Sciences. Laboratory studies were carried out based on methods generally accepted in phytopathology and mycology (Belay 1982, Eyal et al. 1987). These methods were supplemented or modified according to our research.

Potato-glucose agar medium was used to cultivate the *Septoria* leaf spot pathogen. The nutrient medium was prepared in advance, according to the standard method of V. Belay (1982). Modifications were made in the preparation of the medium, which was as follows: the volume of the medium was prepared for 500 ml of water, and not for 1 liter, as indicated in the method; boiled the potato broth for 40 minutes; the amount of glucose and agar was equal to 6 g; sterilization of the medium was carried out at 1.5 atm and a temperature of 120°C, 35 minutes; immediately after sterilization, the nutrient medium was poured into sterile Petri dishes, previously with the addition of 2 ml of antibiotic (gentamicin). The Petri dishes for dispensing the medium were prepared in advance. They were pre-sterilized in a dry heat oven at 160°C for 1 hour. After pouring, the nutrient medium in the Petri dishes was left until it solidified. Next, the dishes with the medium were wrapped with sterile film. This method is less labor-intensive and ensures long-term storage of the nutrient medium in sterility and suitability for use for up to 14 days or more.

Isolates and morphology

Mycological studies were carried out in laminar under sterile conditions. Isolation of the *S. helianthi* pathogen and inoculation on a nutrient medium were carried out based on two stages of standard methods, supplementing them with the author's ones. At first, the isolated spores of the pathogen were transferred onto a nutrient medium and the colony was cultivated for 7–10 days, after the specified time, this colony was transferred to a fresh nutrient medium and cultivated for 30 days. The colony of *S. helianthi* was cultivated on a nutrient medium in the dark with a temperature of 15, 21–24, and 27°C at a thermostat. Identification of the *S. helianthi* pathogen was carried out using a microscope based on the morphological characteristics of the spores and the cultural and morphological characteristics of the mycelium, according to literary sources (Acimovic 1998). Observation and description of the development of *S. helianthi in vitro* were carried out in comparison with data from literature sources (Crous et al. 2013, Monteiro et al. 2018, An Yuan-Yan et al. 2021). The size of *S. helianthi* spores was calculated using standard calculations (Buric et al. 2021).

Next, control of the presence of spores of the pathogen was carried out. To do this, a drop of the resulting suspension was placed on a sterile glass slide and examined under a microscope. The spores of the pathogen were straight, elongated, and transparent with membranes. The size of *S. helianthi* spores was about 65 µm, and the number of septa varied from 3 to 5 (Fig. 2a). According to identification from literature sources, the obtained spores corresponded to pycnospores of *S. helianthi*.

Results

Isolation of *S. helianthi* and obtaining a pure culture of the pathogen

Isolation of the pathogen *S. helianthi* was carried out from fresh cotyledons leaves or true sunflower leaves with *Septoria* leaf spot symptoms on the day of collection in the laboratory. Treatment of affected leaves was carried out under sterile laminar conditions. Affected necrotic particles were cut out from the collected leaves, and previously washed under running water for 5 minutes. The crushed infected material was washed with sterile distillate for 1 minute and disinfected with a solution of 96% alcohol diluted in the proportion of 1 part alcohol solution:3 parts sterile distilled water for 1 minute. To wash off the disinfectant solution, the leaf material was washed in a sterile distillate in a separate Petri dish and dried on sterile filter paper for several minutes. After surface sterilizing, the infected material was soaked in sterile distilled water for 20–30 minutes in a sterile Petri dish (Fig. 1).

Fig. 1 – True sunflower leaves affected by *Septoria* leaf spot, soaked in distilled water to obtain spores of *S. helianthi* pathogen.

If spores pathogen were present, this suspension was transferred onto a nutrient medium. To do this, a drop of the resulting suspension was taken with a sterile loop and sown according to the "stroke" sowing pattern on a solid nutrient medium. The preparation of the nutrient medium was carried out in advance, based on potato broth with the addition of glucose and agar (PGA) in equal quantities (6 g of agar and 6 g of glucose were added per 500 g of potatoes), following the standard procedure with our modifications. The sterile nutrient medium was poured into Petri dishes under laminar conditions. After sowing, the material was placed in the dark in a thermostat for 7–10 days at a temperature of 23°C. After the specified time, the growth of the fungal colony was observed in the direction of the stroke (Fig. 2b).

