Heterokaryosis in *Fusarium oxysporum* f.sp. *lycopersici*

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SUMMARY

Intra-isolate and inter-isolate heterokaryons were synthesized between auxotrophic mutants of *Fusarium* crown rot, 'purple variant' and the wilt isolates. This is the first report of intra- and inter-isolate heterokaryons in *Fusarium oxysporum* f.sp. *lycopersici*. Conidial ratios determined for several heterokaryons between different mutants of the *Fusarium* crown rot organism showed that the ratio is constant for each heterokaryon and that the ratio usually is in favour of one of the mutants.

INTRODUCTION

A new strain of *Fusarium oxysporum* (Schl.) f.sp. *lycopersici* (Sacc.) Snyder and Hansen which causes a generalized root and crown rot of susceptible tomato varieties has been identified and described by Leary & Endo (1971). A characteristic of this race is the segregation of non-pathogenic 'purple variant' clones when the wild-type is propagated by single spore isolations. The mutagenesis and pathogenicity of these strains is the subject of a previous paper (Sanchez, Leary & Endo, 1975).

The present investigation was designed to test the ability of nutritionally marked strains of the crown rot, wilt and 'purple variant' types to form intra- and inter-strain heterokaryons. Conidial ratios were also studied and attempts were made to alter these ratios by adjusting the nutritional conditions.

METHODS

Strains. For the origin and details of the wild-type strains and the procedures used to obtain the mutants, see Sanchez *et al.* (1975).

Heterokaryon formation. With one method, 5 mm agar plugs of two different auxotrophic mutants were placed 2.5 cm apart on a Schizophyllum minimal agar (SMA) (Raper & Miles, 1958) plate supplemented with 10 μ mol of the required growth factors/ml. Another technique employed was a modification of the method of Caten & Jinks (1966). A 0.1 ml drop of a spore suspension containing 0.5×10^5 spores of each mutant was placed in the centre of a SMA plate supplemented with 10 μ mol of the growth factors required by each of the mutants/ml.

Complementation tests for heterokaryons. Agar plugs 1 mm in diameter were transferred from the areas of abundant growth on the supplemented SMA to SMA and Schizophyllum migration complete agar (SCA) plates (Snider & Raper, 1958), and growth of the presumed heterokaryons was compared.

Isolation of heterokaryotic hyphal tips and heterokaryotic mycelium fragments. The degree of complementation and the stability of the heterokaryon was assayed by the following

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procedures: (i) Single hyphal tip fragments 1 to 4 mm in length were cut from a heterokaryon growing on SMA and transferred to SMA and SMA supplemented with the individual growth factors. (ii) Complementing heterokaryons growing on SMA were blended in 25 ml of Schizophyllum minimal broth (SMB) in a Waring blender for two periods of 30 s interrupted by a 15 s interval. A 5 ml sample was pipetted into a 300 ml Erlenmeyer flask containing 25 ml of SMB and incubated at 25 °C for four days until a compact mat of mycelium was formed. The mat was washed five times with sterile water to remove the spores. The mat of mycelium was then blended as described above, the concentration of fragments was adjusted to 30 to 40/ml, and 1 ml portions of the fragment suspension were pipetted into 2 ml of molten 2 % (w/v) SMA. The entire 3 ml suspension was overlaid on SMA, SCA, and on SMA plus each growth factor. These cultures were incubated at 25 °C and assayed for growth after 48 h.

Determination of the conidial ratios of heterokaryons. Microconidia were harvested from complementing heterokaryons growing in SMA. Ten plugs, 5 mm in diameter, were taken at random, suspended in sterile water, agitated on a Vortex mixer and the spores separated by filtration through Whatman No. 1 filter paper. The spore concentration was adjusted to about 300/ml and 1 ml samples were pipetted into 2 ml of 2 % (w/v) molten SMA and overlaid on to SMA plates supplemented differentially with the growth factors required by the mutant components of the heterokaryon. The plates were incubated at 25 °C and the colonies counted after 48 h.

