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Genetic Effects of Griseofulvin on Plant Cell Cultures

F. Lo Schiavo, V. Nuti Ronchi and M. Terzi
Laboratorio di Mutagenesi e Differenziamento CNR, Pisa (Italy)

Summary Griseofulvin induces metaphase arrest and polyploidization in plant cells in culture; after which a process of chromosome segregation follows. During this process spontaneous or induced recessive mutations become expressed through the formation of homozygotes or monosomics. This finding can be of use whenever the isolation of recessive mutations is needed and haploid culture is difficult.

Key words: Chromosome Segregation – Recessive Mutation – 8-Azaguanine resistance – Metaphase arrest

Introduction

Griseofulvin (GF), a fungicidal compound produced by various species of *Penicillium* causes abnormalities in nuclear division in both animal and plant cells (general reference: Léonard et al. 1979). Larizza et al. (1974) described, in populations of human cells treated with the drug, an increased frequency of polykaryocytes and polyploids. This was followed by chromosomal segregation, reducing the spread of chromosome numbers.

Léonard et al. (1979) investigated the mutagenicity of the drug and found no effect on *Salmonella typhimurium* (Ames test) and on the induction of structural chromosome aberrations.

However, as previous experiments conducted in this laboratory (Simi and Terzi 1977; Colella et al. 1979) had shown an increase in the frequency of recessive mutations induced by GF (and other spindle poisons) on animal cells, apparently due to chromosomal variation and not to a change in the base sequence in DNA, we thought it would be interesting to investigate the problem of the genetic and cytogenetic effects induced by griseofulvin more deeply in a suitable cell system.

The results show that GF, weakly- or non-mutagenic in itself, can greatly increase the number of mutants recovered after treatment with a known alkylating agent, Ethyl methane sulfonate (EMS).

Materials and Methods

Chemicals

Griseofulvin was bought from EGA-CHEMIE KG and dissolved at 1,5 mg/ml in N-N'-dimethyl formamide; Ethyl metane sulfonate was obtained from Eastman and 6-benzyl-amino-purine (6-BAP) from Sigma. 2,4-dichlorophenossiacetic acid sodium salt (2,4-D) was a gift from Rumianca S.p.A.

Plant Materials and Growth Conditions

A two-year suspension culture of *Medicago sativa* L. was obtained from Dr. F. Sala, Laboratorio di Genetica Biochimica ed Evoluzionistica, CNR, Pavia (see Nielsen et al. 1979). *Medicago sativa* L. cells were grown in liquid or agarized MS medium (Murashige and Skoog 1962) containing 0.5 mg/l of 2,4-D and 0.25 mg/l of 6-BAP. GF was added at a concentration of 150 μg/ml to a culture at its third day; it was removed at the indicated times by centrifugation at 100xg for 10 min., resuspending the pelleted cells in fresh medium without GF. For chromosome counts, cell samples were treated for 3 hours with colchicine (0.025%) fixed in Carnoy (ethanol acetic acid 3:1 v/v) and prepared as Feulgen-squashes. For DNA cytophotometry, cell samples were fixed in Formalin 4%, treated for 15 min. at room temperature with a mixture of pectinase and cellulase 5% and then squashed and hydrolysed in 5N/HC1 at room temperature for 1 hour, stained with Feulgen reagent, washed with three changes of SO_2 water (10 minutes each) and made permanent with Eukitt. The DNA contents of nuclei and mitoses were measured with the Barr and Stroud integrating microdensitometer, type GM2, and transformed tc C values by taking as standard the DNA content of *Medicago sativa* diploid methaphases (4x = 32). Mutagenesis with EMS (0.5% v/v) was done for two hours on cells grown for two days, filtered and resuspended in fresh medium. Mutants resistant to 8-azaguanine were selected for in Petri dishes containing 3 layers of solid medium. The bottom one was a feeder layer of 10^6 untreated cells, the intermediate layer contained 8-azaguanine at a concentration such

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that all layers eventually reached 3×10^{-5} M; to the top layer the mutagenized cells were added at a cell density of $0.8\text{-}1.4 \times 10^5$ ml^{-1}. The cells to be plated had been left to recover for at least 5 days after mutagenic treatment.

