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Pathogenic Strains Storage of the Main Causative Agents of Cereal Crops Septoriosis (Leaf Spot) in The State Collection of Phytopathogenic Microorganisms in All-Russian Research Institute of Phytopathology

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ABSTRACT

The paper describes various ways of storing strains of three septoriosis (leaf spot disease) pathogens (Stagonospora nodorum, Septoria tritici and Stagonospora avenae f. sp. triticea) included in the State Collection of Phytopathogenic Microorganisms of All-Russian Research Institute of Phytopathology. The advantages and disadvantages of these methods were noted, the results of long-term studies on the viability and pathogenicity of strains under various storage conditions were presented. The most reliable methods and optimal storage times for different types of septoriosis pathogens were determined.

Keywords: *Strains, pathogens, septoriosis, state collection, storage, viability, pathogenicity*

INTRODUCTION

The collection of septoriosis pathogens strains in the All-Russian Research Institute of Phytopathology (ARRIP) was started in 1981. Methods for determination and stabilization on culture-morphological (CM) signs of isolates on nutrient medium, methods for assessment of pathogenicity and virulence of isolates on host plants, and ways of maintenance and long-term storage of cultures were developed by employees of the Institute [1- 8].

Currently, the collection contains 321 strains of the three main types of septoriosis pathogens, common in wheat and barley crops in Russia. They include: 107 strains of Stagonospora nodorum [Berk.] Castellani and E.G. Germano isolated from wheat (98 strains) and barley (9 strains), 212 strains of Septoria tritici Rob. et Desm. and 2 strains of Stagonospora avenae Bissett f. sp. triticea T. Johnson, isolated from wheat. The composition of the species presented in the collection covers 10 regions of the Russian Federation (Central, Central-Chernozem, North-Caucasian, North-Western, Northern, Volga-Vyatka, Volga, West Siberian, East Siberian, Far Eastern), as well as the neighboring countries (Ukraine, Belarus, Moldova, Kazakhstan, the Baltic republics). All strains were included in the State Collection of Phytopathogenic Microorganisms (SCPM) created in ARRIP in 1996 for the development of fundamental, search and applied research.

Collection strains of septoriosis pathogens are used for carrying out immunological studies both in ARRIP and in other research institutions in Russia. At the request of various selection and testing stations, the biomaterial was sent to Krasnodar Krai, Kabardino-Balkaria, Kirov Oblast, Krasnoyarsk Krai, Altai Krai and others to create an artificial

infectious background in testing varieties of wheat and barley for resistance to septoriosis. In addition, the collection strains of Septoria serve as an object for molecular genetic studies conducted at ARRIP.

Preservation of microorganisms without loss of their basic properties is a paramount importance not only for workers responsible for hundreds and thousands of cultures, but for any researcher and practitioner dealing with single microorganisms. The issues of storage are interesting for specialists from different countries. The most widely known and common way to maintain fungi live cultures is replanting to fresh nutrient media. Many organisms in the largest collections of the world have been preserved in this way for many years. However, it is known that the fungi retained in this way are sometimes subjected to significant changes with the loss of important morphological and physiological signs [9].

Like many facultative fungi, the causative agents of septoriosis exhibit considerable variability. With frequent re-sowing, strains can lose not only their cultural and morphological features, but also an important characteristic, such as pathogenicity. In order to maintain or restore pathogenic properties, it is recommended to periodically carry out the passages of strains through the host plant by inoculation [3]. However, as the volume of the collection increases, this becomes an increasingly difficult task. Therefore, it is necessary to select storage methods that allow longer preservation of cultures without significant changes in their basic properties and viability.

