

# A STUDY OF VARIATION IN *NECTRIA STENOSPORA* BERK. & BR.

By ANN GIBSON\* and D. M. GRIFFIN\*

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## Summary

This paper records a preliminary investigation of variation in *Nectria stenospora* Berk. & Br., a species which produces both sexual and asexual spores in culture. The cultural characteristics of the species and methods for isolation of single ascospores and conidia are described. Staining reveals that all cells of the frequently anastomosing mycelium are uninucleate. Two types of variation are described, one being a gene-controlled variation in colony colour, the other a cytoplasmically controlled variation in colony texture. Certain strains are shown to be self sterile, others self fertile although preferentially outbreeding.

## I. INTRODUCTION

Within the last few years the study of variation and genetics in fungi has revealed many features of great interest, such as heterokaryosis (Pontecorvo, Roper, and Forbes 1953; Pontecorvo *et al.* 1953; Buxton 1954), cytoplasmic variation (Jinks 1954; Arlett 1957), and the parasexual cycle (Buxton 1956; Pontecorvo 1956). This paper presents the results of a preliminary investigation of variation in *Nectria stenospora* Berk. & Br. (order Hypocreales) along with other notes on the species. The work has been briefly reported previously (Gibson and Griffin 1958).

## II. MATERIALS AND METHODS

### (a) Vegetative Stage

*N. stenospora* is a common temperate and subtropical saprophyte, the present isolate having been obtained from the bark of a dying *Psoralea* bush at Sydney. The fungus grows on all the usual laboratory media but unless otherwise stated, all experiments were performed on 2 per cent. potato dextrose agar (P.D.A.) at 32°C at which temperature the initial rate of linear extension is c. 10 mm/day (7.5 mm/day at 25°C).

In order to study the assimilative hyphae without the interference of the aerial mycelium, a gently warmed slide was fused onto the surface of the agar so that it overlapped the edge of a vigorously growing colony. The hyphae continued to grow underneath the slide, forming normal assimilative mycelium. When the slide was prised off the agar, it came away bearing the uppermost layer of hyphae and could be passed through the staining process without any change in the orientation of the mycelium. The frequency of anastomosis in the assimilative hyphae was very great, the mature colony being in fact a complex mosaic of interconnected cells rather than an assemblage of radiating hyphae.

\* School of Agriculture, University of Sydney.

These slide cultures, or conidia attached to a slide with albumen fixative, were stained by a modification of Heubschman's (1952) method. The material was fixed in fresh Carnoy's fluid for 30 min, washed, and immersed in 1N HCl at room temperature for about 12 hr, and subsequently stained for at least 30 min in a mixture of 50 ml 2 per cent. aqueous azur 1, 3 ml 10 per cent. NaHSO<sub>4</sub>, and 3 ml 1N HCl. After washing in water, the slides were mounted in glycerine and examined. By this means it was shown that every cell, including the long cell at the tip of each hypha, where the nucleus was always found a little ahead of the last cross wall, contained only one nucleus. The only exceptions were a very few binucleate cells where it seemed reasonable from the position of the nuclei to postulate that mitosis had just occurred. These observations were confirmed by Giemsa staining. *N. stenospora* would appear to be unusual amongst members of the Ascomycetes and the Fungi Imperfecti in having consistently uninucleate cells.

#### (b) Imperfect Stage

On the usual agar media, the fungus typically produces abundant conidiophores and wet phialospores best referred to the genus *Verticillium* Nees ex Wallr. (or to *Acrostalagmus* Corda if these are not considered synonyms). There is considerable variation, however, and some conidiophores resemble those of *Gliocladium* Corda whereas on sterile corncobs the conidia are borne in sporodochia referable to *Dendrodochium* Bon. or *Tubercularia* Tode ex Fr.

Staining with azur 1 revealed that the phialides were uninucleate as were the great majority of conidia, although a few of the largest contained two nuclei. The uninucleate nature of the phialides, however, insured the genetic uniformity of even these binucleate conidia. These observations are in accord with previous ones on fungi producing phialospores.

