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EXPERIMENTAL ARTICLES

The Effect of Some Amino Acids on the Growth and Biosynthesis of Ergot Alkaloids and Quinocitrinins in the Fungus *Penicillium citrinum* Thom 1910

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Abstract—The effect of some amino acids, added to the medium either during inoculation or in the stationary growth phase, on the growth and biosynthesis of ergot alkaloids and quinocitrinins in the fungus *Penicillium citrinum* VKM FW-800 has been studied. Exogenously added amino acids were mostly utilized in primary metabolism. When added during inoculation, tryptophan and leucine virtually did not influence fungal growth and synthesis of the alkaloids, whereas the addition of isoleucine enhanced the biomass accumulation. When added in the stationary growth phase, tryptophan stimulated the synthesis of both ergot alkaloids and quinocitrinins. Leucine added in the stationary growth phase did not influence the synthesis of ergot alkaloids but inhibited the synthesis of quinocitrinins. Isoleucine inhibited the synthesis of both ergot alkaloids and quinocitrinins irrespective of the time of its addition to the medium.

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Key words: *Penicillium citrinum*, secondary metabolites, biosynthesis, alkaloids.

The problem of physiological control over the biosynthesis of secondary metabolites is of considerable interest from the standpoints of both pure science and biotechnology. However, many aspects of the problem still remain poorly understood. In particular, little is known about the regulation of secondary metabolism with nitrogen sources such as ammonium ions and amino acids. Meanwhile, recent progress in the production of many biologically active substances (antibiotics, alkaloids, hormones, etc.) has largely been due to knowledge of the physiology and biochemistry of producers and the regulatory mechanisms of their biosynthetic pathways.

The fungus *Penicillium citrinum* VKM FW-800 synthesizes secondary metabolites of two different classes: the ergot alkaloids agroclavine-1 and epoxyagroclavine-1 and the quinoline alkaloids quinocitrinin A and quinocitrinin B [1, 2]. These metabolites are synthesized from the precursors anthranilic acid and tryptophan [3]. Isoleucine can also serve as a precursor of quinocitrinin A [2]. The enrichment of the cultivation medium with amino acids that can serve as precursors of secondary metabolites may considerably influence the growth and secondary metabolism of producing strains.

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This study was undertaken to elucidate the effect of tryptophan, leucine, and isoleucine on the growth and biosynthesis of alkaloids in the fungus *P. citrinum* VKM FW-800.

MATERIALS AND METHODS

The strain *Penicillium citrinum* VKM FW-800 used in this work was isolated from ancient (1.8–3.0 million years old) Arctic permafrost sediments [4]. The strain was maintained on glucose–potato agar slants. The inoculum was prepared by suspending spores from 14-day-old cultures in water to a density of $(1–2) \times 10^7$ spores/ml. The basal medium contained (in g/l distilled water) mannitol, 50; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.0; and $ZnSO_4 \cdot 7H_2O$, 0.004. The pH of the medium was adjusted to 5.4 with aqueous ammonium hydroxide solution. The fungus was cultivated at 24 ± 1°C in 750-ml Erlenmeyer flasks containing 150 ml of the medium on a shaker (220 rpm).

To study the effect of amino acids on fungal growth and alkaloid production, the basal medium was supplemented with L-tryptophan, L-leucine, or L-isoleucine (Reanal, Hungary) at concentrations of 0.25 and 2 mM. The amino acids were added to the medium either during inoculation or in the stationary growth phase (tryptophan after 10 and 11 days of cultivation; leucine and isoleucine after 10 days). The control culture was