There is a lot of data in the most diverse ways of storing cultures in the literature. In mycological practice, cases of prolonged survival of fungi on naturally dried substrates are known, which made it possible to avoid the need for frequent replanting. Fungi in this state remained alive for 4, 10, 15 and even 30-35 years [9]. In addition, the freezing of cultures is widespread, because, at temperatures below 0 °C, the vital activity of cells slows down, so cultures preserve longer. Spore suspensions, cultures on nutrient media and air-dry spores are usually subjected to freezing [9]. There is only one work we know on the use of freezing for the storage of Septoria isolates, in particular S. nodorum, where fungal spores were in test tubes in the medium consisting of glycerol, dextrose, water, polyethylene glycol and mineral oil and kept frozen at -35 °C for one year [10]. However, after a year of storage, the authors noted a decrease in spore germination and pathogenicity. One way of long-term storage of phytopathogenic fungi without loss of their basic properties is storage in the soil [11, 12]. In the literature there is information about the storage of S. nodorum and S. tritici species as a soil-spore preparation in the dark at 40 °C without loss of sporulation ability and pathogenicity for 20 months or more [13-15]. At the same time, there are reports of 5 years of survival in sterile soil, as a record for some fungal species [16, 17]. Nowadays, in collections of microorganisms cultures, lyophilization is widely used as the main method of storage. In our case, for a long-term storage of fungal isolates, a method for lyophilizing cultures on strips of filter paper is known [18]. The authors, however, do not have data on the maximum survival of Septoria isolates under this storage method.

In ARRIP, the storage of collection strains is carried out in several ways, developed or modified in the course of many years of research. The main ones are storage in test tubes on agar slants, storage in the form of herbarium material of host plants infected with certain strains of fungus, storage in sterile soil, storage in a lyophilized state. In addition, observations of cultures survival duration on dry nutrient medium, on slants filled with a layer of sterile water, and also in tubes with cultures placed in the freezer were made.

In this article, the methods tested for storing strains of Septoria/Stagonospora have been described; the advantages and disadvantages of these methods have been noted. The results of studies on the viability and pathogenicity of strains under various storage conditions have been presented. The optimal storage time for strains on different types of septoriosis pathogens has been determined.

MATERIALS AND METHODS

Storage in test tubes was carried out on sloping potato dextrose agar (PDA) in a refrigerator at a temperature of + 4 - 8 °C. Observations were made on the duration of survival during drying of nutrient medium. For the purpose of the experiment, a small amount of sterile distilled water was poured into individual test tubes, so that it was above the edge of the slant by 1 cm, and stored in a vertical position in the refrigerator at a temperature of + 4 - 8 °C.

To monitor the storage of cultures in a frozen state, the test tubes were placed in a freezer at a temperature of minus 15 - 20 °C after inoculation.

Storage of the fungus on the host plants leaves was carried out as follows: plants in the two leaves phase were artificially infected with separate strains of Septoria and, after the formation of typical lesions (in the case of S. tritici, the spots should be with pycnidia) were cut, herbarized and placed in paper bags indicating the strain number, the date of inoculation and collection and stored in a refrigerator at a temperature of + 4 - 8 °C.

To store the strains in sterile soil crushed and sifted lawn-and-garden soil was taken; 5 grams of this substrate were poured into glass vials, covered with cotton plugs and then sterilized twice for 1 hour at 120 °C with a one-day break, during which the vials were kept at a temperature of 26 - 28 °C. In prepared test tubes with soil, 2 ml of a thick aqueous suspension of spores was poured, then these tubes were shaken to distribute the spores evenly in the soil. After that, this substance was dried at room temperature and stored in a refrigerator at a temperature of + 4 - 8 °C.

For subsequent lyophilization, the fungus was first grown on sterile strips of filter paper (0.5 x 3 cm) placed on a nutrient medium (PDA) in Petri dishes by inoculating them with a spore suspension. Strips with grown fungus culture were placed in cryovials, dried with a lyophilic dryer for about 16 hours and stored in the REVCO freezer at a temperature of minus 80 °C, as well as in the freezer of a household refrigerator at minus 15 - 20 °C.

