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EXPERIMENTAL ARTICLES

Trisporoids under the Stimulation of Carotenogenesis in *Blakeslea trispora*

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Abstract—The patterns of trisporoid synthesis in joint cultivation of *Blakeslea trispora* mates have been studied. The pair of the not-zygospore-forming carotenoid overproducer strains T(+) and T(–) was found to synthesize a large amount of trisporoids, which did not differ in biological activity from those in the wild type strains. While the β-carotene synthesis stimulator β-ionone increased the amount of trisporoids, the share of trisporic acids in their composition decreased considerably. The lycopene synthesis stimulator 2-amino-6-methylpyridine caused a decrease in the content of trisporoid which had no trisporic acids in their composition. Emergence of a new substance with the maximum absorption at 250 nm, which accounted for up to 45% of the sum of trisporoids, was a general regularity in the action of both stimulators. The combined action of these two effectors resulted in additional stimulation of lycopene synthesis and was accompanied by the disappearance of trisporic acids. The aggregate findings indicate that both carotenogenesis stimulators inhibit the synthesis of trisporic acids, i.e., their action is not mediated by stimulation of trisporoid synthesis.

Keywords: Blakeslea trispora, carotenogenesis, trisporoids, trisporic acids, β-ionone

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The fungus *Blakeslea trispora* is an overproducer of carotenoids, in particular, β-carotene and lycopene [1–4]. In biotechnology, joint cultivation of heterothallic (+) and (–) strains is used because sexual interaction stimulates expression of the genes *carRA* (phytoene synthase and lycopene cyclase) and *carB* (phytoene dehydrogenase) [5]. Moreover, expression of the gene *tsp3* (β-carotene oxygenase) is induced when heterothallic strains communicate [6] and the sex hormone, trisporic acids (TSA), which controls the formation of sex structures, stimulates carotenogenesis, and regulates its own feedback type synthesis, is formed as a result of the cooperative sex-specific process [7–9]. TSA and their precursors, the family of trisporoids to which C-18 or C-19 isoprenoid compounds with the C-14 main chain belong, are formed in the joint culture of the (+) and (–) strains of mucoraceous fungi [9].

It was shown for *B. trispora* that carotenogenesis may be stimulated not only by the sex hormone but also by cAMP, hydrogen peroxide, Span 20, retinol acetate, β-ionone, etc. [5, 10–12]. The most common stimulator β-ionone, which is structurally closely related to TSA, significantly enhances carotenogenesis but decreases the amount of TSA [13, 14] and does not induce zygophore formation [15]. A feature of this stimulator is its action on the joint culture alone; no stimulation of carotenogenesis was observed in individual strains [16]. It was established that β-ionone acts at the early stages of carotenoid synthesis—between 5-phosphomevalonic acid and dimethylallyl pyrophosphate, i.e., at the stage of formation of the isoprenoid unit, a structural block of carotenoids and sterols [13]. It was also shown that TSA competed with β-ionone for the action on carotenogenesis, and vice versa [17]. The authors suggest that the action of TSA is linked to depression of the carotenoid synthesis genes at the same stage where β-ionone also operates. The similarity between the action of β-ionone and TSA lies in the fact that both influence the overall terpenoid synthesis, stimulating both carotenoid and sterol syntheses. The study of the effect of β-ionone on carotenoid synthesis in the presence of cycloheximide and actinomycin D, inhibitors of protein and RNA synthesis, made it possible to suggest the stimulation of the de novo synthesis of carotenogenesis enzymes [18]. The action of β-ionone on trisporoid (TS) formation has not been fully understood yet. The data on the competitive character of the action of β-ionone and TSA allow the suggestion that this stimulator appreciably affects the sex hormone synthesis.

