Gibberellins and Carotenoids in the Wild Type and Mutants of *Gibberella fujikuroi*

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A new screening procedure was used to isolate 14 *gib* mutants of *Gibberella fujikuroi* with modifications in the production of gibberellins. The production of carotenoids and gibberellins was investigated in the *gib* mutants and in representative *car* mutants with various modifications of carotenoid biosynthesis. The determinations of gibberellins were carried out with a simplified fluorescence method. One of the mutants lacked both gibberellins and carotenoids. In many mutants the two pathways compensated each other: an increase in the production of one group of compounds was accompanied by a decrease in the production of the other. Under certain conditions the compensation was quantitative when the output of the two pathways was measured in moles of the common precursor, geranylgeranyl pyrophosphate. α-Picoline, an inhibitor of lycopene cyclase in *G. fujikuroi*, inhibits gibberellin biosynthesis. Other agents that affect the accumulation of carotenoids have no noticeable effect on the accumulation of gibberellins; such is the case with diphenylamine and β-ionone, two inhibitors of phytoene dehydrogenation, and visible light, which stimulates carotenogenesis.

The gibberellins are a group of natural plant hormones with various effects on growth and morphogenesis (13) and practical applications in agriculture and brewing. The gibberellins are industrially purified from the culture media of some strains of the ascomycete *Gibberella fujikuroi* (6, 12), a rice pathogen whose imperfect stage is known as *Fusarium moniliforme*.

Terpenoids are a large family of natural compounds that includes the gibberellins, the carotenoids, which are widespread pigments made by plants and by many fungi and bacteria, and the sterols, which are universal components of cell membranes. Gibberellins and carotenoids are made from a common 20-carbon precursor, geranylgeranyl pyrophosphate (11). The mycelia of *G. fujikuroi* contain the orange carotenoid neurosporaxanthin and its precursors, such as torulene, γ-carotene, and phytoene. The carotenoid concentration depends on the culture conditions, particularly the medium, illumination, and temperature (3, 4). The production of carotenoids is inhibited by the addition of diphenylamine, α-picoline, or β-ionone (2).

Many color mutants of *G. fujikuroi* show alterations of carotenoid biosynthesis (1). The deep-orange mutants have much more neurosporaxanthin than does the wild type. Albino mutants lack carotenoids or accumulate the colorless phytoene. Red mutants accumulate torulene. Pale-orange mutants are similar to the wild type in the light and accumulate appreciable amounts of carotenoids in the dark (3).

Industrial researchers have established the conditions for gibberellin production and have looked for high-yielding strains. Very little is known about the genetics of *G. fujikuroi*, and only a few mutants with specific alterations in gibberellin biosynthesis have been described (5).

We developed simplified methods that allowed us to isolate mutants defective in gibberellin production. Analyses of these mutants, the wild type, and mutants with altered carotenoid production illustrate the relationships between the gibberellin and carotenoid pathways.

MATERIALS AND METHODS

Strains and general procedures. The strains used in this work are listed in Tables 1 and 2. Unless otherwise stated, cultures were grown at 30°C in an orbital shaker (120 rpm) in the dark in 500-ml Erlenmeyer flasks with 250 ml of liquid 10% ICI medium (10), a medium for gibberellin production with 0.48 g of NH_4NO_3 per liter. Where indicated, the glass top of the incubator allowed exposure of the culture flasks to white light (fluence rate, 2 W m^{-2}) from five fluorescent lamps (Sylvania F40T121D). α-Picoline was dissolved in distilled water and added to the medium at a final concentration of 2 g liter^{-1} either immediately before inoculation or after 9 days of growth. Diphenylamine was dissolved in 96% ethanol and added to 9-day-old cultures at final concentrations of 20.3 mg of diphenylamine and 0.6 ml of ethanol per liter. β-Ionone was emulsified in a mixture of ethanol and polyoxyethylenesorbitan monooleate (Tween-80) and added to 9-day-old cultures at final concentrations of 0.2 g of β-ionone, 2 ml of ethanol, and 1 ml of Tween-80 per liter.