Cross-feeding experiments. The degree of cross-feeding between different pairs of mutants was determined by placing 5 mm plugs of each of the mutants 5 cm apart on SMA plates supplemented differentially with the growth factors required by the mutants. This was based on the assumption that only one mutant should grow normally throughout the plate, while the growth of the other mutant would not be stimulated unless cross-feeding occurred.

RESULTS

Heterokaryon formation

When combinations of mutants were made on minimal agar the conidia did not germinate and no significant mycelial growth from the agar plugs occurred under such conditions. No significant growth occurred when the medium was supplemented with less than 10 µmol of any required nutrient/ml. This concentration was used routinely in subsequent experiments. When a drop of spores was placed on the centre of a plate of SMA supplemented as described above, presumptive heterokaryons were detected as tiny actively growing colonies (Fig. 1*b*). The same visual indication was observed when two mutants were mated by placing agar plugs 2.5 cm apart on the supplemented SMA plate. Colonies which originated from complementation were easily detected in the area of interaction between the hyphae of the two mutants in the centre of the plate (Fig. 1*a*). Such colonies were visible between 10 and 12 days after the plates were inoculated. Most of the auxotrophic mutants tested showed no growth which could be attributed to cross-feeding, and only a few gave a positive response after the supplemented mutant had grown extensively for several days (Fig. 2*b*).

Growth from 1 mm plugs of several of the presumed heterokaryons synthesized between several pairs of mutants of the *Fusarium* crown-rot wild-type isolate on SMA and SCA is summarized in Table 1. Wide variation in the amount of growth among the different forced heterokaryons was observed. For example, when a mutant of the *Fusarium* crown-rot wild-type isolate (CR) which required inositol and tryptophan (*inos-1 trp-1*) was combined with each of several other mutants, the amount of growth varied depending upon the

Fig. 1. Techniques used to synthesize heterokaryons. (a) Plugs of two mutants were placed 2.5 cm apart on SMA plates supplemented with 10 µmol of both growth factors/ml. Tiny visible colonies formed in the area of mycelial interaction represent the formation of heterokaryons. (b) A drop of a mixture of spore suspension of the mating mutants (top plate) was placed in the centre of a supplemented SMA plate (same as above). Small visible colonies around the drop represented the formation of heterokaryons. No visible growth was observed in either of the two lower plates containing the mutants inoculated alone. Plates were incubated for 12 days at 25 °C.

Fig. 2. Cross-feeding tests. (a) Each plate was inoculated with both mutants (CR *inos-1 ade-1* and W *met-1*) 5 cm apart. The plate on the left was supplemented with adenine and the plate on the right with methionine. (b) Each plate was inoculated with the mutants, CR *inos-1 met-1* and CR *inos-1 arg-1*. The plate on the left was supplemented with methionine and the plate on the right with arginine. Plates were incubated for 10 days at 25 °C. (All plates contained inositol.)

Fig. 3. Compatible and non-compatible heterokaryon formation. Above: Heterokaryons (CR *inos-1 trp-1* + CR *inos-1 leu-4*) growing in SMA (right) and in SCA (left). Below: Mutants (CR *inos-1 trp-1* + P *inos-1 met-1*) which did not form heterokaryons; the plate on the right is SMA and the plate on the left is SCA. Plates were incubated for 6 days at 25 °C.

other mutant. When heterokaryons were synthesized between three different arginine-requiring mutants and CR *inos-1 nic-1* the growth of the heterokaryon was different for each combination. Some heterokaryons, such as those formed between the CR *inos-1 arg-4* + CR *inos-1 trp-1* and the CR *inos-1 arg-4* + CR *inos-1 leu-3* did not grow well when transferred from supplemented SMA to non-supplemented SMA (Fig. 3). Heterokaryons were formed between only a few of the mutants of the *Fusarium* wilt isolate (W), and those that did form had a reduced growth rate on SMA when compared with the growth on SCA (Table 2). Heterokaryons were formed between some pairs of mutants of CR and the *Fusarium* crown-rot 'purple variant' (PV). The growth of these heterokaryons on SMA supplemented with a low concentration of the required nutrients was good (Table 3), but when they were transferred to non-supplemented SMA the growth of the heterokaryons was significantly reduced. In the heterokaryons studied between the CR and PV isolates (Table 3), the nutritional requirements assorted exclusively with the parental phenotypes.

