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A COMPARISON OF SELECTED MEDIA AND INCUBATION TEMPERATURES FOR ISOLATION OF MICROSCOPIC FUNGI FROM DRIED MEDICINAL PLANTS

PORÓWNANIE WYBRANYCH POŻYWEK I TEMPERATUR HODOWLI W IZOLOWANIU GRZYBÓW MIKROSKOPOWYCH Z SUSZONYCH ROŚLIN LECZNICZYCH

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The study compared selected media and incubation temperatures for isolation of fungi from dried medicinal plants (chamomile, peppermint, lemon balm, St. John's wort and two herbal mixtures). The DG18 medium was found to be the most suitable for characterization of the mycoflora at 25°C. The medium selection for 37°C was dependent on the species to be isolated. MEA + 40% sacharose and YpSs were found to be the best media for isolation of thermophilic and thermotolerant fungi from dried medicinal plants.

Key words: microscopic fungi, dried medicinal plants, isolation, media, temperature
Słowa kluczowe: grzyby mikroskopowe, suszone rośliny lecznicze, izolowanie, podłoża, temperatura

INTRODUCTION

Dried medicinal plants are more and more commonly used for prophylaxis or treatment of many diseases. Poland, with 15,000-20,000 tons of herbs processed per year, is the leader on the medicinal plant market. In comparison, 100,000 tons of herbs per year are processed over the world [19]. However, the problem of their microbial contamination and associated effects is still poorly understood. The fungal contamination of dried medicinal plants may result in (1) biodeterioration of plant material, biologically active constituents in particular; (2) production of secondary metabolites, including mycotoxins; and (3) propagation of isolates with potential pathogenic properties to man. It is surprising, therefore, that a relatively small number of studies on fungal contamination of dried medicinal plants has been recently published [1, 2, 3, 4, 5, 7, 12, 21].

Most of the studies have been focused on mesophilic fungi and their mycotoxins in plant material. However, low water activity favors the growth of xerophilic fungi in dried medicinal plants. Surprisingly, no data on this subject have been found in available literature. Additionally, dried medicinal plants have never been surveyed for thermophilic/thermotolerant and actidione-resistant fungi, with potential pathogenic properties to man. The study was to compare selected media and incubation temperatures for isolation of microscopic fungi from dried medicinal plants.

MATERIALS AND METHODS

Dried medicinal plant samples were purchased in herbal shops of Szczecin, Poland. The samples were as follows: chamomile (*Flos Chamomillae*), peppermint (*Folium Menthae piperitae*), lemon balm (*Folium Melissae*), St. John's wort (*Herba Hyperici*), and herbal mixtures 1 (16 constituents) and 2 (13 constituents). The herbal mixture samples were obtained in one portion, while the other samples were prepared by mixing of many infusion bags. The samples consisted of small plant pieces. The amount of each sample was ca. 0.5 kg. The dried medicinal plants examined are commonly used in Poland.

The media used for examination of fungi at 25 and 37°C were as follows: MEA (Malt Extract Agar provided by Oxoid; a_w = 0.99), MEA20 (Malt Extract Agar + 20% sucrose), MEA40 (Malt Extract Agar + 40% sucrose; a_w = 0.89), SAB+ACT (Sabouraud medium provided by bioMerieux, supplemented with cycloheximide in the concentration of 500 mg/l), and DG18 (Dichloran 18% Glycerol Agar provided by Oxoid; a_w = 0.95). The a_w values of the media were taken from Petrovič et al. [24]. The media used for isolation of thermophilic and thermotolerant fungi from plant material were as follows: MEA, MEA20, MEA40, and YpSs (Yeast Powder Soluble Starch) [11]. The final pH of the media ranged between 5.5-6.5. All media were supplemented with chloramphenicol (100 mg/l).

