Letter to Editors

Inhibition of Ergosterol Biosynthesis in Fungal Plant Pathogens by *Bacillus* sp.

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Abstract

Antifungal activity of *Bacillus coagulans* creates the possibility to use this microorganism in biological control of fungal plant pathogens. Activity of *Bacillus coagulans* (No 6), isolated from lupine compost, against seven pathogenic species of indicator fungi: *Bipolaris sorokiniana, Trichothecium roseum, Rhizoctonia solani, Sclerotinia sclerotiorum, Fusatium oxysporum, Fusarium solani* and *Fusarium culmorum* were examined in this work. Ergosterol assessment and counting colony forming units (CFU) carried out determination of fungal growth. The addition of *Bacillus coagulans* to culture of fungi resulted in inhibition of ergosterol biosynthesis in mycelium. The strongest inhibition in all tested fungal plant pathogens was observed when *Bacillus coagulans* was co-inoculated at the beginning of culture. The decrease of ergosterol level did not always correlate with the decrease of CFU.

Keywords: ergosterol, antifungal activity, *Bacillus* sp.

Introduction

Biological control of plant pathogens has been the subject of much research in recent years. It is a potential possibility for the limited use of chemical pesticides which have harmful influence on the environment. Naturally occurring substances found in bacteria, fungi and higher plants are important sources of molecules with antifungal properties. The first major natural products of microbial origin to be commercialised were the antibiotics blasticidin S, kasugamycin and validamycin A. They were developed in Japan to control rice blast *(Pyricularia oryzae)* and sheat blight *(Rhizoctonia solani)* [16].

Plant pathogens have caused an almost 20% reduction in the principal food and cash crops worldwide [11]. These losses may be limited by the use of fungicides, sanitation practices, and crop rotation or by the use of disease-tolerant cultivates. From the ecological point of view natural fungicides are the most important alternative for the use of chemical substances. Literature data 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 [6] isolated from active lupine compost bacteria strains from the genus of *Bacillus* and the majority of them showed fungistatic properties against plant pathogens.

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One of the most popular techniques of determination of fungal growth is based on the measure of ergosterol level. Ergosterol is the primary sterol in the cell membranes of filamentous fungi and is either absent or a minor component in most higher plants. It is a constituent of membranes in mycelia, spores and vegetative cells. Ergosterol content has been widely used as a measurement of fungal biomass in various environments, e.g., in soil and aquatic systems, food and feeds [4, 5].

The aim of this study was to estimate antifungal properties *Bacillus coagulans* (No 6) against 7 pathogenic species of indicator fungi, using ergosterol assessment and comparative, counting colony forming units (CFU).

Materials and Methods

Cultures. *Bacillus coagulans* (strain no 6) was isolated from lupine compost [15]. Fungal indicators: *Bipolaris sorokiniana* (BPR 808), *Trichothecium roseum* (BPR 671), *Rhizoctonia solani* (BPR 635), *Sclerotinia sclerotiorum* (BPR 648), *Fusarium oxysporum* **(BPR** 694), *Fusarium solani* (BPR 718) and *Fusarium culmorum* (BPR 727) were obtained from Collection of Pathogenic Microorganisms (BPR) from Institute of Plant Protection in Poznan, Poland.

Media. The following media were used: for bacterial cultivation - 20% peas extract [2] (it was made using ground peas and kept under the toluene for 5 days at 37°C; after filtration extract was enriched with 1% glucose, 0,5% peptone and 0,5% NaCl; solid medium contained 2% agar addition), for fungal cultures - Czapek-Dox medium.

Cultivation. Bacterial strains were kept on nutrient broth and passed several times through agar slants and liquid medium. 10 ml of the final culture was brought to 90 ml of the medium. The cultures were incubated at 37°C; the shaking speed was 100 rpm.

Fungal cultures were cultivated on slants at 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 at 1:10. The fungi were incubated at 30°C, at 100 rpm shaking. *Bacillus coagulans* and fungal indicators were grown separately in 250 ml flasks containing adequate medium. An equal volume of 6-h-grown bacterial culture of *Bacillus coagulans* containing

