The Effects of Griseofulvin on Diploid Strains of *Coprinus lagopus*

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SUMMARY

Diploid strains of *Coprinus lagopus* grown on medium supplemented with griseofulvin ($20\ \mu\text{g ml}^{-1}$) produced fast-growing sectors of two types after 7 to 28 days incubation: these were diploid somatic recombinants and haploids. A dikaryon grown on the same medium broke down to give one haploid component only; resolution of this dikaryon and a near-isogenic diploid resulted in selection for sectors carrying a gene for resistance to griseofulvin. The use of this antibiotic is suggested as a tool for routine haploidization in *C. lagopus*.

INTRODUCTION

Stable diploid monokaryons are easily selected from common-*A* heterokaryons of *Coprinus lagopus* (Casselton, 1965). Diploids are stable, and have been used for a variety of physiological and genetical studies (Casselton & Lewis, 1967; Day & Roberts, 1969; North & Lewis, 1973).

Haploidization of diploid strains of several fungi has been used as a routine technique to assign new markers to linkage groups, e.g. in *Aspergillus nidulans* (Pontecorvo & Käfer, 1958) and *Ustilago violacea* (Day & Jones, 1969). Haploidization is a useful procedure even in sexually reproducing fungi because it is often quicker than a sexual cross for the initial localization of a new marker and because, as crossing-over is very rare with haploidization, the association of a new mutation with a linkage group is clear even if it is more than 50 map units away from other known markers on the group.

Coprinus lagopus has eight linkage groups although the chromosome number is probably 12; some groups are well marked while others are very poorly labelled (Lewis & North, 1974). *Coprinus lagopus* diploids are very stable, except in dikaryons, even under selective pressure (Casselton, 1965) and there is no established haploidization technique. *p*-Fluorophenylalanine (PFP) is widely used in fungi to cause haploidization or select its products (Day & Jones 1971), but although *C. lagopus* is sensitive to PFP it has no effect on chromosome numbers in diploids (Casselton, 1965). A suggested mechanism for the effects of PFP on diploid fungi involves its incorporation into spindle proteins causing a higher rate of non-disjunction (Day & Jones, 1971).

Griseofulvin is an anti-fungal antibiotic which interferes with mitosis (Gull & Trinci, 1973, 1974) and causes haploidization and mitotic recombination in *A. nidulans* (Kappas & Georgopolous, 1974). The object of this investigation was to look at the effect of griseofulvin on *C. lagopus* to discover if the drug could be used in routine haploidization.

Table 1. *Genotypes of diploid strains*

Strain	Markers					
	<u>A6</u>	<u>B5</u>	<u>+</u>	<u>+</u>	<u>+</u>	<u>ad-3</u>
ARR3866	A6	B6	nic-4	paba-2	ad-me	+
ARR3149	<u>A6</u>	<u>B5</u>	<u>nic-4</u>	<u>+</u>	<u>paba-2</u>	
	A6	B6	+	ad-6	+	
ARR3158	<u>A6</u>	<u>B5</u>	<u>+</u>	<u>+</u>	<u>ad-3</u>	
	A6	B1	nic-4	paba-2	+	
ARR2425	<u>A6</u>	<u>B6</u>	<u>ad-9</u>	<u>+</u>		
	A6	B5	+	me-5		

Table 2. *Genotypes of haploid strains*

Strain	Markers				
	<u>A6</u>	<u>B5</u>	<u>ad-3</u>		
ARR38	A6	B6	nic-4	paba-2	ad-me
ARR33*	A5	B6	nic-4	paba-2	ad-me
WMR66	A6	B6	nic-4	paba-2	ad-me
UVR25-OI	A6	B6	acu-9		

* Product of a cross involving WMR66.

METHODS

Strains. Diploid strains used in this investigation are listed in Table 1 and were selected by the method of Casselton (1965). The haploid strains used are shown in Table 2. Details of the *C. lagopus* linkage map and the gene symbols used have been described (Lewis & North, 1974).

Media. Minimal medium (MM), supplemented MM and complete medium (CM) were as described previously (Lewis, 1961). The sodium acetate medium was that of Casselton (personal communication) and contained (g l⁻¹ in glass-distilled water): agar, 15; sodium acetate (anhydrous), 10; KH₂PO₄, 1; Na₂HPO₄·12H₂O, 2.25; Na₂SO₄, 0.29; MgSO₄·7H₂O, 0.5; ammonium tartrate, 5; and thiamin, 40 µg. All salts were of analytical grade. The medium was sterilized at 121 °C for 15 min and used fresh.

Griseofulvin. This was kindly given by Dr K. Gull, University of Kent. It was dissolved in acetone and added to the appropriate medium to give a final concentration of 20 µg ml⁻¹. Acetone alone had no effect on *C. lagopus* at the concentration used in the medium.