The inoculation manner was that 0.1-g portions of plant material (small pieces) were uniformly spread over the surface of Petri dishes containing isolation media. For each temperature and medium, inoculations were performed in six Petri dishes. Inoculated Petri dishes were incubated in the dark at 25 and 37°C for 7-14 days and at 45°C for 3-7 days for the total fungal population and thermophilic and thermotolerant fungi, respectively. Fungal colonies growing around dry plant pieces were transferred onto MEA medium and purified by several passages or physiological saline dilution method. Pure fungal isolates were identified to the species level based on macro- and micromorphological characteristics and using selected taxonomic monographs [6, 17, 25, 27, 30]. The number of isolates was the sum of isolates obtained from six Petri dishes with a given medium and at a given temperature. The species represented by single isolates were not considered in the study.

Biosafety levels for fungal species identified were determined according to de *Hoog* [14] and de *Hoog* et al. [15].

RESULTS

Altogether, 639 fungal isolates belonging to at least 31 species were obtained from the dried medicinal plant samples examined (Table I). *Rhizopus oryzae* (19.2%), *Aspergillus niger* (18.9%), *Rhizopus microsporus* var. *rhizopodiformis* (16.7%), *Aspergillus fumigatus* (7.7%), *Absidia corymbifera* (6.6%) and *Rhizomucor pusillus* (5.5%) prevailed in the samples. The other species were isolated from the samples with lower quantities (<5% of the total number of isolates). The highest number of isolates was obtained from peppermint (126), followed by herbal mixture 1 (124), lemon balm (117), herbal mixture 2 (110), chamomile (82) and St. John's wort (80). Subsequently, the highest number of species was identified in peppermint and lemon balm (19), followed by herbal mixtures 1 and 2 (14), St.

Table I. The fungi isolated from dried medicinal plants. Data obtained on all media and at all incubation temperatures

Grzyby wyizolowane z suszonych roślin leczniczych. Wyniki otrzymane dla wszystkich pożywek i temperatur hodowli

Fungal species	Number of isolates from:					
	Chamomile *Flos Chamomillae*	Peppermint *Folium Menthae piperitae*	Lemon balm *Folium Melissae*	St John's wort *Herba Hyperici*	Herbal mixture 1	Herbal mixture 2
1	2	3	4	5	6	7
Rhizopus oryzae Went & Prinsen Geerling[1]	21	17	16	23	24	22
Aspergillus niger v. Tiegh.[1]	7	14	27	23	31	19
Rhizopus microsporus v. Tiegh. var. *rhizopodiformis* Cohn[2]	37	7	12	12	15	24
Aspergillus fumigatus Fres.[2]	1	14	7	2	12	13
Absidia corymbifera (Cohn) Sacc. & Trott[2]	-	19	7	3	6	7
Rhizomucor pusillus (Linda) Schipper[2]	7	3	8	3	10	4
Alternaria sp.	2	9	2	4	4	4
Eurotium amstelodami Mangin[1]	-	5	4	5	4	4
Aspergillus flavus Link:Fr.[2]	4	1	5	-	10	-
Aspergillus versicolor (Vuill.) Tiraboschi[1]	-	1	6	-	-	2
Syncephalastrum racemosum Cohn[2]	-	-	5	-	-	4
White yeasts	1	4	-	-	2	1
Fusarium solani (Mart.) Sacc.[2]	-	8	-	-	-	-
Rhizopus stolonifer (Ehrenb.:Fr.) Vuill.[1]	-	4	1	-	1	2
Emericella nidulans (Eidam) Winter[1]	-	3	3	-	-	-
Mucor circinelloides v. Tiegh[1].	-	5	-	1	-	-
Pink yeasts	-	5	-	-	-	-

John's wort (10) and chamomile (9). *Rhizopus microsporus* var. *rhizopodiformis* occurred abundantly in chamomile, whereas *Absidia corymbifera* prevailed in peppermint.

