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CHEMICAL MODIFICATION OF CAROTENOGENESIS IN *GIBBERELLA FUJIKUROI*

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Key Word Index—*Gibberella fujikuroi*; regulation of biosynthesis; carotenoids; neurosporaxanthin; phytoene; inhibitors; diphenylamine; α-picoline; β-ionone; phenols.

Abstract—The wild type of the fungus *Gibberella fujikuroi* has no carotenoids in the dark and only moderate amounts in the light. The deep-orange mutant SG22 accumulates large amounts of carotenoids both in the light and the dark. The predominant compound is always neurosporaxanthin. β-Ionone and diphenylamine inhibit phytoene dehydrogenation, with concomitant loss of mycelial colour. A significant accumulation of lycopene has been observed only in the presence of high concentrations of α-picoline. Ethanol, propanol, dimethyl sulphoxide and α-picoline, but not 4-hydroxymercurybenzoate, result in low-levels of carotenoid accumulation by the wild type in the dark. Carotenogenesis in *Gibberella* is unaffected by many compounds which strongly stimulate or inhibit it in other organisms, e.g. retinol acetate, α-ionone, 2-(4-chlorophenylthio)triethylamine. HCl, nicotine, imidazole, dimethylphthalate, veratrol and cinnamic alcohol. The quantitative results suggest that *Gibberella* lacks a feed-back regulation of carotenogenesis similar to the one found in *Phycomyces*.

INTRODUCTION

Carotenoid accumulation in fungi and plants is strongly affected by a variety of chemicals (for review, see ref. [1]). Diphenylamine at suitable concentrations blocks the production of the unsaturated, coloured carotenoids, because it inhibits phytoene dehydrogenation with accumulation of phytoene and other intermediates in the cells [2–5]. Very large amounts of the same intermediates accumulate in *Phycomyces blakesleeanus* mycelia grown in the presence of cinnamic alcohol and other compounds [6, 7]. Accumulation of the red pigment, lycopene, is caused by chemicals inhibiting the cyclization steps of the pathway, e.g. 2-(4-chlorophenylthio)triethylamine. HCl (CPTA) [8–10], nicotine [11], imidazole [12] and α-picoline [6].

β-Ionone activates carotenogenesis in *Phycomyces* [4, 13], but inhibits it in *Verticillium albo-atrum* [5]. Retinol is a powerful activator of carotenogenesis in *Phycomyces* [14] and other Mucorales [15]. The effectiveness of these molecules may be due to their structural resemblance to each of the ends of the β-carotene molecule. The resemblance is not absolutely required, since many other aromatic compounds, including dimethyl phthalate and veratrol, activate carotenogenesis in *Phycomyces* [7].

Carotenoid biosynthesis is photo-inducible in many organisms [16]. Dark-grown mycelia of *Fusarium aquaeductuum* make carotenoids in the presence of 4-hydroxymercury-benzoate, which substitutes for light [17].

Gibberella fujikuroi (*Fusarium moniliforme*) is well known to phytochemists for its production of the plant hormones, gibberellins [18]. When grown in the light, the mycelia of this fungus are pale orange owing to their carotenoid content, and particularly to neurosporaxanthin. Dark-grown mycelia are white and contain the pigments only as traces. Mutants are readily obtained after exposure of microconidia (uninucleate spores) to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or UV radiation [19]. The deep-orange mutants contain large amounts of carotenoids, whether they are grown in the light or in the dark [19].

We have investigated the action of all the chemicals mentioned above on the accumulation of carotenoids in *Gibberella*. The deep-orange mutants are suitable for the study of inhibition of carotenoid biosynthesis, but might be unresponsive to some activators, as noted with *Phycomyces* mutants superproducing β-carotene [6]. The small amounts of carotenoids present in the wild type, particularly when grown in the dark, make it difficult to detect changes due to inhibitors but facilitate the detection of changes due to activators. We therefore tried each chemical on two different strains, the wild type and a deep-orange mutant, SG22, both grown in the light and in the dark.

RESULTS

The main pigment in the wild type and in the deep-orange mutant is neurosporaxanthin, which usually represents about 75% of total carotenoids. The rest consists of several coloured carotenes, including lycopene, γ-carotene and torulene, which are lumped together under the name of 'intermediate carotenes', and of the colourless phytoene (Fig. 1).

