INTER-ISOLATE HETEROKARYOSIS IN *THANATEPHORUS CUCUMERIS*

II.* BETWEEN ISOLATES OF DIFFERENT PATHOGENICITY

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Abstract

Evidence is presented for heterokaryosis occurring between two mutants derived from different pathogenic strains of *T. cucumeris* from anastomosis group 2. The evidence relates both to the heterokaryon formed and to the recovery in its progeny of a number of markers from the original mutants. Among the progeny were some strains of different pathogenicity from their parents. Synthesis of such a heterokaryon was difficult compared with that between isolates of similar pathogenicity. This would suggest that, in nature, inbreeding of pathogenic strains more commonly operates than outbreeding, resulting in biological isolation of specific pathogenic types. Nevertheless outbreeding, though rare, could be one means whereby new pathogenic strains arise.

I. INTRODUCTION

Our objective was to investigate, in *Thanatephorus cucumeris* (Frank) Donk, the inheritance of pathogenicity with regard to host specificity. Pathogenic isolates of *T. cucumeris* may be host specific attacking only one family of plants, e.g. Cruciferae, or non-specific with respect to host, attacking a wide range of host plants.

Parmeter, Sherwood, and Platt (1969) have classified field isolates of *T. cucumeris* into four anastomosis groups (A.G.) on the basis of their ability to anastomose with each other, viz. anastomosis occurs between isolates from the same group, but not between isolates from different groups. Isolates in A.G.1 and A.G.4 attack a wide range of host plants whereas those tested in A.G.2 are more restricted in their host range, some attacking only one genus. Progeny of wide host-range pathogens tested appear to be the same as their parents in being able to attack the same range of different hosts, any differences being in virulence rather than specificity. We have not been successful in obtaining mutant progeny from A.G.1 and A.G.4 isolates where the host range was more restricted than that of the parent. Therefore isolates of A.G.2 were used that had different host specificities. Recent work (Stretton and Flentje 1972) has shown that inter-isolate heterokaryosis can occur between A.G.2 isolates that are specifically pathogenic to crucifers. This work has now been extended so that these same crucifer pathogens were used with cowpea pathogens that also belong in A.G.2 and which would therefore be expected to have a genetic affinity with each other.

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II. Materials and Methods

The isolates used are listed in Table 1. All fruited on aerated steam-treated soil (Stretton *et al.* 1964) and isolate 296 also fruited on soil-extract agar. Mutant single-basidiospore cultures derived from these isolates were obtained either by spontaneous mutation or following ultraviolet irradiation (Flentje, Stretton, and McKenzie 1967). The general media used [potato–Vegemite–dextrose–agar (P.V.D.A.) (McKenzie *et al.* 1969), soil-extract agar (Flentje 1956), and *Rhizoctonia* medium (R.M.) (Stretton and Flentje 1972)] have been described previously as have methods for single-cell culturing (Flentje and Stretton 1964) for inducing anastomosis and heterokaryon formation (McKenzie *et al.* 1969) and for testing pathogenicity on crucifers (Flentje, Stretton, and McKenzie 1967). The cowpea pathogens were isolated originally from *Phaseolus* leaves but when tested later were found to be non-pathogenic on 10 different *Phaseolus* varieties. However, they are

Table 1

List of isolates of *T. cucumeris* A.G.2 showing origin, reference, and pathogenic specialization

Isolate No.	Origin of isolate	Locality	Reference	Pathogenic specialization
48	Soil	Adelaide, S.A.	Flentje, Stretton, and Hawn (1963)	Stems of Cruciferae
68	Cabbage stem lesion	Hitchin, U.K.	Flentje and Saksena (1957)	
69	Cabbage stem	Clare, S.A.	Flentje and Saksena (1957)	
106	Crucifer stem	Slough, U.K.	Stretton and Flentje (1972)	
296	*Phaseolus* leaves	Costa Rica	As C-296 Parmeter,	Stems of *Vigna sinensis*
297			As C-297 Sherwood,	
298			As C-298 and Platt	
301			As C-301 (1969)	

pathogenic on cowpea (*Vigna sinensis*, cv. Black-eye) stems. Pathogenicity tests on cowpea were carried out in paper cups of soil, previously treated with aerated steam (71°C for 30 min) to rid it of any contaminating rhizoctonias that might be present. The soil was inoculated and planted with five seeds per cup. In all pathogenicity tests, isolates were screened for their ability to attack stems and classified according to the stage reached in infection as described earlier for crucifers (Stretton and Flentje 1972). For biochemical studies, Dodman's minimal medium (Dodman, unpublished data) was used. The method used for electrophoresis studies of soluble proteins from hyphal extracts was that of Chrambach *et al.* (1967) for polyacrylamide gels, the presence or absence of bands observed on the gels and their intensity being used as criteria for comparison.

