

A MUTATIONAL ANALYSIS OF CONIDIAL DEVELOPMENT IN *ASPERGILLUS NIDULANS*

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INDIVIDUAL morphological mutants have been studied in many higher organisms such as *Drosophila* (HADORN 1955) and the mouse (GRÜNEBERG 1952), but a rigorous mutational analysis of the type employed on many biochemical pathways is readily carried out only in microorganisms. The most remarkable study of this type is the work on bacteriophage T4 (EPSTEIN *et al.* 1963) in which the whole of the development of the virus is open to analysis. In more complex organisms the spectrum of morphological mutants obtainable is very broad (e.g., in *Neurospora*: GARNJOBST and TATUM 1967) and analysis can only be carried to any depth if attention is confined to a process such as sporulation (reviewed in HALVORSON, VARY and STEINBERG 1966), or to mutants affecting an individual enzyme (e.g., BRODY and TATUM 1966).

Aspergillus nidulans, unlike most eukaryotes, lends itself to a similar approach since it can be treated as a microorganism and has a well-studied genetic system (PONTECORVO *et al.* 1953). The conidial apparatus is a particularly suitable subject, as the structure is a well-defined one, and the spores possess a distinctive green pigment so that mutants lacking this can readily be picked up and examined for modification or absence of conidia. Most important, however, is the fact that the conidia are dispensable structures, inessential for normal growth, and indeed, inessential for storage and propagation since the sexual cycle in this homothallic organism provides an alternative source of spores.

The structure of the conidial apparatus of *Aspergillus nidulans* is shown in Figures 1 and 2. Like the hyphae, the conidiophores and vesicles are multinucleate, but the sterigmata and conidia are uninucleate (PONTECORVO *et al.* 1953; CLUTTERBUCK 1969).

Initiation of conidiation in ascomycetes is complex (MORTON 1961; TURIAN 1966), and preliminary studies in *A. nidulans* suggest that there is a balance between conidiation and production of cleistothecia or aerial mycelia. Considering this complexity, it was decided to accept for study only those mutants that had a normal density of conidiophore initials; that is, to confine the study to the processes following conidiophore initiation.

A second limitation imposed on this analysis is the rejection of mutants whose linear growth rate on agar is less than that of the wild type. This is done in order to exclude the many mutants in which there is a defect in their normal metabolism that only has a serious effect during conidiation. Since the subject of this

work is differentiation, mutants defective in their basic hyphal growth are not required.

Working within these limits, it was expected that, by analogy with metabolic pathways or with the analysis of T4 morphogenesis, a number of mutants would be found blocking development at each of a series of stages such as vesicle formation, production of primary sterigmata, secondary sterigmata, etc. However, the results obtained suggest that the activities of only two loci, in addition to those normally functioning in the hyphae, are essential for the conversion from hyphal to conidial morphology. Other loci described modify the course of this development.

MATERIALS AND METHODS

General methods are those of PONTECORVO *et al.* (1953).

Strains: All strains are from the Glasgow stocks of *Aspergillus nidulans*; all mutants were obtained in the *bi-1* (biotin requiring) strain, or in mutants derived from it during this study. Master strains MSE and MSF were used for assigning loci to chromosomes (McCULLY and FORBES 1965). Totally aconidial mutants were stored and manipulated in the form of ascospores.

Strains carrying the new markers described here which are suitable for mapping purposes will be deposited in the Glasgow stocks and at the Fungal Genetics Stock Centre, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755.

Media: Minimal medium (MM—PONTECORVO *et al.* 1953) is Czapek-Dox with 1% glucose and 1.5% agar; complete medium (CM) is a simplified medium, made by the addition to MM of peptone: 2 g/l, hydrolyzed casein: 1.5 g/l, yeast extract: 0.5 g/l, biotin: 2 µg/l, *p*-amino-benzoic acid: 100 µg/l, pyridoxine and aneurin: 500 µg/l, and nicotinamide: 1000 µg/l. Incubation is at 37°C unless otherwise stated.

