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Effect of Potassium Sorbate, Sodium Benzoate, and Sodium Nitrite on Biosynthesis of Cyclopiazonic and Mycophenolic Acids and Citrinin by Fungi of the *Penicillium* Genus

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Abstract—The effect of potassium sorbate, sodium benzoate, and sodium nitrite used as preservatives in the food industry in the production of such mytotoxins as citrinin cyclopiazonic and mycophenolic acids by the contaminating fungi *Penicillium citrinum*, *P. commune,* and *P. brevicompactum,* respectively, was investigated. It was shown that the effect of preservatives used at concentrations relevant to the food industry on the synthesis of mycotoxins depended on the species-specific biochemical and physiological features of the cultures. The growth of *P. brevicompactum* was inhibited to the highest degree by sodium nitrite and potassium sorbate, and the growth of *P. commune* was so inhibited by sodium benzoate. It was established that the introduction of 0.015% sodium nitrite into the medium resulted in 1.3- and 1.4-fold reductions of the production of citrinin and mycophenolic acid, respectively, while the production of cyclopiazonic acid did not change in comparison with the control. The introduction of 0.015% sodium benzoate caused a more than 1.5-fold increase of the concentration of citrinin, cyclopiazonic, and mycophenolic acids, and the addition of 0.02% potassium sorbate increased the production of cyclopiazonic and mycophenolic acids by 1.7 and 2.6 times, respectively.

Keywords: Penicillium. citrinum, *P. commune*, *P. brevicompactum*, mycotoxins, potassium sorbate, sodium benzoate and sodium nitrite

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INTRODUCTION

Strategies for the prevention of food contamination by mycelia fungi are considered very important in food industry today. The fungi of the ascomycete affinity, with fungi of the *Penicillium* genus as their main representatives, are considered the major food contaminants [1–3]. It is known that the majority of them produce various secondary metabolites, which are a health concern due to their synergetic toxic effect on humans [4].

Such preservatives as sorbic and benzoic acids and their salts, sodium and potassium nitrites, and other compounds are used to prevent contamination of food contact surfaces in industry and of food products. Some preservatives have been used in the food industry for a long time and are considered safe for consumers at concentrations not exceeding the threshold limit values (TLVs). It is known that the TLVs are 0.02–0.2% for sorbates in food products, 0.015–0.4% for benzoates, and 0.005–0.015% for nitrites [5]. However, it was reported that sorbate concentrations exceeding the TLV by several times did not inhibit fungal growth [5–7] and that mycotoxin formation occurred under these conditions.

The currently available data on the effect of preservatives on biosynthesis of secondary metabolites are contradictory. It was reported that potassium sorbate (PS), sodium benzoate (SB), and sodium nitrite (SN) inhibited the formation of aflatoxins, ochratotoxins, patulin, and fumonisins by producers both in culture medium and in food products [5, 8]. At the same time, the use of these preservatives at subinhibiting concentrations caused stimulation of biosynthesis of aflatoxin B_1 and T-2 toxin [9].

The goal of this work is to study the effect of PS, SB, and SN on the growth of *Penicillium commune*, *P. brevicompactum,* and *P. citrinum* and on the production of α-cyclopianisc (CPA**)** and mycophenolic (MPA) acids, as well as citrinin, by these fungi.

METHODS

The fungi used in this study, *P. commune* VKM F-4500, *P. brevicompactum* VKM F-4480, and *P. citrinum* VKM F-3942, were provided by the All-Russia Collection of Microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. These strains were pre-

viously isolated from the surfaces of sausage and cheese, as well as from the air of a cheese producing factory [2, 3].

The effect of preservatives on the radial growth of fungi at concentrations from 0.01 to 0.20% was studied on an agar medium, the effect on mycotoxin biosynthesis was studied by deep broth cultivation with PS, SB, and SN concentrations of 0.020, 0.015, and 0.015%, respectively. Aqueous solutions of preservatives (10%) were filter-sterilized by filters with a pore diameter of 0.22 μm (Millipore, United States) and introduced into sterile medium before inoculation. Mineral broth of the following composition was used (g/L of distilled water): mannitol—50.0, succinic acid—5.4, $MgSO_4 \cdot 7H_2O$—0.3, KH_2PO_4—1.0, and $ZnSO_4 \cdot 7H_2O$—0.004, pH—5.4. The pH of the medium was adjusted by the addition of 25% NH_4OH solution.