Only two successful heterokaryons were synthesized from the crosses between the several pairs of mutants of the W organism and CR (Table 3).

Combinations of mutants of the PV isolates and also those between mutants of the W isolates produced few heterokaryons, and their growth was generally poor. Heterokaryons were formed in crosses between a few pairs of mutants of the CR and the PV isolates. Since the 'purple variant' was obtained as a segregant from the CR isolate, we assumed that the

Table 1. *Synthesis and growth of heterokaryons between double mutants of the Fusarium crown-rot wild-type isolate (CR)*

Mutants*	Heterokaryon formation	
	Synthesis on supplemented SMA†	Growth after transfer to SMA‡
CR <i>trp-1</i> + CR <i>leu-1</i>	+++++	+++++
CR <i>trp-1</i> + CR <i>leu-4</i>	++++	++++
CR <i>trp-1</i> + CR <i>arg-3</i>	++++	++
CR <i>trp-1</i> + CR <i>arg-4</i>	++++	+
CR <i>trp-1</i> + CR <i>cys-2</i>	+++++	++
CR <i>ade-1</i> + CR <i>leu-2</i>	++++	++++
CR <i>ade-1</i> + CR <i>leu-3</i>	+++++	++++
CR <i>ade-1</i> + CR <i>arg-3</i>	+++++	++++
CR <i>ade-1</i> + CR <i>arg-4</i>	++	+
CR <i>ade-1</i> + CR <i>trp-1</i>	+++++	+++
CR <i>ade-1</i> + CR <i>nic-1</i>	+++++	+++++
CR <i>ade-1</i> + CR <i>cys-2</i>	+++++	++++
CR <i>cys-1</i> + CR <i>leu-2</i>	+++++	+++++
CR <i>nic-1</i> + CR <i>leu-3</i>	+++++	+++++
CR <i>nic-1</i> + CR <i>arg-2</i>	++	++
CR <i>nic-1</i> + CR <i>leu-3</i>	+++++	+++++
CR <i>nic-1</i> + CR <i>trp-1</i>	++++	+++
CR <i>nic-1</i> + CR <i>arg-4</i>	++++	+
CR <i>nic-1</i> + CR <i>arg-2</i>	++	++
CR <i>nic-1</i> + CR <i>arg-3</i>	++++	++++
CR <i>nic-1</i> + CR <i>cys-2</i>	+++++	+++++
CR <i>leu-3</i> + CR <i>arg-4</i>	+++++	+

* All strains used required inositol (*inos-1*) in addition to the requirements listed.

† Minimal agar supplemented with 10 µmol/ml concentrations of the growth factors required by both mutants.

‡ Growth of the heterokaryon —, no heterokaryon formed; +, ++, +++, ++++ and +++++, 10, 10 to 25, 25 to 50, 50 to 75, and 75 to 100% growth, respectively, as compared with growth on SCA.