Results

Cytogenetic Effects

In order to find the best condition for treatment we characterized a few parameters of the cell culture such as fresh weight, dry weight, number of cells and mitotic index. These are reported in Fig. 1. It should be stressed that all these parameters refer to the cell population and not to the single cell: in other words, one doubling of the cell culture does not necessarily mean that all cells divided once but, more likely, that only some of the cells divided more than once.

From this preliminary characterization we decided to add GF at the third day of culture and to leave it for six days (i.e. covering approx. 2 doublings). Microscopically the addition of GF gave evident effects within a few hours and consisted of arrested metaphases with contracted chromosomes scattered through the cytoplasm.

The resulting accumulation of mitoses gave a mitotic index of 8.4% after two days of treatment whereas the control culture never showed a mitotic index higher than 3%. The mitotic index of the treated cells consisted mainly of metaphases (7.9%), the anaphases being almost absent. The most striking effect was evident during the recovery in medium without the drug. Most of the mitoses, after separation of the chromatids, showed the total complement of chromosomes segregating into irregular groups (Fig. 2) which resulted as nuclei of varying sizes within the original cell (Fig. 3). Cell cleavage could follow, giving small mononucleate cells. In other cases, during the subsequent mitoses, the chromosomes fused together to give a restitution polyploid nucleus. Highly polyploid cells and cells with reduced chromosome number were the results of such an effect. Arrested metaphases and irregular anaphases were still visible in the culture after 4-6 days of recovery, showing a persistence of the drug action (Fig. 4).

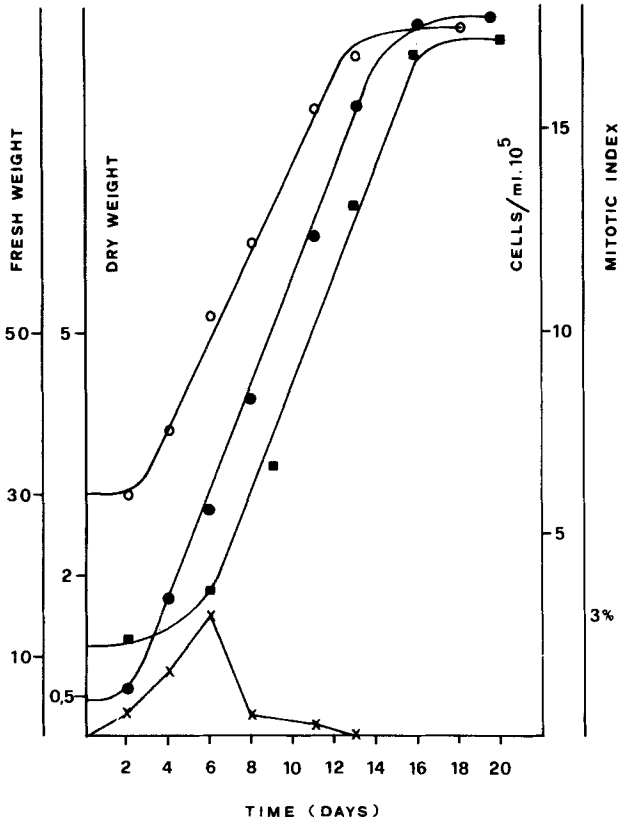

Fig. 1. Variation in weight and cell number shown by *M. sativa* cultures with time. The variation in mitotic index is also reported ○ – ○ fresh weight; ● – ● dry weight; ■ ■ cell concentration (ml^{-1}); X – X mitotic index

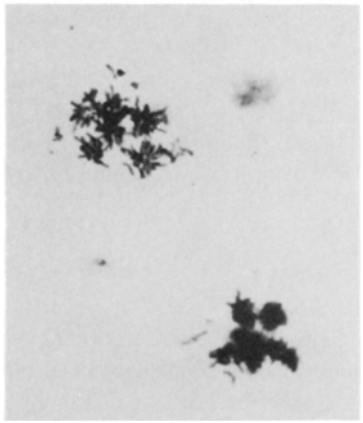

Fig. 2. Two examples of multipolar mitosis

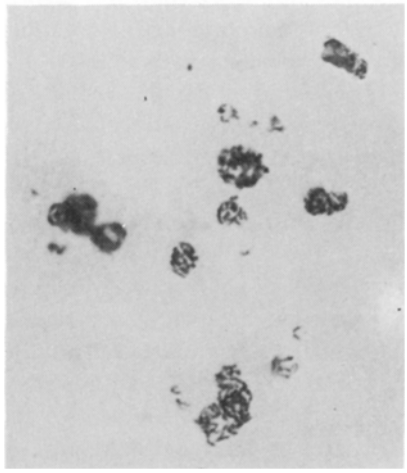

Fig. 3. Multinucleate cells (See text)