In order to obtain lycopene, the last step of the pathway of carotenoid synthesis, lycopene cycling to β-carotene, is inhibited with the resultant accumulation of lycopene in the fungus mycelium. Various pyridine and imidazole derivatives are mainly used as inhibitors [4, 10, 19]. Nicotine, a pyridine derivative, exemplified that the inhibition of lycopene cyclase occurs via irreversible binding to the enzyme [20]. The lycopene cyclase inhibitor proposed by us earlier,

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2-amino-6-methylpyridine (MAP), significantly increases the lycopene share (90–95% of the sum of carotenoids) but, compared with β-ionone, stimulates carotenogenesis much more weakly [9]. Earlier, β-ionone was not used in the biotechnologies for obtaining lycopene. Since MAP and β-ionone have different mechanisms of action, we suggested that it was expedient to use β-ionone combined with MAP for an additional stimulation of lycopene synthesis. From the practical point of view, the study of combined action of the stimulators could increase the level of carotenoid yield, and the investigation of the trisporoid composition would be effective in understanding their role in carotenoid overproduction.

It was shown earlier that the optimum conditions for TSA and carotenoid synthesis in *B. trispora* did not coincide [21]. The maximal amount of carotenoids was formed when the (+) to (–) strain ratio in the inoculum was 1 : 7, whereas a ratio of 1 : 1 was more favorable for TSA synthesis. The study of the trisporoid composition under stimulation of carotenoid synthesis could help to overcome this contradiction.

The goal of the present study was to investigate the action of MAP and β-ionone on the carotenoid and trisporoid composition in a joint culture of *B. trispora* T(+) and T(–) strains.

MATERIALS AND METHODS

The study was conducted with the mucoraceous fungus *Blakeslea trispora* Thaxter, the strains T(+) and T(–) from the culture collection of the Institute of Microbiology, Russian Academy of Sciences. The fungus was maintained on the sporogenous agarized potato–carrot medium; 7–10-day cultures were used for the experiments.

The fungus was grown in submerged culture. The spore suspension ($2–5 \times 10^5$ spores/mL medium) was used for inoculation. The inoculum of the (+) and (–) strains was grown separately in 100 mL of hydrolytic flour medium [19] in 750-mL flasks. The cultivation was carried out for 48 h on a rotary shaker (220 rpm) at 27–28°C. In order to study carotenogenesis, 750-mL flasks containing 50 mL of the flour medium were inoculated with the inocula of the (+) and (–) strains at a ratio of 1 : 7 and in an amount of 20% (vol/vol) with the subsequent addition of 3% (vol/vol) of sunflower oil. The lycopene formation stimulator 2-amino-6-methylpyridine (0.005%) was added at inoculation and after two days, and the carotenogenesis stimulator β-ionone (0.1% (vol/vol)) and sunflower oil (2% (vol/vol)) were introduced after two days of growth; the cultivation lasted four days.

In order to study trisporoid formation, the inocula of the (+) and (–) strains were inoculated at a 1 : 1 ratio and an amount of 10% (vol/vol) with the subsequent addition of 3% (vol/vol) of sunflower oil. The cultures were grown for one to four days under the same conditions.

Chromatographic separation of trisporoids, identification, quantitative analysis of their composition, preparative isolation, and determination of their biological activity were carried out as described earlier [22].

B. trispora T(–) strain was used as the test strain for studying the carotenogenic action of trisporoids. Petri dishes with wort agar 7°B were lawn-inoculated with the spore suspension, and growth was maintained for 24 h until the stage of formation of aerial mycelium without sporangia. A certain amount of trisporoids (30–300 μg) was applied onto disks of filter paper (5 mm in diameter); after ethanol evaporation, they were placed on the lawn surface, and the cultivation was continued for additional 24 h. The stimulation was assessed only qualitatively by the appearance of an orange ring around the circle with TS.

The carotenoid composition was determined by the method developed by us [23].

The experiments were performed in triplicate; the results show the data of the typical experiment. The scatter in the results did not exceed 10%; the main patterns coincided.