To test the S. nodorum and S. avenae triticea strain viability, stored in test tubes on a nutrient medium, inoculation was made with a piece of fungus culture on PDA in Petri dishes. 2-3 days after the formation of the mycelium, the dishes were placed under conditions of constant ultraviolet (UV) illumination by LE-30 sunlamps with a wavelength of 300 - 320 nm and incubated for about 10 days until fungal colonies were formed. In the case of S. tritici, the inoculation was performed by the "stroke" method. To do this, a piece of culture was taken and placed in a test tube with 2 ml of sterile distilled water. Petri dishes with PDA were inoculated by the resulting spore suspension using a wire loop. The cultures stored in the frozen state were first thawed at room temperature for 1 day, and then the inoculation was performed as described above.

To re-isolate the fungus from herbarium specimens in the case of S. nodorum, the fragments of diseased plant tissue after surface sterilization was placed on nutrient medium (PDA) in Petri dishes. 2-3 days after the formation of a small white filamentous mycelium, the dishes were placed under conditions of constant ultraviolet illumination and incubated for about 10 days prior to the formation of the fungal colonies. In the case of S. tritici, fragments of diseased tissue containing pycnidia were placed in a drop of sterile water for 10-15 minutes. After the secession of the spores from the pycnidia, spore suspension was put on nutrient medium (PDA) in Petri dishes by streak plating method with a wire loop. The formation of the fungal colonies after 5 - 7 days served as an indicator of viability.

To check the viability of the soil culture, a small part of it was put into a test tube with 2 ml of sterile distilled water, stirred and the resulting soil-spore suspension was poured onto the surface of the nutrient medium (PDA) in Petri dishes. In the case of S. nodorum and S. avenae triticea, the dishes were placed for 7-10 days under 24-hour UV illumination. The indicator of the strain viability was the formation of characteristic fungal colonies.

Lyophilized cultures were restored after storage by inoculating strips with fungus culture (or fragments) onto PDA in Petri dishes. In the case of S. nodorum and S. avenae triticea, after the formation of a white fluffy mycelium, the dishes were placed for 7-10 days under 24-hour UV illumination. In the case of S. tritici, the dishes were left in the laboratory. The indicator of the strain viability was the formation of typical fungal colonies.

The pathogenic properties of the strains were tested on varieties of native selection plants. For this purpose, 1-2 susceptible varieties from the set of differentiators were used: wheat varieties Moskovskaya 35 or Saratovskaya 29 (for wheat isolates) and barley variety Moskovsky 121 (for barley isolates). Then the plants were inoculated by spraying with the spore suspension of the fungus. Biomaterial was grown on PDA in Petri dishes for 10 days with 24-hour UV illumination (S. nodorum) or for 4-5 days in laboratory conditions (S. tritici). The concentration of the spore suspension is $1x10^6$ spores/ml of S. nodorum and $1x10^7$ spores/ml for S. tritici. The volume was taken at the rate of 5 ml per 1 pot. Infected plants for 2 days were placed in a moist chamber, and then transferred to a growth chamber, where the following conditions were maintained: temperature 18 °C (at night) – 22 °C (in the daytime), relative humidity of air 70-80%, photoperiod 16 hours a day with illumination about 15 thousand lux.

The infection degree of plants with S. nodorum isolates was determined in 14 days after inoculation by visual assessing the percentage of the infected surface on first and second leaves. The pathogenicity level was determined by the average lesion degree of the leaves: I – low pathogenic (degree of lesion less than 20%), II - medium pathogenic (21-50%), III - high pathogenic (more than 50%).

The pathogenicity of S.tritici isolates was determined by two parameters: the degree of plants lesion and the sporulation activity of the fungus in vivo. The infection degree of plants was determined after 20 days from inoculation in the same gradations as for S. nodorum isolates. Sporulation was determined by the number of spores/leaf using the hemocytometer. On the intensity of sporulation in vivo, S. tritici isolates were differentiated into low-sporulating (up to 100 thousand spores/leaf); mid-sporulating (from 100 to 200 thousand spores/leaf); high-sporulating (more than 200 thousand spores/leaf).