The glucose concentrations in the medium were determined with a glucose analyzer (model 27; Yellow Springs Instruments, Yellow Springs, Ohio) or with glucose oxidase (9).

Carotenoid analyses were carried out as described previously (2). The term "intermediate carotenes" designates the sum of colored carotenes; it does not include neurosporaxanthin. For the comparison of carotenoid and gibberellin production, it was assumed that 1 mol of the precursor geranylgeraniol yields 267 g of phytoene, 265 g of intermediate carotenes, or 250 g of neurosporaxanthin (half of a mole in each case) or 345 g of gibberellins (a mole of gibberellic acid).

Fluorimetric determination of gibberellins. Fluorescent derivatives of gibberellins were obtained by shaking together 0.2 ml of culture medium, 0.2 ml of ethanol (96%, vol/vol), and 2 ml of a cooled mixture of equal volumes of sulfuric acid and 96% ethanol and incubating the reaction mixture at 48°C for 30 min. Fluorescence spectra were obtained with an LS-5 Perkin-Elmer fluorimeter. The fluorescence emissions at 464 nm of samples excited at 406 nm were compared with that of authentic gibberellic acid (Sigma Chemical Co., St.

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TABLE 1. Production of gibberellins and carotenoids in the wild-type IMI58289 and in *gib* mutants[a]

Strain	Gibberellins (mg liter^{-1})		Carotenoids (μg g of dry wt^{-1})		
	6 days	12 days	Phytoene	Intermediate carotenes	Neurosporaxanthin
IMI58289	22	79	1	6	3
SG121	3	10	0	11	7
SG122	7	27	2	19	12
SG123	1	15	0	9	3
SG124	2	25	0	5	3
SG127	5	15	0	0	0
SG128	6	32	0	8	4
SG129	7	23	0	3	2
SG133	15	49	0	2	1
SG134	10	57	0	6	1
SG135	4	5	1	18	6
SG136	4	8	2	22	15
SG137	0	0	0	6	1
SG138	0	0	2	19	16
SG139	0	1	2	14	9

[a] All cultures were incubated at 30°C in the dark. Cultures used for gibberellin analyses were grown in liquid medium. Those used for carotenoid analyses were grown on minimal agar for 4 days.

TABLE 2. Gibberellin production in the wild-type IMI58289 and in *car* mutants

Strain	Color, dark/light (main carotenoid)[a]	Gibberellins (%)[b]	Growth (%)[c]
IMI58289	Albino/Orange (NX)	100	100
Mutants derived from IMI58289			
SG2	Albino (none)	117	97
SG4	Albino (none)	118	99
SG53	Albino (none)	112	97
SG54	Albino (none)	97	92
SG43	Albino (phytoene)	108	98
SG1	Deep orange (NX)	1	99
SG19	Deep orange (NX)	55	94
SG22	Deep orange (NX)	66	97
SG36	Deep orange (NX)	54	95
SG37	Orange (NX)	44	95
SG39	Deep orange (NX)	42	98
SG20	Pale orange/orange (NX)	62	107
SG48	Pale orange/orange (NX)	51	99
Mutants derived from SG22			
SG75	Albino (none)	45	90
SG76	Albino (none)	106	97
SG78	Albino (high phytoene)	70	98
SG68	Deep red (torulene)	68	100
SG71	Orange (NX)	55	98
SG72	Pale orange (NX)	110	97
SG73	Very deep orange (NX)	46	96

[a] According to reference 3. Many strains have the same color after growth in the light or in the dark. NX, neurosporaxanthin.

[b] Gibberellins present in the culture medium after 12 days of incubation relative to those of the wild-type controls run in parallel. The wild type produced 76 ± 4 mg of gibberellins per liter (mean and its standard error in 18 experiments).

