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EXPERIMENTAL ARTICLES

Effect of Microelements on the Biosynthesis of Secondary Metabolites by the Fungus *Penicillium citrinum* Thom VKM F-1079

A. G. Kozlovskii, V. P. Zhelifonova, N. G. Vinokurova, and S. M. Ozerskaya

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—*Penicillium citrinum* VKM F-1079 was found to produce clavine ergot alkaloids and citrinin, a secondary *O*-heterocyclic metabolite. Citrinin was produced in the idiophase, whereas the production of ergot alkaloids paralleled fungal growth. The addition of manganese ions to the growth medium stimulated the biosynthesis of both citrinin and ergot alkaloids. Zinc ions stimulated only citrinin synthesis. The presence of these microelements in the growth medium influenced the proportion between the ergot alkaloids synthesized. Copper, manganese, and iron ions slightly affected fungal growth and alkaloid production. The effect of microelements on the main kinetic parameters of growth and alkaloid production was studied.

Key words: fungi, physiology, secondary metabolites, clavine ergot alkaloids, citrinin

Investigation of the regulation of secondary metabolism in fungi is an urgent problem from both theoretical and applied points of view. It is known that mineral nutrients, including microelements, can stimulate the secondary metabolism of fungi [1–5]. However, little is known about the mechanisms by which microelements exert their action on the secondary metabolism as a whole or on its particular stages.

The aim of the present work was to study the effect of some heavy metal ions (microelements) on the synthesis of secondary metabolites by the fungus *P. citrinum* VKM F-1079, in order to elucidate the possible regulatory role of microelements in alkaloid production.

MATERIALS AND METHODS

The fungus *Penicillium citrinum* VKM F-1079 used in this work was obtained from the All-Russia Collection of Microorganisms (VKM). The strain was maintained on glucose–potato agar slants. Material for inoculation was 4- to 5-day cultures grown in the following control (i.e., without the addition of supplementary microelements) medium containing (g/l distilled water) mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 5.4 with 25% NH_4OH. The fungus was cultivated at 24 ± 1°C in 750-ml Erlenmeyer flasks containing 150 ml of the medium on a shaker (200 rpm).

Supplementary microelements in the form of 0.2% sterile solutions were added to the control medium immediately before inoculation to yield the following final concentrations (mg/l): $FeSO_4 \cdot 7H_2O$, 5.0; $ZnSO_4 \cdot 7H_2O$, 4.4; $MnSO_4 \cdot H_2O$, 1.7; $CuSO_4 \cdot 5H_2O$, 3.3; and Na_2MoO_4, 2.4. The amount of microelements that appeared in the medium as a result of their presence in the reagents used for its preparation did not exceed (mg/l) Fe^{2+}, 0.06; Mn^{2+}, 0.01; Mo^{6+}, traces; Cu^{2+}, 0.01; and Zn^{2+}, 0.03. The concentration of microelements was determined using a Hitachi 518 atomic absorption spectrophotometer.

In order to extract secondary metabolites of a basic nature, the culture liquid filtrate was alkalinized to pH 8–9 with 25% NH_4OH and trice extracted with an equal volume of chloroform. The aqueous phase was acidified to pH 3 with 10% HCl, and acidic secondary metabolites present in this phase were trice extracted with chloroform. The extracts were dehydrated above calcinated Na_2SO_4 and completely dried in a rotary evaporator.

Secondary metabolites were analyzed by TLC on 60 F_{254} silica gel plates (Merck) in three solvent systems containing chloroform, methanol, and 25% NH_4OH in proportions of 90 : 10 : 0.1 (system 1), 80 : 20 : 0.2 (system 2), and 90 : 10 : 1 (system 3).

Alkaloids were identified by their cochromatography with the reference samples prepared as described earlier [6] and by UV spectrophotometry. The concentration of citrinin in the culture liquid was measured as follows. An aliquot of the culture liquid was mixed with 2% tartaric acid to yield a the concentration of citrinin within 5–10 μg/ml (pH 2.5–3.5), and the absorbance of

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this mixture was measured at $\lambda = 329$ nm. The amount of citrinin was determined from a calibration curve.