*Isolation of single conidia.*—Well-separated monoconidial colonies were obtained by streaking a suspension of conidia from slime balls over the surface of P.D.A. contained in a 10-cm petri dish.

#### (c) Perfect Stage

In illuminated petri-dish cultures on P.D.A., certain isolates (see below) produced small, hard, brown structures near the edge of the dish. In cultures 9 or more weeks old, these structures had developed into mature, erumpent, ostiolate perithecia, orange in colour and with thin soft walls. They contained asci and typical two-celled hyaline ascospores, *c.* 10 by 3.5  $\mu$ . In some cases the groups of perithecia were stipitate, being borne on the ends of stalks *c.* 3 mm long.

The earliest stage in perithecial formation observed was the occurrence of extensively coiled, robust hyphae which developed into hard knots of tissue and ultimately into perithecia. In the dark, or on Czapek-Dox agar, development did not proceed beyond the formation of the hard knots. Only coiled perithecial initials were developed under any conditions by infertile strains or crosses. Wheeler (1954) has described the blocking of the sexual process at various stages in *Glomerella cingulata*.

Although cytological studies were not made, it is presumed that, as in other fungi with binucleate ascospores, the two cells of the ascospore contain identical

nuclei, the final mitosis occurring after the delimitation of the spore and being followed by the laying down of the spore septum (Backus and Keitt 1940; Adam, quoted in Pontecorvo *et al.* 1953).

*Isolation of ascospores.*—The eight ascospores are obliquely uniseriate within the ascus until shortly before discharge. At full maturity the ascus becomes clavate and just protrudes through the minute ostiole, the spores now being clustered in a mucilaginous group at the top. The asci within a given perithecium discharge successively but the eight spores from each ascus are shot out in a group to adhere to any surface within a few centimetres. Thus well-separated groups of eight spores can be obtained on a surface held for a short time over a group of mature perithecia.

The small size of the ascospores of *N. stenospora*, combined with the mucilaginous matrix surrounding them, made the normal methods of isolation developed for *Neurospora crassa* unsuitable. The features of the sexual stage described above led to the development of an alternative method. Gelatine was sterilized by fumigation with propylene oxide (Snyder and Hansen 1947) before mixing with sterile water. Perithecia were suspended above plates of the very thick jelly and allowed to discharge at 10°C, the low temperature keeping the jelly firm and retarding spore germination. Groups of eight spores were cut out and the gelatine dissolved from them in warm sterile water, the suspension being spread over a P.D.A. plate. Plates developing eight colonies contained the separate spores from one ascus.

### III. EXPERIMENTAL

#### (a) *Variation in Colour*

Analysis of the ascospores from asci I, II, and III showed that, in each case, there had been a 1 : 1 segregation for "yellow" and "pale" colony colour. Both strains invariably bred true on transfer, either by single conidia or mycelium, through many generations and the difference is assumed to be gene-controlled. The colour difference is most conveniently shown on P.D.A., there being no difference on Czapek-Dox agar unless ammonia is substituted for nitrate as the nitrogen source.

In petri-dish cultures, the colour was best seen on the reverse side, the colour being present in the submerged mycelium and the immediately adjacent agar. The conidia varied from yellow to orange in yellow cultures whereas in pale cultures the reverse side and the conidia were pale yellow to buff. However, the difference between conidial colour of the two types was insufficiently constant and distinct to allow classification on this character alone and cultures were always differentiated on colony colour. The difference was best shown when the cultures were grown in the light, for the pigment then tended to fade and the slight yellow colour of the pale colonies was destroyed, leaving the yellow colonies still quite strongly coloured.

Petri dishes containing agar were inoculated at the centre with mixed suspensions of pale and yellow conidia and the resultant colonies were initially yellow. Frequently, however, straight-sided pale sectors appeared at various stages during the growth of the colony. Such colonies were called "mixed" colonies and it might be considered that in them the two strains had grown independently, although closely intermingled, and that the presence of the pale strain had been masked by the local

diffusion of the yellow pigment from the other strain. Only if a considerable area of pure, or practically pure, pale strain occurred would its presence be revealed by a pale sector. However, the very abundant anastomoses between the hyphae make it impossible to assume that hyphae of different genotypes are physiologically independent of each other and it seems best to consider these mixed colonies as physiologically heterokaryotic. As the individual cells of *N. stenospora* are uninucleate, such a use would involve the widening of the concept of heterokaryosis and this is discussed further below.