None of the chemicals tested induced the wild type to produce large amounts of carotenoids in the dark. In view of this finding, the rest of this presentation is confined to the three other experimental systems.

Many chemicals which strongly affect carotenogenesis in other organisms have little or no effect in *Gibberella*, e.g.

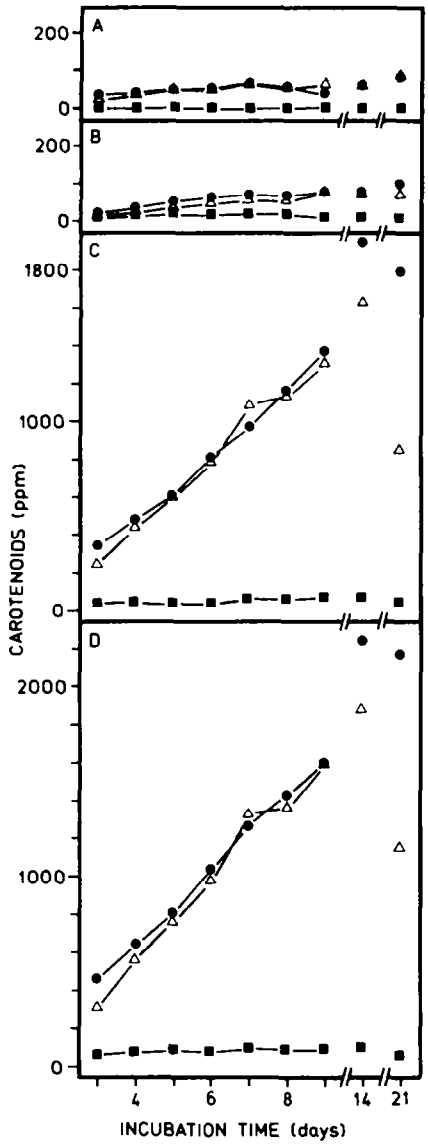

Fig. 1. Phytoene (A), intermediate carotenoids (B), neurosporaxanthin (C) and total carotenoid content (D) of wild type *G. fujikuroi* grown in the light (■) and strain SG22 grown either in the light (△) or in the dark (●).

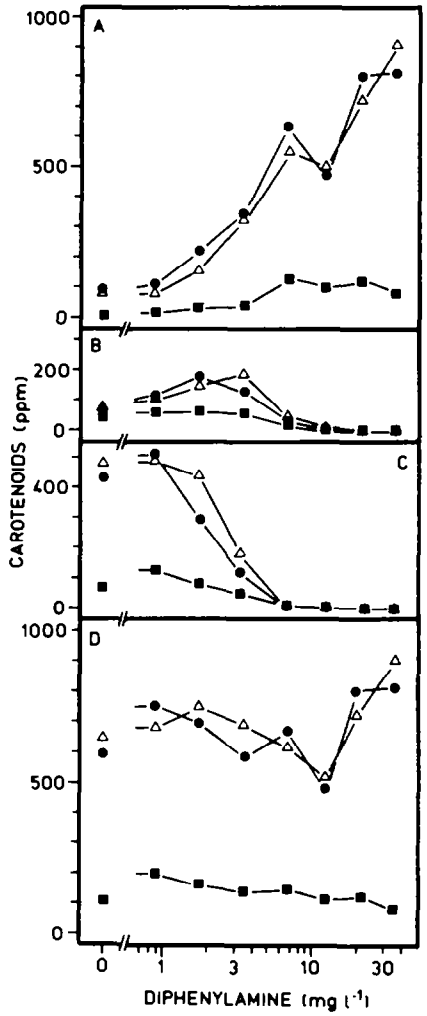

Fig. 2. Effect of diphenylamine on the content of phytoene (A), intermediate carotenoids (B), neurosporaxanthin (C) and total carotenoids (D) of wild type *G. fujikuroi* grown in the light (■) and strain SG22 grown either in the light (△) or in the dark (●).

cinnamic alcohol, CPTA, nicotine, imidazole, α-ionone, retinol acetate, dimethyl phthalate and veratrol (Table 1). These compounds were administered at concentrations which slowed down considerably the outgrowth of the mycelium, so that their failure to affect carotenogenesis cannot be attributed to a lack of entry into the cell.