Clavine ergot alkaloids were determined as described earlier [8].

The UV spectra of metabolites were recorded on a Shimadzu UV-160A spectrophotometer (Japan).

Designations used in this work: X (g/l), biomass concentration; P (mg/l), metabolite concentration in the culture liquid; $Y_{P/X}$ (mg/g), product yield with respect to biomass; μ (h^{-1}), specific growth rate; q_P (mg/(g h)), specific production rate.

RESULTS AND DISCUSSION

P. citrinum VKM F-1079 (formerly *P. gorlenkoanum* VKM F-201) synthesizes alkaloids with unique configurations (5R and 10S), namely costaclavine and epicostaclavine, as well as chanoclavine-I and isochanoclavine-I [7]. A more thorough analysis of the secondary metabolites present in the culture liquid of this strain showed that it also contained an acidic *O*-heterocyclic metabolite, which was identified as citrinin based on the following facts. The spots of this compound on chromatographic plates gave yellow fluorescence when illuminated with visible or UV light and brown color when sprayed with a 3% ethanol solution of $FeCl_3$. The chromatographic mobility, R_f, of this metabolite in system 2 was 0.18. When recrystallized from absolute ethanol, the metabolite showed a melting point of 172–174°C (according to data available in the literature, the melting point of citrinin is 175°C [8]). Thus, *P. citrinum* VKM F-1079 synthesizes at least two types of secondary metabolites, namely citrinin, with acetyl- and malonyl-CoA as precursors [9], and clavine ergot alkaloids, whose precursors are tryptophan and mevalonic acid [1].

Among the five microelements investigated, only manganese and zinc ions raised the concentrations of biomass (X), alkaloids (P_a), and citrinin (P_c) in the culture liquid (see table). The increase in the concentration of citrinin in the culture liquid under the action of Mn^{2+} and Zn^{2+} ions (3.4-fold and 22.1-fold, respectively) was due to the stimulation of not only fungal growth but also citrinin synthesis; its yield with respect to biomass ($Y_{O/X}$) increased in the presence of manganese and zinc ions by 2.7 and 10 times, respectively. These data suggest that manganese and zinc ions stimulate both primary metabolism (this follows from the stimulation of growth) and secondary metabolism (presumably, the microelements directly stimulate the enzymes involved in citrinin biosynthesis).

The addition of zinc ions to the growth medium led to a 1.6-fold increase in the concentration of alkaloids (P_a) in the culture liquid and a 2.2-fold increase in the concentration of biomass (X). As a result, the yield of alkaloids with respect to biomass ($Y_{a/X}$) decreased by 26%. The stimulatory effect of zinc ions on the synthesis of clavine ergot alkaloids was observed by a number of researchers [1, 10–12]. It should be noted that Rosazza *et al.* explained the effect of zinc by the activation of tryptophan synthetase rather than that of the enzymes directly involved in alkaloid synthesis [13].

Effect of microelements on the growth of *P. citrinum* VKM F-1079 and production of secondary metabolites

Microelement	Biomass (X), g/l	Alkaloids		Citrinin	
		P_a, mg/l	$Y_{a/x}$, mg/l	P_a, mg/l	$Y_{a/x}$, mg/l
Control	9.5	3.7	0.39	19	2.0
Cu	9.3	2.2	0.24	17	1.8
Mo	9.3	2.9	0.31	18	1.9
Fe	8.6	3.4	0.39	18	2.1
Mn	11.9	10.5	0.88	65	5.5
Zn	20.9	6.1	0.29	420	20.1

The 2.8-fold increase in the concentration of alkaloids in the culture liquid induced by $Y_{a/X}$ ions was mainly due to the enhancement of the secondary metabolism; the yield of alkaloids with respect to biomass ($Y_{a/X}$) rose 2.3-fold, whereas the biomass increased only 1.2-fold. These results agree with the relevant data of Turner [9] and can be explained by the activation of isopentenyl pyrophosphate isomerase, an enzyme involved in the synthesis of clavine alkaloids [1].