After 10 days of sowing, the colony of the pathogen was transferred to a fresh PGA medium of similar composition, observing sterile conditions. After transfer, the pathogen was cultivated at a temperature of 21–24°C in a thermostat for a month. The pure culture of *S. helianthi* was maintained at a temperature of 22–23°C for preservation, and it was transferred to a fresh nutrient medium, if necessary.

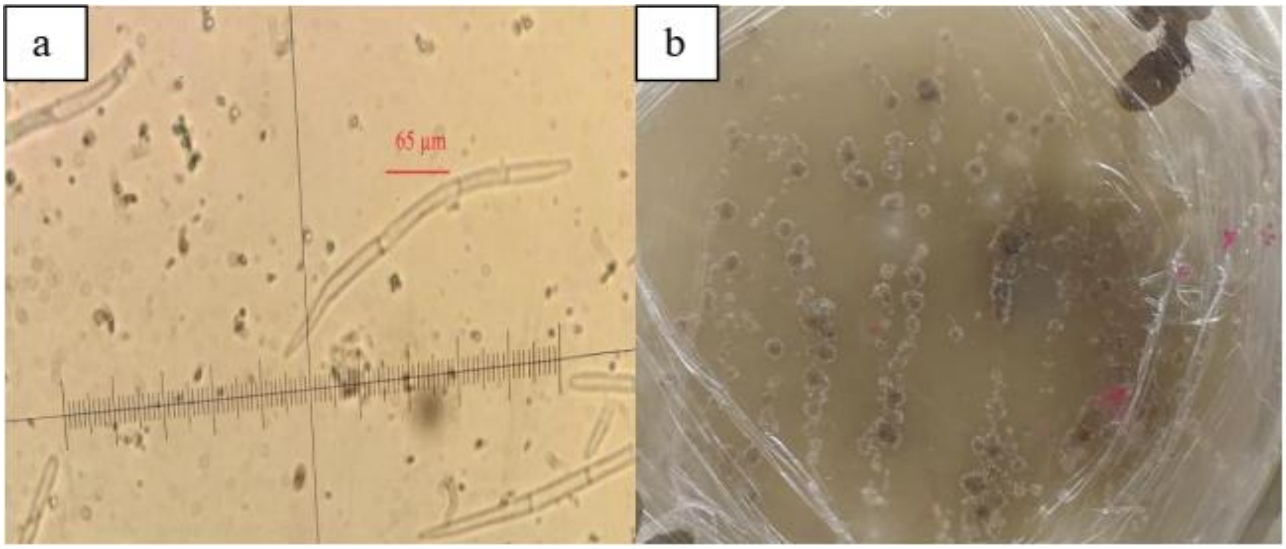


Fig. 2 – Pycnidiospores (a) and culture of *S. helianthi* pathogen. (b) on potato-glucose agar medium.

According to our research, it should be noted that to isolate and subsequently obtain a pure culture of the *S. helianthi* pathogen, one can use both herbarium leaves and fresh true and cotyledon leaves of sunflower with symptoms of damage. However, the isolation of the pathogen from sunflower cotyledon leaves with existing pycnidia in the affected areas was the most successful. After all, it was the cotyledon leaves affected by *Septoria* leaf spot, that were not infected by a foreign infection that was present at the stationary infectious nursery, which in turn ensured obtaining a pure culture of the fungus.

Characteristics of morphogenesis of S. helianthi colonies in vitro

After sowing the spore suspension on the PGA medium, colonies of *S. helianthi* pathogen were located in clear or diffuse rows on the substrate. Colonies glabrous, delimited along the margin, with discontinuous, low air-fibrous mycelium. The mycelium is immersed in the substrate a pale brown, brown or light olive colored. The growth of the *S. helianthi* pathogen was active, but the size of the colonies was quite limited. During microscopic examination of the fibrous mass separated from the pathogen colony for 7–10 days, fungal spores were detected.

The resulting colony was transferred onto a fresh, same in composition nutrient medium, and subsequently, further growth of the fungal colony was noted. It was observed that the size of colonies *in vitro* conditions was different. Thus, some colonies occupied ⅛ of the surface of the medium, others – ½ of the surface, and some occupied the entire surface of the medium.

It should be noted that when a small part of the pathogen colony was transferred to a fresh nutrient medium, its development continued. Approximately from the 10th day after re-seeding, the formation of mycelium on the colonies was noted. In the pure culture, the pathogen has septate, dense, swoolly or heavily pubescent, airy mycelium, the color varied from white to dark gray. On the reverse side of the dish, in places where the colony grew, the dark olive, almost black mycelium was immersed in the substrate in diffuse bunches. Its structure was compact, long hyphae separated by thin walls. The resulting mycelium corresponded to the *S. helianthi* pathogen.