Altogether, 309 isolates belonging to at least 30 species were obtained from the samples at 25°C (Table II). *Rhizopus oryzae* (23.6% of the total number of isolates at 25°C), *Asper-*

1	2	3	4	5	6	7
Eurotium herbariorum (Wiggers) Link[1]	-	-	-	4	-	-
Mycelia sterilia (orange)	-	4	-	-	-	-
Penicillium nigricans Bain. ex Thom	-	-	4	-	-	-
Phoma glomerata (Corda) Wollenw. & Hochapfel[1]	-	1	2	-	1	-
Aspergillus melleus Yukawa	-	-	2	-	-	-
Eurotium rubrum Konig, Spieckermann & Bremer	-	-	2	-	-	-
Gliocladium penicillioides Corda	-	-	-	-	-	2
Mycelia sterilia (brown-gray)	-	-	2	-	-	-
Paecilomyces variotii Bain.[2]	2	-	-	-	-	-
Scopulariopsis brevicaulis (Sacc.) Bain.[2]	-	-	-	-	-	2
Scopulariopsis candida (Guéguen) Vuill.[1]	-	-	-	-	2	-
Trichosporon mucoides Guého & M.Th. Smith[2]	-	-	-	-	2	-
Trichothecium roseum (Pers.) Link ex Gray	-	2	-	-	-	-
Wallemia sebi (Fr.) v. Arx[1]	-	-	2	-	-	-
Number of isolates	82	126	117	80	124	110
Number of species	9	19	19	10	14	14

[1] – BioSafety Level 1 fungi [14, 15]
[2] – BioSafety Level 2 fungi

gillus niger (20.7%), *Rhizopus microsporus* var. *rhizopodiformis* (8.4%), *Alternaria* sp. (8.1%) and *Eurotium amstelodami* (6.8%) prevailed in the samples.

The highest number of isolates was obtained on DG18, followed by SAB-ACT, MEA20, MEA40 and MEA. The highest number of species was identified on DG18, followed by SAB+ACT, MEA20, MEA and MEA40. *Rhizopus oryzae* was successfully isolated on all media. However, higher numbers of this species were obtained on media containing malt extract. The highest number of *Aspergillus niger* isolates was isolated on MEA40 and DG18. The fungal compositions on SAB+ACT and DG18 were more diverse than the compositions on media containing malt extract. On SAB+ACT, *Alternaria* sp. was the predominating species, while DG18 was the only medium on which *Eurotium amstelodami* prevailed. *Rhizopus microsporus* var. *rhizopodiformis* was isolated on MEA, SAB+ACT and DG18.

Altogether, 227 isolates belonging to 11 species were obtained from the samples at 37°C (Table III). *Aspergillus niger* (25.1% the total number of isolates at 37°C), *Rhizopus oryzae* (22%), *Rhizopus microsporus* var. *rhizopodiformis* (16.3%), *Aspergillus fumigatus* (5.3%), *Rhizomucor pusillus* (8.8%) and *Absidia corymbifera* prevailed in the samples. The highest

Table II. The fungi isolated from dried medicinal plants on different media at 25°C
Grzyby wyizolowane z suszonych roślin leczniczych na różnych pożywkach w 25°C

Fungal species	Number of isolates on media:				
	MEA	MEA20	MEA40	SAB+ACT	DG18
Rhizopus oryzae	21	17	20	7	8
Aspergillus niger	8	14	17	6	19
Rhizopus microsporus var. *rhizopodiformis*	8	-	-	10	8
Alternaria sp.	-	4	-	20	1
Eurotium amstelodami	-	-	1	-	20
Aspergillus flavus	-	-	-	3	7
Aspergillus versicolor	-	-	-	5	4
White yeasts	-	-	-	1	7
Rhizopus stolonifer	4	-	-	-	4
Fusarium solani	1	-	1	-	4
Mucor circinelloides	-	1	-	2	3
Syncephalastrum racemosum	-	-	-	5	-
Pink yeasts	-	-	-	1	4
Absidia corymbifera	1	-	-	1	2
Eurotium herbariorum	-	-	-	-	4
Mycelia sterilia (orange)	-	-	-	-	4
Penicillium nigricans	-	-	-	2	2
Phoma glomerata	-	1	-	-	3
Aspergillus fumigatus	-	-	-	2	1
Trichothecium roseum	-	1	-	1	-
Aspergillus melleus	-	-	-	2	-
Eurotium rubrum	-	-	-	-	2
Gliocladium penicillioides	-	-	-	-	2
Mycelia sterilia (brown-gray)	-	-	-	-	2
Paecilomyces variotii	-	2	-	-	-
Scopulariopsis brevicaulis	-	-	-	2	-
Scopulariopsis candida	-	-	-	2	-
Trichosporon mucoides	-	-	-	2	-
Wallemia sebi	-	-	-	-	2
Number of isolates	43	40	39	74	113
Number of species	6	7	4	18	22

