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Gibberella fujikuroi Mutants Obtained with UV Radiation and *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine

J. ÁVALOS, J. CASADESÚS,† AND E. CERDÁ-OLMEDO*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

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***N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) and to a lesser extent UV radiation are very mutagenic for *Gibberella* microconidia. The recommended nitrosoguanidine doses lead to much higher frequencies of mutants than are found in other microorganisms. The frequency of mutants among the survivors increases linearly with the nitrosoguanidine dose (molar concentration × time); the absolute number of viable mutants in a given population reaches a maximum for a dose of ca. 0.7 M · s. The microconidia are uninucleate. The onset of germination brings about increased lethality of nitrosoguanidine, but it does not modify the action of UV radiation. Mycelia are more resistant than spores to both agents. Visible illumination effectively prevents lethality when given immediately after UV irradiation. Auxotrophs and color mutants are very easily obtained. Pink adenine auxotrophs and several classes of color mutants are affected in the biosynthesis of the carotenoid pigment, neurosporaxanthin.**

The industrial interest in the fungus *Gibberella fujikuroi* (as an imperfect fungus, known also as *Fusarium moniliforme*) stems from its abundant production of gibberellins, plant hormones widely used in horticulture and brewing (15). The organism may also have a future in the industrial production of protein and of carotenoids.

Morphological and biochemical mutants of *G. fujikuroi* have been isolated after treatment with different chemical and physical mutagens (1, 10, 11, 18, 24). Not surprisingly, these efforts have been concentrated largely on mutants affecting gibberellin production.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) has been extensively used for the induction of mutations in many organisms (12). The effects of the drug, including the frequency of mutants and their distribution over the genome, vary from one organism to another. In yeasts (8), as in bacteria (5), the mutations are preferentially induced in DNA regions being replicated at the time of mutagen exposure. In bacteria (13), but not in yeasts (2), this results in relatively high rates of closely linked double mutants.

Detailed studies of the application of nitrosoguanidine to the fungi *Saccharomyces cerevisiae* and *Phycomyces* spp. have recently been published by Calderón and Cerdá-Olmedo (2) and by Roncero et al. (21), respectively. We investigated the effects of nitrosoguanidine on *G. fujikuroi* and compared them with those of UV radiation.

MATERIALS AND METHODS

Strain, media, and culture conditions. *G. fujikuroi* IMI58289 was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England.

Minimal medium contained (per liter) glucose, 30 g; $NaNO_3$, 3 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; HBO_4, 10 μg; $CuSO_4$, 100 μg; $FeCl_3$, 200 μg; $MnCl_2$, 20 μg; MoO_4Na, 20 μg; and $ZnSO_4$, 2 mg. Nutrient broth was prepared by adding 4 g of yeast extract (Difco Laboratories, Detroit, Mich.) and 8 g of peptone (Bacto-Tryptone; Difco) to the minimal medium. Solid medium contained additionally 16 g of agar (Analema, Vorquimica, Vigo, Spain). Cultures were incubated at 30°C in the dark. To harvest microconidia, cultures were grown on a special, carbon-limited medium containing (per liter) yeast extract (Difco), 1 g; NH_4NO_3, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and agar, 16 g. The plates were grown for 5 days at 30°C 10 cm away from a battery of 40-W fluorescent lamps (Sylvania Lifeline Daylight F40 V/D), yielding ca. 1.25 W of white light m^{-2}. Microconidia were harvested by washing the sporulated mycelia with sterile distilled water and cleaned by passage through filter paper and low-speed centrifugation. Conidial suspensions were stored at 4°C and titrated by microscopic observation with a Petroff-Hauser chamber. Viable counts were derived from colony-forming ability on nutrient agar. For nuclear staining, mithramycin (a kind gift of Pfizer, Inc., Groton, Conn.) was dissolved at 10 μg ml^{-1} in a mixture of equal volumes of dimethyl sulfoxide and water.