Culture techniques. These were as described previously by Lewis (1961). Growth was measured as mm radius after 72 h.

RESULTS

Growth of a diploid on griseofulvin

Diploid strain ARR3866 was inoculated on to solid MM supplemented with griseofulvin, nicotinamide, adenine sulphate and *p*-aminobenzoic acid. Radial growth after 72 h was 25% of that of the control without griseofulvin. After 7 days incubation, sectors appeared in the colonies. These were isolated on CM and tested for their ability to grow on MM. Auxotrophic sectors were tested for their growth requirements and *B* mating-type allele; they were then re-inoculated on to supplemented MM plus griseofulvin.

Table 3. Sectors from ARR3866 grown on griseofulvin

	No. of sectors	<i>B</i>	<i>nic-4</i>	<i>paba-2</i>	<i>ad-me</i>	<i>ad-3</i>
Haploid	1	5	+	+	+	—
	9	6	+	—	—	+
	2	5	+	—	—	—
Diploid	1	Both	+	+	+	—
	1	Both	+	—	—	+

Table 4. Oidial size of original strains and their sectors isolated after growth on griseofulvin

Strain	Length ($\mu\text{m} \pm \text{S.E.M.}$)
Original haploids	
ARR38	4.86 ± 0.59
WMR66	5.27 ± 0.51
Sectors from ARR3866	
Haploid (i)	5.13 ± 0.59
Haploid (ii)	4.88 ± 0.89
Diploid	7.42 ± 0.75
Original diploid	
ARR3866	7.08 ± 0.77

Sectors continued to arise from isolated sectors for about 28 days from the start of the treatment. After that time the sectors became stable; some stabilized after only 14 days. The phenotypes and ploidy of the isolated sectors (Table 3) indicate that griseofulvin caused haploidization in *C. lagopus*, and also produced a few diploid sectors. As expected, the linked markers *paba-2* and *ad-me* segregated together; the other markers segregated randomly as expected, but none of the sectors carried the *nic-4* allele.

Confirmation of the ploidy of sectors

It is possible that sectors with unmasked auxotrophic markers are in fact aneuploid, particularly those with two *B* alleles. To test this, the growth and stability of sectors were examined and their oidial size was measured.

Aneuploids in fungi are usually slow-growing and unstable. All the sectors from ARR3866 grew at least as vigorously as the parent diploid and maintained a stable phenotype over several months of subculture on CM. Two sectors, one haploid and one diploid, have been kept in paraffin stock vials for 2 years with no change in stability.

Oidial measurements can be used to distinguish between haploids and diploids (Casselton, 1965). Table 4 shows measurements of oidia from ARR3866, its haploid components, and three sectors derived from it. Oidial shape was regular in all strains. The results indicate that the sectors isolated have the ploidy expected from the number of *B* mating-type alleles they carry. This provides a convenient initial test for sectors as well as giving an automatic marker for linkage group II.

Effects of griseofulvin on dikaryons and haploids

Although the physiological behaviour of diploids and dikaryons in *C. lagopus* is often similar, differences might be expected in their response to non-disjunction. A dikaryon can lose chromosomes from one haploid nucleus resulting in a stable sector of the other nuclear

Table 5. *Inhibition of growth of C. lagopus on griseofulvin*

Growth on medium containing griseofulvin ($20 \mu\text{g ml}^{-1}$) was measured as mm radius after 72 h at 37°C . Each value is an average of several experiments.

Strain	% Inhibition
ARR38	86
ARR33	100
WMR66	100
ARR3866	75
ARR38 $\times$ ARR33	100

Table 6. *Sectors from other diploid strains*

Diploid strain	Ploidy	No. of sectors	<i>B</i>	Markers		
				None (prototroph)		
ARR3158	Haploid	1	5			
	Haploid	2	5	+	<i>paba-2</i>	+
	Haploid	1	1	+	+	<i>ad-3</i>
	Haploid	1	5	+	+	<i>ad-3</i>
	Haploid	1	5	+	<i>paba-2</i>	<i>ad-3</i>
	Diploid	1	both	+	<i>paba-2</i>	+
ARR3149	Haploid	2	6	+	+	<i>ad-6</i>
	Haploid	1	6	+	<i>paba-2</i>	+
	Diploid	1	both	<i>nic-4</i>	+	+
	Diploid	1	both	+	<i>paba-2</i>	+
ARR2425	Haploid	2	5	<i>ad-9</i>	+	
	Haploid	4	6	<i>ad-9</i>	+	
	Haploid	1	5	+	<i>me-5</i>	
	Haploid	1	6	+	<i>me-5</i>	

type, whereas a diploid could undergo free recombination of chromosomes resulting in the variety of haploid types described above. The dikaryon $\text{ARR33} \times \text{ARR38}$ is as near-isogenic as possible to ARR3866. Growth of the dikaryon on griseofulvin was completely inhibited after 72 h; but after a week, sectors with haploid morphology began to arise. The sectors were all auxotrophs of the genotype *A6 B5 ad-3* corresponding to the haploid component ARR38, which has the *nic*⁺ allele common to the sectors from ARR3866.

