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COMPARISON OF VARIOUS METHODS FOR DETERMINATION OF ANTIFUNGAL ACTIVITY OF *BACILLUS COAGULANS*

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Abstract: The aim of this study was to compare various methods of antifungal activity determination in model experiments. For the investigations *Bacillus coagulans* (No. 6) isolate with the high antifungal activity, isolated from lupine composts was used. Antifungal activity of the examined isolate was estimated against five pathogenic species of fungi: *Rhizoctonia solani, Bipolaris sorokiniana, Sclerotinia sclerotiorum, Trichothecium roseum* and *Fusarium oxysporum*. Determination of fungal growth was carried out by three methods: ergosterol assessment, counting colony-forming units (CFU) and agar plate diffusion assay.
Statistically significant Spearman's rank coefficients were recorded between ergosterol assessment and two other methods. On the basis of this data the assessment of ergosterol is the best way of determination of antifungal activity and may be used as a reference procedure.

Key words: antifungal activity, pathogenic fungi, *Bacillus coagulans*, ergosterol, CFU, agar plate diffusion assay

INTRODUCTION

In our Department research project on the possibility of employing antifungal properties of composts prepared from various plant materials was conducted. Literature date indicate that composted plant materials are very often characterised by biological activity, which is probably associated with the active microflora developing in these composts. Gulewicz and Trojanowska (1995) isolated from active lupine composts bacteria isolates from the genus of *Bacillus* and majority of them

showed antifungal properties against pathogenic fungi. Some problems are connected with choosing the suitable method for determination of this activity.

For many years tests for the sensitivity of microorganisms to antimicrobial agents have relied on diffusion techniques. Fleming did the first antimicrobial susceptibility test that utilised diffusion of the antibiotic substance through agar media with penicillin against *Staphylococcus aureus* in 1924. Cutting wells into agar that serve as reservoirs for liquid preparations of antimicrobial agents and use sensitivity disks (sterile paper containing antimicrobial substances) have been popular modifications of this technique (Hoover and Harlander 1993).

Another group of techniques of determination of antimicrobial activity is based on plate count method using indicator organisms. The sampling, media and conditions of incubation should be optimal for the indicator strain to insure colony formation by all viable cells also the cells should not form chains or clumps to ensure precision of colony counting. There might be some problems with using these techniques for determination of antifungal activity by two various organisms to each other. In this case some problems are connected with the fact, that colonies usually arise only from spores and heavily sporulating species will be overestimated and less abundantly sporulating species underestimated. Plate count techniques are time- and labour consuming and do not detect dead fungi (Kaspersson 1987).

In the recent years however, other methods have been developed. The most important are based on high performance liquid chromatography (HPLC) – ergosterol assessment (de Ruiter et al. 1993), enzyme-linked immunosorbent assay (ELISA) (Gouarma and Bullerman 1995), abbot TDX polarisation fluoroimmunoassay system and substrate-labelled fluoroimmunoassay assay (SLFA) (de Ruiter et al. 1993) and direct bioautography using thin-layer chromatography (2D-TLC) (Wedge and Nagle 2000).

From the last group of methods the most popular technique is estimation of ergosterol. Ergosterol is the primary sterol in the cell membranes of filamentous fungi and is either absent or a minor component in most of higher plants. It is a constituent of membranes in mycelia, spores and vegetative cells. Ergosterol content has been widely used as an estimate of fungal biomass in various environments, e.g., in soil and aquatic systems, food and feeds (Davis and Lamar 1992; Djajakirana et al. 1996; Kinsey et al. 1999).

The aim of this study was to compare three methods of antifungal determination – counting colony-forming units, level of ergosterol biosynthesis and agar plate diffusion assay.

MATERIALS AND METHODS

Cultures: *B. coagulans* (strain No. 6) was isolated from lupine compost (Gulewicz and Trojanowska 1995). Fungal indicators: *R. solani* (BPR 635), *B. sorokiniana* (BPR 808), *S. sclerotiorum* (BPR 648), *T. roseum* (BPR 671) and *F. oxysporum* (BPR 694) were obtained from the Collection of Pathogenic Microorganisms (BPR) from Institute of Plant Protection in Poznań, Poland.

Media: The following media were used: for bacterial cultivation – pea extract (Czaczyk et al. 2000) (it was made using ground peas and kept under the toluene

for 5 days at 37°C; after filtration an extract was enriched with 10 g/L glucose, 0.5 g/L peptone and 0.5 g/L NaCl; solid medium contained 20 g/L agar addition), for fungal cultures – Czapek-Dox medium.

Cultivation: Bacterial isolates were kept on nutrient broth and were cultivated five times on agar slants and then four times on liquid medium. 10 mL of the final culture was brought to 90 mL of the medium. The cultures were incubated at 37°C, at the shaking speed of 100 rpm.