III. Experimental Details and Results

(a) *Designation of Mutant Genes*

All crucifer field isolates used produced wild-type growth on minimal medium ("wild type" in these studies refers to growth rate and cultural morphology on P.V.D.A. at 25°C for each particular field isolate). Morphological mutants were present in all single-basidiospore cultures used, a brief description of these in the

crucifer isolates having been given previously with evidence that the aberrations are single gene mutations (Stretton and Flentje 1972).

The *Phaseolus* field isolates carried stable heritable biochemical mutants in that they failed to produce wild-type growth on minimal medium. Instead they grew sparsely with absence of sclerotia, although growth rate was the same as wild type. Isolate 297 produced growth as dense as wild type with the addition of thiamine (1 mg/l) to the minimal medium (Fig. 1), while 296, 298, and 301 required both thiamine and nitrite. Morphological mutants obtained in the *Phaseolus* isolates were less stable than the crucifer mutants and tended to revert to the wild type after frequent subculturing. The genetic control of the aberrations in the *Phaseolus* mutants is not known although evidence from reversion is that the morphological and pathogenicity mutations behave as single genes.

The isolates also differed in pathogenicity. The crucifer isolates were virulent pathogens on hypocotyls of radish (*Raphanus sativus* cv. Long White Icicle), and gave no reaction on cowpea (*Vigna sinensis* cv. Black-eye) hypocotyls, while the *Phaseolus* isolates produced large lesions on the stems of cowpea resulting in death of most seedlings, but gave no reaction on radish hypocotyls. The inheritance of these different specificities was followed in "crosses". Some of the mutant cultures used in the crosses, however, were non-pathogenic.

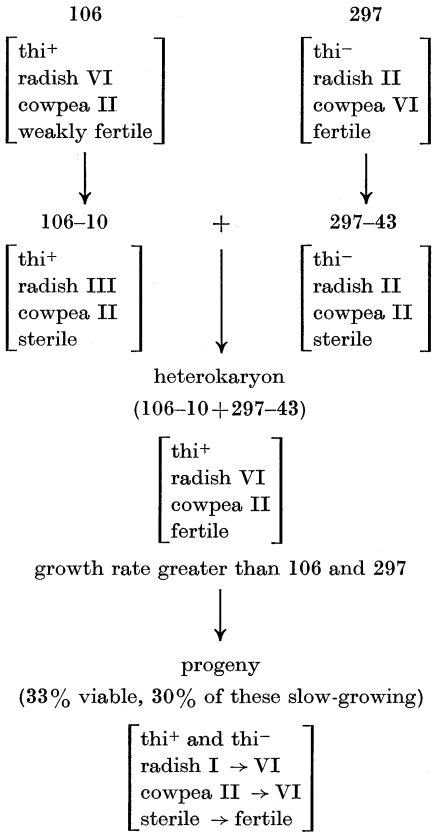
(*b*) *Attempts to Induce Heterokaryosis*

Mutant progeny from different field isolates were macerated in pairs to facilitate anastomosis and heterokaryon formation. Of approximately 100 different pairings involving *Phaseolus* plus crucifer mutants, some repeated many times, only four resulted in wild-type growth which could be maintained by hyphal tip culture. (Sixteen independently isolated morphological mutants from the *Phaseolus* isolates and 17 from the crucifer isolates were used. The instability of the mutants from *Phaseolus* isolates reduced the number of pairings possible and prevented the use of some in further pairings, as many of them reverted to wild type.) Of the four putative heterokaryons so obtained, two were sterile and therefore could not be analysed; one gave progeny which were identical and like one of the original parents in 200 tested, and was probably due to reversion of one of the mutants involved. The fourth was fertile and evidence will be presented to indicate that it was in fact a heterokaryon. Observations of actual cell-wall fusion of the two mutants involved in this cross have been made (from pairings made on Cellophane overlying agar), although violent incompatibility reactions were commonly seen. However, regrowth through dead cells in the anastomosis area was observed on one occasion. Subsequent attempts to reproduce this fourth putative heterokaryon resulted in wild-type cultures which consistently ceased growing on further subculturing.

(*c*) *Evidence for Heterokaryosis between the Original Mutants*

The putative heterokaryon resulted from the maceration of mutant 106–10 with mutant 297–43. Characteristics of these mutants and their parents are described

in Table 2 and illustrated in Figure 2, and may be charted thus:



where thi+ and thi− represent thiamine independence and dependence, respectively.