Nitrosoguanidine mutagenesis. Nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in distilled water at 1 mg ml^{-1}, kept frozen until use, and never refrozen. The safety rules proposed by Ehrenberg and Wachtmeister (9) were adopted.

Freshly collected suspensions of ca. 10^6 conidia per ml of distilled water were exposed to the drug. Several doses of nitrosoguanidine were used. We define the dose as the product of the molar concentration of the drug and the exposure time in seconds. Any dose can therefore be obtained by using different combinations of concentration and exposure. Treatments were stopped by diluting and washing.

UV mutagenesis. Freshly collected microconidia were inoculated onto nutrient-agar plates, placed 49 cm away from a Sylvania G15T8 lamp (UV flux, 0.35 W m^{-2}), and then incubated in the dark. For photoreactivation, UV exposure was followed by a 5-h incubation in the light, as described above, before incubation in the dark.

Characterization of mutants. Growth requirements of auxotrophic mutants were determined following the method of Holliday (14). Carotenoids were extracted with acetone from lyophilized, weighed, and ground mycelial samples and determined with a Bausch & Lomb Spectronic 2000 recording spectrophotometer.

* Corresponding author.
† Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112.

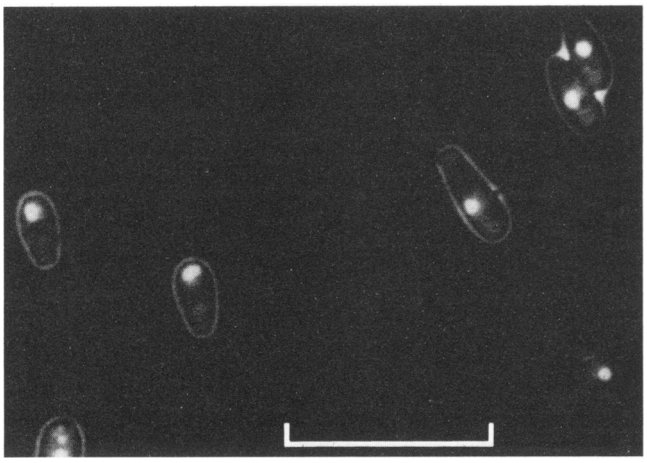

FIG. 1. Spores of *G. fujikuroi* IMI58289 stained with mithramycin and photographed with a fluoroscence microscope. Bar, 10 μm.

RESULTS

Microconidia. Our *Gibberella* strain grows vigorously but conidiates poorly under usual laboratory conditions. Less than 10^3 conidia per plate were found after 5 days of growth in the dark or on nutrient medium in the light. Similar cultures grown on minimal agar in the light produced ca. 2.4 $\times$ 10^4 conidia per plate. Abundant spores (5.1 $\times$ 10^6 per plate) were produced in cultures grown for 5 days on an especially poor agar (conidiation agar) under the same bright illumination.

The spores are microconidia, that is, unicellular, ellipsoidal structures, 4 to 6 μm long and 2 to 3 μm wide, each containing a single nucleus (Fig. 1).