TABLE 1  
CHARACTERISTICS OF STRAINS DERIVED FROM ASCOSPORES OF  
ASCUS III

| Ascospore Strain* | Characteristics (see text)             |
|-------------------|----------------------------------------|
| 1, 2              | Self sterile, pale, uniform growth     |
| 3, 4              | Self sterile, yellow, tasselled growth |
| 5, 6              | Self fertile, pale, ragged growth      |
| 7, 8              | Self fertile, yellow, ragged growth    |

\*The numbering of the strains is solely for reference purposes and no order is implied for the parent ascospores.

(b) *Variation in Colony Texture*

As shown in Table 1, the colonies derived from ascus III differed in their texture, being either "uniform", "ragged", or "tasselled". The character referred to as tasselled is not easy to describe, although very obvious visually (Plate 1, Fig. 1). On P.D.A., tasselled areas begin as variously shaped areas of the colony edge with reduced hyphal density and no conidiophores. From this sparse area, tassel- or fan-shaped areas of normal sporulating mycelium arise, grow for about 5 mm, and then in turn cease growth and give rise to a new tassel. The growth rate of such colonies is normal on P.D.A. Uniform and ragged colonies could only be distinguished in old, staled cultures (when ragged colonies had an uneven margin) and in most experiments they have been grouped together as being of normal appearance.

When subcultured by cutting out pieces of the agar and mycelium, all colonies maintained their characteristics unaltered through many transfers. In particular, the ragged areas at the edge of old colonies of strains 5-8 (Table 1) produced normal parental-type colonies on subculturing whereas the tasselled strains 3 and 4 maintained their abnormal growth form. Since only one pair of ascospores had given rise to tasselled strains it was possible that the character was controlled by mutant genes at two loci, e.g. *a* and *b*. Thus the genotype  $a^+b^+$  might be assigned to strains 1 and 2,  $a^+b$  to 5 and 6,  $ab^+$  to 7 and 8,  $ab$  to 3 and 4. The hypothesis was tested in the following way.

(i) *Nature of Mixed Colonies.*—If the above hypothesis were true, it might be expected that the juxtaposition of nuclei bearing  $a^+b$  and  $ab^+$  would result in a

mixed colony of tasselled phenotype due to interactions through the anastomoses. Such colonies were therefore made by mixing conidial suspensions from strains 6 and 7 and plating them out and also by growing strains 6 and 7 adjacent to one another on the same plate so that anastomosis occurred at the junction. By both means tasselled growth was produced, in the first case at various areas throughout the colony after an initial period of normal growth (Plate 1, Fig. 2) and in the second case along a band marking the line of junction. Similar results were obtained by mixing strains 5 and 7 and, unexpectedly, in a few cases, by mixing strains 5 and 6. No tasselling was observed in mixtures of 1 and 6 but mixed colonies derived from strain 3 and any ascus III strain were uniformly tasselled.

The results are in accordance with expectation except for the production of tasselled areas in mixed colonies derived from the spore pair 5 and 6. This anomaly cast doubt on the accuracy of the initial hypothesis.

TABLE 2  
CHARACTER OF MONOCONIDIAL DAUGHTER COLONIES FROM ASCUS III ASCOSPORE STRAINS

| Ascospore Strain | Character of Monoconidial Daughter Colonies |           |               |
|------------------|---------------------------------------------|-----------|---------------|
|                  | Normal                                      | Tasselled | Tasselled (%) |
| 1 (uniform)      | 48                                          | 0         | 0             |
| 1 (uniform)      | 100                                         | 0         | 0             |
| 4 (tasselled)    | 40                                          | 26        | 39.4          |
| 4 (tasselled)    | 126                                         | 63        | 33.3          |
| 6 (ragged)       | 41                                          | 6         | 12.8          |
| 6 (ragged)       | 235                                         | 30        | 11.3          |

(ii) *Analysis of Colonies Developing from Monoconidial Isolates.*—Monoconidial isolates were made from many colonies derived either from single ascospores or from a mixed inoculum. Many monoascospore cultures were shown to produce monoconidial daughter colonies of two types and the results are shown in Tables 2 and 3.