It was assumed that 297–43 carried the thi− characteristic as did its parent, and that one or more morphological mutations that severely restricted its growth prevented an observable response by 297–43 on minimal medium plus thiamine.

(i) *Characteristics of the Heterokaryon*

The heterokaryon was culturally distinct from either 106 or 297 on P.V.D.A. (Fig. 2) and grew significantly faster than either of them on liquid R.M. (viz. increase in mass at optimum (g/24 hr) of 106, 297, and the heterokaryon were 0·5, 1·0, and 2·75 respectively). It displayed some characteristics from each parent but appeared to be more like one (isolate 106), in that it was specifically pathogenic to crucifers and thiamine-independent (Fig. 3). As mutants 106–10 and 297–43 would not grow sufficiently on liquid R.M. for protein tests, a comparison of soluble protein patterns of 106, 297, and the heterokaryon was made on polyacrylamide gels. Their patterns indicated wide protein differences between all three cultures (Fig. 4), an unlikely result if the putative heterokaryon had arisen by reversion of either one of the mutants to wild-type.

TABLE 2

CHARACTERISTICS OF TWO MUTANTS, THEIR PARENT FIELD ISOLATES, AND THE PUTATIVE HETEROKARYON RESULTING FROM PAIRING THE TWO MUTANTS

Isolate No.	Cultural characteristics on P.V.D.A.	Growth on minimal medium	Growth on minimal medium +thiamine	Pathogenicity on radish	Pathogenicity on cowpea	Fertility
106	fast-growing, dense, light brown, no sclerotia (= wild-type for 106)	wild-type	wild-type	virulent (stage VI)*	non-pathogenic (stage II)	fruits only sparsely
106–10	slow-growing mutant, dense, "feathery" at edges; not known to revert to wild type	slow, dense	slow, dense	non-pathogenic (stage III)	non-pathogenic (stage II)	sterile
297	fast-growing, dense, brown; large dark sclerotia (= wild-type for 297)	fast, sparse	wild-type	non-pathogenic (stage II)	virulent (stage VI)	fruits abundantly
297–43	slow-growing mutant, very sparse, with no actual colony but a few runner hyphae only; unstable, tends to revert to parent-type or die	very slow, a few runner hyphae only	very slow, a few runner hyphae only	non-pathogenic (stage II)	non-pathogenic (stage II)	sterile
Heterokaryon (106–10+297–43)	fast-growing, dense, culturally distinct from 106 and 297	fast-growing, dense	fast-growing, dense	virulent (stage VI)	non-pathogenic (stage II)	fruits abundantly

* Stages of the infection process have been classified (Stretton and Flentje 1972) thus: I, growth inhibited on stems; II, hyphal growth on stems, but no response; III, attachment of hyphae to stems; IV, infection cushion formation; no penetration; V, hypersensitive reaction; VI, pathogenesis.

(ii) *Progeny of the Heterokaryon*

Hyphal tips were isolated from the heterokaryon and the resulting colonies induced to form basidiospores. However, although the heterokaryon fruited readily, only one-third of the spores could be induced to grow and establish cultures while the rest died, irrespective of the medium used. Of over 200 single basidiospores cultured on P.V.D.A., approximately 30% produced very slow-growing colonies. Some were similar in culture to 106–10, and a few to 297–43 but these latter eventually either died or reverted to 297 type. Confirmatory backcrossing tests therefore could not be completed with these 297-type progeny. Progeny could not be classified into four groups on the basis of their cultural morphology as was possible with progeny from heterokaryons synthesized from two crucifer mutants (Stretton and Flentje 1972).

TABLE 3

CHARACTERISTICS OF PROGENY OF PUTATIVE HETEROKARYON (106–10+297–43) THAT PRODUCED FAST, DENSE GROWTH ON P.V.D.A.

Cultural identity	Frequency	Pathogenic stage* reached on		Thiamine dependent (frequency)
		Radish	Cowpea	
106	11	VI	II	1
297	2	II	II	1
	1	II	VI	1
	1	IV	V	
Heterokaryon†	1	I	IV	
	1	I	V	
	1	II	II	
	1	II	IV	
	1	III	V	1
	1	IV	IV	
	2	V	II	
	1	V	III	
	4	VI	III	
	8	VI	IV	1
	13	VI	V	1
	6	VI	VI	1

* Stages of the infection process are listed under Table 2.

† Or similar in cultural appearance.