[c] Glucose consumed after 12 days of incubation, relative to those of the wild-type controls run in parallel. The medium initially contained 80 g glucose per liter, and the wild type consumed 31 ± 1 g (mean ± standard error for 17 experiments). The wild type reached 7.0 ± 0.2 g (dry weight) per liter (10 experiments); the mutants averaged 6.6 ± 0.2 g (36 experiments).

Louis, Mo.) that had been dissolved in 0.2 ml of ethanol and mixed with fresh medium and ethanolic sulfuric acid as described above. The determinations of standard and problem samples were run in parallel. The values given are the averages of at least two determinations.

Rapid screening for gibberellin production. The colonies to be screened were transferred individually to vials with 1 ml of liquid medium and incubated for 9 days at 30°C. A 0.5-ml sample of culture medium was slowly added to a tube with 0.25 ml of sulfuric acid and observed under a "black-light" lamp (Sylvania F18W/BLB/ES). The presence of gibberellic acid in the sample led to a blue-greenish fluorescence.

Mutagenesis. A water suspension of IMI58289 spores was exposed for 1 h at 22°C to 0.2 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml (1), washed, and plated on minimal agar with 1 g of yeast extract per liter. The resulting colonies were inoculated with toothpicks onto minimal agar, incubated for 3 days at 30°C, and then transferred to liquid medium for the rapid screening procedure. The mycelia whose culture media showed reduced or altered fluorescence were transferred to fresh minimal agar and allowed to sporulate for a repetition of the screening procedure.

RESULTS

Simplified method for gibberellin analysis. The available methods for the analysis of gibberellin content are too cumbersome for the quick screening of many samples. We developed a simplified method based on the fluorescence of the reaction products of gibberellins and sulfuric acid. An aliquot of the *Gibberella* culture medium is incubated with an ethanol solution of sulfuric acid. The light emission at 464 nm is determined in a spectrofluorimeter after excitation at 406 nm and compared with that of a pure gibberellic acid standard (Fig. 1).

The emission and excitation fluorescence spectra observed after application of this method to the culture media of the wild-type IMI58289 and the deep-orange mutant SG22 coincided with that of pure gibberellic acid. The results of this method were in good agreement with those obtained

after extraction and separation of the gibberellins by high-performance liquid chromatography (8).

Isolation of mutants with altered gibberellin biosynthesis. An even simpler screening procedure was designed for the rapid identification of strains with possible alterations in the production of gibberellins. This method was applied to 4,100 colonies grown from spores that had been exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 34 gave reduced or altered fluorescence in three successive screenings. A difference from the wild type was maintained by 14 isolates after application of the simplified analytical method of the previous section. These 14 isolates are listed in Table 1. The three-letter code *gib* was chosen for the mutations responsible for modifications in gibberellin biosynthesis; the new strains carry the alleles *gib-1* through *gib-14*.

Carotenoid content of the *gib* mutants. All of the *gib* mutants except SG127 accumulated carotenoids in their mycelia at 30°C in the dark (Table 2). The biosynthesis of carotenoids was photoinducible in these mutants and in the wild type: at 22°C they accumulated 45 to 111 μg of carotenoids per g of dry weight in the light but less than 12 μg/g in the dark. Strain SG127 was devoid of carotenoids under all conditions tested. These carotenoid analyses were carried out with mycelia grown on minimal DG agar (1); these mycelia produced no gibberellins. No correlation was seen
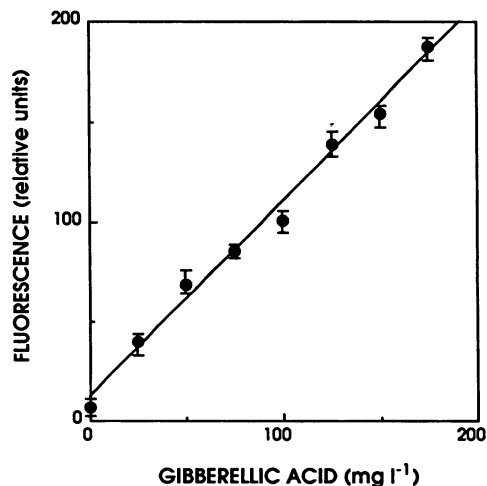
FIG. 1. Fluorescence (emission at 464 nm after excitation at 406 nm; means ± standard errors for two independent determinations) after application of the simplified analytical method to standard solutions of gibberellic acid.