Thus, although all these cultures maintained their characteristics if transferred by mycelium, only uniform strains produced wholly parental-type cultures if transferred by single conidia. Tasselled areas produced only 20–40 per cent. (mean = 31.7) tasselled colonies whereas normal (presumably ragged) areas produced 1–13 per cent. (mean = 9.7) tasselled colonies. The overall ratios for normal: tasselled daughter colonies were 304 : 141 and 409 : 44, for colonies derived from conidia from tasselled and normal (ragged) areas, respectively. These ratios are significantly different at the 0.1 per cent. level of probability ( $\chi^2_1 = 66.2$ ).

(iii) *Proof of Cytoplasmic Inheritance.*—The results given above were strongly indicative that the tasselled character was cytoplasmically inherited and this was

proved by Jinks's (1954) test. Previously this test had only been applied to fungi which are morphologically heterokaryotic but there seems to be no reason why it should not be applied in the present case where, although morphological heterokaryosis was absent, there was ample opportunity for a given nucleus to become associated with the cytoplasm of another strain by virtue of anastomoses. A mixed colony was synthesized from conidia of strains 1 (pale, uniform) and 3 (yellow, tasselled), using colony colour as a nuclear marker. Single conidia from the yellow tasselled mixed colony so obtained were grown and some daughter colonies were both pale and tasselled. The tasselling effect had therefore become dissociated from the yellow-marked nucleus and associated with the pale nucleus. It is, of course, possible that the yellow nuclei had mutated to pale but it seems unlikely that the mutation rate is sufficiently high for this to be a likely explanation.

TABLE 3  
CHARACTER OF MONOCONIDIAL DAUGHTER COLONIES FROM MIXED PARENTAL COLONIES

| Mixed<br>Parental Colony<br>and Area | Character of Monoconidial Daughter Colonies |           |                  |
|--------------------------------------|---------------------------------------------|-----------|------------------|
|                                      | Normal                                      | Tasselled | Tasselled<br>(%) |
| 6 and 7 (normal area)                | 84                                          | 1         | 1.2              |
| 6 and 7 (normal area)                | 49                                          | 7         | 12.5             |
| 6 and 7 (tasselled area)             | 62                                          | 29        | 31.9             |
| 6 and 7 (tasselled area)             | 76                                          | 23        | 23.2             |

(iv) *Selection of Tasselled and Normal Strains.*—Jinks (1954) showed that the abundance of conidia in *Aspergillus nidulans* was controlled cytoplasmically and demonstrated the selection of an asexual strain by consistently subculturing with conidia. In the present experiment, two lines (normal and tasselled) were established from an ascospore 6 colony (ragged) and each line was then propagated for five asexual generations, in each case selecting for the line character. In each generation and line, approximately 50 conidia were taken from a single selected colony and analysed for the percentage of normal and tasselled strains.

It will be noted (Fig. 1) that the percentages in each line remain remarkably constant and after five generations there is no evidence of selection being effective. One conidium, however, from the first transfer gave rise to a normal colony which thereafter never produced tasselled cultures. This conidium had presumably been free of the tasselled factor.

(v) *Effect of Sexual Reproduction.*—From nearly all perithecia produced by strains from ascus III (see below), spores were formed which gave rise to tasselled colonies. One selfed perithecium from strain 6 produced only tasselled colonies from its spores.