RESULTS

The (+) and (–) strain ratio in the inoculum affected the carotenogenic activity of the culture. The greater the (–) strain content in the inoculum, the higher carotenogenesis was. The maximal carotenoid level was observed with the sevenfold predominance of the (–) strain (Table 1). On the contrary, the highest amount of TS was observed at the equal ratio of the heterothallic strains. The major TS components were trisporic acids and trisporins/trisporols; the minor components were methyltrisporates and unidentified substances with the maximum absorption at 260 and 290 nm. The predominance of one of the strains resulted in the same 1.5-fold reduction in the amount of TS; however, the TS composition changed differently. For example, substantially less TSA was formed at the predominance of the (–) strain, compared with the equal strain ratio, which was due to the absence of TSA-A and the low TSA-B content. The prevalence of the (+) strain was accompanied by a decrease in the share of trisporins/trisporols and an increase in the share of TSA at the expense of an increase in the relative TSA-A and TSA-B content.

Investigation of the dynamics of TS formation at the optimal, equal strain ratio showed that after 24 h of the strain interaction, very low amounts of trisporoids (less than 0.06 g/L) were formed, so that no analysis of their composition was made. The maximal amount of TS was observed on day 4. Note that their amount changed in the dynamics of growth of the joint culture, but the change was not uniform. For example, a twofold difference in the TS content was observed between two- and three-day cultures, whereas a fivefold difference was found between three- and four-day

Table 1. Effect of the *B. trispora* T(+) to T(–) strain ratio in the inoculum on carotenogenesis and the trisporoid composition

The (+) to (–) strain ratio	DB, g/L	β-Carotene, % of DB	Trisporoids									
			% of Σ								DB, mg/g	g/L
			NF			AF						
			TN/TL	X_{290}	MTSA	TSA-A	TSA-B	TSA-C	Σ TSA	X_{260}		
7 : 1	24.9	0.073	13.3	ND	1.3	13.6	10.6	29.8	54.0	10.8	77.2	1.92
1 : 1	22.1	0.135	31.1	13.0	3.4	5.6	9.1	31.5	46.2	9.0	148.2	3.28
1 : 7	34.3	0.244	25.3	16.2	5.6	ND	3.2	26.0	29.2	10.6	60.6	2.08

Note: Designations: NF, neutral fraction; AF, acid fraction; TN/TL, trisporins and trisporols; X, unidentified substances; DB, dry biomass; ND, not determined.

Table 2. Dynamics of trisporoid formation in a joint culture of *B. trispora* T(+) × T(–) strains with an equal strain ratio in the inoculum

Cultivation time, days	DB, g/L	β-Carotene, % of DB	Trisporoids									
			% of Σ								DB, mg/g	g/L
			NF			AF						
			TN/TL	X_{290}	MTSA	TSA-A	TSA-B	TSA-C	Σ TSA	X_{260}		
2	36.5	0.199	31.0	0.5	1.6	ND	20.0	13.0	33.0	11.0	10.0	0.37
3	33.4	0.184	37.9	tr.	3.1	2.2	24.6	22.3	49.1	8.5	22.4	0.75
4	36.9	0.190	25.7	1.9	2.5	3.8	18.4	27.1	49.3	11.9	97.7	3.61

Note: See Table 1 for designations.

cultures (Table 2). The share of trisporins and trisporols changed slightly in the dynamics of development, and the relative TSA content increased 1.5-fold due to the TSA-C increase.

It is known that β-ionone inhibits the growth processes. Therefore, it is used in the idiophase in biotechnology. MAP does not exert an inhibitory effect on growth; it may be introduced during inoculation. That is why the technique of introduction of these stimulators with regard to their action on the growth processes was developed. If MAP was introduced during inoculation and β-ionone, after two days the total amount of lycopene increased, but the share of β-carotene increased as well. The best results from the point of view of lycopenogenesis were obtained in the variant when MAP was introduced twice: during inoculation and in a combination with β-ionone, which was introduced in the initial idiophase. As a result, the amount of lycopene increased, compared with the variant with MAP, and was comparable with the amount of β-carotene in the variant with β-ionone (Fig. 1), the lycopene share being about 95%.