Mutagens: Four mutagenic treatments were used (Table 1); ultraviolet irradiation as described in PONTECORVO *et al.* (1953), nitrous acid treatment as in SIDDIQI (1962), and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment as in CLUTTERBUCK and SINHA (1966). Diethyl sulphate was used as a mutagen at a strength of 0.05 M in 0.1M phosphate buffer pH7; treatment of conidial suspensions was for 30 min at 37°C, and was stopped by centrifuging down and washing the conidia. This procedure gave approximately 0.5% survival. Treated conidia were plated on complete medium plus sodium desoxycholate (MACKINTOSH and PRITCHARD 1963) to give about 200 colonies per dish, and mutants were picked by visual selection.

Autonomy: Mutants were tested for cell-localized action in heterokaryons. In a heterokaryotic conidial head, nuclei will be segregated at the point of formation of uninucleate sterigmata. Mutants with autonomous action in the sterigmata or conidia will thus give, in heterokaryons with the wild type, heads bearing both mutant and wild-type structures. If the action is non-autonomous, only the structures typical of the dominant component will be seen in mixed heads.

Tests of complementation and dominance: These were carried out in diploids obtained by normal methods (PONTECORVO *et al.* 1953). Where temperature-sensitive mutants were involved, conidia were collected after incubation at 22°C. Alternatively, some diploids were obtained from shaking cultures of heterokaryons in liquid MM, a method first demonstrated by ROBERTS (1964). Since all diploids described had one temperature-sensitive or leaky component and were themselves temperature-sensitive or leaky, they could be recognized even in the case of aconidial mutants by the normal methods—i.e., conidial size and complementation of the auxotrophic markers of the parent strains.

RESULTS

The search for mutants was concentrated mainly on those totally lacking conidia; two such types of mutant were found. In addition, some mutants with interesting alterations of the conidial system were picked up (Table 1). These

TABLE 1

Mutants obtained with different mutagens in Aspergillus nidulans

Type of mutant	Nitrous acid	Diethyl sulphate	Ultraviolet irradiation	N-methyl-N'-nitro-N-nitrosoguanidine
wet-white	<i>wet-2</i> to 5	<i>wet-6</i>
dark	<i>drk-1</i> to 3
stunted	<i>stu-1</i>	<i>stu-2</i>	<i>stu-3</i>
medusa	<i>med-15</i>	<i>med-16</i> to 19	<i>med-24</i> to 30
abacus	<i>aba-1</i> to 3,5,14	<i>aba-6</i> to 12	<i>aba-22,23,46</i> to 49, 51 to 60, 62 to 66	<i>aba-13,20,21</i> , 31 to 45
bristle	<i>brl-6,12</i>	<i>brl-1</i> to 5, 35	<i>brl-7,11</i> to 21	<i>brl-8</i> to 10, 23 to 34, 37 to 41
ivory	<i>ivo-2</i> to 119

This table should not be taken to indicate the relative frequencies of different types of mutant since these differ in conspicuousness, and were not selected at random. In experiments in which only the two types of truly aconidial mutants were picked up, *abacus* mutants were about four times as frequent as *bristle* mutants.

will now be described under the headings: mutants with modified conidia, mutants with disturbed conidiophore or sterigma morphology, aconidial mutants, and mutants affected in their conidiophore pigmentation. The map positions of the loci described are shown in Figure 12.

1. *Mutants with modified conidia*: Mutants at seven loci affecting spore color are in regular use in *Aspergillus nidulans* (DORN 1967). During this study phenotypes corresponding to all these mutants were regularly observed and in addition, two new types were found. Further examination suggests that these mutants are affected in more than their pigmentation.

a. *Wet-white* (*wet*). In these mutants the conidia are colorless, becoming brown, and water droplets accumulate on the tops of the conidial heads. Although the conidia are normal in microscopic appearance when they are formed, they completely autolyze within a few days (Figure 11). *wet-3* is autonomous in heterokaryons and recessive in diploids. *wet-6*, which is closely linked to *wet-3*, is a temperature-sensitive mutant, producing stable green conidia at room temperature, but the wet-white phenotype at 37°C.

b. *Dark* (*drk*). The conidia of this mutant are darker than normal in color. Following some preliminary electron microscope observations (by P. T. P. OLIVER), conidia were mounted in water for light microscope examination when it was seen that in some instances the outer layer of the conidia, instead of enclosing each conidium separately, forms a continuous sac containing a whole chain of conidia. More frequently, this outer layer appears to be completely shed (Figures 9 and 10). It is suggested, therefore, that the change in the appearance of the conidia is due to a structural defect. *drk-1* is recessive in diploids, but the phenotype is not distinctive enough for identification in heterokaryons.

