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NICOTINE-INDUCED INHIBITION OF LYCOPENE CYCLIZATION IN PHASEOLUS VULGARIS COTYLEDONS

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SUMMARY - The complete nicotine inhibition of lycopene cyclization during light-induced carotenogenesis in excised bean cotyledons was achieved. The inhibitory effect was easily reversible and removal of nicotine has allowed synthesis of the normal cyclic carotenoids.

Nicotine is well known as an inhibitor of cyclic carotenoid formation in microorganisms /1-18/, but such an inhibitory action has also been found in higher plants /19,20/. In latter works it was shown that the nicotine treatment caused the inhibition of carotenoid cyclization resulting in the accumulation of precursors of bicyclic carotenoids, lycopene and δ - and γ - carotenes, which were not found normally in investigated plant tissues. However, this inhibitory action was not very effective and the first cyclization step could not be completely prevented. Besides, it was not reported if the inhibitory effect was reversible and the normal cyclic carotenoids could be formed on nicotine removal.

The present work is therefore intended to examine whether the reversible and complete inhibition of lycopene cyclization in higher plant system is possible. The availability of such a system may provide an opportunity to further studies in vivo cyclic carotenoid formation.

EXPERIMENTAL

Bean seeds /Phaseolus vulgaris L.var. Złota Saxa/ were germinated in the dark in Petri dishes on filter paper moistened with

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distilled H_2O within a thermostat at 24°C. After 48 h the cotyledons were excised under a green safe light, placed in another Petri dishes containing 50 ml of either culture medium /Knop solution/ with nicotine in concentration of 7.5 mM /pH 8.5/ or culture medium without nicotine /control/. The dishes were covered with a glass lid to minimize evaporation and exposed to light /24.8 $W.m^{-2}$/ from fluorescent lamps of the "Daylight" type. After incubation for 12 h at 24°C the samples both of controlled and nicotine treated cotyledons were collected, blotted with paper towels and subjected either to the dry weight determination or the carotenoid analysis. The remaining portion of nicotine-treated cotyledons was washed with distilled H_2O in order to remove nicotine and reincubated with fresh medium without nicotine. After reincubation, for 12 h at 24°C, the cotyledons were again sampled and subjected to the carotenoid analysis. Methods for carotenoid analysis including extraction, purification, identification and spectrophotometric estimation were essentially the same as previously described /21/. The data and criteria used for ketohydroxylycopene /new structure/ identification are given below.

Chromatographic properties. Carotenoid was chromatographically homogenous in three thin - layer chromatographic systems and its adsorption affinity was as follows:

1. MgO /International Enzymes/ with light petroleum-acetone /13:7/ - Rf 0.17
2. Al_2O_3 typ 60/E /Merck/ with light petroleum - benzene - acetone-methanol /40: 15: 6: 3/ - Rf 0.40
3. Silica gel G/Merck/ with light petroleum - Et_2O /9 : 1/ - Rf 0.00

Spectroscopic data. UV/VIS : λ_{max}/acetone - 432, 457, 485; λ_{max}/benzene/ - 442,467,498; λ_{max} /ethanol/ - 429,453,482. IR: ν_{max}/benzene/ - 3580 /OH/, 3420 /H-bonded OH/, 1750 /non-conj. carbonyl/, 1720 /acyclic non-conj.carbonyl/, 1450, 1420, 1370 /gem.dimethyl/, 1250 /enol/, 1220, 1100, 1060 /sec.OH/, 905. MS /the upper part of the spectrum/ m/e: 570 /2; M^+/, 569 /3.7; M-1/, 568 /3 ; M-2/, 552 /2.2; M-18/, 551 /1.1/, 550 /1.5/, 524 /2.5; M-18-28/, 523 /2.2/, 478 /0.6; M-92/, 477 /1.3/, 476 /1.2/, 464 /0.7; M-106/,463/0.8/, 462 /0.3/, 412 /2.2; M-158, 411/2.3/ 409/2.6/, 370 /2.7/, 369 /4.9/, 368 /1.9/, 367 /2.2/, 354 /2.7/, 339 /2.1/, 313 /5.9/, 312 /2.6/, 311 /2.6/.

Electronic spectra were recorded on an Specord UV VIS spectrophotometer from VEB Carl Zeiss Jena. IR spectra were determined in benzene solution on an Zeiss UR-20 in the range 700-5000 cm^{-1}.

MS were obtained on an LKB 2091 GCMS instrument at 70 eV using the direct insertion technique and an ion source temperature of 250°C. The probe temperature was 150°C.