number of isolates was obtained on MEA20, followed by DG18, MEA, MEA40 and SAB+ACT. Subsequently, the highest number of species was identified on MEA20 and SAB+ACT, followed by MEA40, DG18 and MEA. *Aspergillus niger* was isolated on all media. However, the highest number of *Aspergillus niger* isolates was isolated on MEA40. MEA20, MEA40 and DG18 were the best media for isolation of *Rhizopus oryzae*. The predominance of *Rhizomucor pusillus*, *Rhizomucor microsporus* var. *rhizopodiformis* and

Table III. The fungi isolated from dried medicinal plants on different media at 37°C
Grzyby wyizolowane z suszonych roślin leczniczych na różnych pożywkach w 37°C

Fungal species	Number of isolates on media:				
	MEA	MEA20	MEA40	SAB+ACT	DG18
Aspergillus niger	8	6	18	10	15
Rhizopus oryzae	-	14	16	2	18
Rhizopus microsporus var. *rhizopodiformis*	9	22	2	-	4
Aspergillus fumigatus	4	1	-	11	5
Rhizomucor pusillus	15	-	3	2	-
Absidia corymbifera	7	4	2	2	4
Aspergillus flavus	-	5	-	4	1
Emericella nidulans	-	2	-	4	-
Syncephalastrum racemosum	-	-	-	4	-
Fusarium solani	-	-	2	-	-
Eurotium amstelodami	-	1	-	-	-
Number of isolates	43	55	43	39	47
Number of species	5	8	6	8	6

Table IV. Thermophilic and thermotolerant fungi isolated from dried medicinal plants on different media at 45°C
Grzyby termofilne i termotolerancyjne wyizolowane z suszonych roślin leczniczych ma różnych pożywkach w 45°C

Fungal species	Number of isolates on media:			
	MEA	MEA20	MEA40	YpSs
Absidia corymbifera	2	-	14	3
Aspergillus fumigatus	6	7	2	10
Rhizomucor pusillus	3	5	5	2
Rhizopus microsporus var. *rhizopodiformis*	4	12	12	16
Number of isolates	15	24	33	31
Number of species	4	3	4	4

Aspergillus fumigatus was characteristic for MEA, MEA20 and SAB+ACT, respectively. *Rhizopus microsporus* var. *rhizopodiformis* was not isolated on SAB+ACT.

Altogether, 103 isolates belonging to four species were obtained from the samples at 45°C (Table IV). *Rhizomucor microsporus* var. *rhizopodiformis* was isolated from the samples with the highest number of isolates (42.7% of the total number of isolates at 45°C), followed by *Aspergillus fumigatus* (24.3%), *Absidia corymbifera* (18.4%) and *Rhizopus pusillus* (14.6%). The highest number of isolates was obtained on MEA40, followed by YpSs, MEA20 and MEA. *Rhizopus microsporus* var. *rhizopodiformis* was successfully iso-

lated on all media. However, the highest number of this species was obtained on YpSs. YpSs was also the best medium for isolation of *Aspergillus fumigatus*. *Absidia corymbifera* grew best on MEA40. The species was not isolated on MEA20. The best media for isolation of *Rhizomucor pusillus* were MEA20 and MEA40.