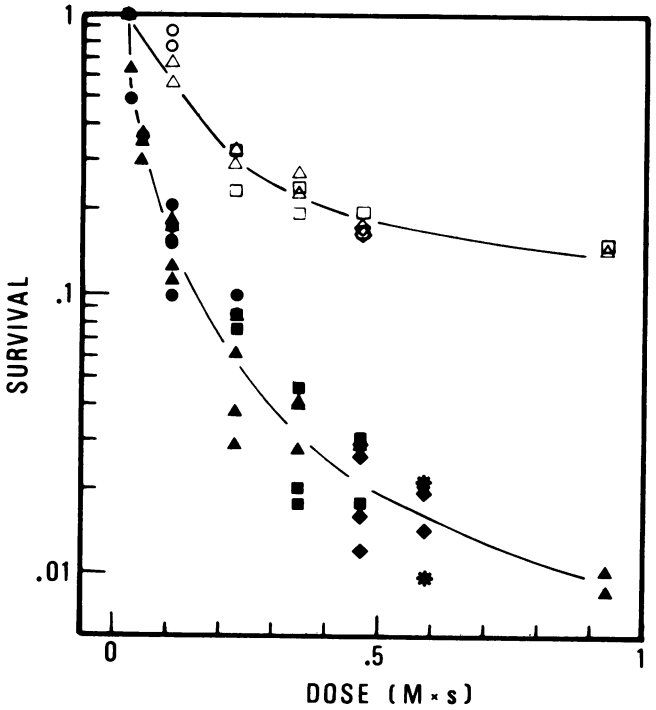

FIG. 2. Survival of *Gibberella* spores exposed to nitrosoguanidine. Open symbols, resting spores (kept in water); closed symbols, spores preincubated for 2 h in nutrient broth. The doses indicated in the abscissa were obtained by exposures of 15 (triangles), 30 (squares), 45 (circles), 60 (diamonds), or 75 (stars) min to the appropriate nitrosoguanidine concentrations.

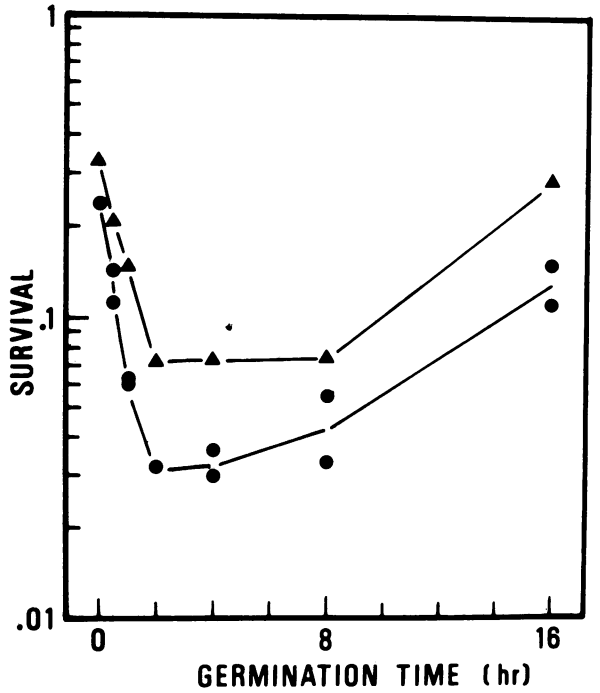

FIG. 3. Effect of germination on the survival of *Gibberella* spores mutagenized with nitrosoguanidine. The spores were preincubated for various periods in nutrient broth and then exposed to nitrosoguanidine at 0.234 M · s (100 μg ml^{-1} for 30 min; triangles) or 0.351 M · s (100 μg ml^{-1} for 45 min; circles).

Nitrosoguanidine lethality and mutagenesis. The conidia are quite sensitive to the lethal effects of nitrosoguanidine. Survival and mutagenesis depended only on the dose of nitrosoguanidine and not on the particular concentrations and times used in each experiment (Fig. 2). Nitrosoguanidine lethality was enhanced by preincubation of the microconidia in nutrient broth, i.e., by the onset of germination (Fig. 2 and 3). The highest sensitivity was reached after a 2-h preincubation, before the appearance of the first gross morphological changes and was maintained through the protrusion of germ tubes. After 6 h of preincubation, 80% of the spores exhibited germ tubes; after 12 h, 99% exhibited germ tubes.

Mutagenesis, as judged by the proportion of auxotrophs among the survivors, increased linearly with the dose. The number of viable mutants induced by nitrosoguanidine in a given initial cell population reached a maximum at a dose of about 0.07 M · s (Fig. 4). Resting spores suffered fewer deaths and mutations than preincubated spores, if the comparison was made at the same dose. If both kinds of spores were killed to the same survival level, they showed the same mutation frequencies (Table 1).

UV lethality and mutagenesis. The *Gibberella* spores were readily killed by UV irradiation at a wavelength of 254 nm. The lethal effect of UV irradiation was reversed by immediate exposure to white light (Fig. 5). The lethality approximately followed first order kinetics with a small shoulder.