Chemical reactions. Saponification. After KOH treatment /22/ the carotenoid could be completely recovered from alkaline phase by ether extraction, e.g. could not be saponified.
Reduction. Under $NaBH_4$ reduction /23/ the carotenoid was converted into compound which was more polar /TLC, MgO/ then the parent compound; the positions of absorption maxima were not changed but the little increase in fine structure of spectrum was observed.
Acid isomerization. Absorption spectra of the carotenoid were not changed by concentrated HCl treatment /24/.

RESULTS AND DISCUSSION

The complete inhibition of lycopene cyclization in excised bean cotyledons incubated in culture medium containing nicotine was achieved. In nicotine treated cotyledons solely lycopene could be formed; the traces of β - carotene and lutein observed, have already been existed in etiolated cotyledons i.e. before inhibitor and light treatment. Absence of the notable amounts of monocyclic carotenoids, δ - and γ - carotenes, confirms the great effectiveness of nicotine action.

It was discovered that the complete inhibition was attainable when the inhibitor was applied to cotyledons of dark-grown /etiolated/ seedlings. No carotenogenesis inhibition was observed when nicotine was administrated to green cotyledons.

Nicotine concentration of 7,5 mM applied, has been proved to be the smallest during which the cyclization was totally prevented. Since nicotine have exerted only slight, insignificant effect on the total carotenoid production /Table 1/, it appears that nicotine action was specific and the cyclization was at least the main object of this.

The previous investigations /19,20/ have indicated that, the inhibitory action was not enough effective and cyclization process

Table 1. Carotenoid changes in Phaseolus vulgaris cotyledons incubated in culture medium containing nicotine and reincubated in culture medium after removal of the inhibitor

Carotenoid	Carotenoid content µg/100g dry weight		
	Cotyledons incubated for 12 h in the light with nicotine		Cotyledons incubated with nicotine and reincubated for further 12 h in the light without nicotine
	Control	Nicotine	
α -Carotene	2.7 ± 0.1	-	7.1 ± 0.4
β -Carotene	17.6 ± 0.4	trace	19.5 ± 0.9
δ -Carotene	trace	-	2.5 ± 0.3
γ -Carotene	trace	-	3.1 ± 0.4
Lycopene	5.8 ± 0.4	40.2 ± 1.5	20.4 ± 1.2
β -Carotene 5,6,5,6-diepoxide	-	-	3.3 ± 0.3
Violaxanthin	1.9 ± 0.2	-	trace
Lutein	9.0 ± 0.3	trace	10.0 ± 0.5
Zeaxanthin	2.5 ± 0.2	-	5.5 ± 0.3
Neoxanthin	1.6 ± 0.2	-	2.5 ± 0.4
Ketohydroxylycopene	-	-	19.1 ± 1.1
Total carotenes	26.1 ± 0.9	40.2 ± 1.5	52.6 ± 3.2
Total xanthophylls	15.0 ± 0.9	-	40.4 ± 2.6
Total carotenoids	41.1 ± 1.8	40.2 ± 1.5	93.0 ± 5.8

For details see section EXPERIMENTAL. Values shown are means of three independent experiments ±SD.

could not be completely blocked at the lycopene stage, even at high nicotine concentrations /50 mM/. In both cases the considerable amounts of γ- and δ - carotenes were formed.
The reasons of the differences between results of previous and present investigations are difficult to explain at present.

The next experiment demonstrated that the inhibitory effect was reversible i.e. on removal of nicotine the formation of normal

cyclic carotenoids could again proceed /Table 1/. Thus, when cotyledons that had been grown in the presence of nicotine were reincubated in culture medium without nicotine the amount of newly produced cyclic carotenoids exceeded the level of previously accumulated lycopene. At the same time this lycopene level decreased by about 50%.

Above results might be therefore interpreted as indicating that cyclic carotenoids were formed at the expense of accumulated lycopene, were it not for the fact, that in reincubated cotyledons considerable amounts of the other acyclic carotenoid, ketohydroxylycopene have been discovered.

Since the sum of these two acyclic pigments was roughly unchanged after the reincubation period i.e. lycopene decrease was approximately the same as the ketohydroxylycopene increase, it may be cosidered that accumulated lycopene was converted into ketohydroxylycopene, whereas the increase of cyclic carotenoids amount resulted mainly from the conversion of newly produced lycopene.
The situation appears to be similar to that for microorganisms in which was elearly demonstrated that postlycopene intermediates were synthetized, on removal of nicotine, not from pool accumulated lycopene but from early precursors /10,11,16,18/.
It was shown however, that accumulated lycopene could be further converted when the carotenogenic pathway was blocked at the level of phytoene desaturation.

In conclusion, the results obtained in this work do not provide yet an opportunity to the utilization of that higher plant system for in vivo investigations of lycopene cyclization. Therefore, further experiments may be similar to those on microorganisms, which should be performed in order to verify the assumptions made in this paper.

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