The Effect of Temperature on the Production of Perithecia by *Neurospora crassa*

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SUMMARY

The wild-type strains of *Neurospora crassa* first isolated by Dodge, Abbott & Chilton were found to differ in the range of temperatures over which protoperithecia were produced; similar results were obtained with two recently isolated wild strains of a species of *Neurospora*. The ability to develop protoperithecia at 30° was found to be controlled by at least two genes in reciprocal crosses between two wild types differing in this character. A correlation of these observations with the reported features of tyrosinase production is discussed. Certain mutant strains backcrossed repeatedly to a wild-type strain gave exceptional results at 30°. Preliminary observations suggest that the part played by nicotinamide in the sexual cycle may be connected with some of these exceptions. Temperature did not obviously affect ascus development in outbred crosses between Lindegren and Abbott wild-type strains. With inbred crosses ascus development was controlled by several factors which were temperature sensitive and differed in degree of effect in reciprocal crosses. In most strains tested 20° was the optimum temperature for normal ascus development. At higher temperatures gross abnormalities were observed including asci with more than eight spores.

INTRODUCTION

During studies on the effect of temperature on sexual reproduction in *Neurospora crassa*, the production of fertile perithecia at the extremes of temperature at which sexual reproduction will occur was found to vary with the strain used as maternal parent. Evidence indicating that the original wild-type strains involved in the ancestry of most mutant strains were the source of this variability prompted an examination of these wild-type isolates for ability to produce fertile perithecia over a range of temperatures. The production of fertile perithecia may be divided into three stages. (1) Differentiation to form protoperithecia. The formation of protoperithecia in a number of wild-type isolates was studied in the present work. Two recently isolated strains of a *Neurospora* species were included to determine whether the observed characters might have been present in these wild-type strains when originally isolated or are a result of continuous laboratory culture. An examination of the inheritance of the observed character variation was undertaken and the effect of certain mutations on the expression of this character studied. (2) Fertilization. Observations throughout the present work indicated that the process of

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fertilization was not affected by temperature. (3) Subsequent development, involving meiosis and the delimitation and maturation of ascospores. These stages were studied over the temperature range 15–30° in crosses between a Lindegren and an Abbott wild-type strain (equivalent to outbreeding) and between two Lindegren or two Abbott wild-type strains (equivalent to inbreeding).

METHODS

In all the crosses listed below, the first-named strain was used as protoperithecial parent and is considered to have provided the main source of cytoplasmic inheritance (Srb, 1958). The following strains were used. *Neurospora crassa*: Lindegren 1A (L1A), wild type; Lindegren 25a (L25a), wild type; Lindegren 1a (L1a), a re-isolate from the cross L1A × L25a backcrossed to L1A six times and then selfed twice; Abbott 4A (A4A), wild type; Abbott 12a (A12a), wild type; Abbott 4a (A4a), a re-isolate from the cross A4A × L25a backcrossed to A4A six times. The derived strains L1a and A4a resembled closely the strains to which they had been backcrossed and were used as opposite mating-type of those strains. Mutant strains of *N. crassa*: *cr* (F945), crisp, morphological mutant; *lys-3* (4545), lysine requiring; *nic-1* (3416), nicotinamide requiring. Other *Neurospora* species: Costa Rica CU5, Costa Rica CU8, single ascospore isolates of opposite mating-type from material recently isolated from the bark of a *Musa* cultivar. Taxonomic status not yet verified.

Stock cultures grown on bacto-peptone medium (Frost, 1962) and stored at 4° were used as the source of conidial inoculum. Crosses and tests for protoperithecial production were made on minimal reproductive medium (Westergaard & Mitchell, 1947) adjusted to pH 6.7 and dried for 5 days at about 18°. This drying resulted in more consistent protoperithecial production with all the strains used. The protoperithecial parent was grown for 7 days at the required temperature before the fertilizing strain was added as a suspension of conidia in sterile water; the culture was then returned to the same temperature.

The testing of progeny for protoperithecial production was done by using a standardized procedure of medium preparation and inoculation. Protoperithecial strains of one mating-type in each experiment were fertilized with samples of the same conidial suspension.

The production of protoperithecia was estimated by looking at the cultures immediately before the addition of the fertilizing strain and then recording the presence of perithecia 1–4 days later. This allowed, to a considerable extent, the detection of protoperithecia which were embedded in hyphal masses or in the medium, or which were not fertilized although produced with a low or a fair frequency.

Ascus structure was examined in fresh material mounted in 2M-sucrose solution. This solution made manipulation easier and prevented excessive bursting of mature asci which occurred in water alone.