Fungal cultures were cultivated on slants at temperature 30°C for 7–10 days.

Assessment of ergosterol: A well-developed fungal culture on a slant was rinsed with 10 mL of sterile water. Fungal suspension (10^5 CFU/mL) was mixed with Czapek-Dox medium in proportion 1:10. The fungi were incubated at 30°C, at the shaking 100 rpm. *B. coagulans* and fungal indicators grown separately in 250 mL flasks containing adequate medium. An equal volume of 6-h-grew bacterial culture of *B. coagulans* containing 10^{10}–10^{11} CFU/mL (from midlog phase culture without any endospores) was co-inoculated into flasks seeded with spores of fungi at 24h. For each sample, the flasks were incubated for 168 h at 30°C, at the shaking speed of 100 rpm. Fungal culture with addition of sterile pea extract was used as a control. The experiments were run in three replications and repeated three times.

The samples from these experiments were used to **determine of colony forming units** (CFU) by dilution plating method.

Sample preparation for HPLC analysis: 20 mL of mixed culture was homogenised for 2 min and blended with 100 mL methanol. After 24 h it was centrifuged, the supernatant was poured off and 50 mL of methanol was added. The two methanol supernatants were combined, mixed with 25 g ethanolic KOH and refluxed for 30 min. The saponified mixture was extracted with 100- and 50- mL portions of hexane. Both hexane extracts were combined and evaporated. These residues were suspended in 5 mL of methanol and used for HPLC analysis (Seitz et al. 1977).

HPLC analysis: Determination was carried out on MERCK-HITACHI system consisted of autosampler (model L–7250), pump (model L-7100) and diode array detector (model L–7455) set at 282 nm. Analysis were performed isocratically at flow rate 1.5 mL/min at 30°C on Adsorbosphere C_{18} column (150 × 4.6mm) – Alltech. Methanol-water (95:5) as a mobile phase was used. The ergosterol amount was quantified by computer integration of peak area (external standard mode).

Agar plate diffusion assay: A well-developed fungal culture on a slant was rinsed with 10 mL of sterile water. Fungal suspension was mixed with Czapek-Dox medium in proportion 1:10, and poured onto Petri dishes (about 15 mL). Then the well (10 mm diameter) was cut in the middle of each inoculated Petri dish. These plates were incubated at the temperature 30°C for 24 h to encourage development. After this time 0.1 mL suspension of bacterial strains (10^{10}–10^{11} CFU/mL) was brought to the well. Antifungal activity was determined as zones of growth inhibition (mm). Membrane sterilised (Millipore 0.22 mm) supernatant of bacterial culture was used as a control. All investigations were carried out in five replications.

Statistical methodology: Due to the procedures applied, the antifungal activity is expressed in different measuring units, i.e. millimeters (agar plate diffusion assay), CFU/mL or mg/L of ergosterol. This is major source of problems with data

comparison. Therefore the results of antifungal activity obtained using ergosterol assessment method were first sorted in descending and ranking order. Identical classification was performed for agar plate diffusion assay and CFU method and correlation coefficients on the ranks were computed.

All statistical analyses were conducted using the Spearman's rank correlation (Johnson 1984). Rank correlation coefficient (r_s) was found using the formula:

$$r_3 = \frac{6\Sigma(d_i)^2}{n(n^2 - 1)} \quad [1]$$

Where:

d_i - difference in the ranking

n – the numbers of pairs of data

We anticipate the existence of either positive or negative correlation because using various methods, antifungal activity was observed as an increase (agar plate diffusion assay) or decrease (ergosterol assessment and CFU) of measure parameter. To use procedure for calculating the r_s statistics critical value of Spearman's rank correlation coefficient was 0.648 (α=0.05; n=10).

RESULTS

The variation of ergosterol content between species was observed in presented study. In control cultures it ranged from 2.54 – 21.93 mg/L (Tab. 1). Addition the log phase culture of *B. coagulans* (No. 6) to 24-h-grown culture of fungi resulted in inhibition of this sterol biosynthesis in mycelium. Strong inhibition of ergosterol biosynthesis against all the tested fungal plant pathogens was noticed (Tab. 1). Complete inhibition was observed for *R. solani* and *T. roseum*. The lowest ergosterol inhibition was observed for *F. oxysporum*. For the remaining variants of the experiments, the level of ergosterol biosynthesis inhibition in plant fungal pathogens by *Bacillus* sp. reached 90%.

Determination of antifungal activity using CFU counting of fungi gave mostly the positive results (decreased of CFU/mL). It was observed for *R. solani*, *S. sclerotiorum*, *T. roseum* and *F. oxysporum* (Fig. 1). In the case of *B. sorokiniana* the number of living cells was on the same level in control and tested samples.

