ACTA MYCOLOGICA
Vol. 39 (2): 139-145
2004

Preservation methods for isolates of ascochyta blight fungi

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Marcinkowska J.: *Preservation methods for isolates of ascochyta blight fungi*. Acta Mycol. 39 (2): 139-145, 2004.

Isolates of ascochyta blight fungi, two of *Ascochyta pisi*, four of *Mycosphaerella pinodes*, and four of *Phoma pinodella* were stored: A - on slants under mineral oil, B - on CN's medium agar disks, and as conidial suspension: C - in glycerine, D - in water. Viability and pathogenicity of recovered cultures after each consecutive year were assesed from 1991 to 1999. The compared parameters were first of all strongly influenced by the preservation method, but fungus species and number of years had a minor importance. The best for longer storage was method "A" because after 9 years the isolates were viable, highly pathogenic, and cultures recovered from them were clean. The method "C" is good for short keeping (2-3 years), as conidia in vials need only small space and gave clean cultures.

Key words: *Ascochytai*, *Mycosphaerella*, *Phoma*, preservation methods, pathogenicity, *Pisum*

INTRODUCTION

A couple of storage techniques have been used for fungi preservation (Buell and Weston 1947; Fennel 1960; Webster and Davey 1976; Smith and Onions 1983; Smith 1990; Dhingra and Sinclair 1995). The kind of applied method depends on an organism nature (Webster and Davey 1976; Dhingra and Sinclair 1995) and a period of keeping it viable (Smith 1990). Any method should be simple, economical, and yield viable cultures that are morphologically and physiologically unchanged (Wilkinson and Murphy 1990; Smith 1990). Some are easy to be followed, like dehydration methods, but preservation in sterile soil (Shearer et al. 1974), sand (Tio et al. 1977) or under anhydrous silica gel (Lange and Boyd 1968, Smith and Onions 1983) is economically reasonable while freeze-drying (Smith, Onions 1983) and freezing in liquid-nitrogen (Dahmen et al. 1983; Smith and Onions 1983) need special equipment what makes them expensive.

Cultures of *Ascochyta pisi* Lib., *Mycosphaerella pinodes* (Berk. et Blox.) Vester., syn. *Didymella pinodes* (Berk. et Blox.) Petrak (an.: *Ascochyta pinodes* L. K. Jones) and *Phoma pinodella* (L. K. Jones) Morgan-Jones et Burch, the causal agents of

ascochyta blight on pea (*Pisum sativum* L.), are easily maintained on slants in tubes by periodic transfer (Marcinkowska et al. 1991). Nevertheless, the long lasting research programmes concern secondary metabolites produced by ascochyta complex fungi (Marcinkowska et al. 1991), their pathogenicity (Marcinkowska and Witkowska 1996), occurrence on pea seeds (Marcinkowska 1997) caused the necessity of maintaining these fungi isolates of similar properties over an extended period of time to obtain repeatable and uniform results in inexpensive way. As majority of fungi can be successfully maintained under mineral oil (Buell and Weston 1947; Little and Gordon 1967; Smith et al. 1970; Wilkinson and Murphy 1990) or in water (Boeswinkel 1976; Ellis 1979) the similar procedures were used in the study to find out simple and economical methods available for any laboratory for longer storage of ascochyta complex fungi.

MATERIALS AND METHODS

The isolates were obtained from seeds, leaves and stems of different pea cultivars or lines planted in 1987 and 1988 at various regions of Poland (Tab. 1) and were kept as monoconidial cultures on PDA (Difco Potato Dextrose Agar) slants in a refrigerator at 5°C. The cultures were transfered once a year. After two years of that way storage conidial suspension (10 5 conidia per ml of water containing 0.1% Tween 20) of each isolate was prepared to inoculate two week-old pea plants of Belinda cv. Reisolation from symptoms of inoculated plants on Coon's agar (CN) (Marcinkowska et al. 1991) in Petri dishes allowed to receive fresh cultures of 10 tested isolates (Tab. 1) for starting this experiment in May 1990. Cultures necessary to settle each method were 10 day-old on CN agar. Methods were as follow: oil storage ('A'), water ('B' and 'D') and glycerine storage ('C'). The method 'A' - cultures on CN agar slants, in cotton plagged tubes, were covered to a depth of 1 cm above the highest point to avoid the possibility of evaporation. The method 'B' - culture disks of 2 cm diam. were cut out from the edge of colony and placed in 50 ml sterile distilled water in a glass flask tightly covered with cotton plag. In each flask were flooded 10 discs. The method 'C' - conidia scraped from culture were suspended in 4 ml of glycerine (104/ml) in 5 ml vials covered by gum plag. The method 'D' - water suspension of conidia obtained after flooding culture disks as in the method 'B'. Fungi preserved by the methods 'A', 'B' and 'D' were kept at temp. 8°C in a refrigerator, and by 'C' method at - 20°C in a freezor.