2. *Mutants with modified conidiophores or sterigmata*:

a. *Stunted* (*stu*). In these mutants the whole conidial head is stunted so that many heads fail to get above the surface of the medium at all (Figure 3). The

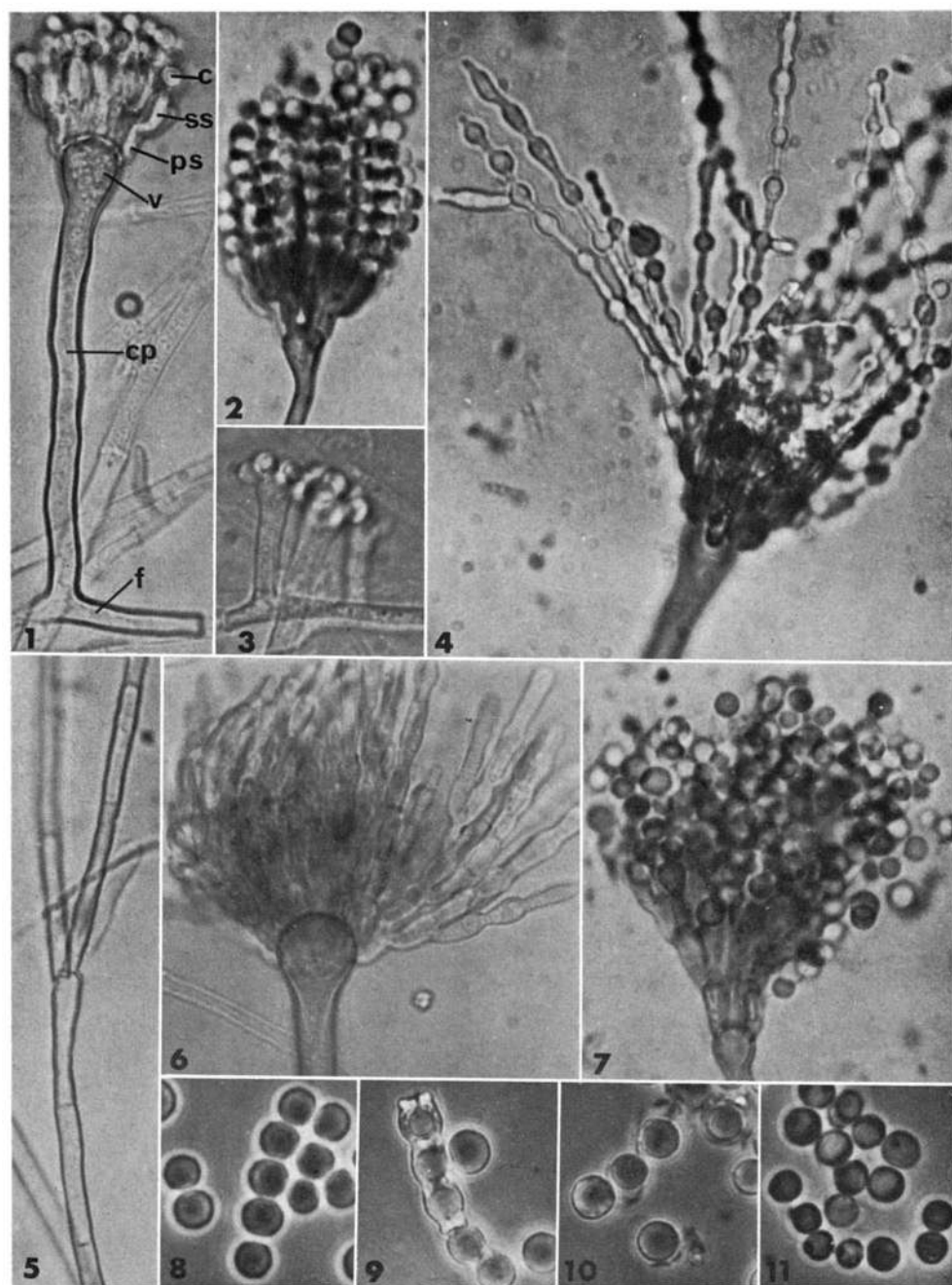


FIGURE 1-11.—Conidial apparatus of normal and mutant strains of *Aspergillus nidulans*. Figure 1.—Wild type. c: conidium, ss: secondary sterigma, ps: primary sterigma, v: vesicle, cp: conidiophore, f: foot. Figure 2.—wild type, small head stained with cotton blue, showing conidial chains. Figure 3.—stunted mutant (*stu-1*). Figure 4.—abacus mutant (*aba-1*). Figure 5.—leaky bristle mutant (*brl-9*). Figure 6.—*brl-9* grown on high salt medium at 22°C. Figure 7.—medusa mutant (*med-15*). Figures 8-11.—conidia; Figure 8.—wild type; Figures 9 and 10.—dark mutant (*drk-1*); Figure 11.—wet-white mutant (*uet-3*), partially autolyzed. Magnifications—Figure 5: $\times 500$, Figures 8-11: $\times 1500$, remaining figures all $\times 1000$.

TABLE 2
Tests of aconidial mutants of Aspergillus nidulans for allelism and dominance

Tester	Mutants tested	Type of test	Result	Conclusions
<i>aba-1</i>	<i>aba-2,3,6</i> to 13,20,21	Recombination in crosses	No wild-type recombinants in more than 100 colonies from ascospores of crossed origin	Mutants closely linked, probably allelic
<i>aba-6</i> (slightly leaky mutant)	<i>aba-3,9,20,21,43</i>	Complementation in diploids	Diploids all have phenotypes similar to <i>aba-6</i>	Mutants allelic
<i>aba-14</i> (temperature-sensitive mutant)	<i>aba-1,2,6,23,32,33,35,36,41,44,46</i> to 49,51 to 53, 55 to 60, 62 to 66	Complementation in diploids	Diploids all have phenotypes similar to <i>aba-14</i>	Mutants allelic and recessive
<i>aba+</i>	<i>aba-1,6,20</i>	Dominance in diploids	Diploids have wild-type phenotype	Mutants recessive