Instead there was a wide range of cultural types (Fig. 2) of varying growth rates, pigmentation, sclerotial production, and morphology. Progeny with fast, dense growth on P.V.D.A. were tested on minimal medium for thiamine dependence, and on radish and cowpea stems for pathogenicity. These tests are summarized in Table 3. Many of these wild-type progeny were like the 106 parent, i.e. pathogenic to radish stem, non-dependent on thiamine, non-pathogenic to cowpea, and lacked dark sclerotia in

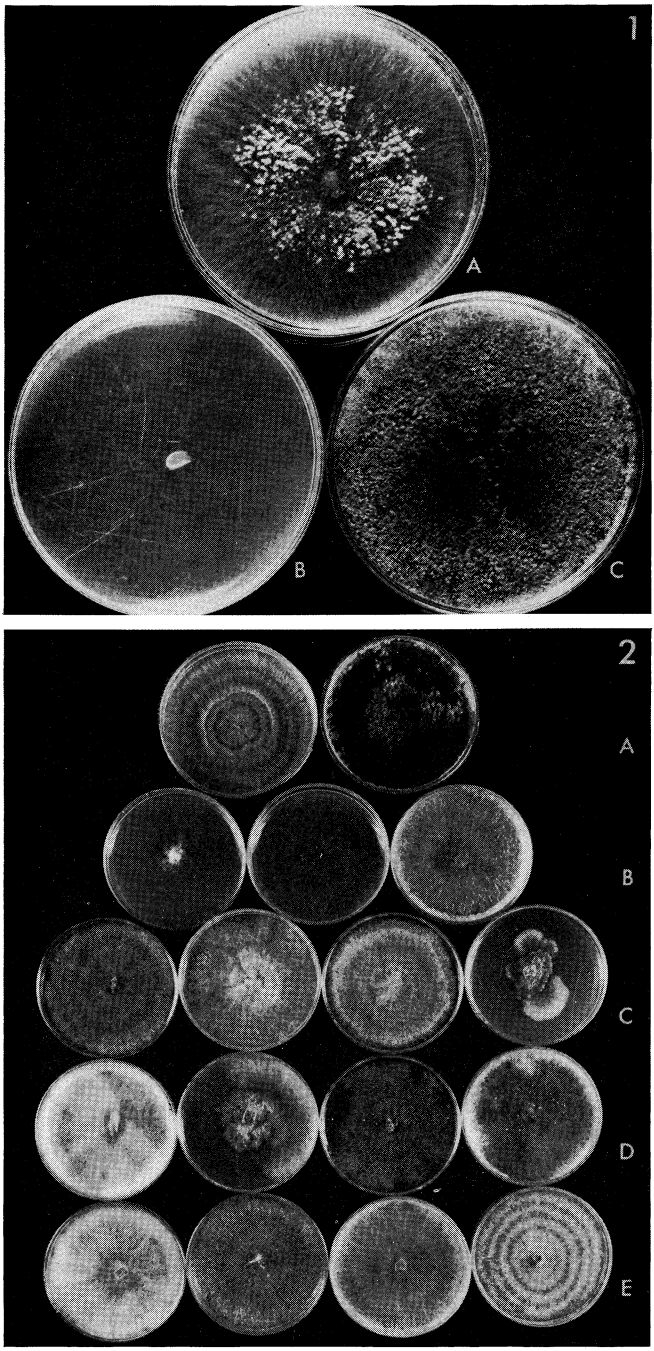
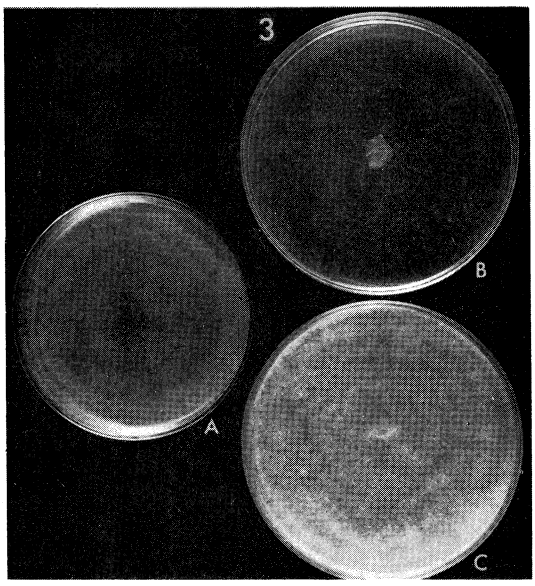
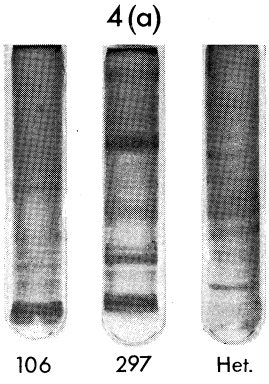
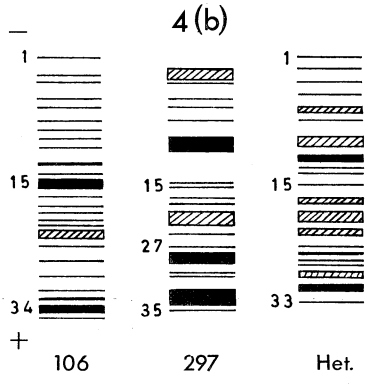