(c) *Variation in the Mating System*

Of the few asci analysed, ascus III was again the most interesting. As may be seen from Table 1, strains 1-4 proved to be self sterile, 5-8 to be self fertile. In contrast, no colony derived from a single ascospore from ascus I proved to be self fertile nor was any cross between the colonies fertile. In a previous paper (Gibson and Griffin 1958) the terms "homothallic" and "heterothallic" were used instead of "self fertile" and "self sterile". On the evidence available, however, it would seem preferable to use the latter terms.

Crosses between the pale, self-fertile strain 6 with either the yellow, self-fertile strain 7 or the yellow, self-sterile strain 3 were made. All crosses produced fertile perithecia and one ascus from each of several perithecia were analysed. Of the  $6 \times 7$

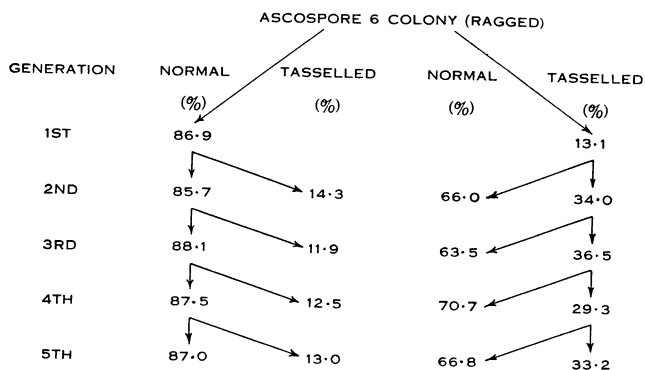


Fig. 1.—Analysis of five generations of monoconidial daughter colonies derived from an ascospore 6 colony.

crosses, three asci showed segregation for pale and yellow, a fourth ascus giving only pale progeny. Of the  $6 \times 3$  crosses, both asci analysed showed segregation for the colour factor. Pure colonies of strain 6 gave only pale progeny from the four asci analysed. Therefore, although the number of asci analysed is admittedly very small, in five cases out of six the self-fertile strain 6 had outcrossed with either a self-fertile or a self-sterile strain. Such an effect has been described by Pontecorvo *et al.* (1953) for *Aspergillus nidulans* and there termed relative heterothallism. Such preferential outbreeding in homothallic fungi has also been described in *Sordaria fimicola* by Olive (1954) and in *Glomerella cingulata* by Wheeler (1954).

## IV. DISCUSSION

The experiments described above suggest that it may be useful to widen the concept of heterokaryosis. The term "heterokaryotic" has generally been applied to a given cell and, by extension, to a colony composed of such cells, although Buxton (1954) has discussed the situation in *Fusarium oxysporum* in which only the cell at each hyphal tip is multinucleate. In the case of *N. stenospora*, individual cells are all uninucleate but the frequency of anastomosis is such that the colony resembles a cell mosaic. Thus although intracellular reactions between nuclei of different genotype

cannot occur, the many anastomoses provide a pathway for intercellular, interhyphal nuclear reactions. The yellow colony formed from the mixed yellow and pale conidia might therefore be considered heterokaryotic rather than consisting of a mixture of two strains with little connection except approximate concurrence.

Such a concept of heterokaryosis is supported by the work of Pittenger and Atwood (1956) working with *Neurospora crassa* heterokaryons formed from biochemically deficient strains. They have shown that the nuclear ratios in individual hyphae at the growth frontier varied widely and that the growth rate of the frontier depended upon the overall nuclear ratio of a large number of hyphae in the area. Thus the growth rate of hyphal tips did not reflect their genotype unless they were isolated from their neighbours. Such facts could only be explained on the assumption of interaction between the nuclei (or their products) from different cells and hyphae. The existence of cytoplasmic translocation between cells provides one means for such reactions.

Thus even with fungi with heterokaryotic cells, intercellular and interhyphal reactions can be the dominating feature. It seems not unreasonable to extend the term heterokaryotic to colonies of fungi with a mixed nuclear population, even if individual cells are uninucleate, so long as there are sufficient anastomoses to provide cytoplasmic continuity for interactions between the cells of different genotype even if they are in different hyphae, and the local colony character depends on the nature of the overall nuclear population in that area.