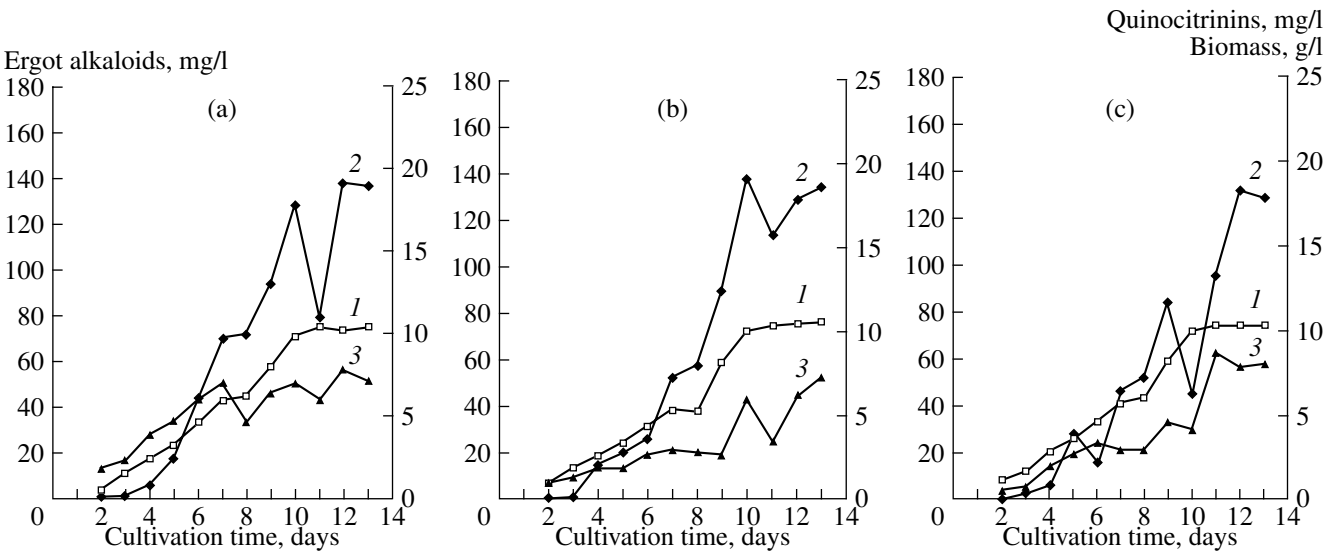

Fig. 1. Growth dynamics and the level of alkaloids in the culture liquid of *P. citrinum* VKM FW-800 grown in (a) the control medium and the same medium supplemented with (b) 0.25 mM tryptophan and (c) 2 mM tryptophan: (*1*) biomass, (*2*) ergot alkaloids, (*3*) quinocitrinins.

grown in the medium containing no amino acids. Samples for analysis were taken at 1- day intervals. Growth was monitored by determining the dry weight of the fungal mycelium.

Secondary metabolites were extracted from the culture liquid filtrate as described earlier [1, 2]. The extracts were qualitatively analyzed by TLC on Silica gel 60 F_{254} plates (Merck, Germany) in three solvent systems containing chloroform, methanol, and 25% NH_4OH in proportions of 90 : 10 : 0.1 (system I), 90 : 10 : 1 (system II), and 80 : 20 : 0.2 (system III). Separated substances were visualized by examining the developed plates under UV light and by spraying the plates with the Ehrlich and Dragendorff reagents.

The total amount of ergot alkaloids was determined by measuring the optical density of methanol solutions at 283 nm. The calibration curve was constructed with an authentic sample of epoxyagroclavine-1 tartrate.

For a qualitative analysis of quinocitrinin A, acid extracts were dissolved in aliquots of a chloroform–methanol mixture (1 : 1) and applied onto Silufol UV-254 plates (Czech Republic). The plates were developed in system III. The spots that corresponded to quinocitrinins were cut out and eluted with methanol. The eluates were filtered and the content of quinocitrinins was determined by measuring the optical density of the filtrates at 314 nm using an SF-26 spectrophotometer (Russia). The calibration curve was constructed with quinocitrinin B.

The intracellular and exogenously added amino acids were analyzed using a T-339 amino acid analyzer (Czech Republic).

RESULTS AND DISCUSSION

The experiments showed that the fungus *P. citrinum* VKM FW-800 grew with a diauxie both in the control and in the experimental media with the amino acids tryptophan, isoleucine, and leucine added during inoculation (Figs. 1, 2). The diauxie was obviously due to the presence of two carbon sources (succinate and mannitol) in the media. Ergot alkaloids and quinocitrinins were synthesized almost concurrently with the fungal growth. During the second lag phase (7–9 days of growth) and in the early stationary phase (11–12 days of growth), the alkaloid content in the culture liquid showed cyclic changes (a drastic fall followed by an increase within one cultivation day).

The addition of tryptophan and leucine during inoculation at concentrations of 0.25 and 2 mM influenced fungal growth and alkaloid synthesis very little. In contrast, as is evident from Figs. 1 and 2, isoleucine at these concentrations enhanced the biomass accumulation and inhibited ergot alkaloid synthesis by approximately 25% and quinocitrinin synthesis by 40 and 60% (0.25 and 2 mM isoleucine, respectively).