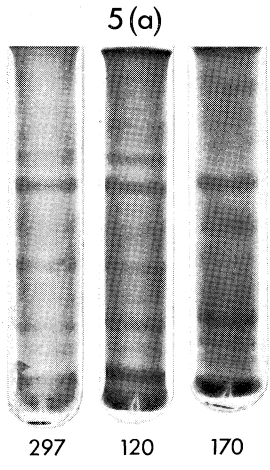
Fig. 1.—Isolate 297 on three different media showing its requirement for thiamine: *A*, P.V.D. agar; *B*, minimal medium; *C*, minimal medium + thiamine.

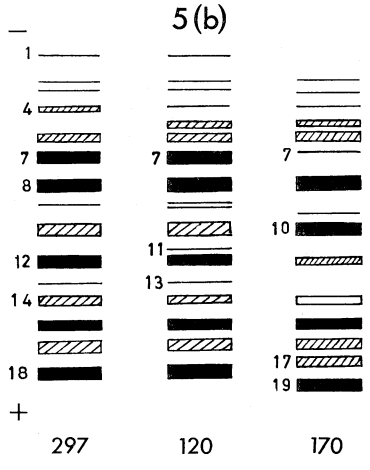
Fig. 2.—Cultural characteristics (after 10 days) on P.V.D. agar of the putative heterokaryon (106–10+297–43), its parent isolates, and its progeny. Row *A*, from left: 106, 297; row *B*, from left: 106–10, 297–43, heterokaryon (106–10+297–43); rows *C*, *D*, *E*: progeny from the heterokaryon.


3
A
B
C


4 (a)
106
297
Het.


4 (b)
1
15
34
35
27
33
106
297
Het.


5 (a)
297
120
170


5 (b)
1
4
7
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10
17
19
297
120
170

culture. One was identical with the 297 parent, i.e. non-pathogenic to radish, thiamine dependent, pathogenic to cowpea (Fig. 6) and produced dark sclerotia in culture. Recombination of parental characteristics was observed, e.g. some progeny were pathogenic to both hosts. Progeny with fast, dense growth on P.V.D.A. displayed a range of different stages reached in the infection process, on radish (Fig. 7). Besides these wild-type isolates, 20 others were "near" wild type or "intermediate" in growth rate and cultural appearance. None of these was identical culturally with 106, 297, or the heterokaryon but two were thiamine-dependent, and again pathogenic reactions ranged from non-pathogenic on either host to pathogenic on either or both.

Since characteristics of both parents involving cultural morphology, nutritional requirement, and pathogenicity were identified in the progeny of this culture (106–10+297–43) it is evident that heterokaryosis between the two original mutants had in fact occurred.

Mycelial protein patterns of two progeny (120 and 170) from the heterokaryon were compared with that of parent 297. The two cultures were identical in appearance with 297, and thiamine-dependent, but 170 differed from its sib 120 and parent 297 in being non-pathogenic to cowpeas. The spectrum of soluble proteins of spore culture 120 appeared to be almost identical with that of parent 297 while 170 differed from these two by a few bands only, the most obvious difference being in the intensity of band number 7 (Fig. 5). This is consistent with results of Clare, Flentje, and Atkinson (1968) using isolate 69, in which protein patterns of two progeny differing in virulence from their parent were found to be very similar.