Diphenylamine strongly inhibits the accumulation of neurosporaxanthin with a simultaneous increase in the concentration of phytoene (Fig. 2) and a pronounced inhibition of mycelial growth (Fig. 3). The intermediate coloured carotenes are always minor components and are virtually undetectable at the higher diphenylamine concentrations. Variations in the total are much less pronounced than variations in the individual carotenes.

β-Ionone is another inhibitor of carotene biosynthesis in *G. fujikuroi* (Fig. 4 and Table 2). The effects of β-ionone

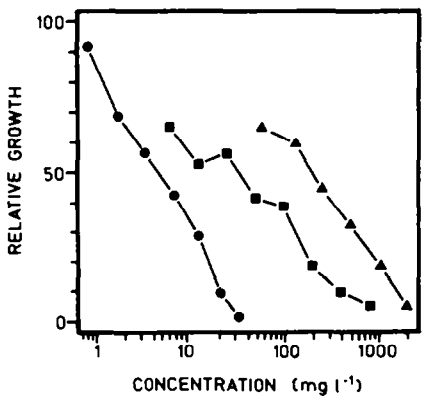

Fig. 3. Dry weight of SG22 colonies grown for 4 days in the dark in the presence of diphenylamine (●), β-ionone (■) and α-picoline (▲). In each experiment the weight of colonies grown in the absence of these chemicals was taken as 100.

Table 1. Lack of effect of different compounds on carotenoid content of *G. fujikuroi* mycelia

Additions (mg l^{-1})	Carotenoid ($\mu g\, g^{-1}$ dry wt)			
	Phytoene	Intermediate carotenes	Neurospora-xanthin	Total
Strain SG22 grown in the light				
None	61	61	556	678
CPTA (200)	105	109	474	688
CPTA (600)	102	151	418	671
Nicotine (649)	107	78	273	458
Nicotine (1622)	130	94	293	517
Imidazole (300)	75	57	365	497
Imidazole (1000)	60	112	347	519
Retinol (400)	105	74	599	778
Strain SG22 grown in the dark				
None	71	87	583	742
CPTA (200)	123	99	508	730
CPTA (600)	125	114	525	764
Nicotine (649)	102	69	432	603
Nicotine (1622)	178	107	465	750
Imidazole (300)	80	53	398	531
Imidazole (1000)	64	160	754	978
Retinol (400)	136	107	543	786
Veratrol (691)	311	338	725	1374
Dimethyl phtalate (388)	277	222	1166	1665
Cinnamic alcohol (27)	179	173	1075	1427
Wild strain grown in the light				
None	< 10	36	60	95
CPTA (200)	< 10	17	29	46
CPTA (600)	< 10	11	13	24
Nicotine (649)	< 10	36	23	59
Nicotine (1622)	< 10	20	33	53
Imidazole (300)	< 10	29	62	91
Imidazole (1000)	< 10	32	81	113
Retinol (400)	< 10	64	64	128
Veratrol (691)	37	53	47	137
Dimethyl phtalate (388)	28	58	63	149
Cinnamic alcohol (27)	19	49	67	135

Table 2. Effect of β-ionone and α-picoline on carotenoid content of *G. fujikuroi* mycelia

Additions (mg l^{-1})	Carotenoid ($\mu g\, g^{-1}$ dry wt)			
	Phytoene	Intermediate carotenes	Neurospora-xanthin	Total
Strain SG22 grown in the light				
None	70	71	604	745
α-Picoline (500)	89	84	527	700
α-Picoline (2000)	51	248	269	568
β-Ionone (400)	504	96	34	634
Strain SG22 grown in the dark				
None	84	86	563	733
α-Picoline (500)	107	89	459	655
α-Picoline (2000)	53	334	290	677
β-Ionone (400)	916	125	62	1103
Wild strain grown in the light				
None	< 10	41	60	101
α-Picoline (500)	< 10	41	54	95
α-Picoline (2000)	< 10	48	36	84
β-Ionone (400)	61	5	1	66

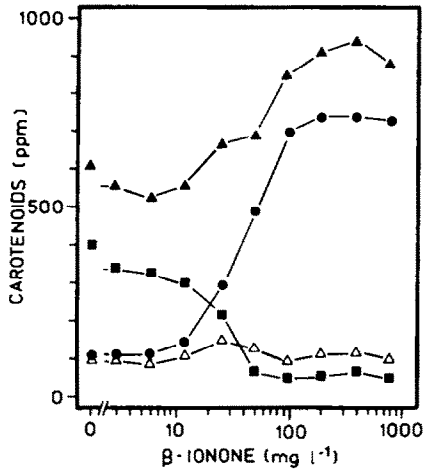

Fig. 4. Effect of β-ionone on the content of phytoene (●), intermediate carotenoids (△), neurosporaxanthin (■) and total carotenoids (▲) of strain SG22 grown in the dark.

on carotenoid content and growth inhibition (Fig. 3) largely coincide with those of diphenylamine. The only differences are that a little of the final product is made even at the higher concentrations of β-ionone and that the concentrations of the intermediate coloured carotenoids are practically unaffected by this compound.

