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Influence of the medium in bioautography-TLC screening test to the reliability of the results.

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Abstract: *Influence of the medium in bioautography-TLC screening test to the reliability of the results*. To the preliminary determination of the biological activity of selected chemicals used screening tests, allowing for quick identification of the fungistatic properties, such as bioautography. Because some factors (culture medium composition, tested microorganisms, extractive method, pH, solubility of the sample in the culture medium, etc.) can change results. The aim of the present work was to analyze the minimum inhibitory concentration (MIC) of selected fungicides (IPBC, 4,5-dichloro-2-octyl-2H-isothiazol-3-one, dichloro-2-n-octyl-4-isothazolin-3-one and 2-octyl-2H-isothiazol-3-one, N-alkyl(C12-18)-N,N-dimethyl-N-benzylammoniumchloride, chalcone) using various culture medium. This would allow to determine whether the change of the medium affects the results of screening test. The aim was also predicting the most preferred culture medium for screening - bioautography.

Keywords: bioautography, screening test, fungicide

INTRODUCTION

The degradation of wood is mainly caused by biotic factors decreasing its lifetime. Wooden objects most often are attacked by wood decaying fungi (*Basidiomycetes)* and microfungi, commonly called molds. A high air humidity or influence of precipitation foster the growth of microorganisms, therefore wood used in such condition needs preventive treatment. To protect wood mainly are used preservatives, expecting from them, long-lasting effectiveness and environmentally friendly

The global interest in the exploration and use of natural products as fungicides is rapidly growing. Various natural substances are potentially suitable for the wood protection against attack by fungi. Among plant extracts, essential oils, and other products from natural sources chalcone is one of compounds with many biological properties. They have found numerous applications as pesticides, food additives as well as anti-inflammatory and anticancer agents [Nowakowska 2007]. For the preliminary determination of the biological activity of biocides thequick laboratory tests – screening have been used.

There are many methods to measure the chemicals impact on the fungi growth but bioautography belongs to the simplest and fastest methods [Homans and Fuchs 1970].
A simple bioautography technique for detection of potential fungicide substances has been in use for many years in mycological laboratory. Bioautography microbiological testing is a commonly used screening to identify antibacterial and antifungal compounds. It is a simple measurement providing a “yes/no” response [Munoz-Olivas 2004]. The sensitivity of this method might be increased. It does not need expensive sophisticated techniques and is adequate to screening of fungicide [Choma, 2005]. Performed bioautography screening method is more sensitive than other methods for determination of biocides properties, and it’s simple, inexpensive and saves your time [Jesionek et al., 2013].

The procedure in bioautography methods is similar to agar diffusion methods. The difference is that the tested compounds diffuse to inoculated agar medium from the chromatographic layer which is thin-layer chromatography plate or paper. Must be considered that some factors (e.g. culture medium composition, tested microorganisms, extractive method, pH, solubility of the sample in the culture medium) can change results [Rios et al.,

1988]. The aim of the present study was to analyze the growth of fungal hyphae on various agar medium composition in order to use it in bioautography screening test; improve the quality of mycelium growth on agar medium as well as give credibility to the obtained results.

MATERIALS AND METHODS

Fungicides: in the study included four commonly used synthetic fungicides: IPBC (Iodopropynyl butylcarbamate) **I**, 4,5-dichloro-2-octyl-2H-isothiazol-3-one (Forchem) **II**, dichloro-2-n-octyl-4-isothazolin-3-one and 2-octyl-2H-isothiazol-3-one (Acima Rhom&Haas) **III**, N-alkyl(C12-18)-N,N-dimethyl-N-benzylammoniumchloride (Lonza) **IV** and one of natural origin: chalcone **V**. To determine the minimal inhibitory concentrations (MIC) each substance was prepared at concentrations in the range of 0.1% to 0.00001% and then applied on the TLC plates in the amount 8µl in three replication for each preparation.
Tested microorganism: *Aspergillus niger* van Tiegen ATCC 6275 (BAM 4)
Culture medium composition: was prepared six microbiological growth media:

A: 60ml of stock solutions, 10 ml of glucose;
B: 60ml of stock solutions, 10 ml of malt extract;
C: 60ml of stock solutions, 10 ml of glucose, 1% agar;
D: 60ml of stock solutions, 10 ml of malt extract, 1% agar;
E: 39g/l of potato dextrose agar (Merck);
F: 4g/l of Czapek-Dox salts (Sigma-Aldrich), 30g/l of malt extract, 20g/l of agar (Merck).