<i>brl-1</i>	<i>brl-3,5</i> to 10,15,16,18, 20,24,26,42	Recombination in crosses	No wild-type recombinants in more than 100 colonies from ascospores of crossed origin	Mutants closely linked, probably allelic
<i>brl-42</i> (temperature-sensitive mutant)	<i>brl-1</i> to 12, 14 to 21, 23, 29-33, 35, 37-41	Complementation in diploids	Diploids show bristle phenotypes at 37°C, conidiate poorly at 22°C.	Mutants allelic and recessive
<i>brl+</i>	<i>brl-1,7,12,15,18,19,42</i>	Dominance in diploids	Diploids have wild-type phenotype	Mutants recessive

carrying *med+*. On the other hand, crosses of eleven independent medusa mutants with *med-15* gave no cleistothecia. This is taken to indicate allelism of these mutants.

3. *Aconidial mutants*:

a. Abacus (aba). Mutants of this type bear rod-like structures with swellings at intervals in place of chains of conidia (Figure 4). These structures may be branched and are grey in color. This color is affected by ivory mutants (see below) but not by mutants affecting conidial pigmentation. The phenotype appears to be very similar to those described as "Cladosarum" in *A. niger* (YUILL and YUILL 1938) and "fuzzy" in *A. fonsaceous* (RAPER and FENNELL 1953). 51 mutants of this type have been isolated in this work (Table 1). Nine of these are leaky or temperature-sensitive to some degree. 40 *abacus* mutants have been tested for allelism by recombination or complementation (Table 2) and all appeared to be allelic, while the 29 mutants tested for dominance are all recessive. The abacus phenotype, however, is autonomous in heterokaryons.

b. Bristle (brl). In these mutants the conidiophore fails to develop a vesicle or any subsequent structures, but continues to grow as a stiff bristle for 2–3 mm—i.e., 20–30 times the normal length. The bristles can be identified as conidiophores rather than sterile aerial hyphae by their thickened walls and foot cells.