RESULTS

The effect of temperature on protoperithecial production

Under the conditions of medium and culture used, the wild-type strains showed a marked difference in the range of temperatures over which protoperithecia were produced (Table 1). The Costa Rican strains also showed this variation, suggesting

that the differences observed between the Lindegren and Abbott strains had not arisen during continuous vegetative culture. The observations made on the Lindegren and Abbott strains were confirmed in several experiments and were made from crosses known to be very fertile at 25° and to produce a high frequency of eight-spored asci. The crosses were duplicated and at temperatures borderline for sexual reproduction up to four replicates were made.

Table 1. *The range of temperatures over which protoperithecia were produced by several wild-type strains of Neurospora*

Wild-type strain	Temperature					
	10°	15°	20°	25°	30°	35°
	Protoperithecial production					
Lindegren 1A	—	+	+	+	+	—
Lindegren 25a	—	+	+	+	+	—
Abbott 4A	—	+	+	+	—	—
Abbott 12a	—	—	(+)	+	+	—
Chilton a	nt	+	+	+	+	—
Costa Rica CU5	nt	+	+	+	+	—
Costa Rica CU8	nt	—	—	+	+	—

+, Protoperithecia produced; —, no protoperithecia produced; (+), abortive protoperithecia occasionally produced; nt, not tested.

More detailed observations were made on the Lindegren and Abbott crosses, including cytological examination of ascogenous hyphae and developing asci in material fixed at intervals after conidiation and stained with acetocarmine. Asci were found to develop with time in three (sometimes four) waves with the oldest asci at the periphery. The results are given in Table 2. Observations of L25a ♀, conidiated by A4A, gave results similar to L1A × A12a (Table 2), except that the rates of development of protoperithecia, perithecia, asci and spores were about 1 day longer than in L1A. In both sets of these Lindegren crosses, broad asci with eight overlapping spores, tending to be two-ranked in arrangement, were observed only with continuous incubation at 30° (Pl. 1, fig. 2). In all four strains, protoperithecial formation at 25° transferred to 30° after conidiation gave similar results to continuous incubation at 25°. Throughout these crosses at all temperatures at which asci with spores were produced, the frequency of abortive or abnormal asci was nil or very low with spore abortion varying from 1 to 5% (cf. Table 4).

Other characteristics were observed to differ between the Abbott and Lindegren strains. Linear growth rate measurements (made by the method of Ryan, Beadle & Tatum, 1943) indicated a more rapid conidial germination and higher growth rate for the Abbott strains, particularly A4A, than for the Lindegren strains. A characteristic, perhaps associated with a higher metabolic rate in A4A, was the more rapid pigmentation of two of the four spore pairs in each ascus in crosses involving A4A as either the maternal or paternal parent (Pl. 1, fig 3). This character was rather difficult to score owing to its transient nature, and the pattern of segregation in each ascus was not always visually obvious. Nevertheless, it appeared from the segregation patterns in clearly scorable asci that a single gene might control this character. Of 225 asci scored from the cross A4A × L1a, 63 showed second division

Table 2. *The effect of temperature on the production of protoperithecia, perithecia, asci and ascospores in certain Neurospora wild-type strains*

Cross ♀ × ♂	Temperature			
	10°	15°	20°	30°
	Production of protoperithecia, perithecia, asci and ascospores			
L14 (× A12a)	Loose hyphal clumps; no perithecia develop	Protoperithecia form in 6 days, very numerous and large, normal in colour, many sterile; ascogenous hyphae develop in 4 days; asci slow to mature; spore discharge normal	Protoperithecia form in 3 days, numerous, normal in size, pale in colour, some sterile; ascogenous hyphae develop in 5-6 days; spores not discharged	Protoperithecia form in 6 days, fewer in number, normal in size and colour; fewer perithecia develop rapidly, necks short; fewer asci; spores mature rapidly, normal discharge
A4.4 (× L25a)	Loose hyphal clumps; no perithecia develop	Protoperithecia form in 5 days, very numerous and large, normal in colour, many sterile; perithecia and asci less slow to mature; spore discharge normal	Protoperithecia form in 2 to 3 days, numerous, normal in size and colour, some sterile; perithecia and asci develop rapidly; spore discharge normal and early	Loose hyphal clumps or protoperithecia not produced
A12a (× L14)	Very loose hyphal clumps; no perithecia develop	Loose hyphal clumps; no perithecia develop	Protoperithecia numerous, small, normal in colour; perithecia and ascogenous hyphae develop, abort, no spores formed	Protoperithecia fairly numerous, large; a few perithecia devoid of spores; spores mature rapidly, normal discharge

segregation for this spore-maturing factor giving a centromere distance of 14.0. A very low frequency of asci with 5:3, 6:2 or 7:1 ratios were also observed.