For each sterilized medium was added 5µl of spore suspension. Stock solutions contained: 7g KH_2PO_4, 3g $Na_2HPO_4 \cdot 2H_2O$, 4g KNO_3, 1g $MgSO_4 \cdot 7H_2O$, 1g NaCl per 1l of tap water [Homans and Fuchs, 1970].
Direct bioautography: 2 x 4 cm silica gel 60 F_{254} sheets (Merck) were used. TLC adsorbents were preconditioned by heating at 120˚C for 3 h. Without this procedure adsorbent layers became partly detached when soaked. TLC plates with added formulation placed in a Petri dish on the agar medium and then coated with agar medium with spores used in the test. Layers were incubated at 28˚C and above 95% RH in darkness. Fungal growth was evaluated macroscopically for four days. From our previous studies we concluded that it is sufficient time for estimation the activity of preservatives. Each experiment was performed with triplicates of each compounds compare to control plates. Visual evaluation of *A.niger* growth on samples was made according to the four-point scale of intensity mycelium growth set out in table 1.

Table 1. Scale of fungal growth

0	no visible growth under the microscope
1	invisible growth with the naked eye but are clearly visible under the microscope
2	visible growth with the naked eye, growth of hyphae without spores
3	visible growth with the naked eye, sporulation mycelium
4	intensive growth, covering the entire surface of the test

RESULTS

Aspergillus niger was used in the mycological assay because its fast growth, easy-sporulating and is rarely infections during the test. As it is shown in table 2, mycelium growth rate was different on various media for control plate. After 24 hours has been observed a visible growth of mycelium what means that the conditions for their growth is an optima one. As we can see in table 2, the results of MIC for each fungicides at various media are very close to each other.

For the most of culture media, MIC of first fungicide (IPBC) was equal to 0.0001%. But the most effective concentration of this fungicide throughout the duration of the test is 0,1%. Minimal inhibitory concentration of 4,5-dichloro-2-octyl-2H-isothiazol-3-one (**II**) is equal 0,001%, for dichloro-2-n-octyl-4-isothazolin-3-one with 2-octyl-2H-isothiazol-3-one (**III**), and N-alkyl(C12-18)-N,N-dimethyl-N-benzylammoniumchloride (**IV**) is equal to 0,1%.