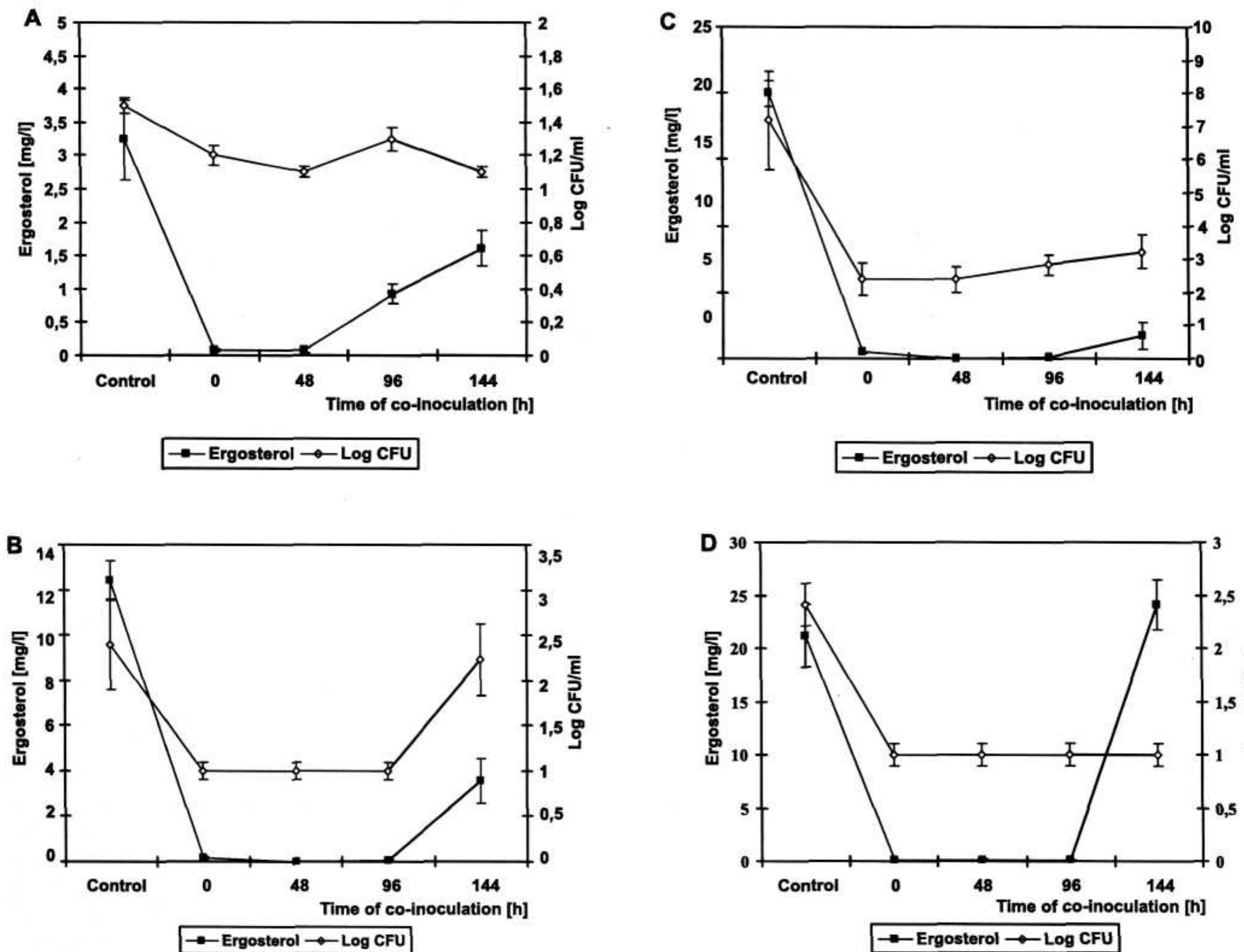

Fig. 1. Effect of co-inoculation fungal cultures by *Bacillus coagulans* on ergosterol biosynthesis and CFU (A - *Bipolaris sorokiniana,* B - *Trichothecium roseum,* C - *Rhizoctonia solani,* D - *Sclerotinia sclerotiorum).*

10^{10}-10^{11} CFU/ml (from midlog phase culture without any endospores) was co-inoculated into flasks seeded with spores of fungi at different time intervals (0, 48, 96, 144 h - for *Bipolaris sorokiniana, Trichothecium roseum, Rhizoctonia solani* and *Sclerotinia sclerotiorum;* 0, 24, 48, 72 h - for *Fusarium oxysporum, Fusarium solani and Fusarium culmorum).* For each sample, the flasks were incubated for 168h at 30°C, at 100 rpm shaking speed. Fungal culture with addition of sterile pea extract was used as a control. The experiments were run triplicate and repeated two times.

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

HPLC analysis. 20 ml of mixed culture was homogenised for 2 min and blended with 100 ml methanol. After 24h it was centrifuged, the supernatant was poured off and 50 ml of methanol was added. The two methanol supernatants were combined, mixed with 25g 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 [14].