Once a year in May storaged isolates were transfered to CN's agar in Petri dishes of 7 cm. diam. to check their viability, pathogenicity and biological cleanness. Small amount of fungal colony on a mounted needle for the method 'A' and a piece of disk for the method 'B' or a drop of conidial suspension in case of the methods 'C' and 'D' were put on 6 places in a dish. All treatments were repeated twice (2 dishes) for 10 tested isolates. A week after transfer viability of an isolate was assessed on the base of clean (without contaminating saprobes) subculture number grown in 2 dishes, with max. of 12 and min. of 0. The 2 week-old clean subcultures were used as source of inoculum. Conidial suspension, of concentration as above, was sprayed onto 14-16 pea seedlings (grown in 2 pots) of susceptible cv. 'Diament'. The ten day-old inoculated plants were covered with plastic bags over 2 nights to keep 100% RH and temperature of 22°C. Disease intensity was determined eight days after inocula-

Table 1
Origin of *Ascochyta pisi*, *Mycosphaerella pinodes* and *Phoma pinodella* isolates

Isolate Nr. Symbol	Location	Cultivar or line Nr.	Part of plant
A. pisi			
1 B21	Oleśnica M.	Delisa II	seed
2 RB50	Oleśnica M.	Delisa II	seed
M. pinodes			
3 2	Radzików	Belinda	leaf
4 B81	Brzozów	Delisa II	seed
5 B221	Błonie	Delisa II	seed
6 RB255	Oleśnica	line 2432	leaf
P. pinodella			
7 B240	Brzozów	Delisa II	seed
8 B271	Radzików	line 754	stem
9 274	Oleśnica	Belinda	leaf
10 B280	Radzików	line 729	leaf

tion and two days after the symptoms appeared. Disease index (0-3) was based on the intensity of lesions: 0 - symptomless; 1 - single, small necrotic spots on leaves and stems; 2 - more numerous necrotic spots on plants; 3 - small necrotic and confluent flecks covering much of leaf and stem surface. Disease index indicated on the pathogenicity of a tested isolate.

Data were statistically evaluated with Statgraphics Plus programme. A multifactor analysis of variance using F test was performed for viability and disease index data to determine which factors had a statistically significant effect on each of them. Means were separated with multiple test for 95.0 percent confidence intervals.

RESULTS

The methods applied for preservation ten isolates of 3 species, namely: *A. pisi*, *M. pinodes* and *P. pinodella* affected their viability (Tab. 2) and pathogenicity (Tab. 3, Fig. 1), but also biological cleanness of recovering subcultures. The viability was significantly different for the each method so four homogeneous groups were distinguished (Tab. 2). All isolates under mineral oil (method 'A') kept their viability during the whole period of nine (1991-1999) years study and gave clean subcultures. The method 'B' was the next (4 years), but after 3 years contaminants occurred on single subcultures. The recovering subcultures of the method 'C' were without contaminants, but only some isolates survived to 1993 and 1994. The worse was the method 'D' because 100% of clean subcultures were only recovered during the first two years, and since 1993 majority of recovered cultures had been contaminated with saprobiotic Bacteria and Fungi (e.g. *Penicillium* spp., *Alternaria* spp.). However, a weak growth of a few contaminated cultures was observed for the whole period.

The data concern viability in dependance of year shown three homogeneous groups (Tab. 2). The highest viability had cultures after one year of storage, but the lowest after 4-10 years depend on an isolate.