Inheritance of the ability to form protoperithecia at 30°

The progeny of reciprocal crosses between L25*a* and A4*A* were analysed for the ability to produce protoperithecia at 30°. Parallel crosses were then made between a single isolate, 44*a* (from the cross L25*a* × A4*A*), which produced abundant protoperithecia at 30°, and either L1*A* or A4*A*, the latter strains acting as protoperithecial parent. The progeny of these crosses were tested for the ability to produce protoperithecia at 30°. Three replicates were made at 30° and one in the controls at 25°. A few strains which showed a marked decrease in protoperithecial production at 25° were rejected from the analysis; the numbers rejected are given in Table 3.

Subsequent to the addition of the fertilizing strain, an assessment of perithecial frequency was made by scoring on an arbitrary scale: 3, 2, 1, rare, very rare, absent; from this an indirect assessment of protoperithecial frequency was made.

Two features emerged showing good agreement between replicates. First, the progeny may be divided primarily into two classes: protoperithecia present or protoperithecia absent. Secondly, among strains in which protoperithecia were produced there was a less distinct subdivision into the presence of a high frequency or a low frequency of perithecia. The results are presented in this form in Table 3.

The results obtained from the progeny of the reciprocal crosses between L25*a* and A4*A* showed marked agreement, indicating the absence of a cytoplasmic effect. A good fit to a 3:1 ratio between the two classes, perithecia present to perithecia absent, was observed although the sample sizes were small (Table 3).

In early experiments to test the progeny of these reciprocal crosses, L1*A* and L25*a* were used as the fertilizing strains since they were known to give fertile crosses even when inbred. However, in some cases with these strains there was the possibility of overgrowth and subsequent protoperithecial production by the conidiating parent so that young perithecia, although absent at 4 days, appeared 7 days after addition of the fertilizing parent. To prevent this, A4*A* and 44*a* were used as the fertilizing strains in subsequent experiments since these strains do not form protoperithecia at 30°. However, as suggested by Barbesgaard & Wagner (1959), a bisexual heterocaryon may have formed after conidiation in which the two component strains were blocked at different stages in sexual differentiation. Complementation in such a heterocaryon would result in an eventual formation of perithecia. The possibility of delayed protoperithecial production by the maternal strains was tested by conidiating two replicates after 7 days and two after 14 days. In no case did cultures in which no perithecia were present in 7 days produce them after 14 days.

The results obtained from the reciprocal crosses between L25*a* and A4*A* suggested that backcrossing a strain which showed a high frequency of protoperithecia at 30° to A4*A* would result in a higher proportion of progeny unable to form protoperithecia at 30° than were able to form them, while the same strain backcrossed to L1*A* would result in the converse. Strain 44*a* (from the cross L25*a* × A4*A*) was used for these backcrosses and one replicate was made at each temperature when testing the progeny. The results from these backcrosses are presented in Table 3

Table 3. *The frequency of perithecial production at 30° among the progeny of Neurospora wild-type parents differing in this character*

Wild-type parents crossed at 25°	Number of progeny rejected*	Progeny crossed to tester strains at 30°					χ^2 for a 3:1 ratio present: absent	P (D.F. = 1)
		Perithecia						
		Frequent	Rare	Absent	Present	Absent		
L25a × A4A	11	33	23	18	56	18	0.018	0.80-0.90
A4A × L25a	9	14	11	6	25	6	0.527	0.80-0.50
L1A × 44a†	6	35	20	24	55	24	1.219	0.20-0.30
A4A × 44a	18	3	0	12	3	12	nt	nt

* Progeny which showed a marked decrease in protoperithecial production at 25° were rejected from the analysis; † strain 44a was a single spore isolate from the cross L25a × A4A and showed a high frequency of protoperithecia at 30°; nt, not tested as the sample size was small.

The predicted trend appears to hold in the ratio of perithecia present to absent obtained.

Strain 44*a* had been chosen because it had produced a high frequency of protoperithecia at 30° in previous experiments. However, when used as a control during the testing of the progeny of the above backcrosses, no protoperithecia were produced at 30°. It was then realized that conidia of 44*a* stored at 4° for 8 months had been used, whereas in all previous experiments conidia stored at 4° only for a short while had been used. Horowitz & Fling (1953) during the investigation of the inheritance of tyrosinase production in *Neurospora crassa* at 35° observed that 'the initial classification of a culture changed when the test was repeated at a later date suggesting that uncontrolled physiological factors are important in the expression of this character in some strains'. The possible correlation between tyrosinase activity and protoperithecial production to be discussed later suggests that the anomalous results from 44*a* may have been due to the operation of similar physiological factors.