Deep-broth cultivation was conducted in 750-mL flasks containing 150 mL of medium on a shaker (220 rpm) at 24 ± 1°C. The medium was inoculated with aqueous suspension of conidia of 7-day culture grown in tubes on wort agar slants, $1–2 \times 10^7$ spores/mL. Sampling for analysis was conducted daily from the third to the tenth day of cultivation.

Metabolites were isolated from the culture liquid filtrate by triple extraction with chloroform at low pH. Extracts were analyzed with TLC on Silica gel F_{254} plates (Merck, Germany) in a chloroform–methanol–25% aqueous ammonia (80 : 20 : 0.2) system. CPA was identified by reaction with Ehrlich reagent, MPA was identified with 5% solution of $FeCl_3$, and citrinin was identified from the yellow fluorescence in UV light at 254 nm. The quantitative determination of citrinin, MPA, and CPA in extracts was performed spectrophometrically with a UV-160A spectrophotometer (Shimadzu, Japan) at 320, 305, and 280 nm, respectively, with the use of calibration curves.

In order to evaluate the growth of fungi and biosynthesis of metabolites, the following parameters were used: biomass content (x), maximum specific growth rate (μ_{max}), maximum concentration of metabolites in the medium (p), and product yield with respect to biomass content ($Y_{p/x}$) [10].

RESULTS AND DISCUSSION

The effect of the content of all preservatives used at concentrations in culture medium from 0.01 to 0.20% on the radial growth rate of *P. brevicompactum* VKM F-4480, *P. citrinum* VKM F-3942, and *P. commune* VKM F-4500 was studied. It was shown that fungi did not grow when the concentration of introduced preservative was above 0.05%.

It is known that the contaminating microflora becomes resistant to sorbate at those food-processing facilities, where it is used as a preservative. For example, some strains of the fungi of the *Penicillium* genus isolated from cheeses containing PS grew at a concentration of 0.71% [6]. It was also shown that PS at a concentration of 0.3% did not suppress the growth of 70% of fungal strains isolated from cheeses contaminated with mold [7]. This indicated that these mold strains were insensitive to the action of PS at concentrations not exceeding the TLV.

It was shown previously that the fungi *P. brevicompactum* VKM F-4480, *P. citrinum* VKM F-3942, and *P. commune* VKM F-4500 are capable of producing CPA, MPA, and citrinin, respectively [11]. The content of these mycotoxins is not regulated in foods and feed, but they can present a real health hazard for both humans and animals. The toxic effect of CPA is manifested as the rapid development of symptoms of central nerve system damage and necrotic changes in internal organs. This is explained by the specific inhibition of calcium-dependent ATPase, which is capable of changing the normal intracellular calcium flux [12]. In light of the consequences of induced pathologies caused by CPA in laboratory animals, it was suggested to limit the maximum allowable consumption to 700 μg/day.

MPA is also a physiologically active compound exhibiting pronounced immunosuppressive action. There are known cases in which MPA was found in cheeses in France, Germany, and Spain, which raised justified concern among health professionals and veterinarians.

Citrinin exhibits antibiotic, bacteriostatic, antifungal, and antiprotozoal activities. Its toxic effect is manifested as nephro- and hepatotoxicity. Citrinin is stable in cheeses and up to 600 mg/kg can be accumulated upon *P. citrinum* contamination [13].

The effect of preservatives on the growth and biosynthesis of mycotoxins during deep-broth cultivation of fungi was investigated in the presence of 0.02% of PS, 0.015% of SB, and 0.015% SN, with these concentrations being both subinhibiting and TLV, respectively. PS and SN demonstrated the highest effect on the growth of *P. brevicompactum,* with the maximum specific growth rates of the fungus being 38 and 35% below the control, respectively (Table). The introduction of SN resulted in lengthening of the lag period to 5 days in comparison with the control (1 day). Only SB affected the growth of *P. commune*; μ_{max} was 18% below the control. The investigated preservatives did not affect significantly the growth indicators of *P. citrinum*. Hence, the effect of preservatives on the growth of fungi depended on the species-associated physiological and biochemical features of the cultures.