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 282nm. Analysis were performed isocratically at a flow rate of 1.5 ml/min at 30°C on Adsorbosphere C_{18} column (150 x 4.6 mm) - 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).

All the results were statistically analysed using linear correlation analysis (Microsoft Excel).

Results

In the first part of experiments the level of ergosterol biosynthesis, dependent of the time of co-inoculation fungal cultures by *Bacillus coagulans,* was measured. The results of our investigations are presented as ergosterol content [mg/1] and log CFU/ml in Figure 1 (A - *Bipolaris sorokiniana,* B -*Trichothecium roseum,* C - *Rhizoctonia solani,* D - *Sclerotinia sclerotiorum)* and in Figure 2 (A - *Fusarium oxysporum,* B - *Fusarium solani,* C - *Fusarium culmorum).*

Addition of the log phase culture of *Bacillus coagulans* (No 6) to culture of fungi resulted in inhibition of this sterol biosynthesis in mycelium. The strongest inhibition in all tested fungal plant pathogens was observed when *Bacillus coagulans* was co-inoculated at the beginning of culture. Complete inhibition was observed for *Bipolaris sorokiniana* (after co-inoculation in 0 and 48 h) and for *Trichothecium roseum, Rhizoctonia solani, Sclerotinia sclerotiorum* (after co-inoculation in 0, 48 and 96 h) (Figure 1). For these fungi decrease of ergosterol synthesis was evident during the entire culture period.

Lower ergosterol inhibition was observed for *Fusarium* sp. (Figure 2). In mixed cultures *Fusarium solani* or *Fusarium culmorum* and *Bacillus coagulans* decrease of ergosterol biosynthesis was observed in all variants of

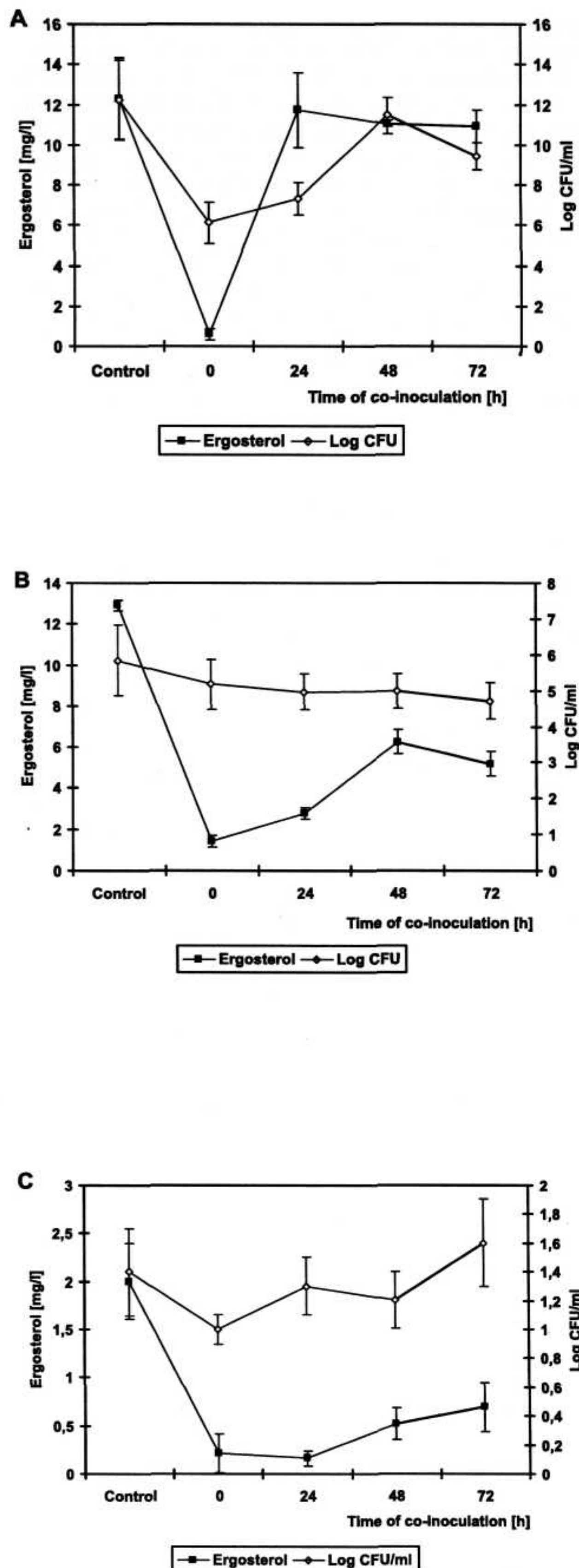

Fig. 2. Effect of co-inoculation fungal cultures by *Bacillus coagulans* on ergosterol biosynthesis and CFU (A - *Fusarium oxysporum,* B - *Fusarium solani,* C - *Fusarium culmorum).*