The effect of certain mutant genes on the production of protoperithecia at 30°

A series of mutant and non-mutant progeny from the fourth or fifth backcross of three mutant strains to either L1*A* or A4*A* were tested for protoperithecial production at 30°. The mutant strains used were: *cr* (F945), *lys-3* (4545), *nic-1* (3416). In the case of the biochemical mutants, the specific growth requirement was added to the medium for both mutant and non-mutant progeny.

In the case of the crisp strain, both mutant and non-mutant progeny from a sixth backcross of A4*A* showed absence of protoperithecia at 30° as expected. However, from a sixth backcross to L1*A*, while non-mutant progeny (with one exception) produced protoperithecia at 30°, twenty mutant progeny produced protoperithecia in ten cases but none in the other ten cases. After conidiation of the former cultures, small perithecia were produced devoid of ascospores. Similar results were obtained with crisp progeny incubated at 15°.

Non-mutant progeny from backcrosses of the lysine strain behaved as expected with few exceptions. However, none of the mutant progeny from a fifth backcross to either L1*A* or A4*A* would produce protoperithecia at 30° and at this temperature vegetative growth was sparse with the production of a yellowish coloration which rapidly permeated the medium.

In contrast, both mutant and non-mutant progeny of the nicotinic strain from a fifth backcross to L1*A* and a fourth backcross to A4*A* showed high fertility at 30°. It is suggested that the addition of nicotinamide to the medium (at 60 mg./l.) overcame the block in protoperithecial production at 30° present in A4*A*. The effect of nicotinamide on several wild-type strains, including wild strains of *Neurospora sitophila*, was tested; in each case protoperithecial production was enhanced.

The effect of temperature on the production of asci and ascospores in inbred crosses of wild-type strains

In contrast to crosses between an Abbott and a Lindegren strain, crosses of two Abbott or two Lindegren strains showed marked effects of temperature on ascus and ascospore development. The results are presented in Table 4 and illustrated in Pls. 1 and 2.

The crosses of the Abbott strains (Table 4 and Pl. 1) showed a high frequency of abortive asci, asci with less than eight spores and the occurrence of misshapen ascospores at all temperatures tested. Such abnormalities were at a minimum at 20°.

Table 4. *The effect of temperature on the frequency of production of abnormal asci and ascospores in crosses between two Abbott or two Lindegren wild-type strains*

Cross ♀ × ♂	Abnormal condition of asci	Temperature				
		15°	20°	25°	25→30°	30°
		Frequency of abnormal asci				
A4A × A12a	Abortive	a	f	a	a	*
	< 8 black, normal spores	All	All	All	All	*
	Misshapen spores	f	o	f	f	*
A4A × A4a	Abortive	f	o	f	a	*
	< 8 black, normal spores	f	o	All	All	*
	Misshapen spores	f	r	r	r	*
L1A × L25a	Abortive	*	o	f	a	va
	< 8 black, normal spores	*	f	f	a	All
	> 8 spores	*	Nil	Nil	f	f
	Broad; overlapping spores	*	Nil	Nil	f	a
	Misshapen spores	*	vr	vr	f	a
L25a × L1A	Abortive	a	Nil	o	a	a
	< 8 black, normal spores	va	Nil	f	a	va
	> 8 spores	Nil	Nil	Nil	Nil	o
	Broad; overlapping spores	Nil	Nil	Nil	f	a
	Misshapen spores	r	Nil	r	f	a

*, No fertile perithecia produced; 25 → 30°, protoperithecia formed at 25° transferred to 30° after conidiation; a, abundant; f, frequent; o, occasional; r, rare; v, very.

Reciprocal crosses of L1A and L25a (Table 4 and Pl. 2) showed differences at 20° indicating cytoplasmic control of some stage in ascus development. Crosses of L1A × L1a were also made and these gave similar results to L1A × L25a (Table 4). In each set of these Lindegren crosses, asci with more than eight spores were observed with continuous incubation at 27.5° or 30° and, in the case of L1A only, with protoperithecial formation at 25° transferred to 30° on conidiation. It was at these three particular temperature régimes too that all the Lindegren crosses showed some asci which were broader than normal with overlapping spores which tended to be two-ranked in arrangement. Again, abnormalities were at a minimum or absent at 20°.

Crosses of Costa Rica CU5 with Costa Rica CU8 were fertile with normal eight-spored asci at 25°. At 30° almost total abortion was observed with short distorted asci containing misshapen thin-walled ascospores.