The amino acids added to the medium during inoculation were completely consumed within 3–5 days of cultivation, depending on the concentration added (Table 1). The consumption of leucine began to considerably increase on day 2 of cultivation and the consumption of tryptophan and isoleucine, on day 3 of cultivation. These data agreed well with the results of microscopic examination, which showed that the inoculated conidia had completely germinated by the end of the first day of cultivation and that the addition of the amino acids did not influence the germination process. The active growth of the culture (that is, its transition to

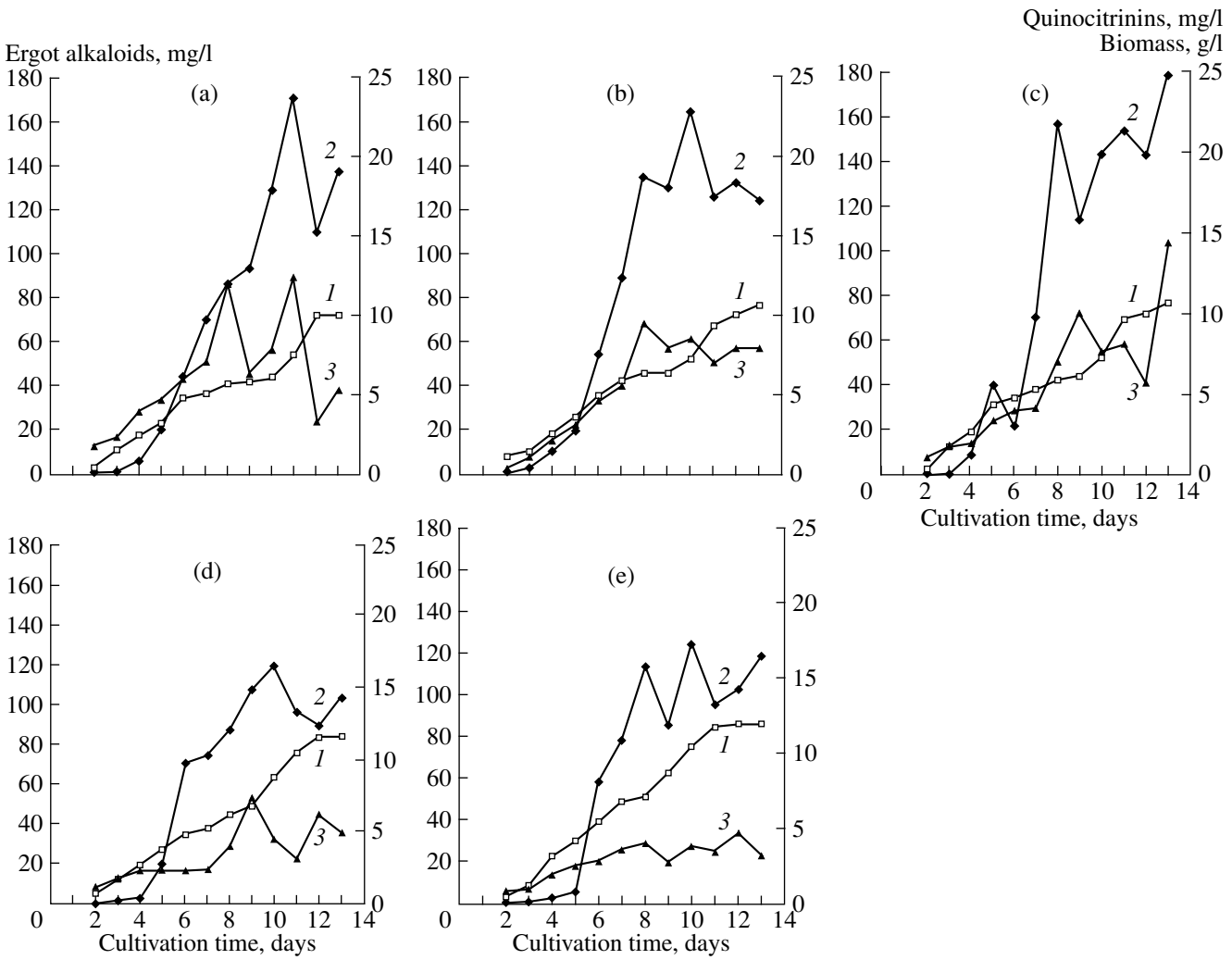

Fig. 2. Growth dynamics and the level of alkaloids in the culture liquid of *P. citrinum* VKM FW-800 grown in (a) the control medium and the same medium supplemented with (b) 0.25 mM leucine, (c) 2 mM leucine, (d) 0.25 mM isoleucine, and (e) 2 mM isoleucine: (*1*) biomass, (*2*) ergot alkaloids, (*3*) quinocitrinins.

the logarithmic growth phase) began on the third day of cultivation.