The study revealed the appearance of formations in the shape of tortuous, accumulated formations along the margin with or without woolly mycelial (Fig. 3a). According to the literature,

these formations were identified by us as coal-black pycnidial conidiomas. Pale watery droplets were noted in the center of the formations. On the surface of the mycelium of the *S. helianthi* colony, a protruding pale pink mass was observed in some places (Fig. 3b). We determined, based on literary sources, that this is a sporulation colony of the *S. helianthi* fungus.

The development of the *S. helianthi* colony under *in vitro* conditions changed under the influence of cultivation temperature. Certain differences were identified in the nature of the formation of the mycelium of the *Septoria* leaf spot pathogen when it is cultivated at different temperatures on a nutrient medium (Table 1).

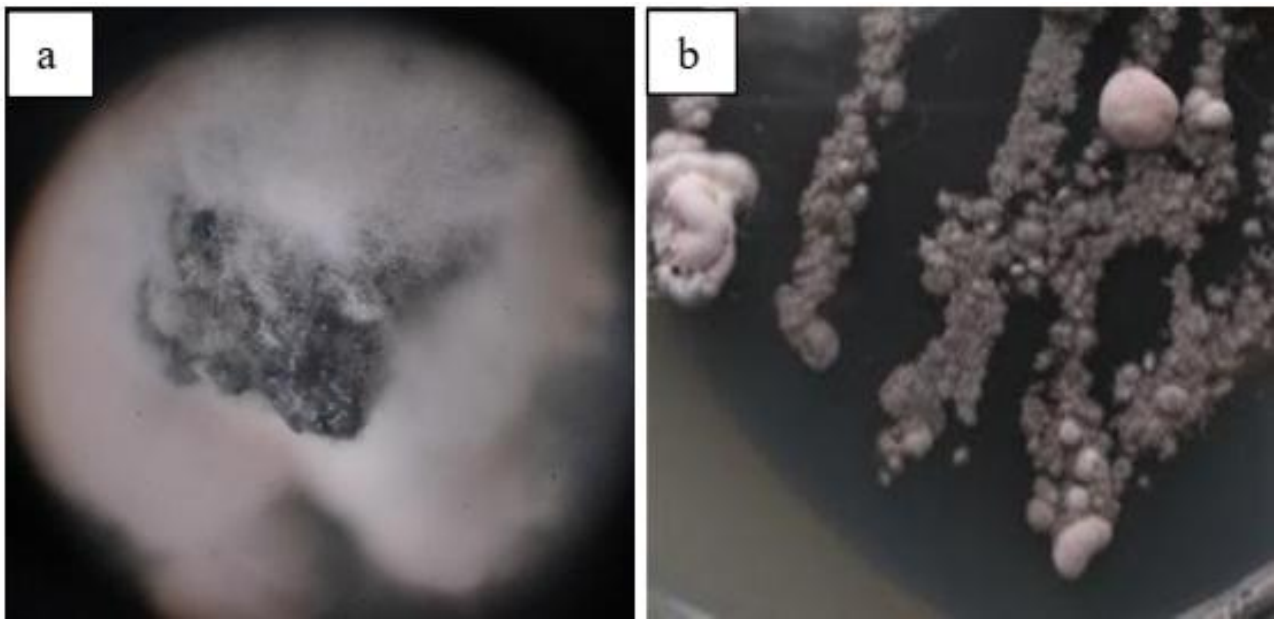


Fig. 3 – Pycnidial conidiomas in a woolly mycelium. (a) and fungal spore mass. (b) formed in a pure culture of *S. helianthi.*

Table 1 Characteristics of *Septoria helianthi* on potato-glucose agar medium when cultivated at different temperatures.