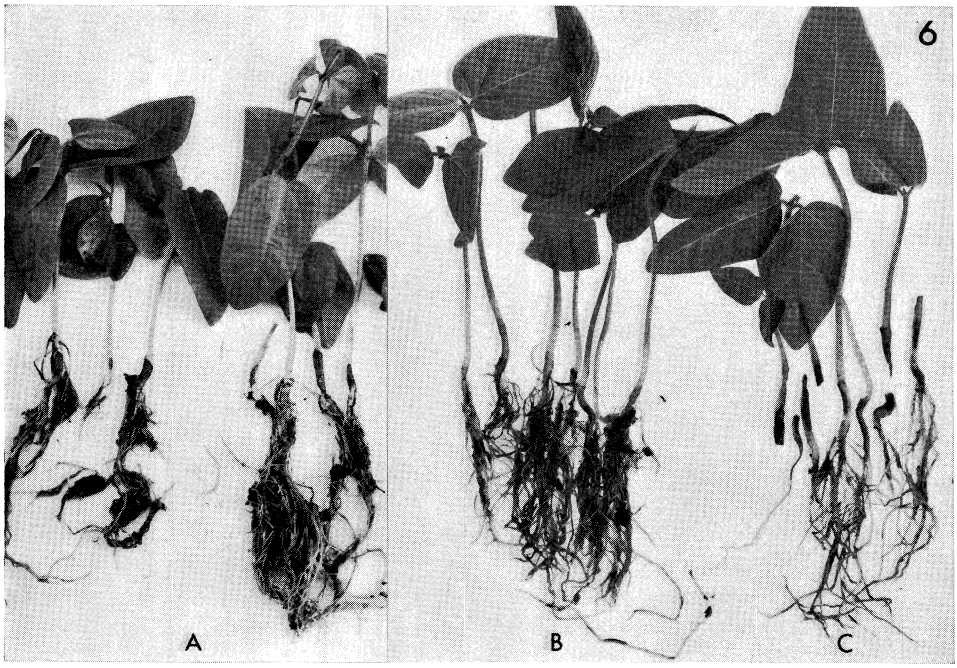
(iii) *Single-cell Cultures of the Heterokaryon*

Secondary septation occurs in *T. cucumeris*, independently of nuclear division, and thereby reduces cell size and number of nuclei down to 1 or 2 per cell (Flentje, Stretton, and Hawn 1963). Small agar disks removed from cultures of the heterokaryon were macerated in a tissue grinder and 450 small single cells were isolated and cultured on P.V.D.A. Some of these single cells would be expected to be heterokaryotic, but others should have contained only one nuclear type. The resulting cultures showed a wider variation than that displayed by similar cultures of either parent and the results are summarized in Table 4. Of the 394 dense fast-growing cultures, 370 were culturally similar to the heterokaryon and 24 were different. Eighty, including the 24 which were different were studied further. Pathogenically they were all like isolate 106 although a few were less virulent, but there were cultural differences which are summarized in Table 4.

Fig. 3.—Cultural characteristics on minimal medium. *A*, isolate 106 (dense); *B*, isolate 297 (sparse); *C*, heterokaryon (dense).

Fig. 4.—(*a*) Soluble protein patterns (pH 8·3) on polyacrylamide gels and (*b*) diagrammatic representation of (from left): isolate 106, isolate 297, heterokaryon (106–10+297–43). All show wide differences in protein spectra.

Fig. 5.—(*a*) Comparison of protein patterns (pH 8·3) of two progeny (120, 170) from heterokaryon (106–10+297–43) with parent isolate 297. (*b*) Diagrammatic representation. All show similarities in protein spectra.