FINDING AND DISCUSSION

The effect of storage methods on the viability of cultures

Storing cultures in test tubes on a nutrient medium, agar, as a rule, have been dried out after 2-3 years. The isolates of S.nodorum and S. avenae triticea remained alive for 5 years from the moment of plating, but the sporulation ability was often lost during storage on dried agar. They formed mycelial colonies without pycnidia when sowing on a fresh nutrient medium. S. tritici isolates quickly lost their viability as the nutrient medium dried out. Storage in this condition for more than 3 years for most of them was fatal.

The method of storage on a nutrient medium under a layer of sterile water was tested for S. tritici cultures. Observations exhibited that after filling the test tubes after 2 years from inoculating with sterile water, which prevented the drying of the agar, the viability of the isolates was prolonged for 2-3 years.

The storage of S. tritici cultures in frozen test tubes exhibited that in 1-2 years after removing from anabiosis the cultures actively grew on the nutrient medium. If they were stored in these conditions in 3-4 years, most of them perished. Out of 40 isolates only 14 ones retained viability. A longer period of freezing usually gave only negative results. Thus, 4 years is a critical period for the storage of frozen cultures of this fungus species.

Studies have shown that the species S. nodorum is able to survive longer in dried plant tissues compared to S. tritici when storing cultures in the form of herbarium material. Thus, isolation of the fungus in a pure culture from leaves stored for 3 years was successful for 94.1% of S. nodorum isolates, after 4 - 5 years of storage - for 73.5%, after 6 - 7 years - for 69.2%, after 8 - 10 years - for 46.7% of isolates. S. tritici isolates recovered significantly worsen after 5 years of storage. There was re-isolated only 7.7% strains isolated from herbarium specimens collected 6-7 years ago (Table 1).

Table 1 - Preservation of Septoria / Stagonospora isolates in herbarium specimens

Storage period	Number of tested isolates	Number of viable isolates	% of viable isolates
S. nodorum			
3 years	17	16	94.1
4-5 years	49	36	73.5
6-7 years	26	18	69.2
8-10 years	15	7	46.7
S. tritici			
3 years	28	23	82.1
4-5 years	22	11	50.0
6-7 years	26	2	7.7

As for S. avenae triticea, isolation of this fungus into a pure culture was successful when using 1-2 years' herbarium specimens. With a longer storage period of the leaves (up to 6 years), the fungus re-isolation almost did not have a positive effect.

The collection contains strains of S. nodorum, which were sown in sterile soil in 1985-1988. The sowing of the remaining isolates, as well as the repeated sowing of isolates from the batches of 1985-88, was carried out beginning in 2001. Their viability was periodically checked in order to establish the maximum storage period.

Result exhibits most of S. nodorum isolates can be well stored in the soil for up to 25 years. At the same time, 100% viability of the cultures was observed up to 10 years of age. With a storage period of 10-25 years, the number of viable strains was still quite high and amounted to 80.0-86.4%, but further the viability of the strains dropped sharply (35.3%) (Table 2).

Table 2 - The viability of S. nodorum strains stored in sterile soil

Storage period	Number of tested isolates	Number of viable isolates	% of viable isolates
Up to 10 years	34	34	100
10-15 years	125	108	86.4
16-20 years	40	32	80.0
21-25 years	39	33	84.6

26-29 years	17	6	35.3
10-20 years	165	135	81.8
More than 20 years	56	39	69.6

S. tritici isolates, in contrast to S. nodorum, can be stored in the soil for a shorter period of time. The maximum storage period of this fungus species in this way was 4-5 years, more rarely 6 years as shown by observations (Table 3).

Table 3 - The viability of S. tritici strains when stored in sterile soil

Storage period	Number of tested isolates	Number of viable isolates	% of viable isolates
2 years	6	6	100
4 years	4	3	75.0
5 years	7	3	42.8
6 years	7	1	14.3

The species S. avenae triticea, like S. nodorum, is also capable of surviving in sterile soil for a long time. Presently the storage period of one of this fungus strains is 25 years. Verification of the strain viability, conducted after 22 years of storage, gave a positive result.