A number of suggestions have been offered to explain the mechanism of PS fungicidal effect [14, 15]. For example, since PS is an unsaturated fatty acid, it can inhibit the functions of a number of membrane enzymes of such dehydrogenases, similarly to the action of crotonic acid. Being a lipophilic compound, it is capable of uncoupling respiration and oxidative

Table 1. Effect of preservatives on indicators of maximal fungal growth

Fungi	Indicator	Control	PS	SB	SN
P. brevicompactum	x, g/L	15.5	14.9	14.9	10.8
	μ_{max}, h^{-1}	0.026	0.016	0.024	0.017
P. citrinum	x, g/L	16.5	15.9	16.0	16.5
	μ_{max}, h^{-1}	0.031	0.030	0.030	0.031
P. commune	x, g/L	19.6	21.4	20.4	20.1
	μ_{max}, h^{-1}	0.055	0.051	0.045	0.055

phosphorylation and affecting cellular membranes, disrupting the process of transport of a number of compounds including amino acids.

The antifungal activity of SB is associated with inhibition of the enzymes of glycolysis, of which phosphofructokinase is affected the most [16, 17]. This activity increases with decreasing pH values. For example, the penetration of SB into yeast cells in an undissociated form resulted in a shift in the intracellular pH by more than one unit, which resulted in the inhibition of glycolytic enzymes (phosphofructokinase was inhibited to the greatest extent). Thus, SB is used as the most efficient preservative in fruit juices, wines, and carbonated beverages, as well as in pharmaceutical and cosmetic products. SB can be formed spontaneously in cheeses and fermented milk products as a result of natural enzymatic processes in lactic acid bacteria [18]. High concentrations of it can be found either on the surface of or inside ripened cheeses. The SN-based preservative is used mainly as an antioxidant and antibacterial agent, preventing, among other things, the growth of *Clostridium botulinum,* the causative agent of botulism [5].

The introduction of PS and SB into the *P. brevicompactum* culture medium increased MPA biosynthesis more than 2.6 and 1.7 times, respectively, in comparison with the control (Fig. 1a). A 30% decrease of the MPA content in the medium was observed in the presence of SN due to the inhibition of biomass growth. The CPA biosynthesis in *P. commune* increased more than 1.6 times upon the introduction of PS and SB in comparison with the control, and it did not change in the presence of SN (Fig. 1b). The indicators of citrinin biosynthesis in *P. commune* increased 1.5 times upon the introduction of SB, and they decreased by 13 and 20% in the presence PS and SN, respectively (Fig. 1c).

Hence, it was established that the PS concentrations subinhibiting the growth of the studied fungi stimulated the biosynthesis of MPA and CPA, while SB stimulated the biosynthesis of MPA, CPA, and citrinin. The introduction of SN at the maximum allowable concentration resulted in decreased citrinin biosynthesis but did not affect the biosynthesis of MPC and CPA. These data are in agreement with the results reported in [9], where it was shown that the use of PS concentrations subinhibiting the growth of *Aspergillus flavus* and *Fusarium acuminatum* fungi caused stimulation of the production of aflatoxin B_1 and T-2 toxin, respectively. This effect was explained by the decreased activity of the enzymes of tricarboxylic acid (TCA) cycle, which, in turn, resulted in the accumulation of intracellular level of acetyl coenzyme A—an important intermediate participating in the biosynthesis of compounds of the polyketide class, and mycotoxins falls into this category. It can be suggested that the stimulating effect of PS on biosynthesis of CPA by *P. commune* and MPA by *P. brevicompactum* is also associated with the decreased activity of the TCA cycle enzymes and the increase of intracellular content of acetyl coenzyme A, which is a precursor of both CPA and MPA.