All 38 bristle mutants isolated were examined in heterokaryons with *brl*⁺ strains. In all cases the bristle phenotype was autonomous. Even in mixed heads the heterokaryons bore mixtures of bristles formed by the *brl* component and conidia of the *brl*⁺ strain (recognizable *in situ* by spore color markers or by other markers after plating out). No conidia of the *brl* genotype were formed. On the hypothesis that the bristle mutants are defective in vesicle formation, this is an unexpected result since in a heterokaryon the vesicle could be formed by the *brl*⁺ component, after which the *brl* component would be expected to be able to complete the process of conidiation. The simplest explanation of the result actually found is that bristle mutants are also defective in stages after vesicle formation when the two types of nuclei in a heterokaryon have been segregated into the uninucleate sterigma initials.

Partially defective and temperature-sensitive bristle mutants confirm this interpretation. The majority of mutants have only unforked, colorless bristles and are regarded as nonleaky, but the phenotypes of other mutants can be arranged in a series postulated to correspond to increasing *brl*⁺ activity (Table 3). First in the series are mutants with forked bristles (Figure 5); these have only a rudimentary vesicle with a few branches which rebranch after some distance. More leaky mutants have more branches on a well-developed vesicle, and the length before rebranching is reduced until the branches resemble sterigmata (Figure 6). It is this stage which demonstrates that mutants at the bristle locus may be defective in sterigma formation whether or not they are able to form a vesicle. In the stage nearest to wild type, conidia are borne on multiple tiers of sterigmata in a manner similar to *medusa* mutants.

Many mutants in this series are shifted along the scale towards wild type (Figures 5 and 6 and Table 3) by a reduction of the incubation temperature or an increase in the osmolarity of the medium (cf. HAWTHORNE and FRIIS 1964). *brl*-12 is unusual in this respect since it does not show any of the intermediate phenotypic features of the other leaky mutants, but instead, a mixture of nearly

TABLE 3

The phenotypes of bristle mutants of Aspergillus nidulans

<i>Bristle mutant</i>	Medium	Incubation temperature	Forking	Brown pigment	Vesicle	Multi-tiered sterigmata	Conidia
1-5,8, 11, 14-32, 37-41	all media	22-37°C	—	—	—	—	—
6	all media	22-37°C	—	+	—	—	—
10	MM	22-37°C	+	—	—	—	—
	MM + M.KCl	22-37°C	++	—	—	—	—
9,33	MM	37°C	+	+	—	—	—
	MM + M.KCl	37°C	++	+	—	—	—
	MM	22°C	—	+	+	—	—
	MM + M.KCl	22°C	—	+	+	+	—
7,35	MM	22-37°C	—	+	+	—	—
	MM + M.KCl	22-37°C	—	+	+	+	+
42	MM	37°C	—	+	+	+	—
	MM + M.KCl	37°C	—	+	+	+	+
	MM	22°C	—	+	+	—	+++
12	MM	22-37°C	—	—/(+)	—/(+)	—	—/+
	MM + M.KCl	22-37°C	—	—/+	—/+	—	—/+++
wild type	all media	22-37°C	—	+	+	—	+++

normal conidial heads and unmodified bristles. This mutant is believed to be showing a variegated position-effect (CLUTTERBUCK 1968a).

Brown pigmentation of the top of the conidiophore and the sterigmata is shown by the wild type and all mutants (with the exception of *brl-10*) that show any signs of leakiness. The bristles of nonleaky mutants are faint pink or colorless.

36 bristle mutants have been tested for complementation in diploids with *brl-42* (Table 2): all are allelic and recessive.

4. *Mutants affecting conidiophore pigmentation: Ivory (ivo)*. The first mutant of this series (*ivo-1*) arose spontaneously in a strain carrying *aba-38*. The mutant lacks the grey-brown pigmentation of the sterigmata and upper part of the conidiophore of the wild type, which is seen more readily in the abnormal structures produced by *abacus* and leaky *bristle* mutants. As noted in the previous section, all nonleaky *bristle* mutants lack this pigment. For this reason an attempt was made to isolate mutants at the *bristle* locus which lacked the pigment without showing the bristle morphology. 118 colorless mutants were produced by mutagenic treatment of conidia from slightly leaky *abacus* strains and 109 of these were crossed to a *bristle* strain. In no case was the *ivory* mutant closely linked to the *bristle* locus. The mutants, however, fell into at least two classes by linkage and complementation tests, and in addition, a group of five partial mutants all gave yellow-green conidia when outcrossed to remove the *abacus* mutant. These five did not complement in diploids with the spore color mutant *yg-6* (CLUTTER-

BUCK 1968b), and it was found on reexamination of strains carrying *yg-6* or other yellow-green mutations that these also were partially deficient in conidiophore and sterigma pigmentation.