As in the case with *Claviceps* [5], the absence of a significant effect of tryptophan on the synthesis of ergot alkaloids and quinocitrinins could be due to the rapid metabolism of this amino acid at the stage of active growth. To confirm this suggestion, we studied the effect of the addition of amino acids in the stationary

Table 1. Consumption dynamics (in mg/l) of amino acids added to the cultivation medium of *P. citrinum* VKM FW-800 during inoculation

Culture age, days	Tryptophan		Leucine		Isoleucine	
	0.25 mM (51.55 mg/l)	2 mM (408.44 mg/l)	0.25 mM (32.80 mg/l)	2 mM (262.36 mg/l)	0.25 mM (32.80 mg/l)	2 mM (262.36 mg/l)
0	51	408	32.75	262	32.75	262
1	50	360	30	240	30	230
2	40	350	0	150	20	220
3	0	110	0	110	0	100
4	0	50	0	0	0	60
5	0	0	0	0	0	0

Table 2. The effect of tryptophan added to the cultivation medium in the stationary growth phase on the level of free tryptophan, ergot alkaloids (EAs), and quinocitrinins (QCs) in the *P. citrinum* VKM FW-800 mycelium

Day of addition	Culture age, day	Control			0.25 mM			2 mM		
		Tryptophan, mg/g	EAs, μg/g	QCs, μg/g	Tryptophan, mg/g	EAs, μg/g	QCs, μg/g	Tryptophan, mg/g	EAs, μg/g	QCs, μg/g
10	10	1.1	1770	140	1.1	1770	140	1.1	1770	140
	11	0	970	60	1.4	1160	80	1.6	1650	110
	12	0.7	960	70	1.9	3820	180	1.5	3900	330
11	11	0	970	60	0	970	60	0	970	60
	12	0.7	960	70	1.3	2880	220	1.1	3670	160
	13	2.7	1310	50	2.8	1710	70	3.0	1480	60

Table 3. The dynamics of tryptophan consumption and alkaloid level in the medium after the addition of tryptophan to the cultivation medium of *P. citrinum* VKM FW-800 in the stationary growth phase

Day of addition	Culture age, days	Control		0.25 mM			2 mM		
		EAs, mg/l	QCs, mg/l	Tryptophan, mg/l	EAs, mg/l	QCs, mg/l	Tryptophan, mg/l	EAs, mg/l	QCs, mg/l
10	10	130	7.0	44	130	7.0	410	130	7.0
	11	80	6.0	0	100	8.2	350	90	9.0
	12	140	7.8	0	100	6.6	210	90	6.5
11	11	80	6.0	44	80	6.0	410	80	6.0
	12	140	7.8	0	80	11.0	300	100	9.6
	13	140	7.2	0	110	6.5	120	110	8.2

growth phase, when the culture growth had already ceased but alkaloid synthesis continued at a sufficient rate. As mentioned above, in the early stationary phase (11–12 days of growth), the alkaloid content in the culture liquid first shows a drastic fall and then an increase within one cultivation day.

It is known that some strains of *Claviceps* fungi show a correlation between the abilities to produce ergot alkaloids and to transfer tryptophan into cells [5]. Rechacek and Saidl suggested that ergot alkaloid synthesis is regulated by the pool of endogenous tryptophan in the producer. However, the study of the intensity of ergot alkaloid synthesis versus the pool size of free intracellular tryptophan in two species, *Penicillium sizovae* and *P. aurantio-virens*, gave contradictory results [6, 7]. Namely, in *P. sizovae*, the active synthesis of alkaloids began after the intracellular concentration of tryptophan had decreased to a certain value, whereas, in *P. aurantio- virens*, the active synthesis of ergot alkaloids coincided with the period of high tryptophan content in the mycelium.