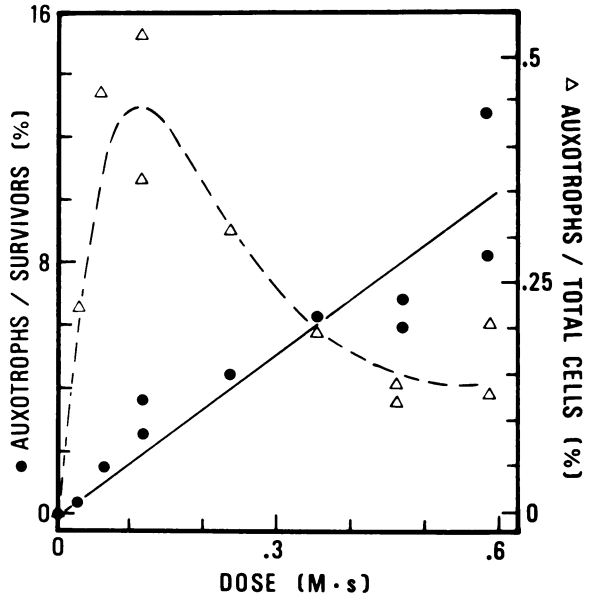

FIG. 4. Percentage of auxotrophs among the survivors of nitrosoguanidine exposure (●). The spores were preincubated for 2 h in nutrient broth, exposed to nitrosoguanidine, and plated on nutrient agar; the resulting colonies were tested for auxotrophy. The same data are replotted as the percentage of viable auxotrophs relative to the total number of cells, living or dead (△).

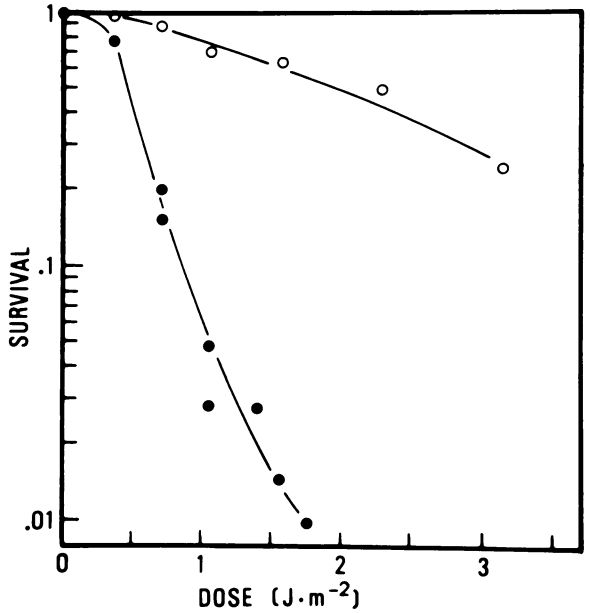

FIG. 5. Killing of *Gibberella* spores by UV radiation and photoreactivation by visible light. After exposure to the dose given in the abscissa, samples were exposed (○) or not (●) to visible light and then incubated in the dark.

UV lethality was not affected by germination up to the massive production of germ tubes; mycelia were more resistant to UV irradiation than were conidia (Fig. 6).

UV exposure was mutagenic, but less so than was nitrosoguanidine treatment, leading to comparable survival levels (Table 1).

***Gibberella* mutants.** The survivors of nitrosoguanidine treatment were not always identical to each other and to the wild type. Colony size, surface texture, color, and overall morphology were often aberrant. The same was true, to a lesser extent, for the survivors of UV irradiation.

Nitrosoguanidine-induced auxotrophs showed a wide variety of nutritional requirements: we identified seven arginine, five methionine, four leucine, three lysine, two biotine, two nicotinic acid, one tryptophan, and one phenylalanine auxotroph.