Table 2. Results of the fungal growth
C – concentration, cp – control plates

	C [%]	I				II				III				IV				V			
		1d	2d	3d	4d	1d	2d	3d	4d	1d	2d	3d	4d	1d	2d	3d	4d	1d	2d	3d	4d
A	0,1	0	0	0	0	0	0	0	0	0	0	0,33	1,33	0	0	0,33	0,33	0	0	1	2
	0,01	0	0	0,33	0,67	0	0	0	0	1	2	2	3	0	2,67	2,67	3	0	2	2	3
	0,001	0	0	1,67	3	0,33	0,67	1,33	2	1	2,33	2,33	3	0	1,67	2,33	3	1	2,67	2,67	3
	0,0001	0	0	2,33	4	1	2,33	3	3	1,67	2,67	2,67	3	0	2,33	3	4	1	3	3	3
	0,00001	1,33	2,33	3	4	2	3	3	3	1,67	3	3	3	1,33	2,33	3	4	1,33	3	3	3
	cp	2	3	4	4	2	3	4	4	2	3	4	4	2	3	4	4	2	3	4	4
B	0,1	0	0	0,33	0,67	0	0	0	0	0	0	0,33	1,33	0	0,33	0,33	0,67	0	0	1	2
	0,01	0	0	0,67	1,33	0	0	0,33	1,33	1,33	2,67	2,67	3	1,67	2,67	3,33	4	1	3,33	3,33	3,33
	0,001	0	0	1,67	2,67	0	0,33	1,33	2,33	2	4	4	4	1,67	3,67	3,67	4	2	4	4	4
	0,0001	0	0	2,33	3,33	1,67	3,67	3,67	3,67	2	4	4	4	2	3,67	3,67	3,33	2	4	4	4
	0,00001	0,33	1,33	2,67	3,67	2	4	4	4	2	4	4	4	2	3,67	3,67	3,67	2	4	3,67	4
	cp	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4
C	0,1	0	0	0	0	0	0	0	0	0	0	1,67	2,67	0	0	0,67	2	0	0	2	4
	0,01	0	0	0	0	0	0	1	2,33	1	2,67	3	3,67	0	2	2,67	3,67	0	1	2,67	3,33
	0,001	0	0	1,67	2,67	0,67	1,33	2	3	1	3	3	4	1	3	3	4	1	2	3	4
	0,0001	0	0	2	4	0,67	1,67	2,33	3,67	0,33	2,67	3	4	1	3	3	4	1	3	3	4
	0,00001	0,33	1	3	4	1,67	3	3	4	1,33	3,33	3,33	4	1	3	3	4	1	3	3	4
	cp	1	2	3	4	2	3	3	4	2	3	3	4	2	3	3	4	2	3	3	4
D	0,1	0	0	0,67	1,33	0	0	0	0,33	0	0	0,67	1,67	0	0,33	1,33	2,67	0	1	2,33	4
	0,01	0	0	1	1,67	0	0,33	1	1,33	1	3	3,67	4	1	3	3	4	1	2,67	3	1,67
	0,001	0	0	2,33	4	0,67	1,67	1,67	2	2	4	4	4	2	4	4	4	1,67	3,67	4	4
	0,0001	0	0,67	2,67	4	1	3	3,33	4	2	4	4	4	2	4	4	4	2	4	4	4
	0,00001	1,67	3,67	3,67	4	1,67	4	4	4	1,67	4	4	4	1,67	3,67	3,67	4	1,67	4	4	4
	cp	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4
E	0,1	0	0	0,33	0,67	0	0	0	0,33	0	0	1	2	0,67	1,67	1,67	2	0	0	2,33	3
	0,01	0	0	0,33	0,67	0	0	0,33	1,33	0	1,33	2,33	4	1,33	3	3,33	3,33	0	0,67	2,33	4
	0,001	0	0	1,67	3,67	0	1,33	2	3,67	1	2	3	4	2	4	4	4	1	1,33	2,33	4
	0,0001	0	0	2	4	0,67	2,33	3	4	1	2	3	4	2	4	4	4	1,67	2,33	3	4
	0,00001	1	2	3	4	1	3	3	4	1	2	3	4	2	4	4	4	1	2	3	4
	cp	1	2	3	4	1	3	4	4	1	2	3	4	2	3	4	4	1	2	3	4
F	0,1	0	0	0	0,33	0	0	0	0	0	0	1	2	0	0,33	1	2	0	0,33	1,67	3
	0,01	0	0	0	1	0	0	0	0,33	1,67	3,67	3,67	3,67	0	2	3	3,67	2	4	4	4
	0,001	0	0	0	4	0	1	2	2,67	2	4	4	4	1	2,67	3	4	2	4	4	4
	0,0001	0	0	0	4	1,67	3,67	3,67	4	2	4	4	4	1,33	3,33	3,33	4	2	4	4	4
	0,00001	1,67	2,67	3	4	2	4	4	4	1,67	4	4	4	1,33	3,33	3,33	4	1,67	4	4	4
	cp	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4	2	4	4	4

Mentioned fungicides (**I-IV**) can be the reference products to compare their fungistatic activity with activity of natural compounds like chalcone. MIC for chalcone is equal to 0,01%. We found that the direct covering thin-layer chromatograms with the spore suspensions of the test fungi in a mineral salts medium was the most effective technique and gave the most reliable results. Among the tested surfaces A-F (containing stock solutions) to cover the TLC plates during bioautography the culture medium composition D, have improved the quality of the tests and shorten the time waiting for results.

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Streszczenie: *Wpływ podłoża mikrobiologicznego na miarodajność wyników bioautografii-TLC.* W celu wstępnego określenia biologicznej aktywności wybranych związków chemicznych, stosuje się testy przesiewowe, które pozwalają na szybką identyfikację np. właściwości fungistatycznych. Jednym z takich testów jest bioautografia-TLC. Niestety, niektóre czynniki (skład podłoża hodowlanego, pH, rodzaj mikroorganizmu) mogą wpływać na wyniki testów. Przedmiotem niniejszej pracy była analiza minimalnego stężenia wybranych fungicydów (IPBC, 4,5-dichloro-2-oktylo-2H-izotiazol-3-on, dichloro-2-n-oktylo-4-izotazolin-3-on and 2-oktylo-2H-izotiazol-3-on, Chlorek N-alkilo(C12-18)-N,N-dimetylo-N-alkilodimetylobenzyloamoniowy, chalkon), które hamuje wzrost grzybni na pożywkach hodowlanych o różnym składzie. Tego typu badanie pozwoli to na określenie, czy zmiana pożywki wpływa na wiarygodność wyników badań skriningowych. Celem jest także wytypowanie najbardziej korzystnego podłoża w badaniu przesiewowym – bioautografii.

Słowa kluczowe: bioautografia, badanie przesiewowe, fungicyd

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