between the production of carotenoids and that of gibberellins by mycelia grown in liquid medium (Fig. 2).

The mutants SG133 and SG134 produce a mixture of gibberellins that is different from that produced by the wild type (7). The other 12 *gib* mutants produce less gibberellin than does the wild type; the substrates thus saved were not diverted into carotenoid production (Fig. 2).

Gibberellin production in mutants defective for carotenogenesis. The wild type can mutate (3) to increased carotenogenesis (orange, deep-orange, and pale-orange mutants in the dark), to decreased carotenogenesis (albino mutants
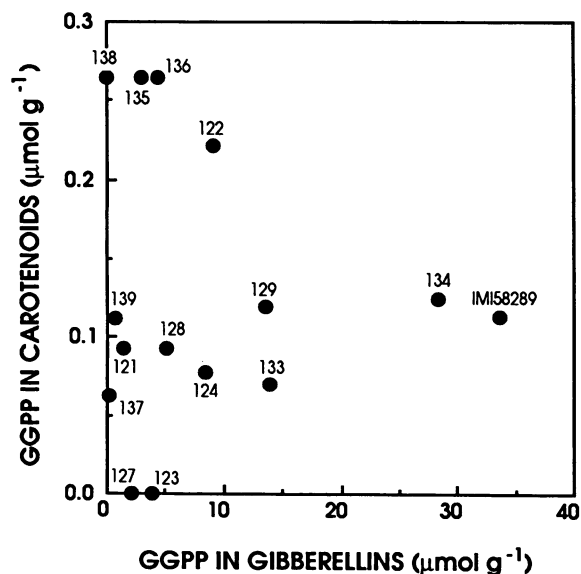
FIG. 2. Gibberellin and carotenoid production by the *gib* mutants after 15 days of incubation at 30°C in liquid medium. To facilitate the comparison, the contents of both groups of terpenoids are expressed in terms of the amount (moles per gram of mycelial dry weight) of the required common precursor, geranylgeranyl pyrophosphate.
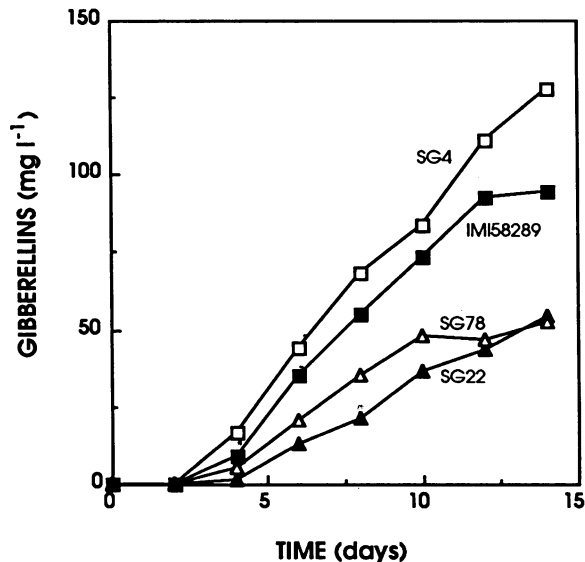

FIG. 3. Gibberellin production by strains IMI58289 (wild type), SG4 (no carotenoids), SG22 (superproducer of neurosporaxanthin), and SG78 (superproducer of phytoene).

without carotenoids), or to the same amount of a different carotenoid (phytoene). The *car* mutants differ in the production of gibberellins (Table 2 and Fig. 3). All of the increases in carotenoid production were accompanied by decreases in gibberellin production. Three of four mutants that lacked carotenoids seemed to produce slightly larger amounts of gibberellins than did the wild type. The phytoene-accumulating mutant SG43 produced about the same amount of gibberellin as did the wild type. The culture medium of one of the deep-orange mutants, SG1, was practically devoid of gibberellins but accumulated a red pigment with an unknown chemical nature.