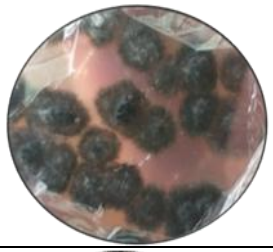
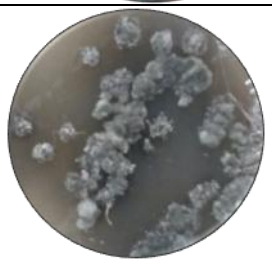
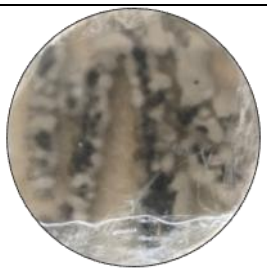
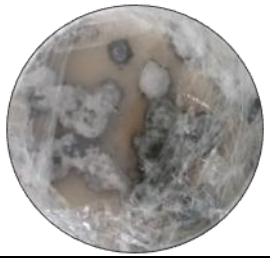
Cultivation temperature	Characteristics of colonies	nutrient medium Appearance of colonies on a
≥ 15°C	The colonies acquired a gray-black color, clustered or scattered on the substrate. On the reverse side of the dish, a black color of the substrate was noted under the areas of the colony. The formation of a pink pigment was observed, spreading into the surrounding agar medium. Mycelium wasn't observed.	
21–22°C	Colonies were brown, gray or black. Conidiomas were formed on the colonies, which were coal-black in color with uneven edges, free of mycelium. Then, low air-fibrous mycelium gradually formed on them.	

Table 1 Continued

Cultivation temperature	Characteristics of colonies	Appearance of colonies on a nutrient medium
23°C	The mycelium is usually gray, less often white, septate, woolly, located along the margin or in the center of the colony. Subsequently, conidiomas were formed and gradually became covered with mycelium along the margin. In some places, a pale pink mass was noted. Mycelial growth continued and was quite productive.	
24–25°C	The mycelium acquired a white color, it was heavily pubescent, dense, and covered the entire colony. Conidiomas were round in shape, completely pubescent with mycelium. The growth of the colony was quite slow.	
≤ 27°C	Colonies had previously formed white or gray mycelium that completely or partially covered the colony. No further development of the colony was observed.	

The table shows that the optimal temperature is 21–23°C range for cultivating the *Septoria* leaf spot pathogen.

The growth of *S. helianthi* colonies at different cultivation temperatures also showed differences (Fig. 4).

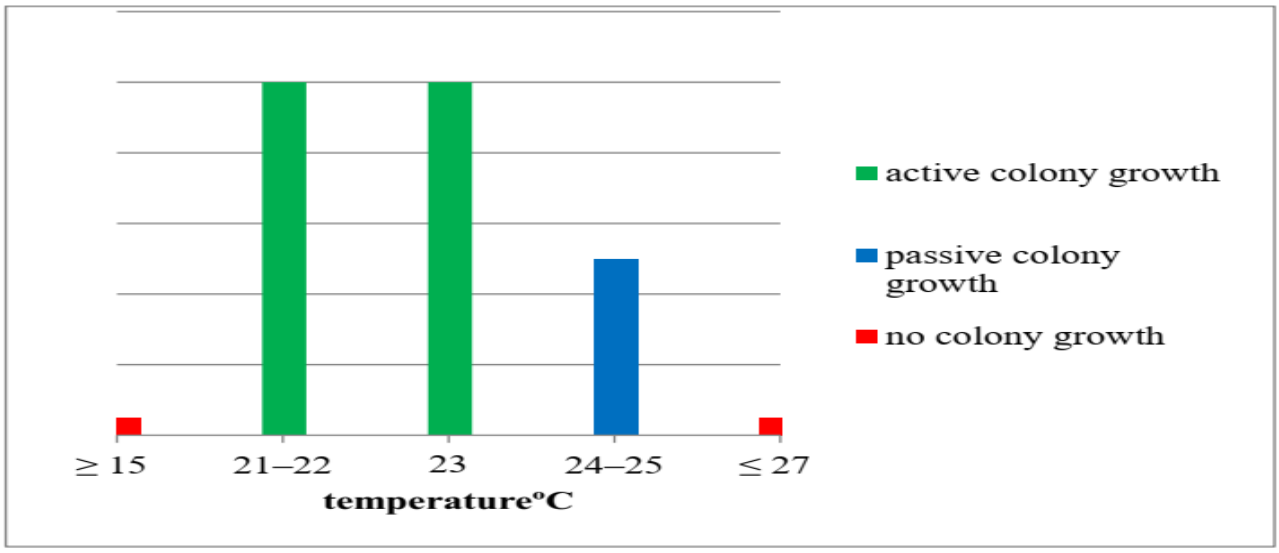


Fig. 4 – Growth of *Septoria helianthi* colony *in vitro* at different temperatures of cultivation.