What happens to the trisporoid composition under the conditions of carotenogenesis stimulation—at the (+) to (–) strain ratio 1 : 7, an increase in the oil concentration up to 5%, and the introduction of stimulators? By changing the carotenoid ratio towards lycopene, i.e., by decreasing the amount of β-carotene (TSA precursor), MAP insignificantly decreased the amount of TS, while β-ionone, a carotenogenesis stimulator, increased their level fourfold compared to the control variant (Table 3). Moreover, noticeable changes in the trisporoid composition were observed. A sharp reduction in the amount of all TSA forms and the emergence of a new substance with the absorption maximum at 250 nm (X_{250}), which was absent in the control variant, was the general pattern resulting from the effect of MAP and β-ionone on carotenogenesis (Fig. 2). The share of X_{250} could be as high as 45%, and it was present in both the acid and neutral fractions. It should be pointed out that only a small amount of TSA-C was found in the variant with β-ionone, whereas none of the TSA forms were revealed in the presence of MAP and in its combination with β-ionone. Furthermore, the minor substances absorbing at 240–250 and 275–280 nm, which could not be associated with the known trisporoids by their spectral characteristics and the results of the biological test, were present in all experimental variants in contrast to the control variant.

It was important to understand whether the TS isolated from the joint culture of the (+) and (–) strains not forming zygospores differed in biological activity from those of the wild-type zygote-forming strains.

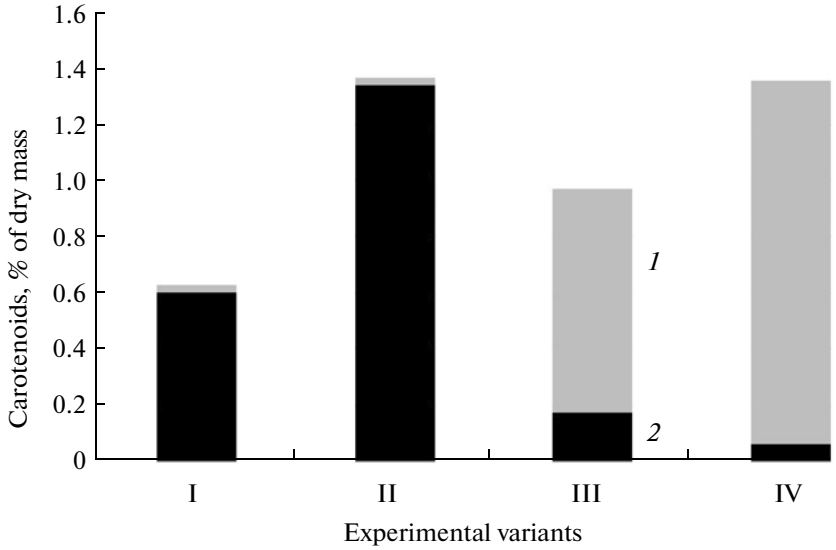

Fig. 1. Effect of the introduction of β-ionone and MAP on *Blakeslea trispora* carotenogenesis: I, control; II, β-ionone (48 h); III, MAP (0 h); IV, MAP (0 h; 48 h) + β-ionone (48 h); (*1*) lycipene; (*2*) β-carotene.

The results of the biological test of trisporoids (Table 4) showed that the biological activity of TSA and trisporins/trisporols did not fundamentally differ from that in the wild strains [22]. The new substance emerging under carotenogenesis stimulation (X_{250}) did not exhibit biological activity with any of the *Mucor mucedo* test strains in the broad range of concentrations (30–300 μg).

The test for the carotenogenesis activity showed that all the TSA forms stimulated carotenogenesis in the T(–) strain, whereas trisporins/trisporols, X_{250}, and the substances absorbing at 240–250 and 275–280 nm did not produce such an effect. It is important to note that TSA stimulation of carotenogenesis in the T(–) test strain occurred at the same concentrations that caused zygophore formation in *M. mucedo* test strains.