Table 2. *Synthesis and growth of heterokaryons between single mutants of the Fusarium wilt isolate (W)*

Mutants	Heterokaryon formation	
	Synthesis on supplemented SMA*	Growth after transfer to SMA†
W <i>met-2</i> + W <i>inos-1</i>	+	+
W <i>met-2</i> + W <i>arg-3</i>	—	—
W <i>arg-3</i> + W <i>inos-1</i>	+	—
W <i>arg-3</i> + W <i>met-3</i>	—	—
W <i>arg-3</i> + W <i>trp-1</i>	—	—
W <i>arg-1</i> + W <i>trp-1</i>	—	—
W <i>met-2</i> + W <i>trp-1</i>	++	+
W <i>met-3</i> + W <i>trp-1</i>	—	—
W <i>cys-1</i> + W <i>trp-1</i>	—	—
W <i>inos-1</i> + W <i>trp-1</i>	—	—
W <i>inos-1</i> + W <i>arg-1</i>	++	+
W <i>inos-1</i> + W <i>met-3</i>	—	—
W <i>inos-1</i> + W <i>cys-1</i>	—	—

* Minimal agar supplemented with 10 µmol/ml concentrations of the growth factors required by both mutants.

† Growth of the heterokaryon (as in Table 1).

Table 3. *Synthesis and growth of heterokaryons between mutants of the Fusarium crown-rot wild-type (CR) and the 'purple variant' (PV) isolates, and between mutants of the Fusarium crown-rot wild-type and Fusarium wilt isolates (W)*

Mutants*		Heterokaryon formation	
		Synthesis on supplemented SMA†	Growth after transfer to SMA‡
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 arg-1</i>	—	—
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 arg-4</i>	—	—
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 trp-1</i>	+++	+
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 leu-4</i>	++	+
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 nic-1</i>	++++	+
PV <i>inos-1 met-1</i>	+ CR <i>inos-1 ade-1</i>	++++	+
PV <i>trp-1</i>	+ CR <i>inos-1</i>	++	++
PV <i>trp-2</i>	+ CR <i>met-1</i>	—	—
PV <i>trp-2</i>	+ CR <i>pan-1</i>	—	—
CR <i>ade-1</i>	+ W <i>arg-3 met-3</i>	+++	—
CR <i>ade-1</i>	+ W <i>arg-3</i>	++++	++
CR <i>leu-3</i>	+ W <i>arg-3 met-3</i>	—	—
CR <i>leu-1</i>	+ W <i>arg-4 met-1</i>	—	—
CR <i>leu-4</i>	+ W <i>arg-3 met-2</i>	—	—
CR <i>nic-1</i>	+ W <i>arg-3 met-5</i>	—	—
CR <i>trp-1</i>	+ W <i>arg-3 met-2</i>	—	—

* Each of the CR double mutants required inositol (*inos-1*) in addition to the requirements listed.

† Minimal agar supplemented with 10 µmol/ml concentrations of the growth factors required by the two mutants.

‡ Growth of the heterokaryon (as in Table 1).

formation of heterokaryons between the two would occur readily. For unexplained reasons, this was not true. Heterokaryons were also synthesized in crosses between some mutants of the CR isolate and the W isolate.

Conidial ratios and growth of several heterokaryons of the Fusarium crown-rot wild-type mutants

Conidial ratios were determined for the CR *inos-1 leu-4* + CR *inos-1 trp-1* heterokaryons growing on supplemented SMA (Table 4). The spore ratio was several fold in favour of the tryptophan requirer over the leucine requirer, even though the heterokaryons were growing on SMA supplemented with leucine. These heterokaryons, when transferred to SMA, showed the same conidial ratios as reported in Table 4. Conidial ratios from other heterokaryons growing on SMA indicated that in the heterokaryons studied, one of the mutants predominated (Table 5). In the heterokaryon CR *inos-1 ade-1* + CR *inos-1 leu-1*, the leucine mutant (*leu-1*) predominated. However, when CR *inos-1 ade-1* was used in a cross with CR *inos-1 leu-4*, the conidial ratio of the heterokaryon changed so that the adenine mutant predominated.

The different heterokaryons varied greatly in colony diameter after a fixed growth period. In heterokaryons formed between CR *inos-1 nic-1* and three other mutants, the growth of each heterokaryon was obvious upon macroscopic examination.