Mutants with new color changes were derived (3) from one of the deep-orange strains, SG22. In some cases the gibberellin production remained low, but it returned to the wild-type level in two mutants with decreased carotenoid production.

The variations in gibberellin production cannot be attributed to differences in growth, as shown by the analysis of the remaining glucose (Table 1).

The very large variations in the content of gibberellins and carotenoids in different strains approximately compensate each other. In the strains given in Fig. 4, the variation coefficients are 109% for the carotenoid contents and only 12% for the sum of both groups of terpenoids.

Effect on gibberellin production of agents that affect carotenogenesis. Light had no effect on the production of gibberellins. This is shown in Fig. 5 for the wild type under conditions that normally allow or repress gibberellin production and for strain SG4, a white mutant unable to make carotenoids either in the light or in the dark. Under the same conditions there was no photoinduction of carotenogenesis: after 15 days in liquid medium in the light or in the dark, the wild-type mycelia contained a total of 24 to 29 μg of carotenoids per g of dry weight.

The wild type does not lend itself to the study of inhibitors of carotenogenesis because of its low carotenoid content. The deep-orange strain SG22 had been used previously in its place (3). α-Picoline inhibits lycopene cyclization and delays
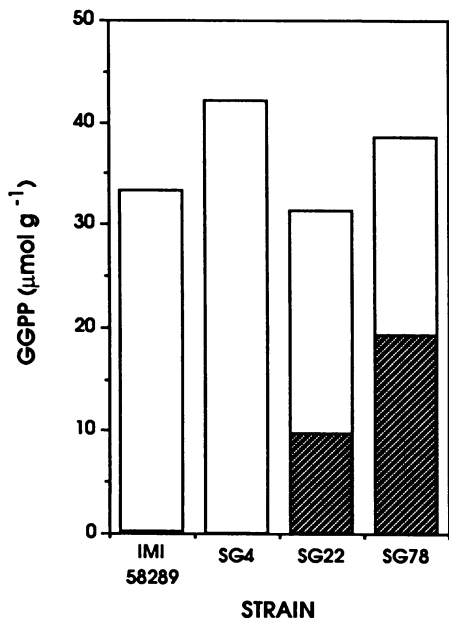
FIG. 4. Production of gibberellins (▢) and carotenoids (▨) by wild-type IMI58289 and some carotenoid mutants after 15 days of incubation at 30°C in liquid medium. The contents of both groups of terpenoids are expressed in terms of the amount (moles per gram of mycelial dry weight) of the required common precursor, geranylgeranyl pyrophosphate.

growth in *G. fujikuroi*. α-Picoline completely blocked gibberellin production when added at a concentration of 2 g liter^{-1} either before the inoculation of the cultures (Fig. 6) or after gibberellin production had started (Fig. 7).