The T(+)/T(–) pair was a carotenoid overproducer, but the role of these strains was different. In our previous work, we discovered that the T(–) strain obtained by mutagenesis did not form zygospores in a pair with any of the seven wild (+) strains or the T(+) strain [22]. On the contrary, T(+) was an active zygote former. However, it was surprising that the T(+) and T(–) strains formed a substantially larger amount of trisporoids in submerged culture than the pairs of wild strains with active zygote formation. In biotechnology, the joint culture of the (+) and (–) strains and the stimulators MAP and β-ionone are used. It is the first time we have shown that the use of MAP in combination with β-ionone resulted in an appreciable stimulation of the synthesis of carotenoids and the predominance of lycopene in their composition. Thus, the yield of lycopene comparable to that for β-carotene was attained when β-ionone was used.

Table 3. Trisporoid composition in the joint culture of *B. trispora* T(+) × T(–) strains under MAP and β-ionone stimulation of carotenogenesis

Experimental variant	DB, g/L	Trisporoids								
		% of Σ							DB, mg/g	g/L
		NF	AF							
		TN/TL	X_{280}	TSA-B	TSA-C	Σ TSA	X_{260}	X_{250}		
Control	43.1	24.2	3.0	10.3	19.5	29.8	10.2	ND	29.8	1.28
β-Ionone	46.1	9.7	5.1	ND	10.3	ND	11.4	33.4	117.0	5.39
MAP	50.3	19.9	8.8	ND	ND	ND	8.5	44.5	19.3	0.97
MAP + β-ionone	44.4	7.8	3.6	ND	ND	ND	3.3	41.2	43.2	1.92

Note: See Table 1 for designations.

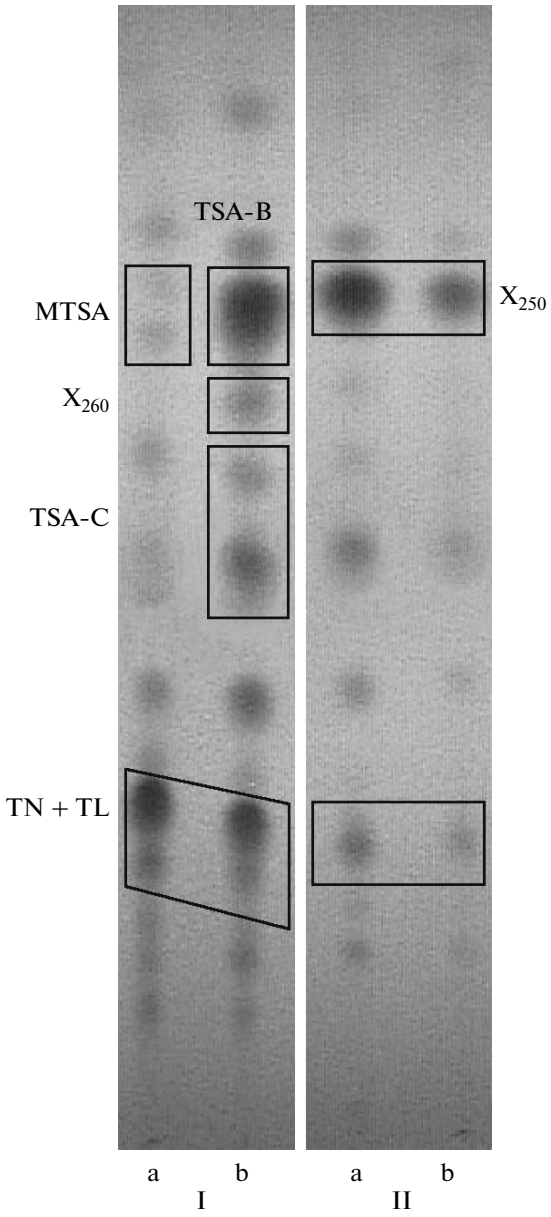

Fig. 2. Chromatogram of the trisporoids formed under carotenogenesis stimulation: I, control; II, in the presence of β-ionone and MAP; (a) neutral fraction; (b) acid fraction.