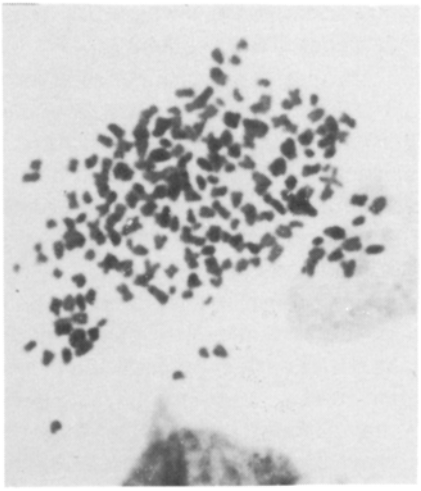

Fig. 4. highly polyploid metaphase

Figure 5 shows the effect of GF on the distribution of the chromosomal numbers in *M. sativa* control and treated cells. Control cells were mainly diploid (4x = 32) with only 10% tetrapoloids. A small percentage showed higher ploidy. In treated cultures, the GF induction of restitution nuclei correlated well with increased frequency of polyploid cells. Small mononucleate cells showed a widening in the distribution of chromosomal numbers ranging from 10 to 64 with small peaks at the haploid (16) and diploid (32) values. With progress of time the distribution of chromosomal numbers narrowed again, diploid and tetraploid numbers comprising, two months after treatment, more than 60% of mitoses (data not shown).

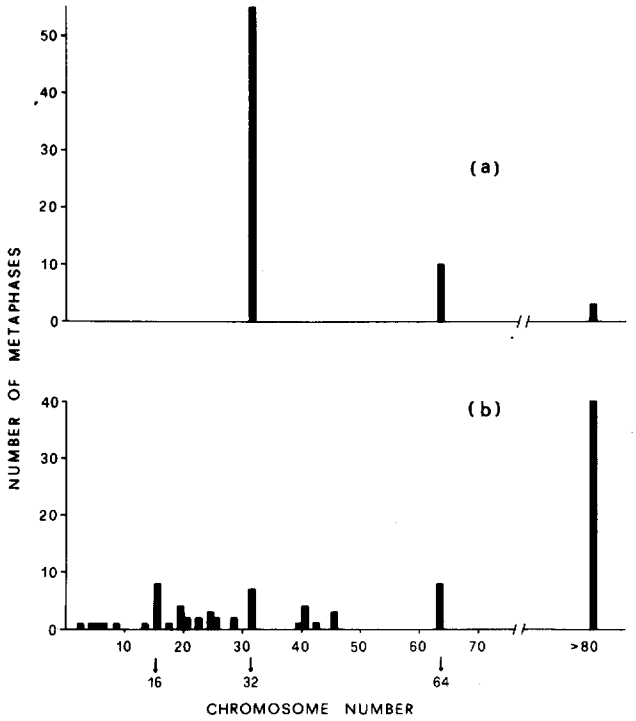

Fig. 5a and b. Frequency distribution of chromosomal numbers a in untreated control, b in cultures treated with GF for 5 days after 5 days of recovery

Microdensitometric Analysis

The same cell population, treated with GF, was submitted to microdensitometric analysis and it showed (Fig. 6) a large scattering of DNA content per nucleus of treated cell.

The resolving power of microdensitometry, although sufficient to give an estimate of the mixoploidy of the total population, is not enough to measure with accuracy variation at low DNA contents – and evaluate the rate of chromosomal segregation. However, with this technique, the entire population, not only the fraction entering mitosis, was analyzed.