Table 2
Analysis of variance and multiple range tests for comparison of isolates viability

Sources of variation	D. f.	Mean square	Significance level	H. gs [1]	Means[2]
method	3	601.8	0.0000	4	4.9 for „D" - 11.9 for „A"
year	8	50.4	0.0000	3	6.7 for 1994 - 10.7 for 1991
isolate	9	18.0	0.0208	3	6.5 for B 81 - 9.0 for B 221

Explanations: D. f. - Degrees of freedom; [1] Number of homogeneous groups according to the Lowest Significant Differences (LSD); [2] Means are presented from the lowest value to the highest one

Table 3
Analysis of variance and multiple range tests for comparison of isolates pathogenicity

Sources of variation	D. f.	Mean square	Significance level	H. gs [1]	Means[2]
method	3	1.3	0.0001	2	2.3 for „D" - 2.6 for „A"
year	7	0.5	0.0049	3	2.3 for 1997 - 2.6 for 1992
isolate	9	2.6	0.0000	5	1.9 for B21 - 2.8 for B221

Explanations: D. f. - Degrees of freedom; [1] Number of homogeneous groups according to LSD; [2] Means are presented from the lowest value to the highest one

The viability was also influenced by an isolates, being the highest for B221, B21, 274 and RB50 and the lowest for B81, B240 and B271 (Tab. 2). The isolates of *A. pisi* (B21, RB50) proved to be more viable to isolates of two other species.

The significant differences in pathogenicity were only proved between isolates preserved under mineral oil (method 'A') and the other used methods (Tab. 3, Fig. 1). When years were compared, three homogeneous groups were noted. The highest pathogenicity shown isolates after 2 and 4 years of storage and the lowest after 7 years, but in the same group were cultures storaged 3, 6, 5, 8 and 9 years. Pathogenicity of tested isolates after preservation was very much differentiated and so divided into five homogeneous groups. The lowest disease index was noted for isolates of *A. pisi* (B21, RB50) and the highest for *A. pinodes*.

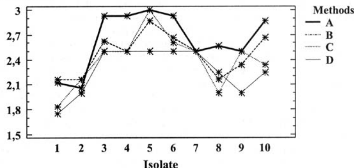

Fig. 1. Influence of a method on an isolate pathogenicity.

DISCUSSION

Most of fungal species can be successfully maintained for a year on agar medium slants in tubes stored in a refrigerator (Smith 1990). The same way of *A. pisi*, *M. pinodes* and *P. pinodella* preservation was used by the author (Marcinkowska et. al. 1991). However, when cultures are necessary for longer-time study, periodic transfer is not recommended since first of all increases the risk of losing pathogenicity, and culture contamination, or failing sporulation with time, but is also laborious and time consuming (Scharen and Krupinsky 1970; Wilkinson and Murphy 1990). The risk of applying repeatable transfer was a reason for testing another economically reasonable, simple, easy to perform in any laboratory, and so useful method. Among several procedures of fungi storage already developed (Dhingra and Sinclair 1995) only cheaper preservation techniques, namely culture storage under mineral oil (Smith and Onions 1983) and in water (Boeswinkel 1976) were tested for ascochyta complex fungi. Paralelly, preservation of spores, as a conidial suspension, in water or in glycerine, kept at different temperature was investigated. The results showed that the applied methods could be used for storage of the tested species but the longevity of keeping organisms viable varied from 2 to 9 years (the whole period of study). The dependence of storage longevity on a method was already proved for different fungi (Smith and Onions 1983). The viability and pathogenicity was influenced by the method, but also a fungus species. The obtained data are in agreement with other results indicating on higher pathogenicity of *M. pinodes* than *A. pisi* (Wallen 1974; Marcinkowska and Witkowska 1996). The recovered culture purity depended on a method. It may be supposed that contaminants presence was caused by methodological failure. The cultures from slants under oil and from conidia in glycerine were always clean, but conidia kept in water recovered clean cultures only after first two years, even some conidia survived 9 years and gave a few culture but contaminated. The period of conidia viability in this test was similar to obtained by Boesevinkel (1976) for different cultures in water. Comparison of the applied methods for longer storage in the presented studies revealed as preferrable preserving cultures under mineral oil. This is an additional proof for well working procedure already stated by earlier researches (Little and Gordon 1967; Smith and Onions 1983; Smith 1990). However, any pathogen should be preserved by more than one procedure, what was already recommended in special studies for some *Septoria* species (Shearer et al. 1974; Wilkinson and Murphy 1990) and other species (Smith et al. 1970, Tio et al. 1977) as well as for the big world collections of fungi (Smith and Onions 1983; Smith 1990).