DISCUSSION

Mesophilic *Rhizopus oryzae* and *Aspergillus niger* along with thermophilic/thermotolerant *Rhizomucor microsporus* var. *rhizopodiformis*, *Aspergillus fumigatus*, *Absidia corymbifera* and *Rhizomucor pusillus* prevailed in the medicinal plant samples examined. Except for *Aspergillus fumigatus*, other fungi with abilities for production of hazardous mycotoxins, e.g. *Aspergillus flavus*, *Aspergillus versicolor*, *Aspergillus melleus* were isolated from the samples with low numbers of isolates. However, the fungi from the genus *Rhizopus* have also been proved to produce mycotoxins, i.e., aflatoxin-like compounds, ergot alkaloids and rhizonin [22, 23, 28]. The mycoflora examined shows many similarities to the mycofloras of medicinal plants in Egypt and Portugal [2, 3, 21]. However, *Penicillium* should, but did not, colonize the samples. This phenomenon is difficult for explanation.

The predominating species have been proved to produce a variety of enzymes, e.g. lipases, proteases, amylases, glucoamylases, cellulases and others [20, 26, 31]. It is likely that these fungi utilize plant organic compounds and contribute to the biodeterioration of dried medicinal plants.

A separate problem is the explanation of the mesophilic and thermophilic species predominance in the samples examined. It can be supposed that the improper storage conditions, e.g., high room temperature, humidity and plant material self-heating, could have resulted in the abundant growth of these fungi in the samples. It is unlikely that the fungi were airborne contaminants, since meso- and thermophilic/thermotolerant species in indoor and outdoor air in Poland are rather poorly represented. The dried medicinal plant mycoflora shows rather similarities to the mycofloras of self-heated municipal wastes and composts [9, 18].

Water activity a_w is the critical factor affecting the growth and activity of fungi in dried food [29]. However, no data have been found on the influence of water activity on fungal populations in dried medicinal plants. The fungal population of the samples examined was composed of species with variable moisture requirements [13]. This indicates that the water activity presumably fluctuated to a high degree in the samples during storage. It must be also stressed that xerophilic *Eurotium* species produce a variety of secondary metabolites and can cause serious food spoilage problems. The activity of these fungi and their influence on the quality of dried medicinal plants are separate problems to be studied.

Some of the species isolated from dried medicinal plants are opportunistic fungi [14, 15]. *Scopulariopsis brevicaulis* has been proved to have keratinolytic property [8]. It is likely, therefore, that the consumption of moldy herbs may pose a risk to immunocompromised individuals. This is another problem to be studied.

The DG18 medium was recommended for isolation of fungi from dried food [11]. In the present study, the numbers of isolates and species were the highest on DG18. Also, the isolation of xerophilic species (*Eurotium* spp. and *Wallemia sebi*) from plant material was possible on this medium. Thus, the results demonstrated that the medium was the

most suitable for isolation of fungi from dried medicinal plants at 25°C. However, the growth of *Rhizopus oryzae* was restricted on DG18 compared with the media containing malt extract. It was also observed that the higher sucrose content the higher number of *Aspergillus niger* isolates on malt extract media. The diversity of the fungal population on SAB+ACT was lower than the diversity on DG18, but still much higher than those on malt extract media. The population of actidione-resistant fungi was found to be rich in dried medicinal plants. The SAB+ACT medium was especially suitable for isolation of *Alternaria* sp., *Syncephalastrum racemosum* and some other actidione-resistant species.

At 37°C, the highest fungal diversity was observed on MEA20 and SAB+ACT. However, no general recommendations can be given for fungal isolation from dried plant material at this temperature. A medium should be chosen depending on the species to be isolated. For instance, MEA, MEA20 and SAB+ACT are recommended for isolation of *Rhizomucor pusillus*, *Rhizopus microsporus* var. *rhizopodiformis* and *Aspergillus fumigatus*, respectively. Subsequently, MEA40 and YpSs were found to be the best media for isolation of thermophilic and thermotolerant fungi from dried plant material. However, better growth of *Absidia corymbifera* on MEA40 and *Aspergillus fumigatus* on YpSs must be mentioned.