DISCUSSION

It is now well established that the different wild-type strains of *Neurospora* show considerable biochemical and genetical variability. In the present work, the differing ability of the wild-type strains to produce protoperithecia over the range of temperature 15° to 30°, and the differing effects of temperature on ascus and

ascospore development in inbred and outbred crosses of the wild types, provide further evidence of variation between the wild strains.

The non-production of protoperithecia at 30° by *Neurospora crassa* A4A in contrast to A12a and the Lindegren wild-type strains parallels the report by Horowitz & Fling (1953) that A4A did not produce tyrosinase at 35° whereas L25a and A12a did. The report by Hirsch (1954) showing a correlation between the appearance of tyrosinase and protoperithecial formation suggests that the correlation above may have some significance. Markert (1950) and Horowitz, Fling, Macleod & Sueoka (1960) reported an association of female sterility with inability to produce tyrosinase. The enzyme was induced by addition to the medium of an aromatic amino acid, which, however, did not affect the female sterility. These results suggest that the two characters may be related through a common biochemical defect, and that it is some stage in the production of this precursor that is temperature sensitive in A4A but not in A12a or the Lindegren wild-type strains. Thus the presence of the enzyme may be used as an indication of ability to reach a specific, but as yet unidentified, stage in sexual reproduction. The production of protoperithecia at 30° by strains repeatedly backcrossed to A4A and tested on minimal medium + nicotinamide suggests that nicotinamide might be involved in the pathway which leads to protoperithecial formation and that A4A is deficient in this compound when incubated at 30°.

The observations reported here on sexual differentiation at the higher temperatures suggest a possible explanation of the anomalous results with the mutant *al-2* (15300) reported by Fox & Gray (1950) and by Horowitz & Shen (1952). The former workers reported the presence of tyrosinase at 30° in *al-2a* but not in *al-2A*. The latter workers found the enzyme present in both strains at 25°. Possibly different re-isolates were used by the two sets of workers such that the 'A' strain of Fox & Gray was derived from A4A whereas the other strains were not.

The 3:1 ratios observed in the L25a × A4A reciprocal crosses suggest that the production of protoperithecia at 30° is controlled by two genes. However, one of the progeny from these crosses, 44a, which showed a high production of protoperithecia at 30°, when crossed to L1A, which also showed a high production of protoperithecia at 30°, gave in the progeny a 3:1 ratio of perithecia present to absent at 30°. These results suggest that 3 or more genes may be involved. This apparent multifactorial inheritance of protoperithecial production at 30° correlates with the observations of a number of workers on the genetic control of tyrosinase synthesis (Markert, 1950; Fox & Burnett, 1959; Horowitz & Fling, 1953; Lewis & Lewis, 1961).

The phenomenon of asci with more than eight spores was first observed in *Neurospora* in crosses between strains of unknown ancestry by Frost & Greenhill (1963), who called such asci 'multi-spored'. The present work indicates that the production of multi-spored asci occurs at temperatures of 27.5° or above and is associated only with crosses between two Lindegren strains. Furthermore, the size and distribution of the spores in multi-spored asci suggest meiosis is irregular with spore walls deposited around nuclear material which in some cases may represent only a single chromosome. Similar observations were made in *Neurospora* by Perkins (1962) in crosses of *bis* × *bis* (morphological mutant, biscuit, B6). Rizet & Engelmann (1949) reported that in *Podospora anserina* asci with more than the normal number of spores were produced in crosses particularly at higher temperatures.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Asci produced by the cross Abbott 4.4 × Abbott 12a incubated at different temperatures: (1a) removed from 25° to 30° at the time of conidiation, (1b) 25°, (1c) 20°, (1d) 15°.

Fig. 2. Overlapping spores in asci from the cross Lindegren 25a × Abbott 4.4 incubated at 30°.

Fig. 3. The slow maturation of four spores in an ascus from the cross Abbott 4.4 × Lindegren 25a incubated at 25°.

PLATE 2

Fig. 4. Asci produced by the cross Lindegren 1.4 × Lindegren 25a incubated at different temperatures: (4a) removed from 25° to 30° at the time of conidiation. A multi-spored ascus containing nineteen spores not all of which are visible here (phase-contrast illumination). (4b) as in 4a but ascus containing nine spores. (4c) 25°, (4d) 20°.

Fig. 5. Asci produced by the cross Lindegren 25a × Lindegren 1.4 incubated at different temperatures: (5a) 30°. A multi-spored ascus is indicated by the arrow (phase-contrast illumination). (5b) 25°, (5c) 20°, (5d) 15°.