DISCUSSION

In the present work, we established for the first time that addition of β-ionone and MAP to the joint culture of the (+) and (–) strains of *B. trispora* resulted in appreciable changes in the composition of TS. A general pattern was a decrease in the amount of TSA and emergence of the new compound X_{250} that was not present in the control variant. The difference in the action of MAP and β-ionone showed up in a change in the amount of trisporins and trisporols. MAP affected their content slightly, whereas β-ionone decreased their content 2.5–3-fold. These findings show that both effectors inhibited TSA synthesis in the joint culture of the fungus. Proceeding from contemporary notions of the mechanism of action of MAP and β-ionone, it may be suggested that they have different mechanisms of regulation of TSA synthesis. MAP, by inhibiting lycopene cyclase (Fig. 3), substantially decreases the synthesis of β-carotene, which is a TSA precursor, resulting in a decrease in the level of all the TSA forms. However, the fact that, in the presence of MAP, X_{250} was accumulated and the share of trisporins/trisporols varied insignificantly against the background of disappearance of all the TSA forms suggests that the influence of this inhibitor at both the early and late stages of TSA synthesis should not be excluded. Strong stimulation of β-carotene formation was observed as a result of the action of β-ionone with an appreciable increase in the amount of trisporoids. However, the share of trisporins/trisporols and TSA in their composition was substantially decreased. In combination with MAP, a still stronger inhibition of trisporoid synthesis by β-ionone was observed: the amount of trisporins/trisporols decreased threefold and the TSA disappeared. Taking into account the accumulation of the X_{250} precursor in an appreciable amount, our findings suggest that β-ionone inhibits TSA synthesis in the joint culture at the stage prior to trisporins/trisporols formation.

It was previously shown that β-ionone competes with TSA for the action on carotenogenesis [17]. The site of its action in the pathway of carotenoid synthesis—from 5-phosphomevalonic acid to dimethylallyl pyrophosphate—was convincingly shown (Fig. 3). However, there is no consensus about the mechanism of action of β-ionone. Some authors suggest that β-ionone acts on the pre-existing enzymes stimulating their activity [13]; others are of the opinion that this effector stimulates the de novo enzyme synthesis [18] because the stimulation of RNA and protein syntheses was observed simultaneously. However, all the investigators agree that β-ionone acts at the level of translation, which is confirmed by inhibitory analysis. The TSA are also known to stimulate their own synthesis of the feedback type [7], inducing the expression of the β-carotene oxygenase gene [6]. The fact that β-ionone competes with TSA for the action on carotenogenesis suggests that it might also complete with TSA for the regulation of trisporoid synthesis following the feedback pattern. In this case, we observe the situation opposite to the stimulation of carotenogenesis, i.e., the inhibition of TSA synthesis. However, in the final analysis, the action of β-ionone leads to a considerably greater accumulation of carotenoids than in the control variant. Considering that this stimulator does not affect certain fungal strains and its action manifests itself only at the translation level, the necessity of sexual interaction, which leads to the induction of expression of the carotenogenic genes and the formation of the carotenogenic enzyme complex, becomes obvious. The findings of β-ionone-mediated inhibition of TSA

Table 4. Biological activity of trisporoids in the joint culture of *B. trispora* T(+) × T(−) strains

Trisporoids, fraction	*M. mucedo* (−) strain		*M. mucedo* (+) strain	
	trisporoids, μg	zygophore number	trisporoids, μg	zygophore number
Trisporines/trisporols, NF	105	ND	105	++
X_{260}, NF	60	ND	30 60	+ ++
TSA-A, AF	35	++	35	++
TSA-B, AF	60 120	++ +++	60 120	++ ++
TSA-C, AF	60 120	+ ++	60 120	+ ++
X_{250}, AF	30–300	ND	30–300	ND

Note: the zygophore number: + fewer than 10; ++ 10–50; +++ 50–100; ND—not determined.