α-Picoline behaves very differently from the other compounds tested, but its effects are obvious only at very high concentrations (Fig. 5 and Table 2), which are very toxic (Fig. 3). The increase in phytoene content is very small in comparison with the increase in lycopene content. The chemical therefore may inhibit lycopene cyclization.

Some of these compounds were dissolved in ethanol or polyoxyethylene sorbitan monooleate ("Tween 80"). In our experimental conditions Tween 80 had no effect on carotenoid content or mycelial growth and ethanol was slightly stimulatory: wild-type mycelia grown in the dark in the presence of ethanol (2% v/v) contained 13 μg neurosporaxanthin g^{-1} dry wt and 4 μg other carotenoids g^{-1} dry wt. Figure 6 compares the effects of

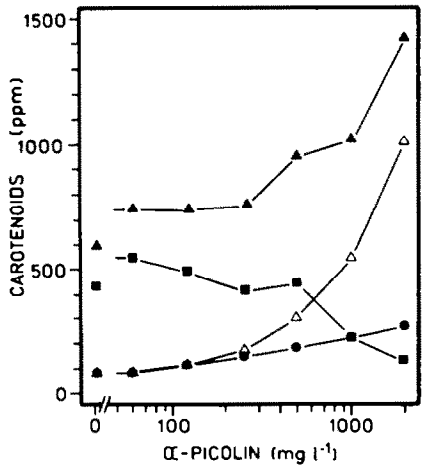

Fig. 5. Effect of α-picoline on the content of phytoene (●), intermediate carotenoids (△), neurosporaxanthin (■) and total carotenoids (▲) of strain SG22 grown in the dark.

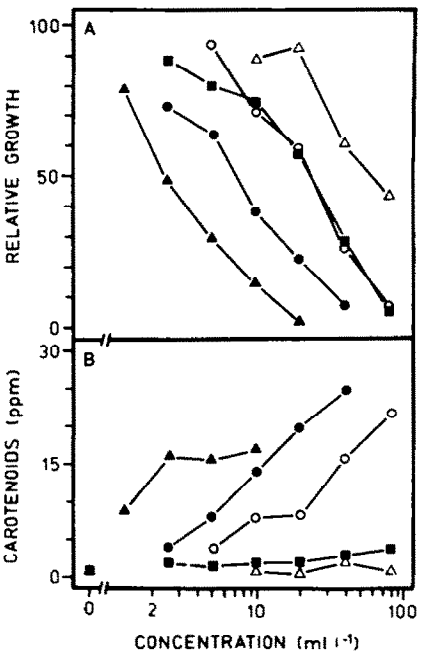

Fig. 6. Effect of methanol (■), ethanol (●), propanol (▲), dimethylsulphoxide (○) and Tween 80 (△) on the growth (A) and carotenoid content (B) of the wild type grown in the dark. In each experiment the weight of colonies grown in the absence of the chemicals was taken as 100.

several alcohols, dimethyl sulphoxide and Tween 80 on carotenogenesis in the dark.

Another minor stimulation of carotenogenesis in the dark was obtained with α-picoline: 2 g l^{-1} of this compound in the medium led to the presence of 12 μg carotenoids g^{-1} dry wt, mostly intermediate carotenes, in the mycelium. However, 4-hydroxymercurybenzoate, tested by following either our protocols or those of Rau *et al.* [17], was totally ineffective.

DISCUSSION

The regulation of carotenogenesis in *Gibberella* is unique in that it remains unaffected by most of the chemicals found to be active in *Phycomyces* and other organisms.