experiments. For *Fusarium oxysporum* a clear decrease of ergosterol level was observed only when *Bacillus coagulans* was co-inoculated in 0 h. During the co-inoculation at 24, 48 and 72 h biosynthesis of this sterol was on the same level as in control samples. In most variants of the experiments, the degree of ergosterol 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). This was observed for *Bipolaris sorokiniana, Trichothecium roseum, Rhizoctonia solani, Sclerotinia sclerotiorum* (Figure 1) and *Fusarium oxysporum* (Figure 2). For the rest of the tested fungal plant pathogens the level of CFU/ml during whole culture was independent of time of co-inoculation by *Bacillus coagulans.*

Discussion

The identification and biological characterisation of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, is of great interest for modern and eco-compatible agriculture. Some problems are connected with selecting the suitable method for determination of activity of these natural antagonisms. Antifungal testing of filamentous fungi generally suffers from incompatibility 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 a small amount of this sterol is accumulated in fungal necromass [3], the efficiencies of extraction are relatively high [12], an exactly defined substance is determined using chromatographic methods, and the results show good reproducibility (in contrast to traditional microbiological methods) [10]. The use of ergosterol assessment as a method of measure fungal growth showed antifungal activity *Bacillus coagulans* against all tested species of fungi in these investigations (Figures 1 and 2). The strongest inhibition of this sterol biosynthesis was observed when bacterial culture was added in initial stages of fungal growth. Similar results were obtained by Podile and Parkash [6], which observed that susceptibility of *Aspergillus niger* to inhibition was more than 90% when *Bacillus subtilis* AF1 was added within 12 h of the growth of fungi. There is no data in literature about determination of antifungal activity using measurement of ergosterol level. The most popular techniques are estimation of dry matter [13] or disc plate diffusion assay [2, 6]. Determination of fungistatic activity based on dry matter of culture is useful only in model experiments, where fungal biomass is the main component. Disc plate diffusion assay also has some disadvantages because it is difficult to create optimal growth conditions simultaneously for bacteria and fungi.

Determination of antifungal activity, based on plate count method, may give false results because fungal colonies usually arise from spores and heavily sporulating species will be overestimated and less abundantly sporulating species underestimated. The plate counting method is a time- and labour-consuming technique, and does not detect dead fungi. This is especially important in analyses where health hazards are to be evaluated. Some authors have 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 [5, 9]. In the present experiments fungistatic activity of *Bacillus* sp. measured as a decrease of CFU/ml was observed in most cases (but not in all). Considering the disadvantages of this method, ergosterol content as a measure of antifungal activity appears to be better and more reliable method than plate counting. Statistically significant correlation between determination of antifungal activity by CFU counts and ergosterol assessment for *Trichothecium roseum* (r^2=0.9999) and *Rhizoctonia solani* (r^2=0.9927) was proven in these investigations. A still significant but moderate correlation exists for *Bipolaris sorokiniana* (r^2=0.7479), *Fusarium oxysporum* (r^2=0.7001) and *Fusarium solani* (r^2=0.7248).

The potential of *Bacillus* sp. to synthesise a wide variety of metabolites with antifungal activity is known and in recent years it has been a subject of experiments [1, 7, 8]. Most of these substances belong to lipopeptides, especially from surfactin, iturin and fengicin classes. Not so much is known about the mechanism of antifungal activity of these substances produced by *Bacillus* sp. Some of them (iturin and surfactin) are able to modify bacterial surface hydrophobicity and, consequently, microbial adhesion to surfaces (to mycelium) [1]. Antibiotics of the iturin group were found to act upon the sterol present in the cytoplasmic membrane of the fungi [8, 16]. Biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1 was also investigated by Podile and Parkash [13]. They demonstrated that the bacterial cells initially adhered to the fungus, multiplied and extensively colonised the surface. Rapid growth of bacterial cells on the surface resulted in damage of fungal cell walls.

These aspects appear essential in association with the antifungal properties of *Bacillus* sp. used in the biological control of plant diseases. *Bacillus coagulans* may be a factor with unique activity against fungicide resistant pathogens with novel mechanism of action.

In conclusion, ergosterol content as a determinant of antifungal activity is worth consideration and may be used as a reference procedure for other methods.

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