On the basis of agar plate diffusion assay antifungal activity was estimated as zones of growth inhibition fungi by bacterial culture. Differences in pathogen sen-

Table 1. Average ergosterol content in control samples and in samples co-inoculated by *Bacillus coagulans* (standard errors in brackets)

Pathogenic fungi	Ergosterol content [mg/L]	
	Control	Co-inoculation by *B.coagulans*
Rhizoctonia solani	5.08 (±2.45)	0
Bipolaris sorokiniana	3.04 (±0.02)	0.082 (±0.034)
Sclerotinia sclerotiorum	21.93 (±1.59)	0.078 (±0.012)
Trichothecium roseum	2.54 (±012)	0
Fusarium oxysporum	17.31 (±2.01)	11.70 (±1.87)

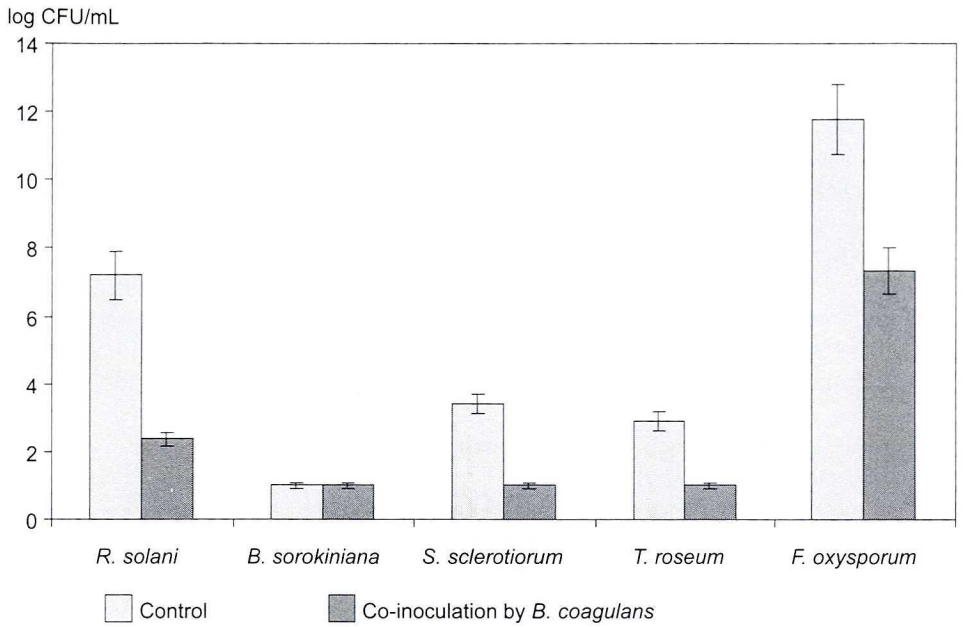

Fig. 1. Determination of antifungal activity of *Bacillus coagulans* using CFU counting

sitivity were observed by direct comparison of zone of fungal inhibition by *B. coagulans* against membrane sterilised supernatant of bacterial culture. According to this method *B. coagulans* inhibited strongly the growth of *T. roseum*, middling in the case of *R. solani*, *B. sorokiniana* and *S. sclerotiorum* and did not inhibit *F. oxysporum* (Fig. 2).

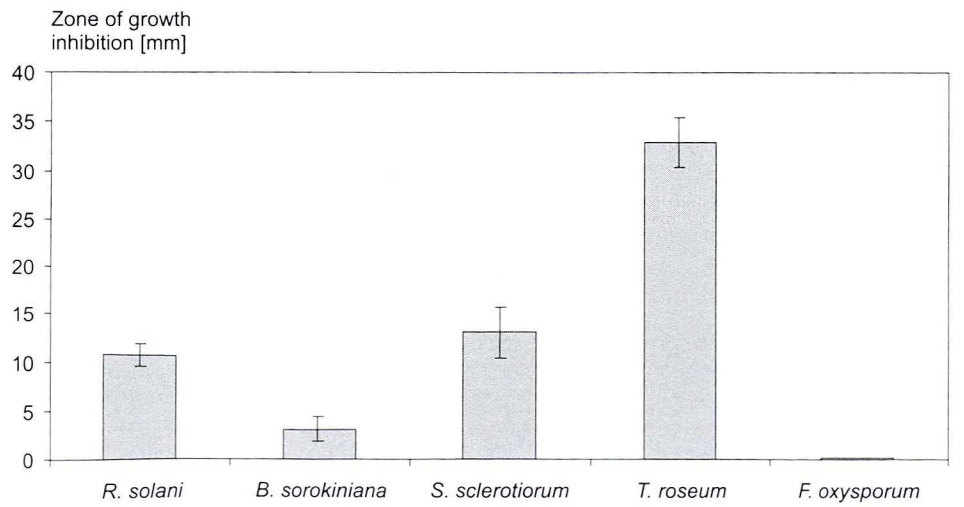

Fig. 2. Antifungal activity (zone of growth inhibition) of *Bacillus coagulans* against pathogenic species of fungi