We studied two pink mutants, which contained the same carotenoids as the wild type and an additional water-soluble pink pigment. These mutants were auxotrophic for adenine. The synthesis of the pink pigment was repressed by adenine: mycelia grown with 20 mg of adenine liter^{-1} in the dark were deeply colored; with 200 mg liter^{-1}, rather faded; and with 2 g liter^{-1}, white. All of these features are typical of some adenine auxotrophs in other fungi, such as the mutants of

TABLE 1. Mutagenic effects of nitrosoguanidine and UV radiation on *G. fujikuroi*[a]

Mutagen	Dose	Preincubation in nutrient broth (h)	Survival (%)	Auxotrophs among the survivors (%)
Nitroso-guanidine	0.5 M · s	0	15	2.5
	0.1 M · s	2	14	3.1
	0.05 M · s	2	33	1.4
UV radiation	0.7 J m^{-2}	0	20	0.8

[a] A total of 1,040 colonies were tested for auxotrophy in each case. The auxotroph frequency for UV radiation is significantly lower than those for nitrosoguanidine, as judged by the *t* test after the arcsin transformation (23). For example, if the mutagenicities are assumed to be equal, the probability of a difference as large as or larger than that between the lower two lines is $P < 0.04$.

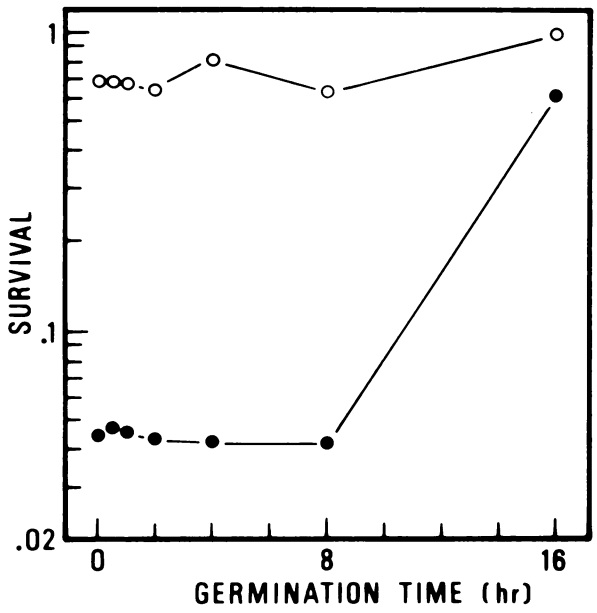

FIG. 6. Effect of germination on the survival of *Gibberella* spores exposed to UV radiation. The spores were preincubated in nutrient broth for the period given in the abscissa, exposed to 1 J m^{-2} UV radiation, exposed (○) or not (●) to visible light, and incubated in the dark.

genes *ad1* and *ad2* in *S. cerevisiae* (22) and *ade1* and *ade2* in *Candida albicans* (19).

Other mutants were purple when grown in either the light or the dark. They were phototrophic, and their carotenoid content coincided with that of the wild type. The nature of the pigment remains unknown.

The rest of our color mutants differed from the wild type in neurosporaxanthin content. The wild type was orange when grown in the light, containing 100 to 150 μg of neurosporaxanthin g^{-1} (dry weight) (ppm). In the dark, the synthesis of neurosporaxanthin was repressed, and the mycelium was white (less than 10 ppm of neurosporaxanthin). The carotenoid mutants can be classified into three phenotypic classes. One of them is made up of mutants with little or no pigment in either the light or the dark. Another class is indistinguishable from the wild type in the light, but is orange in the dark, due to accumulation of 30 to 100 ppm of neurosporaxanthin. The remaining carotenoid mutants are deep orange when grown in either the light or the dark, and their carotenoid content increases linearly with time, up to ca. 2,000 ppm of neurosporaxanthin.

DISCUSSION

G. fujikuroi is a highly suitable organism for the induction and isolation of mutants. The uninucleate microconidia readily allow the expression of recessive mutations. To appreciate the convenience of *Gibberella* microconidia, consider the difficulties encountered in the isolation of mutants in multinucleate cells, such as *Phycomyces* spores (21). Not all strains of *G. fujikuroi* are equally favorable for these studies, because many strains produce multicellular macroconidia (16).