Diphenylamine (20.3 mg liter^{-1}) and β-ionone (200 mg liter^{-1}) inhibited growth when added before inoculation of the cultures. When they were added to 9-day-old cultures, there was a partial inhibition of gibberellin accu-
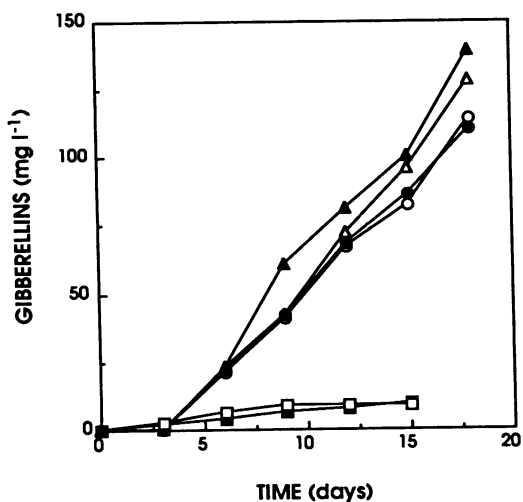

FIG. 5. Effect of light on gibberellin production. Symbols: ○, ▢, and △, cultures grown in the light; ●, ■, and ▲, cultures grown in the dark; △ and ▲, strain SG4 grown in liquid medium; ○ and ●, wild-type IMI58289 grown in liquid medium; ▢ and ■, wild type grown in liquid medium with 4.8 g of NH₄NO₃ per liter.
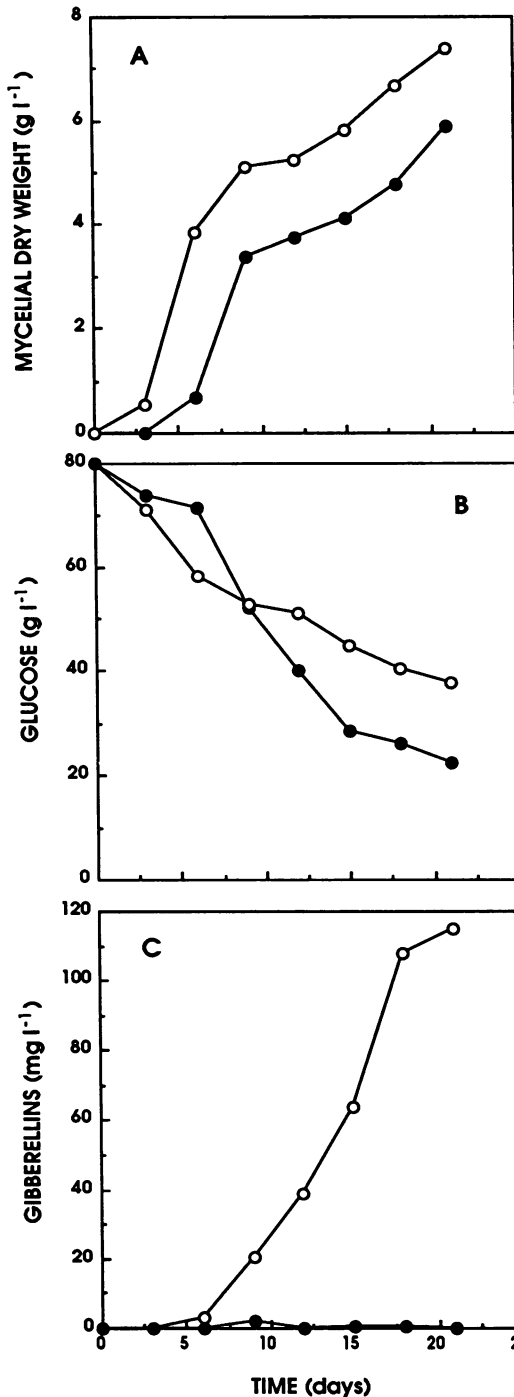

FIG. 6. Effect of α-picoline on the mycelial dry weight (A) of strain SG22 and on the glucose (B) and gibberellin (C) contents of the medium. Symbols: ●, α-picoline (2 g liter^{-1}) added to the medium before inoculation; ○, cultures without α-picoline.