Fe^{2+}, Cu^{2+}, and Mo^{6+} ions at concentrations of 1.0, 0.85, and 1.0 mg/l, respectively, virtually did not affect fungal growth but inhibited (especially copper ions) the synthesis of both citrinin and ergot alkaloids (see table). These findings are inconsistent with the relevant data available in the literature. For instance, copper ions, even at higher concentrations (5 mg/l), stimulated the growth of *Claviceps* and *Aspergillus fumigatus* and their alkaloid production [1, 12]. As for iron ions, they are thought to be necessary for both primary and secondary metabolism [4, 12]. In particular, iron is a constituent of the active centers of the cytochrome *P*-450 of various monooxygenases involved in the biosynthesis of clavine alkaloids [1]. Data available in the literature on the effect of different concentrations of iron on the synthesis of clavine alkaloids are ambiguous, although it is thought that secondary metabolism requires greater amounts of iron than primary metabolism [4, 12].

Thus, the trace amounts of Fe^{2+}, Mn^{2+}, Mo^{6+}, Cu^{2+}, and Zn^{2+} ions present in the control medium satisfied the major requirements of the primary and secondary metabolism of *P. citrinum* for microelements. However, the levels of fungal growth and alkaloid synthesis were determined by the concentration of manganese and zinc ions in the medium.

Costaclavine and epicostaclavine were the major alkaloids of the fungus grown in the control medium,

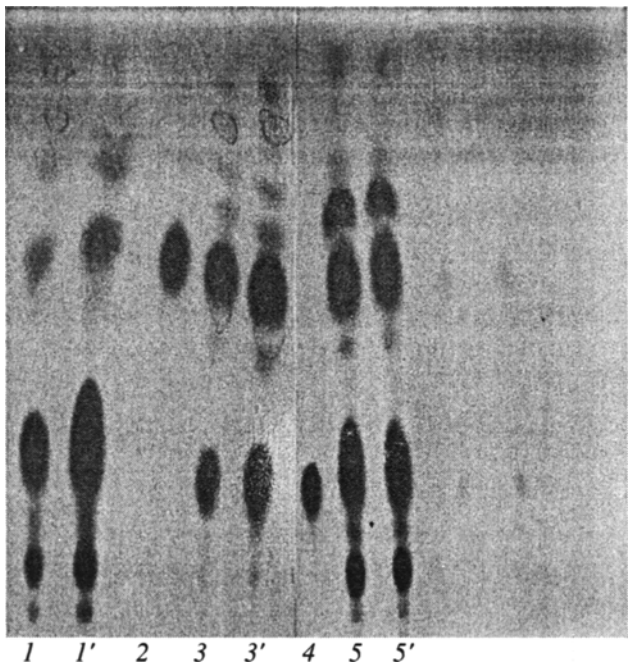

Fig. 1. Effect of manganese and zinc ions added to the growth medium on the proportion between alkaloids synthesized by *P. citrinum* VKM F-1079. Lanes: *1* and *1'*, extract of culture liquid after growth in Zn-supplemented medium; *2*, epicostaclavine; *3* and *3'*, extract of culture liquid after growth in the control medium; *4*, costaclavine; *5* and *5'*, extract of culture liquid after growth in Mn-supplemented medium.

whereas chanoclavine-I and isochanoclavine-I were synthesized in minor amounts (Fig. 1). Unlike iron, copper, and molybdenum ions, zinc and manganese ions influenced the range of the alkaloids synthesized; the addition of Zn^{2+} ions to the medium suppressed epicostaclavine synthesis and enhanced chanoclavine-I synthesis. The effect of manganese ions was much less pronounced than that of zinc ions. When added together, zinc and manganese ions promoted the synthesis of costaclavine, epicostaclavine, and chanoclavine-I.