Nitrosoguanidine and UV radiation kill *Gibberella* microconidia about as effectively as they kill the microorganisms most often used in genetics. The onset of germination increases the sensitivity of microconidia to nitrosoguanidine. In a similar way, actively replicating bacteria and yeast and germinating *Phycomyces* spores are more readily killed by nitrosoguanidine than the corresponding resting cells (3, 4, 21). Increased cell permeability and changes in nuclear structure and function are presumably responsible for this difference. Permeability changes may be the most important, since UV radiation makes no distinction between resting and germinating conidia. The mycelia, after the protrusion of the germ tubes, are much more resistant to both lethal agents than are spores. *G. fujikuroi* shows a most active photoreactivation of UV damage, comparable to that of *Phycomyces* (6) and *Saccharomyces* spp. (17).

As with other organisms, nitrosoguanidine is a more effective mutagen than UV radiation for *G. fujikuroi*. The auxotroph frequencies reported here are among the highest reported for any organisms. Auxotroph frequencies depend to a certain extent on subjective assessment, depending on the criteria for inclusion of leaky mutants. Even so, our frequencies are much higher than those usual for *Phycomyces* spp. (less than 0.1%) or yeast cells (ca. 1%) under similar conditions.

The recommended nitrosoguanidine dose for practical applications depends on the nature of the desired mutants. For rare, nonselectable mutants, the dose should be high, to improve the chances of success at the risk of accumulating multiple mutations in the same genome. For selectable mutants, particularly those intended for physiological studies, the dose should be as low as possible, to avoid multiple mutations. The status of *Gibberella* genetics does not yet allow a direct test for whether a phenotype is due to one or more mutations.

Nitrosoguanidine mutagenesis in *Escherichia coli* quickly reaches saturation: further exposure increases lethality but not the frequency of mutants (4). In *G. fujikuroi*, as in *Phycomyces* spp. (21), the frequency of mutants increases linearly with the dose. This suggests a fundamental difference in the way nitrosoguanidine acts in the two fungi and the bacterium.

The white and deep-colored *Gibberella* mutants resemble similar mutants in *Phycomyces* spp. (7). Most attractive are the mutants that synthesize neurosporaxathin in the dark but that are similar to the wild type in the light. These novel mutants should contribute to the studies of photoinduction of carotenoids, quite developed in a related fungus, *Fusarium aquaeductuum* (20).

Our results encourage the search for all kinds of *Gibberella* mutants, including those of potential industrial interest.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Bearder, J. R., J. MacMillan, C. M. Wels, M. B. Chaffey, and B. O. Phinney.** 1974. Position of the metabolic block for gibberellin biosnthesis in mutant B1-41a of *Gibberella fujikuroi*. Phytochemistry **13**:911–917.
2. **Calderón, I. L., and E. Cerdá-Olmedo.** 1982. Simultaneous induction of multiple mutations by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in the yeast *Saccharomyces cerevisiae*. Curr. Genet. **6**:237–243.
3. **Calderón, I. L., and E. Cerdá-Olmedo.** 1983. Induction by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine of nuclear and cytoplasmic mutations in *Sacharomyces cerevisiae*. Mutat. Res. **108**:133–146.
4. **Cerdá-Olmedo, E., and P. C. Hanawalt.** 1968. The replication of the *Escherichia coli* chromosome studied by sequential nitrosoguanidine mutagenesis. Cold Spring Harbor Symp. Quant. Biol. **33**:599–607.
5. **Certá-Olmedo, E., P. C. Hanawalt, and N. Guerola.** 1968. Mutagenesis of the replication point by nitrosoguanidine: map and pattern of replication of the *Escherichia coli* chromosome. J. Mol. Biol. **33**:705–719.
6. **Cerdá-Olmedo, E., and P. Reau.** 1970. Genetic classification of the lethal effects of various agents of heterokaryotic spores of *Phycomyces*. Mutat. Res. **9**:369–384.
7. **Cerdá-Olmedo, and E. S. Torres-Martinez.** 1979. Genetics and regulation of carotene biosynthesis. Pure Appl. Chem. **51**:631–637.
8. **Dawes, I. W., and L. A. Carter.** 1974. Nitrosoguanidine mutagenesis during nuclear and mitochondrial gene replication. Nature (London) **250**:709–712.
9. **Ehremberg, L., and C. A. Wachtmeister.** 1977. Safety precautions in work with mutagenic and carcinogenic chemicals, p. 401–410. *In* B. J. Kilbey, M. S. Legator, W. Nichols, and C. Ramel (ed.), Handbook of mutagenicity test procedures. Elsevier/North-Holland Biomedical Press, Amsterdam.
10. **Erokhina, L. I.** 1969. Certain characteristics of *Fusarium moniliforme* mutants. Genetika **5**:143–147.
11. **Erokhina L. I., and B. D. Efremov.** 1970. Biochemical mutants of *Fusarium moniliforme* (Sheld.). Genetika **6**:170–172.
12. **Gichner, T., and J. Veleminsky.** 1982. Genetic effects on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and its homologs. Mutat. Res. **99**:129–242.
13. **Guerola, N., J. L. Ingraham, and E. Cerdá-Olmedo.** 1971. Induction of closely linked multiple mutations by nitrosoguan-