The method of fungi storage in the freeze-dried state at low temperatures was first used since 2009 and is used for strains of all three species of Septoria / Stagonospora available in the collection. Today the storage period of individual strains has reached 8 years. Observations of S. nodorum and S. tritici cultures stored in the lyophilized state at a temperature of -20 °C exhibited that strains of both species can be stored during this period under the given conditions. Virtually all isolates remained viable in the first 5 years of storage (86.5 - 98.1% survival). Then this indicator gradually decreased, at that the number of viable S. tritici strains was lower than S. nodorum after 8 years of storage (50 and 70%, respectively) (Table 4).

Table 4 - Viability of S. nodorum and S. tritici strains in the lyophilized state at -20 °C

Number of strains	Storage period							
	1 year	2 years	3 years	4 years	5 years	6 years	7 years	8 years
S. nodorum								
Tested	29	54	67	37	35	18	47	10
Viable	28	53	65	36	34	16	38	7
%	96.5	98.1	97.0	97.3	97.1	88.9	80.8	70.0
S. tritici								
Tested	36	91	161	76	52	28	52	32
Viable	35	87	145	66	45	19	23	16
%	97.2	95.6	90.1	86.8	86.5	67.8	44.2	50.0

Lyophilized cultures of 7 S. nodorum and 12 S. tritici strains stored during deep freezing in the REVCO chamber at minus 80 °C, confirmed their viability by 85.7 and 91.6%, respectively (one strain from each species perished). The storage period for these cultures was 5 years at the time of inspection. Thus, it can be assumed that the temperature storage conditions (-20 °C and -80 °C) approximately affect lyophilized cultures of septoriosis causative agents' viability.

The strains of S. avenae triticea confirmed viability three years after freeze-drying and storage at minus 20 °C. Further observations will be made for all strains of Septoria pathogens that stored in lyophilized state.

Strains pathogenicity assessment

The pathogenicity of the collection strains of the two main types of septoriosis causative agents S. nodorum and S. tritici stored in different ways was evaluated (in test tubes on the nutrient medium slants (PDA), in sterile soil, herbarium specimens, the lyophilized state at -20°C and -80 °C). Strains possessing II and III pathogenicity groups were taken for research. The evaluation of the tested strains exhibited the unequal influence of the storage methods and storage period on this indicator. The number of S. nodorum strains, which preserved pathogenicity at the same level, varied within the limits of 57.1 - 100%. In this case, pathogenicity was best preserved in the variant with sterile soil. The strong degree of leaves lesion was noted when the plants were inoculated regardless of the culture age. A marked decrease in pathogenicity of S. nodorum isolates was observed during prolonged storage in test tubes on the nutrient medium slants. Pathogenicity has changed in half of the tested strains when stored in herbarium specimens. Storage of lyophilized S. nodorum cultures at a temperature of minus 80°C for 5 years resulted in a decrease in pathogenicity in all tested strains, while storage at minus 20°C had a lesser effect on the level of pathogenicity (Table 5).

Table 5 - Changes in the pathogenicity of S.nodorum strains at different methods and periods of storage

Storage method	Storage period	Number of tested strains	Number of strains with reduced pathogenicity	% of strains with preserved pathogenicity
In test tubes on PDA	1 year	1	0	100
	3 years	7	3	57.1
In herbarium specimens	1-3 years	7	3	57.1
	5-6 years	7	3	57.1
In sterile soil	3-10 years	13	1	92.3
	23-24 years	5	1	80.0
In lyophilized state at -20 °C	1 -2 years	12	4	66.6
	3-4 years	7	2	71.4
In lyophilized state at -80 °C	1 year	4	0	100
	5 years	4	4	0

The pathogenicity of S. tritici strains was more susceptible to changes in the storage process than S. nodorum in all the tested methods. The number of strains that preserved pathogenicity at the previous level was significantly lower and was 33.3 - 66.7%. The greatest decrease in pathogenicity was noted during storage in test tubes on a nutrient medium for more than 1 year. In all other variants, the proportion of strains with reduced pathogenicity was approximately the same (Table 6).