Investigation of the relationship between microbial growth and the production of secondary metabolites can provide insight into their ecological role and physiological significance for producers, as well as contribute to the optimization of the production of secondary metabolites [14]. As can be seen from Figs. 2a and 2b, *P. citrinum* VKM F-1079 exhibited a diauxic growth with two peaks of the specific growth rate (two μ_{max} values) and a transient stationary phase between them with an almost zero value of μ. The transient stationary phase is probably associated with a metabolic adaptation of the fungus to the second growth substrate (mannitol) after the first substrate (succinate) has been exhausted in the medium [5, 8]. The addition of Mn^{2+} and Zn^{2+} ions to the medium increased the μ_{max} on succinate by 1.6 and 1.7 times, respectively, and shifted the time of culture transition from growth on succinate to growth on mannitol (Fig. 2b).

The synthesis of secondary metabolites in submerged fungal cultures occurs either in the idiophase, when the specific growth rate is close to zero, or parallels growth, or fits a diphase curve, such as in the case of citrinin production by the fungi *P. notatum* and *P. janthinellum* [14, 15]. Two maxima in the accumulation of citrinin by these fungi were observed in the growth retardation and stationary phases. It should be noted that Patterson and Damoglou [16] related the nonmonotonic pattern of citrinin production by *P. vitidicatum* to its conversion into dihydrocitrinon and ochratoxin.

As is evident from Fig. 2c, the production of citrinin by *P. citrinum* VKM F-1079 did not fit a diphase curve: citrinin was synthesized throughout the growth period, with two peaks of the specific production rate (q_C^{max}) occurring in the growth retardation and transition phases, when μ was close to zero (Fig. 2d). A similar dependence was observed earlier for the fungus *Monoscus ruber* [17]. The threefold increase in the q_C^{max} of *P. citrinum* VKM F-1079 culture under the action of zinc ions supports the suggestion about the stimulating effect of these ions on the enzymes involved in citrinin synthesis.

Manganese ions influenced but little q_C^{max}. In this case, the decrease in q_C was described by a more gently sloping curve than in the control; therefore, manganese lengthened the period during which the fungus synthesized citrinin.

Unlike citrinin synthesis, which mainly occurred when the specific growth rate of *P. citrinum* VKM F-1079 was close to zero, the synthesis of clavine alkaloids exhibited two maxima of the specific production rate, q_a^{max} (Figs. 2e and 2f). The first μ_{max} coincided with the first maximum of the specific growth rate, when succinate was extensively utilized, and the other was observed in the transient stationary phase at $\mu = 0$, when the fungus implemented adaptive metabolic changes related to the transition from one growth substrate (succinate) to the second (mannitol). Manganese and zinc ions increased the first maximum of the alkaloid production rate by 3.3 and 2.3 times, respectively, but did not affect the second maximum (Fig. 2e). This implies that the biosynthesis of alkaloids by *P. citrinum* VKM F-1079 is associated with growth processes and depends on the growth substrate used.

Thus, Mn^{2+} and Zn^{2+} ions at concentrations of up to 1 mg/l stimulated the synthesis of both citrinin and clavine alkaloids by *P. citrinum* VKM F-1079. The mechanisms of action of these microelements on the production of secondary metabolites are different, as follows from their different effects on the yield of metabolites