In a previous study [16], the same dried medicinal plant samples were analyzed for fungal quantities. The dilution method with DG18 and MEA media supplemented with chloramphenicol (100 mg/L) and incubation at 25°C were used. The fungal quantities ranged from $5x10^3$ to $7.8x10^6$ CFU/g in chamomile and peppermint, respectively. It is difficult to compare fungal quantities obtained by the dilution method with isolate counts obtained with the method used in this study. Also, fungal qualitative compositions were found to be different. Only 15 species were isolated from the samples using the dilution method. The higher number of species isolated by the method used in this study could have resulted from higher numbers of media and incubation temperatures. In addition, this method allows isolation of fungi occurring in plant material in small quantities. These fungi are usually lost using the dilution method.

Microbial quantitative limits for medicinal plant material can be found in the literature [10, 32]. Except for the fungal quantity in peppermint, fungal quantities were below the recommended limit for plan material to which boiling water is added before use (10^4 CFU/g). Yeasts being the natural component of plant mycoflora occurred in abundance in peppermint [16]. However, none of the microbiological quality regulations imposes determination of fungal qualitative composition in plant material. In the context of considerable differences in fungal qualitative compositions in dried plant materials, fungi identification is strongly recommended for assessment of potential health effects caused by these microorganisms.

K. Janda, K. Ulfig

A COMPARISON OF SELECTED MEDIA AND TEMPERATURES FOR ISOLATION OF MICROSCOPIC FUNGI FROM DRIED MEDICINAL PLANTS

Summary

The study was to compare selected media and incubation temperatures for isolation of fungi from dried medicinal plants. The samples examined were as follows: chamomile, peppermint, lemon balm, St. John's wort and two herbal mixtures. The media used were MEA, MEA + 20% sucrose, MEA +

40% sucrose, DG18, Sabouraud supplemented with cycloheximide (500 mg/l) and YpSs. 0.1-g portions of plant material (small pieces) were uniformly spread over the surface of Petri dishes containing the media. Incubation was carried out at 25, 37 and 45ºC. Altogether, 639 fungal isolates belonging to at least 31 species were isolated from the samples. Mesophilic, thermophilic and thermotolerant species, i.e. *Rhizopus oryzae*, *Aspergillus niger*, *Rhizopus microsporus* var. *rhizopodiformis*, *Aspergillus fumigatus*, *Absidia corymbifera* and *Rhizomucor pusillus* prevailed in the samples. The DG18 medium was found to be the most suitable for characterization of the total mycoflora at 25ºC. The medium selection for 37ºC was dependent on the species to be isolated. MEA + 40% sacharose and YpSs were found to be the best media for isolation of thermophilic and thermotolerant fungi from dried medicinal plants.

K. Janda, K. Ulfig

PORÓWNANIE WYBRANYCH POŻYWEK I TEMPERATUR HODOWLI W IZOLOWANIU GRZYBÓW MIKROSKOPOWYCH Z SUSZONYCH ROŚLIN LECZNICZYCH

Streszczenie

Celem badań było porównanie wybranych pożywek i temperatur hodowli w izolowaniu grzybów mikroskopowych z próbek suszonych roślin leczniczych (rumianku, mięty, melisy, dziurawca i dwóch mieszanek ziołowych). Wykorzystano następujące pożywki stałe: MEA, MEA + 20% sacharozy, MEA + 40% sacharozy, DG18, Sabouraud z dodatkiem cykloheksymidu w ilości 500 mg/l oraz YpSs. 0,1-g naważki materiału roślinnego rozsypywano równomiernie na powierzchni pożywek w szalkach Petriego. Inkubację prowadzono w 25, 37 i 45°C. Łącznie wyizolowano 639 szczepów grzybowych należących do 31 gatunków. W badanych próbkach dominowały gatunki mezofilne, termofilne i termotolerancyjne, tj. *Rhizopus oryzae*, *Aspergillus niger*, *Rhizopus microsporus* var. *rhizopodiformis*, *Aspergillus fumigatus*, *Absidia corymbifera* oraz *Rhizomucor pusillus*. Najwięcej szczepów i gatunków wyodrębniono na pożywce DG18. Wybór pożywki do izolowania grzybów w 37ºC uzależniony był od gatunku. Najlepszymi pożywkami do izolowania grzybów termofilnych i termotolerancyjnych z suszonych roślin leczniczych były MEA + 40% sacharozy i YpSs.

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