In the early stationary phase of the fungus *P. citrinum* VKM FW-800 grown in the control medium, the level of free tryptophan in the mycelium fell to zero on the 11th day of cultivation and then tended to increase (Table 2). The content of ergot alkaloids and quinocitrinins in the culture liquid and in the fungal mycelium was also at a minimum on the 11th day of cultivation (Tables 2, 3). Such concurrent changes in the concentrations of intra- and extracellular tryptophan and alkaloids suggest that alkaloid biosynthesis may be involved in the regulation of the intracellular pool of tryptophan in the fungus *P. citrinum* VKM FW-800 occurring in the stationary growth phase.

In order to verify this suggestion, tryptophan was added, at concentrations of 0.25 and 2 mM, to the medium on the 10th day of cultivation (i.e., a day before the concentration of alkaloids in the medium began to decrease) and on the 11th day (Table 3). During the next 2 days of cultivation, tryptophan was consumed from the medium and accumulated in the mycelium, although the biomass did not increase (Table 3). In the experiment with 2 mM tryptophan, this amino acid was consumed at a higher rate when it was added on the 11th day of cultivation than when it was added on the 10th day.

When tryptophan was added, at concentrations of 0.25 and 2 mM, on the 10th day of cultivation, the decrease in the concentration of ergot alkaloids in the medium was observed on the 11th day (Table 3). The decrease comprised 23 and 30% (at the tryptophan concentrations of 0.25 and 2 mM, respectively), as com-

pared to 40% in the control. Two days after the addition of tryptophan (on the 12th day of cultivation), the concentration of ergot alkaloids in the medium did not increase (in the control, the concentration of ergot alkaloids in the medium increased by 1.7 times as compared to its level on the 11th day of cultivation). In contrast to ergot alkaloids, the concentration of quinocitrinins in the control medium decreased somewhat. In response to the addition of 0.25 and 2 mM tryptophan to the cultivation medium on the 10th day of cultivation, the concentration of quinocitrinins in the medium increased by 17 and 30%, respectively.

The addition of 0.25 and 2 mM tryptophan to the medium on the 10th day of cultivation raised the concentration of tryptophan in the mycelium to 1.4 and 1.6 mg/g, respectively within one cultivation day. In the control mycelium, free tryptophan was not detected on the 11th day of cultivation (Table 2). In this case, the concentration of ergot alkaloids in the mycelium diminished, but to a lesser degree than in the control. Two days after the addition of tryptophan (on the 12th day of cultivation), the concentration of ergot alkaloids in the mycelium increased to 3.8 and 3.9 mg/g (in the control, the content of ergot alkaloids in the mycelium was 1.0 mg/g). As mentioned above, the concentration of ergot alkaloids in the medium did not increase in response to the addition of tryptophan to the medium. Consequently, the artificial increase in the concentration of tryptophan in the mycelium on the 11th day of cultivation obviously enhanced the synthesis of ergot alkaloids but inhibited their excretion from the cells to the medium.

The high level of intracellular free tryptophan on the 11th and 12th day of cultivation correlated with the enhanced synthesis of quinocitrinins. Thus, two days after the addition of 0.25 and 2 mM tryptophan, the concentration of intracellular quinocitrinins increased by 2.7 and 5 times, respectively, as compared to the control. The high level of free tryptophan in the mycelium on the 11th day of cultivation did not affect the excretion of quinocitrinins from the mycelium. Indeed, their concentration in the medium (8.2 and 9.0 mg/l upon the addition of 0.25 and 2 mM tryptophan, respectively) was higher than in the control experiment (6 mg/l quinocitrinins). Two days after the addition of tryptophan (i.e., on the 12th day of cultivation), the concentration of quinocitrinins in the medium decreased to 6.5 mg/g. Consequently, the artificial increase in the concentration of tryptophan in the mycelium on the 11th day of cultivation obviously enhanced the synthesis of quinocitrinins and inhibited their excretion from the cells to the medium.