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Przechowywanie izolatów grzybów powodujących zgorzelową plamistość grochu

Streszczenie

Porównywano przydatność czterech metod przechowywania grzybów w warunkach laboratoryjnych na przykładzie dwóch izolatów *Ascochyta pisi* Libert, czterech *Mycosphaerella pinodes* (Berk. et Blox.) Vesterg., syn. *Didymella pinodes* (Berk. et Blox.) Petrak (anamorfa: *Ascochyta pinodes* Jones) oraz czterech *Phoma pinodella* (Jones) Morgan-Jones et Burch. Przydatność oceniano na podstawie długości okresu zachowania żywotności izolatów oraz ich patogeniczności. Zastosowano następujące metody: A - 10 dn. kultury (wyjściowe) na skosach pożywki CN zalane olejem mineralnym; B - krążki takich kultur (wyjściowych) umieszczone w wodzie sterylizowanej, C - zawiesina konidiów w glicerynie; D - zawiesina konidiów w wodzie sterylizowanej (woda w kolbach, w których zatopione były krążki kultur wyjściowych metody B). Kombinacje A, B i D przechowywano w lodówce o temp. 6°C. Fiolki z zawiesiną konidiów w glicerynie (kombinacja C) przechowywano w zamrażalniku, temp. - 10°C. Doświadczenie założono w maju 1990 roku, a zakończono po dziewięciu latach. W maju każdego kolejnego roku badano żywotność izolatów, a następnie patogeniczność wyrosłych kultur. Żywotność określano na podstawie liczby kultur wyrosłych z 12 wykładanych na pożywkę CN (2 szalki) fragmentów wyjściowych kultur (metody A i B) lub kropli zawiesiny (metody C i D). Patogeniczność oceniano w stopniach porażenia (0-3) po wykonaniu inokulacji 14 - 16 roślin grochu (w 2 doniczkach) będących w fazie 4-5 węzłów widocznych nad podłożem. Wyniki poddano analizie wariancji dla zobrazowania istotności różnic pomiędzy danymi analizowanych czynników zmienności.

Zastosowane metody przechowywania wpływały zarówno na żywotność badanych izolatów grzybów, jak i ich patogeniczność. Przy tym żywotność izolatów była istotnie różna dla każdej z metod, zaś statystycznie udowodnioną wyższą patogenicznością odznaczyły się tylko izolaty

przechowywane na skosach zalanych olejem mineralnym. Ta właściwość, a także żywotność zachowana przez cały okres badań na podobnym poziomie, najwyższym w porównaniu z innymi metodami, oraz czystość odszczepianych kultur wyróżniają metodę skosów pokrytych olejem jako najlepszą. Najszybciej (po 2-4 latach) żywotność utraciły konidia w glicerynie. Natomiast konidia w wodzie przeżywały 4-9 lat, ale uzyskiwane z nich kultury były zanieczyszczone saprotroficznymi bakteriami oraz grzybami, jak np. *Penicillium* spp., *Alternaria* spp., w pojedynczych przypadkach nawet już po dwóch latach. Kultury na krążkach agaru w wodzie badane były przez 4 lata, ale niektóre wyrastające z nich młode kultury uległy zanieczyszczeniu saprotrofami już w trzecim roku doświadczenia.

Żywotność i patogeniczność izolatów zależne były również od ich właściwości oraz liczby lat przechowywania. Najbardziej żywotne były kultury po pierwszym i drugim roku przechowywania, a spadek żywotności zależał przede wszystkim od metody. Zastosowane metody nie spowodowały wprost proporcjonalnego do lat przechowywania obniżenia patogeniczności izolatów. Izolaty *M. pinodes* wyróżniły się najwyższą patogenicznością, natomiast *A. pisi* porażały rośliny grochu najsłabiej. Z kolei żywotność kultur ocenianych izolatów różniła się statystycznie, ale nie stwierdzono tej zależności pomiędzy trzema badanymi gatunkami grzybów.

Zastosowane metody można wykorzystywać do przechowywania izolatów grzybów, ale przez różny okres czasu. Z konidiów w wodzie czyste kultury uzyskiwano tylko po roku, a z kultur na krążkach po dwóch latach, tak jak i z konidiów w glicerynie. Najdłużej, w warunkach tego doświadczenia przez 9 lat, przechowały się kultury na skosach zalane olejem mineralnym.