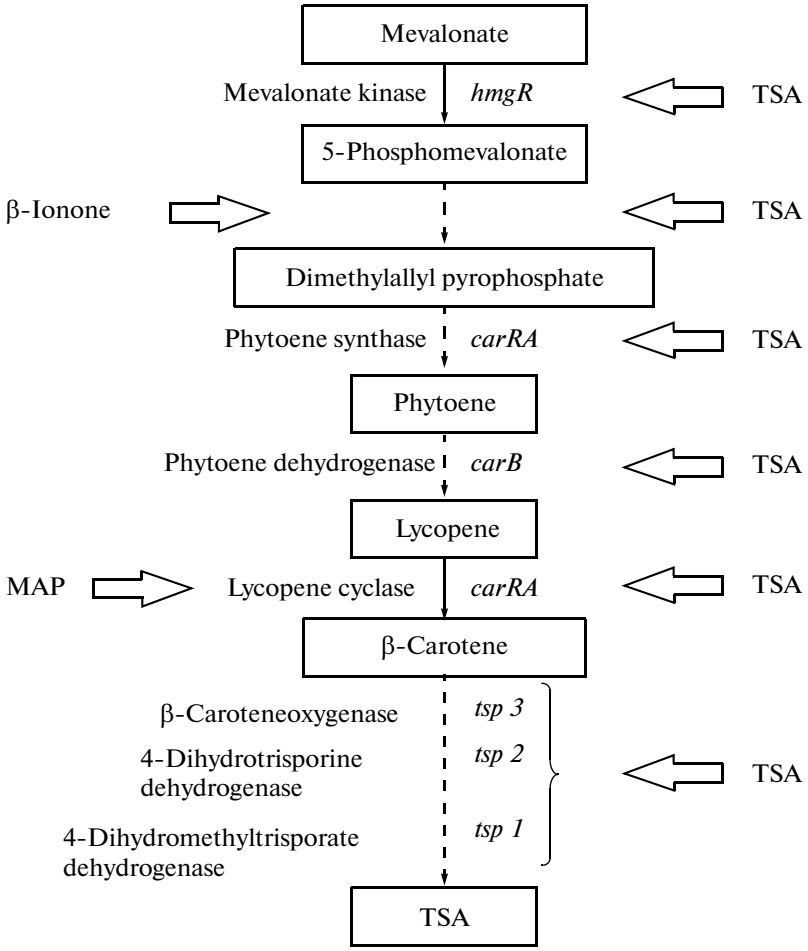

Fig. 3. Scheme of the regulation of carotenoid synthesis.

synthesis indicate that the predominance of the (–) strain required for intense carotenogenesis in the joint culture is associated not with a certain TSA concentration, as it was suggested earlier [22], but with this strain being more carotenogenic than the (+) strain.

The fact of the increased amount of trisporoids in the presence of β-ionone, with a considerably decreased share of trisporols/trisporins and TSA, is noteworthy. The appearance of a large amount of X_{250} and of the multitude of minor compounds not attributable to the typical trisporoids by their spectral characteristics, biological action, and not revealing any biological activity, gives evidence of a possible increase in the processes of double β-carotene cleavage, because, in this case, the appearance of both C_{18}-trisporoids and C_{15}- and C_7-apocarotenoids can be explained [24]. It is not excluded either that, with increased β-carotene yield, the increase in its nonenzymatic cleavage by reactive oxygen species is possible [25].

Thus, this study showed that the combined action of β-ionone and MAP resulted in an additional stimulation of lycopenogenesis. The inhibition of trisporoid synthesis under the conditions of carotenogenesis intensified by these stimulators was shown for the first time, which indicates that the action of these stimulators on carotenogenesis is not mediated by the intensification of TSA synthesis. However, since these stimulators function at the translation level, preliminary sexual interaction (mating), which induces the formation of trisporic acids and carotenogenic enzymes, is required for their stimulating action to be become manifest. In this context, the predominance of the (–) strain in the joint culture required for intense carotinogenesis is associated not with the maintenance of a certain TSA concentration but only with the fact that this strain is more carotenogenic than the (+) strain. Another possible variant of efficient carotenogenesis, i.e., the use of only the (–) culture preliminarily stimulated by TSA, is also known in biotechnology [26].

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