On the 12th day of cultivation, i.e., one day after the addition of tryptophan to the medium at concentrations of 0.25 and 2 mM, the intracellular level of free tryptophan was 1.9 and 1.6 times higher than in the control. In this case, the concentration of extracellular ergot alkaloids in the experiment on the addition of 0.25 mM tryptophan did not change. In the experiment with the addition of 2 mM tryptophan, this concentration did increase, but the increase was 30% lower than in the control. In this case, the concentration of intracellular ergot alkaloids was 2.9 and 3.7 higher (2.9 and 3.7 mg/g, respectively) than in the control (1 mg/g). Two days after the addition of 0.25 and 2 mM tryptophan (i.e., on the 13th day of cultivation), the concentration of ergot alkaloids in the mycelium decreased to 1.7 and 1.5 mg/g, respectively, whereas their concentration in the control experiment increased further. Thus, the addition of 0.25 and 2 mM tryptophan to the medium on the 11th day of cultivation augmented its concentration in the mycelium, enhanced the biosynthesis of ergot alkaloids, and somewhat inhibited their excretion from the mycelium to the medium. These changes were observed 24 h after tryptophan addition.

As for quinocitrinins, one day after the addition of 0.25 and 2 mM tryptophan, the intracellular concentration of these alkaloids was 3.4 and 2.4 times higher than in the control. The extracellular concentration of quinocitrinins (11 and 9.6 mg/l after the addition of, respectively, 0.25 and 2 mM tryptophan) was also higher than in the control (7.8 mg/l), suggesting that exogenously added tryptophan did not affect the excretion of quinocitrinins from the mycelium, at least one day after its addition. On the 13th day of cultivation (i.e., two days after the addition of tryptophan), the concentration of quinocitrinins decreased both in the medium and in the mycelium.

Thus, the addition of tryptophan to the medium on the 10th or 11th day of cultivation augmented the concentration of free tryptophan in the mycelium, enhanced the biosynthesis of ergot alkaloids, and somewhat inhibited their excretion from the mycelium to the medium.

The artificial increase in the concentration of free tryptophan in the mycelium on the 11th day of cultivation enhanced the synthesis of quinocitrinins with a one-day delay. The excretion of quinocitrinins from the cells to the medium fell only two days after the addition of tryptophan.

The data obtained in this study confirm the suggestion that the processes of biosynthesis, excretion, and consumption of ergot alkaloids and quinocitrinins are involved in the regulation of the intracellular pool of tryptophan in the fungus *P. citrinum* occurring in the stationary growth phase.

The presently available data show that tryptophan plays an important role in the synthesis of ergot alkaloids. Tryptophan is the major precursor of the ergoline molecule [5, 8]. It may be involved in the induction, repression, and derepression of the ergot alkaloid synthesis enzymes [8–10]. The particular role of tryptophan in the synthesis of ergot alkaloids depends on the physiological and biochemical specificity of alkaloid producers. For example, the addition of tryptophan at concentrations of up to 2 mM inhibits the synthesis

Table 4. The effect of leucine and isoleucine added to the cultivation medium in the stationary growth phase on the dynamics of free tryptophan, leucine, and isoleucine in the *P. citrinum* VKM FW-800 mycelium

Culture age, days	Control			Leucine						Isoleucine					
				0.25 mM			2 mM			0.25 mM			2 mM		
	Tryptophan	Leucine	Isoleucine	Tryptophan	Leucine	Isoleucine	Tryptophan	Leucine	Isoleucine	Tryptophan	Leucine	Isoleucine	Tryptophan	Leucine	Isoleucine
10	1.10	8.6	3.4	1.1	8.6	3.4	1.1	8.6	3.4	1.1	8.6	3.4	1.1	8.6	3.4
11	0	10.1	4.5	1.0	6.4	1.9	1.2	3.6	2.4	1.0	6.7	1.7	1.0	5.8	1.6
12	0.71	14.2	5.2	1.1	7.5	2.3	1.0	2.9	2.1	1.1	7.2	2.2	0.9	4.6	1.4
13	2.70	11.1	5.2	1.0	5.6	1.9	1.0	2.8	2.1	1.1	6.0	2.0	0.9	5.7	1.8

Table 5. The dynamics of leucine and isoleucine consumption and alkaloid level in the medium after the addition of leucine and isoleucine to the cultivation medium of *P. citrinum* VKM FW-800 in the stationary growth phase