It can be concluded that while griseofulvin causes near random chromosome loss from diploids, it resolves the corresponding dikaryon by the total suppression of one component. As the *nic-4*⁺ allele is retained by all haploid segregants, it is probably linked to a gene involved in the selection of sectors.

Table 5 shows the results of growth tests of the diploid and dikaryon used and their haploid components on medium containing $20 \mu\text{g}$ griseofulvin ml^{-1} . Clearly, ARR38 is resistant to the antibiotic; this resistance is dominant in the diploid but not the dikaryon, and it is retained in all sectors which were all resistant to griseofulvin.

Linkage analysis of griseofulvin resistance

In order to test the resistant allele for linkage to *nic-4*, the dikaryon $\text{ARR33} \times \text{ARR38}$ was fruited and basidiospores were analysed. They showed a 1:1 segregation of resistance to sensitivity indicating the involvement of a single gene locus, but this locus (*gris-1*) segregated independently of *nic-4*. A second cross of a *gris-1* strain was made with UVR25-01 carrying *acu-9* on group IV; *gris-1* segregated independently of this locus too. As some fungal linkage groups are over 250 map units long, these negative results do not rule out the possibility that *gris-1* is on group IV.

Growth of other diploids on griseofulvin

As the effect of griseofulvin on ARR3866 appears to depend partly on the *gris-r*^r allele it carries, the haploidization technique might not apply generally to *C. lagopus*. To test this, three other diploids that did not contain strains closely related to ARR38 were inoculated on to supplemented MM plus griseofulvin, and sectors were isolated and tested as before. All sectors remained stable for at least 1 month. The diploids used are described in Table 1; the phenotypes of the sectors are shown in Table 6.

Griseofulvin appears to have a similar effect on all the diploids tested, although the proportion of haploid to diploid sectors varied and some strains produced very few. The *ad-9* and *me-5* markers of ARR2425 behaved as expected for linked markers.

Thus griseofulvin is suitable for routine haploidization analysis in *C. lagopus*. Apart from the time taken to synthesize the diploids, the analysis should take about 5 weeks. No 'master strain' is available at present, but two or three diploids can be processed together to give the equivalent result.

DISCUSSION

Polymorphic resistance to griseofulvin

The *C. lagopus* population so far examined is polymorphic for a variety of markers including resistance to growth inhibitors such as ethionine and azetidine-2-carboxylic acid (Lewis & North, 1974); thus the resistance to griseofulvin found in ARR38 is not unexpected. Griseofulvin is a naturally occurring compound so the presence of resistance genes in the gene pool of *C. lagopus* could confer a selective advantage on the species. Although *gris-r*^r is recessive in dikaryons, exposure of a heterozygous dikaryon to the inhibitor causes rapid resolution to a resistant haploid; in a natural population such haploids will occur in all mating-types, allowing the establishment of fully resistant dikaryons. The change in dominance between the diploid and the dikaryon may be an indication of the mechanism of resistance, e.g. the gene might code for a microtubular component synthesized near the nucleus. On the other hand, the dominance effect may be the result of the presence of a modifier gene like that reported by Senathirajah & Lewis (1975).

Mechanism of action of griseofulvin

The haploid sectors arising from ARR3866 were resistant to griseofulvin, like ARR3866 itself and the haploid strain ARR38. It is difficult to distinguish between the effect of griseofulvin as an agent causing loss of chromosomes and its effect as a selective agent for resistant sectors. However, previous attempts to select haploid sectors from *C. lagopus* diploids using recessive suppressors of methionine auxotrophs and recessive resistance to *p*-fluorophenylalanine failed to produce any sectors (Casselton, 1965); it is therefore likely that griseofulvin acts primarily as a causative agent of chromosome loss and only secondarily as an agent selecting the sectors so caused. This has the disadvantage that *gris-r*^s sectors cannot be isolated by the method described above.

Chromosome loss during haploidization in *C. lagopus* is rapid in a few cases, although in most sectors stability is not reached until 4 weeks after the start of treatment; the gradual loss of chromosomes and unmasking of markers resembles the pattern shown during haploidization in *A. nidulans* (Käfer, 1961).

True mitotic crossing-over produces sectors homozygous for all markers distal to the point of chromosome breakage. As *paba-2* is close to its centromere, diploid recombinants homozygous for *paba-2* would be expected rarely; but three out of five diploid sectors from

diploids carrying *paba-2* express the marker. This suggests that the diploid sectors may be the result of a non-disjunction event. Kappas & Georgopolous (1974) suggest that this is the mechanism by which griseofulvin produces diploid sectors in *A. nidulans*.

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