Table 6 - Changes in the pathogenicity of S.tritici strains at different methods and periods of storage

Storage method	Storage period	Number of tested strains	Number of strains with reduced pathogenicity	% of strains with preserved pathogenicity
In test tubes on PDA	1 year	3	1	66.7
	2-3 years	3	2	33.3
In herbarium specimens	1-3 years	10	4	60.0
	4-6 years	2	1	50.0
In lyophilized state at -20 °C	2 years	4	2	50.0
	3-4 years	26	14	46.1
In lyophilized state at -80 °C	5 years	4	2	50.0

Table 7 - Optimal storage periods for Septoria / Stagonospora strains by different methods

Storage method	Septoria / Stagonospora species		
	S. nodorum	S. tritici	S. avenae triticea
In test tubes on PDA at +4 – 8 °C	up to 5 years	3 years (5 – under layer of water)	up to 5 years
In test tubes on PDA at -20 °C	4 years (supposedly)	4 years	4 years (supposedly)
In herbarium specimens	5 years	3 years	5 years
In sterile soil	20-25 years	5 years	20-25 years
In lyophilized state:			
at -20^{0}C	8 years *	8 years *	3 years *
at -80^{0}C	5 years *	5 years *	did not investigate

* research continues

CONCLUSION

Thus, for the pathogenic Septoria strains, all the mentioned storage methods are acceptable. However, the storage methods of the strains depend on the fungus species. In general, S. nodorum cultures retain their viability longer in comparison with S. tritici, as they are better suited to saprophytic life. Table 7 presents the optimal, in our opinion, storage period for the strains by all the methods we have tested.

Storage in test tubes on a nutrient medium is a classic way of phytopathogenic fungi keeping. The ability of the causative agents of septoriosis to survive on a dried nutrient medium greatly facilitates the work of maintaining strains since there is no need for frequent re-plating. However, it should be mentioned that with storage period of more than 3 years, the sporulation activity of individual S. nodorum isolates and the viability of S. tritici isolates may decrease. Pouring slants with a layer of sterile water lengthen the storage period of the culture, preventing its drying, which is especially important for strains of S. tritici that tend to lose their viability faster. However, studies have shown that storage on a nutrient medium has the most effect on the level of pathogenicity of the fungus.

The method of freezing test tubes with cultures in the freezer, which was tested on S. tritici strains**,** can be presumably used for storing the strains of other fungus species. But in terms of duration, this method practically does not differ from usual storage in test tubes. In addition, after defrosting, a considerable number of bacteria were often noted in the cultures, so antibiotics were used when plating.

The best method for the septoriosis causative agent (S. nodorum and S. avenae triticea) is the method of storage in sterile soil, in which fungi can survive for a very long time without loss of pathogenicity. The optimal storage period for cultures can reach 20-25 years. For S. tritici, this method is also possible, but the storage period is limited to 5-6 years.

One of the reliable methods for long-term storage of the isolates of all Septoria species is the herbarium material of plants infected with individual strains. As studies have shown, the minimum storage period of septoriosis causative agents in this way was 3 years; the maximum could reach 10 years. Thus, the optimal storage period for strains without significant loss of pathogenicity is 3 - 5 years. With a longer storage period, the success of the fungus re-isolation is significantly reduced, especially with respect to S. tritici.

Method of lyophilizing cultures on strips of filter paper is simple and convenient, but it has so far been little research in terms of the duration of fungus survival. To date, the maximum storage period for Septoria strains in this way has reached 8 years, but research in this direction must continue.

In any case, to ensure the safe preservation of strains collection, several different methods of storing them should be used simultaneously.

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