Table 4. *Conidial ratios of several heterokaryons of mutants of the Fusarium crown-rot wild-type isolate recovered from minimal agar supplemented with leucine*

Heterokaryon CR <i>inos-1 leu-4</i> + CR <i>inos-1 trp-1</i>	No. of colonies on SMA plus leucine*	No. of colonies on SMA plus tryptophan*	Conidial ratio <i>leu/trp</i> †
I	117	415	1:4
II	73	250	1:3
III	58	291	1:5
IV	140	328	1:2
V	26	125	1:5

* Total number of colonies from five plates.

† Spore ratio determined directly from the heterokaryons formed on SMA plus leucine.

Table 5. *Conidial ratios and growth of several heterokaryons synthesized between mutants* of the Fusarium crown-rot wild-type isolate*

Heterokaryon	Conidial ratio†	Colony diameter (%)‡
CR <i>ade-1</i> + CR <i>nic-1</i>	1:1	84.0
CR <i>ade-1</i> + CR <i>leu-1</i>	1:6	58.8
CR <i>ade-1</i> + CR <i>leu-4</i>	7:1	42.2
CR <i>ade-1</i> + CR <i>arg-1</i>	6:1	22.6
CR <i>trp-1</i> + CR <i>nic-1</i>	1:7	36.3
CR <i>leu-4</i> + CR <i>nic-1</i>	1:8	54.4
CR <i>leu-4</i> + CR <i>trp-1</i>	1:3	57.1

* Each of the mutants required inositol (*inos-1*) in addition to the requirements listed.

† The first number of the ratio corresponds to the first mutant of the heterokaryon.

‡ Colony diameter of the heterokaryon in SMA, compared with the growth in SCA after 7 days at 25 °C.

Table 6. *Induced alteration in the nuclear ratios and colony diameters of the heterokaryons of Fusarium crown-rot wild-type isolate*

Leucine or tryptophan added to minimal agar (μmol/ml)	Heterokaryons			
	<i>nic-1</i> + <i>trp-1</i> plus tryptophan		<i>nic-1</i> + <i>leu-4</i> plus leucine	
	Nuclear ratio	Colony diameter (%)	Nuclear ratio	Colony diameter (%)
SMA	1:40	40.2	1:8.3	46.1
SMA + 0.1	1:51	42.0	1:4.6	47.4
SMA + 1.0	1:41	41.3	1:7.3	46.7
SMA + 10	1:26	55.8	1:4.0	48.5
SMA + 100	1:13	91.6	1:8.7	47.2
SCA	1:39	100.0	1:250	100.0

Induced alterations in the conidial ratios and colony diameters of heterokaryons

The conidial ratio of heterokaryons remained constant and was specific to the mutants used to establish the heterokaryons. The conidial ratios of several heterokaryons frequently and reproducibly showed that one spore type greatly outnumbered the other. Heterokaryons were grown on SMA supplemented with increasing concentrations of the growth requirement of the mutant not favoured in the conidial ratio, in an attempt to modify that ratio. In one of two heterokaryons studied, the conidial ratio and growth of the colonies was modified significantly (5 % level) by supplementing the SMA. In the other heterokaryon CR *inos-1 nic-1* × CR *inos-1 leu-4*, colony growth was modified only slightly (Table 6).

DISCUSSION

The synthesis of heterokaryons between races of *F. oxysporum* f.sp. *pisi* has been reported by Buxton (1956), Tuveson & Garber (1959, 1961) and Fleischmann (1963); between form species by Garber, Wyttenbach & Dhillon (1961); and between species by Singh & Hoffman (1969). Whether this last report dealt with true species is questionable. The present report is the first of heterokaryon formation between different races of *F. oxysporum* f.sp. *lycopersici*. We have demonstrated that heterokaryons can be formed between mutants manifesting different degrees of pathogenicity and between mutants of the pathogenic *Fusarium* crown-rot wild-type (CR) strain and mutants of the non-pathogenic *Fusarium* crown-rot 'purple variant' (PV). Heterokaryons were easily formed between mutants of the CR isolate using several different mutant combinations.