DISCUSSION

The identification and the biological characterisation of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, is of great interest for the modern and eco-compatible agriculture. The biggest problem is selection of the suitable method for determination of activity of these natural antagonisms. Antifungal testing of filamentous fungi generally suffers from incomparability of results.

The use of ergosterol measurement as a biomarker for fungal growth has some advantages in comparison with other methods. Ergosterol is specific for fungi, only small amount of this sterol is accumulated in fungal necromass (Davis and Lamar 1992), the efficiencies of extraction is relatively high (Padgett and Posey 1993), an exactly defined substance is determined using chromatographic methods, the results show good reproducibility (in contrast to traditional microbiological methods). Mycelial age and availability of nutrients are known to influence ergosterol synthesis (Newell et al. 1987).

Plate counting method needed no special apparatus, but it is time- and labour-consuming technique and did not detect dead fungi. Fungal colonies usually arise from spores and heavily sporulating species will be overestimated and less abundantly sporulating species underestimated (Kaspersson 1987).

In the diffusion techniques (agar plate diffusion assay) antimicrobial agents may not have been absorbed properly and is thus not achieving an insufficient level in the cultivation media. It is well known, however, that a number of other factors will influence the size of the zone of inhibition (choice of medium, pH, size of inoculum, incubation times e.g.) and these factors will require control (Hadacek and Greger 2000).

To use procedure for calculating the r_s statistics Spearman's rank correlation coefficients between using methods are presented in table 2.

Statistically significant rank coefficients were observed between ergosterol assessment and both other methods. This arises from the data in table 2, that ergosterol content is very well associated with CFU method of measurement of antifungal activity. The degree of association as calculated in % ($100r^2$), is 65.1%. Still significant, but moderate association exists between ergosterol content and agar plate diffusion assay – $100r^2 = 43.6\%$. The results obtained with CFU counting method are not comparable those obtained by agar plate diffusion assay. Therefore, the ergosterol content as a measure of antifungal activity is worth the consideration as a reference procedure for both CFU counting and agar plate diffusion assay.

Table 2. Spearman's rank correlation and statistical significance between ergosterol assessment (method I), CFU counting (method II) and agar plate diffusion assay (method III)

Method	I	II	III
I	X	0.8069*	–0.6606*
II	0.8069*	X	–0.4545
III	–0.6606*	–0.4545	X

Note: *statistically significant (α=0.05)

It is important to remember that the quantitative analyses should be combined with the other especially when health hazards are to be evaluated. On the basis of this data the best way of determination of antifungal activity is use more than one method. Gouarma and Bullerman (1995) also found that ergosterol assessment has good potential in studying the production of secondary metabolites such as mycotoxins and in predicting the onset of mould activity before formation of these substances.

The methods for determination of antifungal activity must be easy to run, should not require specialised equipment and also should well suit to chemically complex natural product-rich samples. The most important efforts in the improvement of sensitivity tests have been direct towards a standardised method that is generally acceptable.

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POLISH SUMMARY

PORÓWNANIE RÓŻNYCH METOD OZNACZANIA AKTYWNOŚCI ANTYGRZYBOWEJ *BACILLUS COAGULANS*

Celem przedstawionych badań było porównanie różnych metod oznaczania aktywności antygrzybowej *Bacillus coagulans* wobec grzybowych patogenów roślin. Do badań wykorzystano izolat o silnych właściwościach antygrzybowych – *Bacillus coagulans* (Nr 6), wyizolowany z kompostów łubinowych. Jego aktywność antygrzybową określano wobec 5 gatunków grzybów powodujących choroby roślin: *Rhizoctonia solani, Bipolaris sorokiniana, Sclerotinia sclerotiorum, Trichothecium roseum* i *Fusarium oxysporum*. Wzrost patogenów grzybowych określano na podstawie oznaczenia poziomu syntetyzowanego ergosterolu, liczby jednostek infekcyjnych tworzących kolonie i wielkości stref hamowania wzrostu. Na podstawie analizy statystycznej otrzymanych wyników stwierdzono istotną korelację pomiędzy oznaczeniem aktywności antygrzybowej poprzez pomiar poziomu ergosterolu a pozostałymi metodami. Na tej podstawie wytypowano tą metodę jako referencyjną do pozostałych.