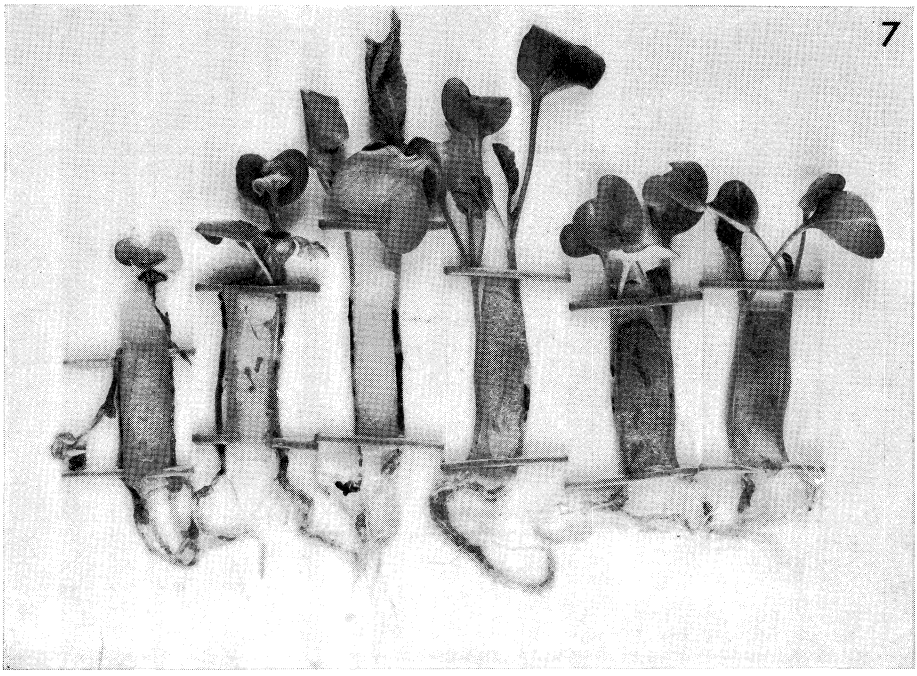


Fig. 6.—Pathogenicity tests on cowpeas of three cultures: *A*, isolate 297; *B*, heterokaryon (106–10+297–43); *C*, single-spore culture 120 from this heterokaryon.

Fig. 7.—Pathogenicity tests on radish hypocotyls of five wild-type progeny from heterokaryon (106–10+297–43) showing a range of reactions from virulent (at left) to hypersensitive lesions (fourth and fifth from left), to non-pathogenic (at right).

In some single-cell cultures, sectors representing both parent types in cultural appearance were produced suggesting that nuclear dissociation had occurred. However, these sectors did not remain constant through a series of subcultures, suggesting that both parent nuclear types were still present in the cells, but that different nuclear ratios were influencing cultural appearance. Nuclear ratios may also influence auxotrophy and virulence.

TABLE 4

THIAMINE REQUIREMENT OF CULTURAL TYPES FROM SINGLE-CELL CULTURES OF THE PUTATIVE HETEROKARYON ON P.V.D.A.

Of a total of 450 cultures, 8 died, 30 were very slow growing, 18 were slow growing, and 394 were dense, fast-growing cultures. Eighty of the latter type were selected and checked for thiamine requirement

Requirement	106 type	297 type	Heterokaryon type	Total
thi$^+$	4	18	44	66
thi$^-$	—	2	12	14
Total	4	20	56	80

IV. DISCUSSION

The above studies present evidence that a heterokaryon was formed between two mutants (106–10 and 297–43) derived from two A.G.2 pathogens of different host specificity. The evidence relates to characteristics of the putative heterokaryon, the recovery in its progeny of characters present in the original mutants or their parents, and the comparison of protein patterns of the parents, the heterokaryon, and selected progeny.

However, a number of problems arise from these studies, and to explain these we have attempted to develop a simple overall hypothesis which fits the data and provides a basis for further experiment and discussion. The infrequency of successful anastomosis between isolates of different pathogenicity was emphasized by the difficulties encountered in attempts to repeat heterokaryon syntheses and to establish hyphal tip cultures from them. Two putative heterokaryons were infertile. Infertility has been noted as a feature of interspecific crosses in *Neurospora* (Fincham 1951) and *Helminthosporium* (Nelson 1963). Emerson and Wilson (1954) report that viability of progeny spores from interspecific crosses of *Allomyces* decreased to one-tenth of that of their parents. It may be expected therefore that a cross between such widely different parents as in the present studies (seen from a comparison of cytoplasmic proteins) would be frequently infertile and incompatible. In the one fertile heterokaryon that was obtained, pathogenicity to crucifers was restored but not the ability to attack cowpeas. We offer one possible explanation for this, based on the assumption that different genes control the different stages of infection. We have evidence for this occurring in crucifer pathogens (Flentje, Stretton, and McKenzie 1967) and it may be expected that this is the pattern in cowpea pathogens also. As the

297–43 mutant in these studies is blocked at stage III for both hosts (i.e. only stage II is observed) and 106–10 mutant is blocked at stage IV for radish and stage III for cowpea, then in the heterokaryon each blockage for radish pathogenesis, being different, is complemented by the corresponding allele and the infection process is able to proceed. However, in both mutant isolates the infection process for attacking cowpea is blocked at the same stage, preventing complementation and subsequent infection on cowpeas from proceeding.