DISCUSSION

The most striking feature of these results is the paucity of mutants completely blocked in conidium formation. Despite the fact that the search was concentrated on such mutants, only the two types, *bristle* and *abacus*, have been found, and in each case, complementation tests have shown that all tested mutants of similar phenotype are at a single locus.

Clearly no search for mutants can claim to be exhaustive. Four mutagens have been used and although there may be quantitative differences in the spectrum of mutants from different treatments, there is no indication that there are qualitative differences such that new mutagens might be expected to give new types of mutant. In this work, no precautions were taken to avoid clones of spontaneous mutants. Such mutants are rare, and in any case it should be impossible to pick up clones of aconidial mutants. Even if this assumption were incorrect, at least 28 of the *abacus* mutants and 18 of the *bristle* mutants must be of independent origin.

Some types of aconidial mutant might have been missed if they either lacked a diffusible material that could be supplied by neighboring colonies, or as a result of failure of conidiation, produced a toxic side product that interfered with hyphal growth.

One feature of the mutants, however, gives strong support for the idea of the economy of loci required for the basic processes in conidiation. This is the complex set of phenotypes given by mutants at the *bristle* locus. It appears that this one locus governs the change from hyphal-type growth, as found in the conidiophore, to the yeast-like growth form of the sterigmata. Such a yeast/hyphal switch is well-known as a dimorphism in fungi (ROMANO 1967) and could well play a part in conidial morphogenesis.

If this is the correct interpretation of the activity of the *bristle* locus, then it must be supposed that the vesicle results from blocking elongation of the conidiophore, whose growth is diverted into forming a swelling. This swelling gives rise on its upper surface to a number of buds in proportion to its surface area. The phenotypes of leaky *bristle* mutants reflect a balance in each mutant between the ability of the *bristle* locus to promote swelling and subsequent budding, and the normal hyphal tendency to elongate.

Once the yeast-like sterigmata are formed, the main change required in the formation of conidia is the introduction of interstitial, rather than apical budding. A secondary sterigma, unlike a primary sterigma, must produce a succession of buds from the same point at its apex—i.e., from the same bud scar. We may postulate that the *abacus* locus is responsible for this change in budding pattern, and that mutants at this locus, unable to make the change can only repeat the production of sterigma-like structures which continue to grow apically.

The economy of loci concerned with development is also illustrated by the two types of mutant: *stunted* and *medusa*, that produce apparently normal conidia on

abnormal conidial apparatus. In both cases, the mutants are also defective in cleistothecium formation, showing that the normal activities of these loci, although not required for vegetative growth, are brought into play in two different situations involving differentiation.

This work, therefore, suggests that few, perhaps only two, gene activities, *brl*⁺ and *aba*⁺, are required to be added to those already active in order to convert conidiophores into conidia. The other loci described add refinements. These genes may be structural genes of some sort, or may act by regulating, in a pattern specific for this developmental situation, the activities of other genes whose functions are not confined to this situation. It is to be assumed that such regulation must be an important feature of development, and it is hoped that some further study of it can be made in this system.

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SUMMARY

A study of the spectrum of mutants affecting the development of the conidial apparatus of *Aspergillus nidulans* suggests that there are very few loci specifically concerned with this development. Mutants affecting only the stages of development after the initiation of the conidiophore have been studied, thus excluding mutants simultaneously defective in hyphal growth and sporulation. Within these limits mutants of six phenotypes have been found. In two of these only the structure of the conidium itself is affected. Two others conidiate, but in an abnormal manner, and are also defective in sexual reproduction. Despite the fact that search was concentrated on aconidial mutants, only two loci which give such mutants have been found. These are designated *abacus* and *bristle* loci: 40 allelic mutants of the former type have been studied, and 37 of the latter. The behavior of *bristle* mutants shows that this locus affects both vesicle and sterigma development and it is suggested that its function may be related to the phenomenon of hyphal/yeast dimorphism. Some mutants involving conidiophore pigmentation are also described.

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