Mutagenesis

In order to get quantitative results, one would like to have the cells completely disaggregated or at least in groups of

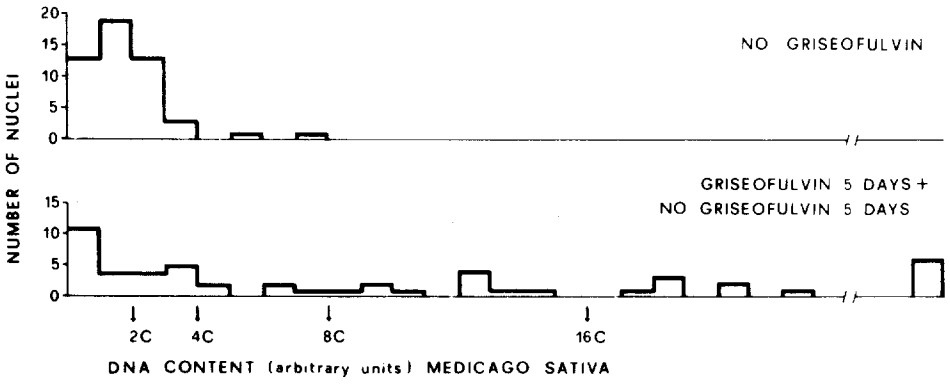

Fig. 6. Microdensitometry of *M. sativa* nuclei: top untreated, bottom treated with GF

uniform size. As this is not always the case, we decided to filter our cell culture at the second day through a tissue with pore size 250 μm.

This way the ratio of total number of cells/number of cell clusters was brought to approximately 2.5 (2.3-2.8) and, moreover, large clusters were eliminated. On this filtered population, treatment with EMS was performed according to the Methods section. The efficiency of plating (i.e. number of clusters giving rise to colonies in agar) was 0.12 immediately after mutagenesis compared with 0.3, that of the untreated control.

With GF the numbers of colonies was not calculated immediately after treatment because 9 days of recovery were allowed. During these 9 days the cell culture underwent two doublings, showing that the lethality induced by the treatment was not very extensive. This conclusion was supported by the fact that virtually all cells arrested in metaphase were able of completing their cycle when the drug was removed.

Therefore, we thought that the frequency of mutants should be referred to the number of treated cells, thus assuming no lethality; we also assumed no differences in the growth constant of the mutants as compared to the wild type during the recovery period before plating in selective conditions.

Having made it clear what are the limits to the quantitative analysis of the mutagenic effectiveness, we can now consider Table 1, which gives the results in terms of number of colonies resistant to 8-azaguanine divided by the number of plated cells (corrected for lethality in the case of EMS). The mutagenic increase compared to the control made equal to 1, is reported in brackets.

Discussion

We have shown that griseofulvin added to a proliferating culture of *M. sativa* cells causes arrested metaphases. When the drug is removed abnormal mitoses take place that cause a large spreading of chromosomal numbers in an otherwise rather homogeneous diploid population.

The abnormal constitutions thus generated will also consist of hypodiploid cells, monosomic for one or more chromosomes, and the possibility exists that parasexual cycles (Martin and Sprague 1969) have also taken place: with this mechanism heterozygous cells may have become homozygous. In both cases, recessive mutations that normally would go unnoticed may become expressed.

We have exploited the chromosomal variation caused by griseofulvin to increase the frequency of EMS-induced mutants resistant to the purine analogue 8-azaguanine. Resistance to 8-azaguanine is caused by an enzyme defect and as such has been shown recessive in several eukaryotic systems (For a review see Seegmiller 1976). It is likely that in *M. sativa* we obtained the same recessive mutation as it shows the same sensitivity to the counter-selective agent, aminopterin (data nor presented).

We feel that the magnitude of the synergism shown by the combined GF + EMS treatment is such that it could make this protocol of mutagenesis very useful (in spite of the limitations we discussed on the exact quantitation of the mutagenic effectiveness) and probably instrumental for isolating mutants whenever the haploid culture method is inapplicable. Its applicability is not limited to *M. sativa* cells as some results were obtained in our laboratory on mammalian cells (Colella et al. 1979) and on another plant system (*Nicotiana* spp. unpublished).

Table 1. Synergic effect of EMS and GF on mutant induction

		EMS –		EMS +	
GF –	$\frac{0}{3 \cdot 10^8}$	(–)	$\frac{30}{3.2 \times 10^8}$	(≥ 28)
GF +	$\frac{1}{7.4 \times 10^7}$	(≥ 4)	$\frac{160}{1.2 \times 10^8}$	(≥ 400)

Ratio expressing number of 8-azaguanine resistant colonies vs. plated cells with or without GF and EMS treatment (See text). The numbers in brackets give the magnitude of induced mutagenic effects for the various treatments relative to the untreated control

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Dr. F. Lo Schiavo
Laboratorio di Mutagenesi e
Differenziamento, CNR
Via Svezia, 10-56100 Pisa (Italy)