It is known that SB affects the cells of aerobic yeasts as a strong peroxidant, causing oxidative stress [19]. The relationship between oxidative stress and mycotoxin biosynthesis has been discussed actively in recent years [20]. The reactive compounds containing reactive oxygen species (ROS) are formed in fungi as a result of metabolic processes, as well as due to the action of environmental factors. The cells activate antioxidant enzymes in response to ROS, which neutralize the excess of oxidants. However, oxidative stress not only initiates antioxidant stimulation but also induces important mechanisms in the fungal life cycle, including the differentiation process. It was noted that some secondary metabolites were synthesized in fungi during morphological and metabolic transitions, which resulted in ROS accumulation. It was shown that the processes of oxidative stress and biosynthesis of aflatoxin are interrelated. Indeed, the addition of oxidants to the medium resulted in the stimulation of *A. flavus* aflatoxin production. The introduction of natural antioxidants in the culture medium almost completely suppressed the expression of all genes of the aflatoxin biosynthesis pathway. Similar results were obtained also for the producers of such mycotoxins as ochratoxin, patulin, and others. It could be suggested that the introduction of SB into the medium resulted in an increased ROS content, which stimulated the biosynthesis of CPA, MPA, and citrinin in *P. commune*, *P. brevicompactum,* and *P. citrinum,* respectively. The decreased production of MPA and

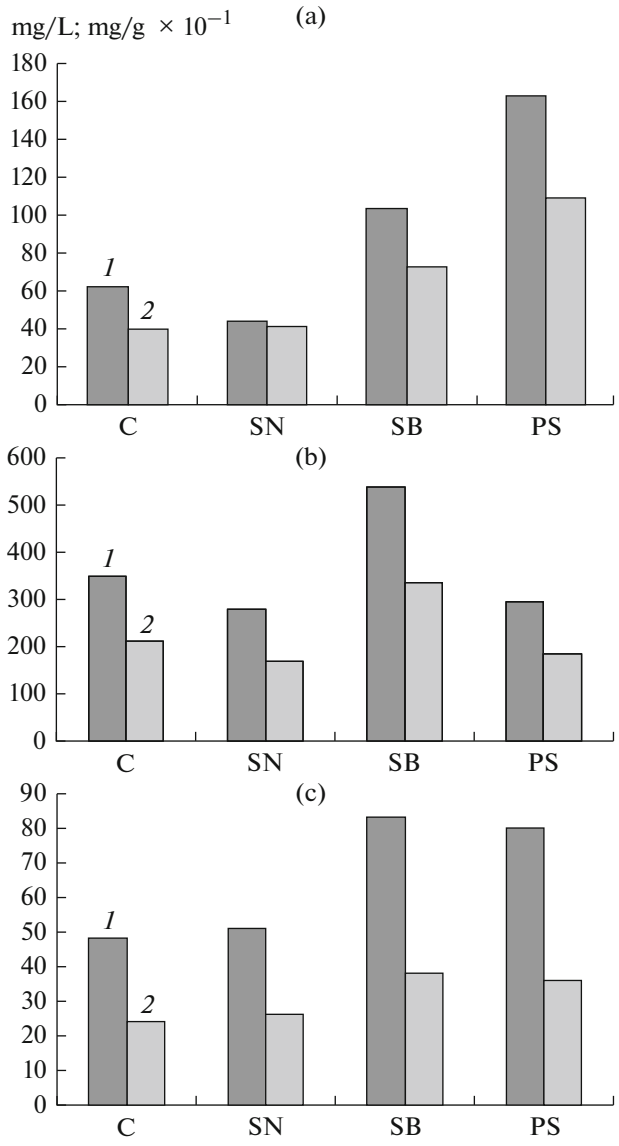

Fig. 1. Effect of preservatives on the concentration of mycotoxins (*1*, mg/L) and yield of their biomass (*2*, mg/g). (a) MPA, (b) citrinin, (c) CPA. C—control without preservatives. SB—sodium benzoate, PS—potassium sorbate, SN—sodium nitrite.

citrinin in the presence of SM is likely related to its antioxidant properties.

Hence, the results demonstrated that the use of such preservatives as PS and SB at subinhibiting concentrations resulted in the stimulation of mycotoxin production by the contaminating fungi.

Information has been revealed recently on the adverse effect of preservatives on humans. It was shown that PS and SB exhibited clastogenic, mutagenic, and genotoxic effects on human peripheral blood lymphocytes [21]. The consumption of preservatives with food could also cause such side effects as eczema, rhinitis, angio-neurotic edema, bronchospasm, and others. On the other hand, the results obtained in this work indicate that a decrease of the concentration of preservatives in food products to concentrations subinhibiting the growth of a microorganism could cause other adverse effects, such as mycotoxicosis.

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