We have detected two inhibitors of phytoene dehydrogenation in *Gibberella*. One of them, diphenylamine, exhibits similar actions in many organisms [2–5]. The similarity extends to the concentrations of the drug which are required for the accumulation of phytoene and the inhibition of growth. The other one, β-ionone, appears to have a similar action in *Verticillium* [5], but a totally different one, the overall activation of the pathway, in *Phycomyces* and *Blakeslea*.

α-Picoline is the only compound leading to an accumulation of lycopene in *Gibberella*. The high concentrations needed for a detectable effect are not very different from those needed in other organisms [6].

There is a noteworthy constancy in the behaviour of *Gibberella* strains, both wild type and deep orange, towards the chemical inhibitors of carotenogenesis: the concentrations of the individual carotenoids may vary enormously, but their sum remains relatively unaffected.

This is at variance with the situation in wild-type *Phycomyces*, where any interruptions in the pathway vastly increase the total content of C_{40} compounds [10], i.e. the *Phycomyces* pathway is stimulated by the scarcity of the final product, but this feed-back regulation is not apparent in *Gibberella*.

Feed-back regulation in *Phycomyces* also explains why undisturbed cultures reach a saturating β-carotene content in a few days and maintain it thereafter. In contrast, the *Gibberella* deep-orange strain SG22 accumulates carotenoids linearly with time for many days, long after growth has stopped, as if the order to cease carotenoid production cannot be given.

The total carotenoid content g^{-1} dry wt of strain SG22 increases moderately (i.e. up to a doubling) in the presence of very toxic concentrations of any chemicals. This may be attributed to the uncoupling of mycelial growth and carotenoid synthesis, with the former being stopped before the latter.

The slight induction of carotenogenesis by methanol, ethanol and propanol correlates with their respective chain lengths, which in turn determine their volatility and solvent partition. We made no special effort to prevent evaporation of these chemicals in our Petri dishes; in similar experiments by A. Aguilera in this laboratory about 50% of the ethanol remained in the plates after 7 days incubation. Membrane modification by the alcohols and dimethyl sulphoxide appears as the most likely mechanism for their action [20, 21].

EXPERIMENTAL

Strains. The wild-type *Gibberella fujikuroi* strain IMI58289 was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England, and the deep-orange mutant SG22 was derived from it by chemical mutagenesis [19].

Culture conditions. Unless otherwise stated, the fungus was grown for four days at 23° on minimal agar as described by Avalos *et al.* [19]. Incubation was either in the dark or under 0.5 W m^{-2} white light from a battery of five fluorescent tubes (Sylvania F40T12ID, 120 cm long). Mycelial dry wts were determined after lyophilization. The dry wt of a colony averaged 27.1 mg with an overall standard deviation of 7.6 mg, but the average standard deviation within each experiment was 1.5 mg.

Chemicals. The chemicals to be tested were added to the medium immediately before pouring the plates and the medium was inoculated 12–24 hr later. Retinol acetate, α-ionone and β-ionone were dissolved in EtOH and Tween 80 and added to the medium at final concns of 8 ml l^{-1} EtOH and 4 ml l^{-1} Tween 80. Diphenylamine, veratrol, dimethyl phthalate and cinnamic alcohol were dissolved in EtOH and added to the medium at a final concn of 20 ml l^{-1} EtOH. CPTA (kindly given to us by Dr. Henry Yokoyama, Agricultural Research Service, Pasadena, CA, U.S.A.), nicotine, imidazol and α-picoline were added directly to the melted medium.

Determination of carotenoids. The Me_2CO extract of lyophilized mycelia was vacuum dried, redissolved in petrol (40–60°), and added to a 2-cm-wide column containing a 1-cm layer of Al_2O_3 [grade II-III containing 3% (v/w) H_2O]. Addition of 12 ml petrol eluted the phytoene; subsequent addition of 10 ml Et_2O eluted the intermediate carotenes; final addition of 16 ml of a soln of 50 g KOH and 50 ml H_2O/l 96% EtOH eluted the neurosporaxanthin. This fraction was mixed with 8 ml 2M HCl and washed with petrol until the H_2O soln had become colourless. Final fractions were vacuum dried and dissolved in *n*-hexane for the spectrophotometric determination of phytoene (maximal E (1 mg l^{-1}, 1 cm) = 91.5 [22]), intermediate carotenes (average maximal E = 250) and neurosporaxanthin (maximal E = 171.5 [2]). To detect lycopene, the Et_2O fraction was added to a similar 10-cm long column and eluted with increasing concns of Et_2O in petrol.

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