A second feature of interest is the variation in colony form in the cultures derived from ascus III, which is similar in some ways to that described by Stover (1956) in *Thielaviopsis basicola* in that two methods of subculturing, by single spores or mass transfers, result in colonies of different phenotypes. It seems clear that this variability is under cytoplasmic control. In only two strains (3, 4) derived from ascus III are the cytoplasmic particles controlling tasselling in sufficient quantity to permit expression of the effect in rapidly growing colonies, although they are present in four other strains (5-8). In these four strains the presence of the particles is only shown by the ragged edge of old colonies or by tasselling in daughter colonies derived from conidia which, by chance, incorporate a greater-than-average number of particles (Arlett 1957).

There are, however, three points of note in connection with this cytoplasmically controlled variation. Firstly, the expression of the character is limited to those strains derived from ascus III. Thus although a mixed colony derived from strains 1 and 3 is tasselled, one between strain 3 and a strain from another ascus is normal. It would therefore seem either that the nucleus or the cytoplasm of these other strains suppresses the phenotypic effect of the particles even if they are present in sufficient numbers to cause its expression in combination with strain 1, itself completely or practically deficient in the particles.

Secondly, the distribution of the particles within ascus III must have been irregular, for one pair of spores had incorporated within them sufficient particles for phenotypic expression in their derivative colonies, two other pairs insufficient for expression, and one pair very few or no particles at all. That the number, or ratio, of particles may be altered after sexual reproduction is shown by the tasselled progeny from the perithecia of the self-fertile strain 6.



Finally, the percentage of normal and tasselled monoconidial daughter colonies produced in each generation remained unexpectedly constant, even under a selective pressure. There is, however, insufficient experimental evidence to postulate the cause.

Whilst further experiments will be needed for a better understanding of these problems, this investigation has revealed aspects of interest in the mechanism of variation in *N. stenospora*.

#### V. ACKNOWLEDGMENTS

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## VARIATION IN NECTRIA STENOSPORA

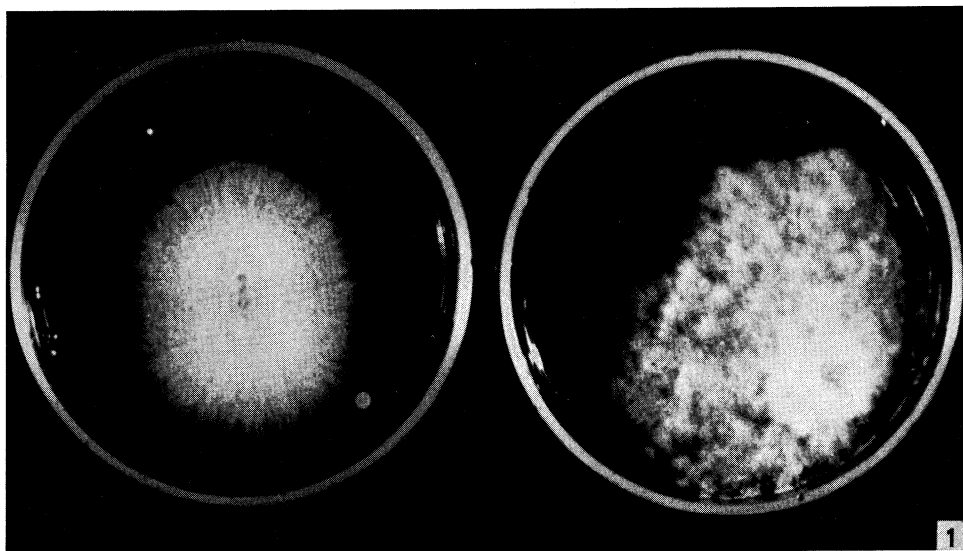


Fig. 1.—Colonies of *N. stenospora* of normal (left) and tasselled (right) morphology.  $\times 0.65$

Fig. 2.—Tasselled areas occurring in a colony of *N. stenospora* after a period of normal growth, the colony originating from a mixed suspension of conidia from two ragged strains.  $\times 0.9$ .