idine. Nature (London) New Biol. **230**:122–125.
14. **Holliday, R.** 1956. A new method for the identification of biochemical mutants of micro-organisms. Nature (London) **178**:987.
15. **Jeffreys, E. G.** 1973. The gibberellin fermentation. Adv. Appl. Microbiol. **13**:283–315.
16. **Kuhlman, E. G.** 1983. Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section Liseola. Mycologia **74**:759–768.
17. **Parry, J. M., and B. S. Cox.** 1968. The effects of dark holding and photoreactivation on ultraviolet light-induced mitotic recombination and survival in yeast. Genet. Res. **12**:187–198.
18. **Phinney, B. O., and C. Spector.** 1967. Genetics and gibberellin production in the fungus *Gibberella fujikuroi*. Ann. N.Y. Acad. Sci. **144**:204–210.
19. **Poulter, R. T. M., and E. H. A. Rikkerink.** 1983. Genetic analysis of red, adenine-requiring mutants of *Candida albicans*. J. Bacteriol. **156**:1066–1077.
20. **Rau, W.** 1980. Blue light-induced carotenoid biosynthesis in microorganisms, p. 283–298. *In* H. Senger (ed.), The blue syndrome. Springer-Verlag, Berlin.
21. **Roncero, M. I. G., C. Zabala, and E. Cerdá-Olmedo.** 1983. Mutagenesis in multinucleate cells: the effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on *Phycomyces* spores. Mutat. Res. **125**:195–204.
22. **Silver, J. M., and N. R. Eaton.** 1969. Functional blocks of the *ad1* and *ad2* mutants of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **34**:301–305.
23. **Sokal, R. R., and F. J. Rohlf.** 1969. Biometry. W. H. Freeman Publications, San Franscisco.
24. **Spector, C., and B. O. Phinney.** 1966. Gibberellin production: genetic control in the fungus *Gibberella fujikuroi*. Science **153**:1397–1398.

ERRATUM

Gibberella fujikuroi Mutants Obtained with UV Radiation and *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine

J. ÁVALOS, J. CASADESÚS, AND E. CERDÁ-ÓLMEDO

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

Volume 49, no. 1, p. 187, abstract, line 5: ". . . reaches a maximum for a dose of ca. 0.7 M · s" should read ". . . reaches a maximum for a dose of ca. 0.1 M · s."

Page 187, abstract, line 9: "Pink adenine auxotrophs and several classes of color mutants are affected . . ." should read "Some adenine auxotrophs are pink, and several classes of color mutants are affected. . . ."

Page 187, column 2, lines 2–6: An ingredient should be added to the description of the carbon-limited medium: "glucose, 1 g."