Culture age, days	Control		Leucine						Isoleucine					
			0.25 mM			2 mM			0.25 mM			2 mM		
	EAs, mg/l	QCs, mg/l	Leucine, mg/l	EAs, mg/l	QCs, mg/l	Leucine, mg/l	EAs, mg/l	QCs, mg/l	Isoleucine, mg/l	EAs, mg/l	QCs, mg/l	Isoleucine, mg/l	EAs, mg/l	QCs, mg/l
10	130	7.8	33	130	7.8	262	130	7.8	33	130	7.8	262	130	7.8
11	170	12.5	0	170	6.0	36	150	10.3	0	160	7.0	24	80	1.8
12	110	3.3	0	90	3.4	0	90	4.0	0	100	4.2	0	90	1.6
13	140	5.3	0	110	7.0	0	100	5.9	0	120	5.1	0	100	4.2

of ergot alkaloids but stimulates the growth of *P. sizovae* [6]. At the same time, exogenously added tryptophan virtually does not influence alkaloid synthesis in *P. kapuscinskii* [11] but induces the synthesis of ergot alkaloids in some fungi of the genus *Claviceps* when added to the medium at concentrations of 0.25 to 2 mM during inoculation [8–10].

Further experiments were carried out to elucidate the role of two other amino acids, leucine and isoleucine, in the synthesis of quinocitrinins. These amino acids were added to the medium on the 10th day of cultivation. In the control medium, the intracellular level of leucine and isoleucine in the stationary-phase fungus did not show significant changes (Table 4). Leucine and isoleucine added at concentrations of 0.25 and 2 mM on the 10th day of cultivation were consumed within 1–2 days, depending on the concentration added (Table 5). The level of leucine and isoleucine in the mycelium was considerably lower than in the control for at least three days of observations (Table 4). For example, one day after the addition of 0.25 and 2 mM leucine to the medium, the concentrations of leucine and isoleucine in the mycelium were, respectively, 1.6 and 2.8 times (leucine) and 1.9 and 2.3 times (isoleucine) lower than in the control. Similarly, one day after the addition of 0.25 and 2 mM isoleucine, the concentrations of leucine and isoleucine in the mycelium were, respectively, 1.5 and 2.4 times lower than in the control. In this case, the concentration of free tryptophan in the mycelium did not change in response to the addition of leucine or isoleucine to the medium.

Thus, exogenously added leucine and isoleucine are probably metabolized by the fungus and serve as negative allosteric regulators of the key enzymes of their synthesis, 2-isopropylmalate synthase and threonine deaminase, respectively. The rise in the concentration of free tryptophan in the *P. citrinum* VKM FW-800 mycelium in response to the addition of leucine or isoleucine to the medium may indicate the activation of cross-pathway control over amino acid synthesis [12].

The addition of 0.25 and 2 mM leucine and 2 mM isoleucine on the 10th day of cultivation almost did not influence the level of ergot alkaloids in the medium over an observation period of three days (Table 5). However, the addition of 2 mM isoleucine did diminish the concentration of ergot alkaloids in the medium almost twofold. Both amino acids also inhibited the synthesis of quinocitrinins, the greatest decrease in the concentration of quinocitrinins in the medium being observed in response to the addition of 2 mM isoleucine. This fact suggests a direct correlation between the intracellular level of free isoleucine and the intensity of quinocitrinin synthesis in the fungus *P. citrinum* VKM FW-800.

To conclude, we studied the effect of L-tryptophan, L-leucine, and L-isoleucine, added to the medium either during inoculation or in the stationary growth phase, on growth and biosynthesis of ergot alkaloids and quinocitrinins in the fungus *Penicillium citrinum* VKM FW-800. The exogenously added amino acids are mostly utilized in primary metabolism. When added during inoculation, tryptophan and leucine influence neither fungal growth nor the alkaloid synthesis. When added in the stationary growth phase on the 10th day of cultivation (i.e., before ergot alkaloids are consumed), tryptophan inhibits the consumption of ergot alkaloids and delays their active synthesis. When added on the 11th day of cultivation (the period of maximal consumption of ergot alkaloids and the beginning of a new cycle of ergot alkaloid synthesis), tryptophan delays the active synthesis of ergot alkaloids and diminishes its intensity. At the same time, tryptophan added to the medium in the stationary growth phase stimulates the synthesis of quinocitrinins. Isoleucine inhibits the synthesis of both ergot alkaloids and quinocitrinins irrespective of the time of its addition to the medium. Leucine added in the stationary growth phase does not influence the synthesis of ergot alkaloids but inhibits the synthesis of quinocitrinins.

The data obtained in this study provide new information on the regulatory mechanisms of secondary metabolism in producing fungi and, hence, may be of theoretical importance. These data can also be used to optimize media for the production of biologically active secondary metabolites with a broad range of action.

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