This same hypothesis could explain also the lack of pathogenicity to cowpeas amongst the wild-type, single-cell cultures obtained from the heterokaryon. But expression of thiamine-dependence by a few of these cultures, and a wide cultural variation amongst them all, is difficult to explain. Theoretically, only two nuclear types, i.e. 297–43 and 106–10, should be present in the heterokaryon apart from spontaneous mutations that might have occurred. But again the instability arising from co-habitation of diverse nuclei may lead to irregularities in conjugate division that would be reflected in cultural morphology. Otherwise nuclear ratios or somatic recombination may need to be considered as an explanation for this variation. Observation of meiotic pairings in basidia of the heterokaryon could give some indication of the extent of genetic disruption resulting from a wide outcrossing, but we could not observe meiotic figures sufficiently clearly in this organism. Poor chromosomal pairing at meiosis leading to non-disjunction may be the cause of high lethal rates among progeny and wide cultural differences amongst those that do survive. We have no expectation for particular segregations of any of the characters in the cross because we do not know the nature of their genetic control in cowpea, although this is known in the "crucifer" parent. This, however, is not important; it is their occurrence in the progeny rather than their frequency which provides ample evidence of heterokaryosis followed by crossing. Amongst the progeny cultures tested for their pathogenic ability on the hosts (Table 3) the range of different stages may be taken to indicate that the genes controlling these stages are not closely linked. Ability amongst the wild-type progeny to be fully pathogenic on crucifers could result from recombination between 106–10 and 297–43 at the crucifer stage IV locus in the 106–10 genome. However, pathogenicity to cowpea amongst the progeny could be explained by a reversion of the gene blocking stage III on cowpeas in the mutant 297–43 genome, a phenomenon commonly observed in this mutant. Fast-growing, dense cultures non-pathogenic to both hosts could have resulted from recombination also. However, blockages at the other stages controlling pathogenesis cannot be easily accounted for by simple recombination. Haskins and Mitchell (1952) have described a situation in *Neurospora crassa* where modifiers influenced gene action when different mutant cultures were crossed. An apparent block in the biosynthesis can be shifted to other positions in the synthetic pathway when diverse genotypes interact. We suggest that blockages in pathogenicity among the progeny additional to those in each parent or recombinations of them may be the result of some such interaction.

It would seem therefore that inter-isolate synthesis of heterokaryons in *T. cucumeris* A.G.2 becomes increasingly difficult as genotypes become more diverse, and in particular where differences in host specificity are involved. This supports the suggestion of Parmeter *et al.* (1969) and Sherwood (1969) that A.G.2 is diverging into

two non-interbreeding populations. These may be referred to loosely as the crucifer and non-crucifer subgroups. The fact that nuclei from each subgroup can complement in the heterokaryon suggests that they represent populations within the same species although diverging ones. The polyacrylamide gel studies also support this divergence. Clare, Flentje, and Atkinson (1968) found relatively large differences between protein patterns of two field isolates of *T. cucumeris* belonging to different anastomosis groups, and a similarly wide range of differences was reflected in the patterns of 106 and 297 isolates (and also when each was compared with the heterokaryon).

Blaich and Esser (1970) report that as a result of the incompatibility reaction between two strains of *Podospora anserina*, a new protein was formed in the heterokaryon which did not exist in the single strains. In our heterokaryon, it appears from comparisons of protein spectra that a number of new proteins may have been formed. Certainly the incompatibility reaction resulting from anastomosis of the two original mutant cultures is severe and may be similar to the heterogenic incompatibility reaction described by Esser and Kuenen (1967) and reviewed by Esser (1971) in interracial crosses in *Podospora*. However, in their studies the phenomenon was related to geographic differences in the isolates; in our studies it appears not to be correlated with geographic differences but with differences in pathogenicity. Such a correlation, however, does not mean that the genes controlling pathogenicity are necessarily directly responsible for the incompatibility and further investigation is needed to resolve this. The incompatibility would restrict heterokaryosis between different strains of *T. cucumeris*, promoting inbreeding and the retention of distinct pathogenic strains, provided that production of pathogenic variants by mutation is at low frequency. The fact that specific pathogenic strains do coexist in some soils with no apparent intermediate types supports this hypothesis, and therefore on this evidence it would seem unlikely that free anastomosis of different pathogenic strains in soil to produce more violent strains, as suggested by Daniels (1963), commonly occurs. On the other hand, from our laboratory studies, it can be seen that such a cross is possible, giving rise to new pathogenic strains, e.g. a progeny culture capable of attacking both radish and cowpea. Earlier evidence suggests that basidiospores do not establish in soil (Flentje and Stretton 1964). However, although the frequency of such inter-isolate crosses occurring in nature may be very rare and the mechanisms for establishment of subsequent recombinants of the new genome inefficient, nevertheless this may be one way in which new pathogenic strains do arise in nature in time. If so, it follows that care must be exercised to prevent the introduction to an area of new isolates with the potential to interact genetically with local ones, and produce new strains of more destructive pathogenic abilities.

V. Acknowledgments

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