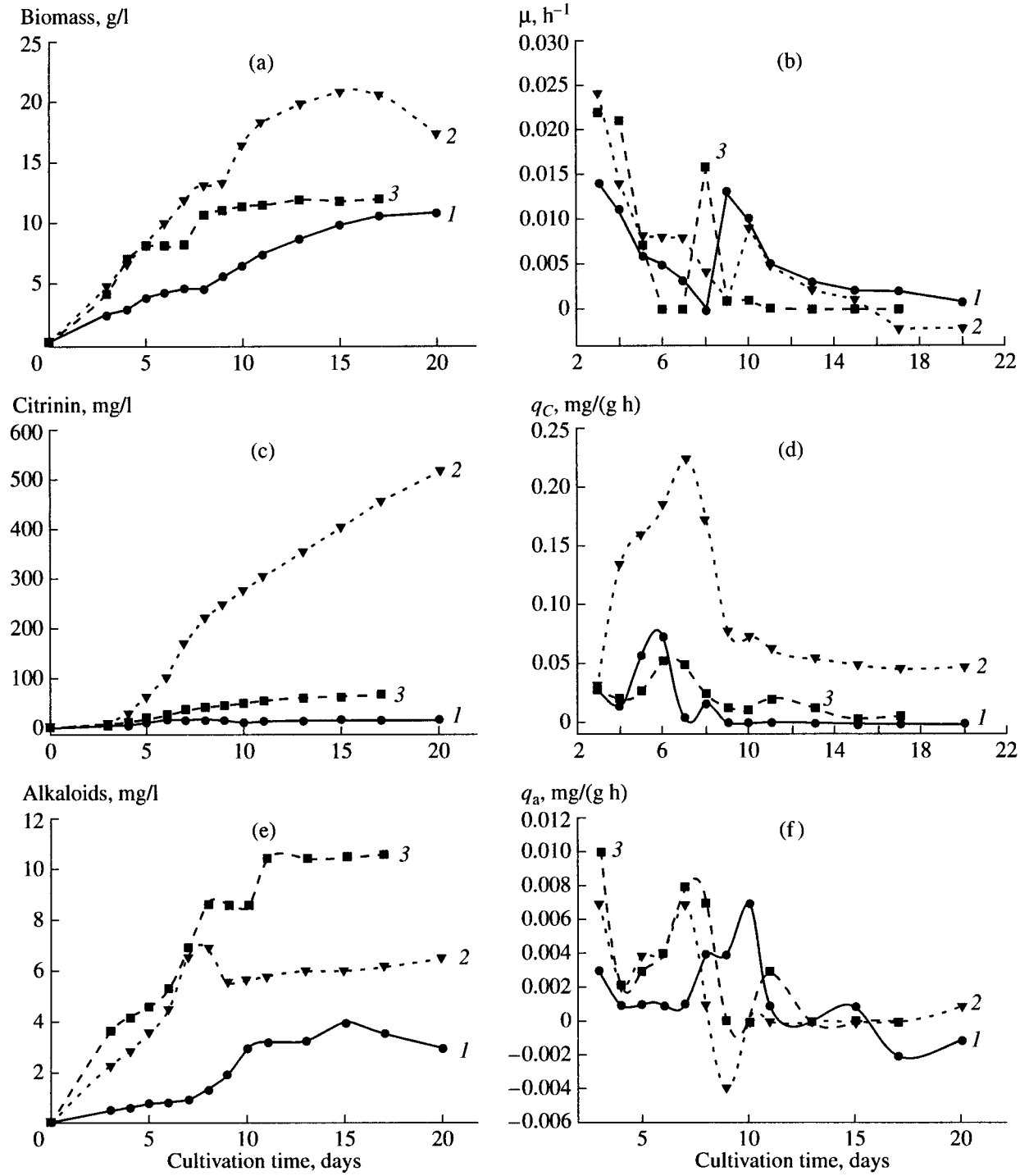

Fig. 2. Effect of manganese and zinc ions added to the growth medium on the parameters of growth and secondary metabolite production by *P. citrinum* VKM F-1079: (*1*) control medium; (2) Zn-supplemented medium; (*3*) Mn-supplemented medium; (a) growth, (b) specific growth rate, (c) citrinin synthesis, (d) specific rate of citrinin synthesis, (e) alkaloid synthesis, and (f) specific rate of alkaloid synthesis.

($Y_{P/X}$) and their specific production rate (q_P). The effect of manganese and zinc ions on the synthesis of citrinin is related to their action on both primary and secondary metabolism. The stimulatory effect of manganese ions on the synthesis of alkaloids is associated with an increase in their yield with respect to the biomass accumulated.

Unlike manganese and zinc ions, the other ions investigated (copper, iron, and molybdenum) virtually did not affect fungal growth and citrinin production. At the same time, copper and molybdenum ions slightly suppressed the production of clavine alkaloids.

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