mulation (Fig. 7), which was probably nonspecific because it was accompanied by a similar inhibition of growth. The conserved fluorescence spectra indicated that these chemicals produced no gross changes in the mixture of gibberellins.
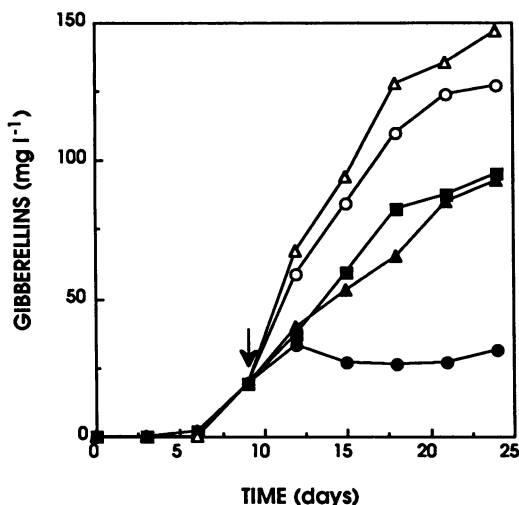
FIG. 7. Effect on gibberellin production by SG22 mycelia of various chemicals added to 9-day-old cultures (arrow). Symbols: ○, cultures without chemical additives; △, emulsifiers ethanol and Tween-80; ●, α-picoline; ■, diphenylamine; ▲, β-ionone.

DISCUSSION

New methods. The isolation of the gibberellin mutants and the other results in this paper would hardly have been possible without the development of two new methods. The fluorescence of the reaction products of gibberellins with sulfuric acid is the foundation of traditional analytical methods (14). Our procedure avoids the extraction and purification steps with their concomitant losses of gibberellins. The procedure might give false-positive reactions with chemicals other than the gibberellins. That this is not the case for our *Gibberella* strains is shown by the negligible fluorescence observed with the wild type in the presence of α-picoline and with mutants devoid of gibberellins. Our rapid screening procedure may be used not only in the search for mutants but in other experiments as a rapid first test to identify the samples that deserve precise analysis.

Relationship between the biosyntheses of gibberellins and carotenoids. Strain SG127 produced negligible amounts of both terpenoids. A single mutation could block the transfer of a prenyl residue to farnesyl pyrophosphate to produce geranylgeranyl pyrophosphate or shut off a possible common regulation of both pathways. The mutational incapacity to produce farnesyl pyrophosphate should be lethal for lack of sterols.

The increased carotenogenesis observed in the deep-orange mutants is quantitatively compensated by a decreased production of gibberellins. The same seems to be the case, in the opposite direction, for the albino mutants devoid of carotenoids. These mutations do not vary the supply of precursors but divert them to the gibberellin or the carotenoid branch. The diversion may occur at the level of geranylgeranyl pyrophosphate or any previous precursor. The albino mutants may be unable to synthesize phytoene from geranylgeranyl pyrophosphate.

The compensation rule does not apply to other mutations, which seem to affect only one of the pathways. This is the case particularly with mutations that block the production of gibberellins. A diversion of the gibberellin precursors into the carotenoid pathway would result in impressive pigmentation levels (about 10 mg g of dry weight^{-1}).

White illumination does not affect the production of gibberellin and carotenoids in our liquid cultures. The light intensity used is not very high but is enough to bring about considerable photoinduction of carotenogenesis on agar cultures.

α-Picoline blocks the biosyntheses of both neurosporaxanthin (2) and gibberellins in *Gibberella*. It does not prevent the production of the common precursor geranylgeranyl pyrophosphate, since neurosporaxanthin is replaced by lycopene. Thus, α-picoline inhibits the formation of the β ring of the carotenoids and is likely to inhibit the cyclizations in the gibberellin pathway.

Both pathways include dehydrogenation steps. Diphenylamine and β-ionone inhibit the dehydrogenations from phytoene to torulene but do not seem to have any specific effect on gibberellin biosynthesis.

The three chemical inhibitors stimulate the consumption of glucose but inhibit growth. They appear to uncouple the catabolism of glucose from its incorporation into fungal biomass.

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