Active growth of the colony of the *Septoria* leaf spot pathogen *in vitro* was observed at temperatures from 21 to 23°C, while it was passive at a temperature of 24–25°C. At temperatures of 15°C and below or 27°C and above, there was no colony growth. That is, the optimal temperature for cultivation of this pathogen is 21–23°C.

The resulting pure culture of the pathogen *S. helianthi* was maintained in a thermostat at a temperature of 22°C, and in winter – 23°C on a nutrient medium for up on 3 months. At the end of the specified period, the colony was transferred. Colonies were transferred to fresh nutrient medium with identical composition and cultivated under the described conditions.

Spores of *S. helianthi* were isolated after the appearance of the first colony for 7–10 days.

Discussion

It should be noted that subsequently, the pathogen retained its pathogenicity and infection with mycelium for a long time, which confirms the method of artificially infecting sunflower seedlings with a 30-day culture of the *Septoria* leaf spot pathogen in phytotron conditions (Levitskaya & Lyakh 2021).

According to researchers who worked with the *Septoria* leaf spot pathogens in different crops, colonies of the pathogen germinate well on potato dextrose agar medium (Eyal et al. 1987, Xie-Jing & Bao-Nan 1988, Joshi et al. 2010). In our studies, we used a PGA medium, which ensured the active growth of colonies of the *S. helianthi* pathogen and their support for 3 months.

In studies by Xie-Jing & Bao-Nan (1988) on sunflower, it was found that the range of favorable temperatures for the development of the pathogen *in vitro* ranged from 10 to 32°C, and the most optimal temperatures were 10–28°C. However, our studies have shown that active growth of colonies of the sunflower *Septoria* blight pathogen *in vitro* was observed only at temperatures from 21 to 23°C. At temperatures of 15°C and below or 27°C and above, colony growth was not observed.

Xie-Jing & Bao-Nan (1988) also studied the influence of various environmental resources and the relationship between pH value and germination of pycnidiospores and indicated the ability of the pathogen to overwinter and still possess vital force until the next season.

Our studies were aimed at the modification of the method of isolating the pathogen from the affected material, the cultivation, describing the morphogenesis of the resulting colonies and maintaining the fungus on a nutrient medium. For culturing the fungus, we used a nutrient medium (potato-glucose agar), which was not mentioned in the work of previous authors. Our studies describe in detail the morphological features of the pure fungal culture *in vitro*, but not the characteristics of pycnidiospores germination.

During our experiment, when cultivating the *S. helianthi* pathogen, coal-black pycnidial conidiomas were formed on the colonies. And in some places of the *S. helianthi* colony, the presence of a pale pink mass protruding above the surface was observed. Similar formations were described by some scientists in their studies of different species of *Septoria* pathogens on different plant species (Monteiro et al. 2018, An Yuan-Yan et al. 2021).

It should be noted that the methods described in the literature for isolating *Septoria* pathogens in different crops are mostly based on obtaining spores for sowing in a moist chamber, isolating pycnidia from the affected leaf and transferring them to a nutrient medium, or placing affected leaves containing pycnidia directly on the nutrient medium for spore germination. However, these methods are not suitable for isolating *S. helianthi* due to the biology of the pathogen and the morphological characteristics of the sunflower leaf. We have observed that the use of a moist chamber for pathogen isolation provokes rapid growth of contaminants or other pathogens that require less time to develop than the *S. helianthi* pathogen. Other methods were not effective for isolating this pathogen. The method we described for isolating the *S. helianthi* fungus provides a simple and fast way to obtain a pure culture of the pathogen and subsequently maintain colonies in an active state for a long time.

Conclusions

The method of isolating and cultivating the *S. helianthi* pathogen was modified, in which fresh or herbarium cotyledons, as well as true sunflower leaves affected by the disease, were used to obtain spores, which were subsequently sown on a nutrient agar medium. Inoculating a suspension of spores of the *S. helianthi* pathogen provides a pure pathogenic culture. It has been determined that potato-glucose agar medium with the addition of an antibiotic is suitable for cultivating the *Septoria* leaf spot pathogen. In addition, the optimal temperature for cultivating the pathogen *in vitro* conditions is from 21 to 23°C is established. It was determined that the development of the *S. helianthi* colony under *in vitro* conditions changes under the influence of cultivation temperature, which is manifested in the nature of the formation of the pathogen mycelium and colony growth. Furthermore, it has been determined that maintaining a culture of *S. helianthi* on a nutrient medium in a thermostat under optimal temperature conditions for a long time ensures the preservation of the pathogenic ability to infect.

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Competing interests

The authors declare that they have no competing interests.

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