Cross-feeding tests conducted with several pairs of mutants of the three fungal strains demonstrated that growth due to cross-feeding did not occur with most of the mutants tested.

The finding that some pairs of mutants from the same isolates can form heterokaryons while others cannot, suggests three possible explanations: (1) that some pairs of mutants are unable to complement the reciprocal nutritional requirements; (2) that heterokaryon incompatibility loci are involved; (3) that the strains used are intrinsically incapable of forming heterokaryons for some other reason.

Evidence for hypothesis (1) is as follows: (i) Some heterokaryons grew well on supplemented SMA, but when transferred to SMA the growth was poor. (ii) Most of the presumed heterokaryotic hyphal tips were unable to grow on SMA. However, when hyphal tips were isolated on SCA, spores having both parental requirements were recovered. (iii) The recovery of heterokaryotic hyphal fragments from minced mycelium was increased several times when the fragments were overlaid on SMA supplemented with the growth factor of the minority mutant in the conidial ratio. (iv) In one of the heterokaryons studied (Table 1), the colony diameter was increased from 40 to 91 % of that on SCA when the heterokaryon was grown on SMA supplemented with the amino acid of the mutant low in the conidial ratio. Therefore, the growth of the heterokaryon was limited by one of the component mutants. (v) The fact that the only heterokaryon studied which had a 1:1 nuclear ratio (Table 1) also exhibited growth most comparable to the wild-type suggests that the nuclear ratio, the growth rate and the reciprocal nutritional complementation of the component mutants of the heterokaryon are intimately related.

Evidence for hypothesis (2), heterokaryon incompatibility, is based on mutants that failed to form heterokaryons in certain combinations but were able to form heterokaryons when mated with other mutants having the same growth requirements. This reaction was observed with intra- and inter-isolate crosses. If such changes in heterokaryon incompatibility loci result from induced mutations, then a similar type of incompatibility may arise in nature by spontaneous mutation. The existence of such incompatibility mechanism in nature would limit the variation of a fungus by preventing the formation of heterokaryons (Caten, 1972).

No attempt was made to differentiate between these possibilities and an intrinsic inability to form heterokaryons.

Both conidial types were recovered from all of the heterokaryons studied (Table 4), and usually one outnumbered the other. Conidial ratios proved to be characteristic of the particular heterokaryon under study. Determinations made at several different times and from many different isolations always produced similar nuclear ratios. The ratio of the two different nuclei is apparently stabilized at a certain level to provide for optimum growth of

the heterokaryotic mycelium (Warr & Roper, 1966). The nuclear ratio in *Neurospora crassa* heterokaryons appears to be characteristic for particular heterokaryons (Prout *et al.*, 1953) and the nuclear proportions remain stable during growth of the heterokaryon (Pittinger & Atwood, 1956). In species such as *F. oxysporum*, where the conidia are uninucleate, the nuclear ratio is usually obtained by determining the conidial ratio of the heterokaryon after growth on minimal medium. Assuming that the conidial ratio of a heterokaryon represents the true population of nuclei in the mycelium, it should be possible to manipulate the nuclear proportion of a mutant in a heterokaryon by mating it with different mutants that result in different ratios. This technique would provide valuable information for studies of phenotypic expression of individual nuclei in heterokaryons.

Finally, the data taken together argue against the existence of heteroplasmon as an explanation for the segregation of the 'purple variant' and indicate that the 'purple-variant' phenotype and the nutritional requirements are both nuclear. Therefore, the most reasonable explanation for the segregation of the 'purple variant' as a constant proportion of the single conidia is that the 'potential' isolates exist as heterokaryons and that the ratio of 'purple-variant' nuclei to wild-type nuclei is under endogenous control similar to that exerted over the nuclear ratio of different auxotrophic mutants in forced heterokaryons.

These studies were submitted by L.E.S. to the University of California, in partial fulfillment of the requirements for a Ph.D.

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