Chemical Mutagenesis of *Fusarium oxysporum* f. sp. *lycopersici*: Non-selected Changes in Pathogenicity of Auxotrophic Mutants

By L. E. SANCHEZ, J. V. LEARY AND R. M. ENDO*

*Department of Plant Pathology, University of California, Riverside,
California 92502, U.S.A.*

(Received 10 October 1974)

SUMMARY

Single and multiple auxotrophic mutants of the *Fusarium oxysporum* f. sp. *lycopersici* strains which cause Fusarium crown-rot and Fusarium wilt of tomato were obtained by chemical mutagenesis with nitrous acid, nitrosoguanidine and ethyl-methanesulphonate. The mutagenesis and selection procedures, adapted for use with a plant pathogenic fungus, are described. Changes in pathogenicity were observed when the auxotrophs were compared with the wild type but no correlation was observed between changes in pathogenicity and the particular nutritional requirement.

INTRODUCTION

A new strain of *Fusarium oxysporum* (Schl.) f. sp. *lycopersici* (Sacc.) Snyder and Hansen which causes a generalized dark-brown rot of the crown and stem tissues of infected tomato plants has been described (Leary & Endo, 1971). An intriguing feature of this new strain is the continued segregation of variants when the wild type is cloned by single-spore isolation (Leary, 1972). The variants differ from the wild type in colony and hyphal morphology, produce large quantities of a purple pigment, produce macroconidia almost exclusively at normal growth temperatures, and are uniformly low in pathogenicity. They are called 'purple variants'.

The segregation of such atypical, non-pathogenic, single-spore variants of *Fusarium oxysporum* had been reported previously (Brown, 1926; Haymaker, 1928; Wellman, 1943). Most of these reports attributed this segregation to a high rate of mutation and suggested that changes in the degree of pathogenicity are a result of pleiotropy. Changes in pathogenicity coincident with auxotrophy and attributed to auxotrophy have been described (Garber, Shaeffer & Goldman, 1956; Tuveson & Garber, 1959; Coplin, Sequeira & Hanson, 1974). This suggests that a pathogen which required a specific substrate for growth would not be successful in establishing a compatible host-parasite relationship unless supplied with that substrate (Garber, 1954, 1956; Tuveson & Garber, 1959).

To investigate this question, a large number of chemically induced mutants were obtained which required the same growth substance, and these mutants were assayed for change in pathogenicity. There are few reports on the use of chemical mutagens in genetic studies on plant pathogenic fungi, and previous workers apparently have relied on mass-isolation of surviving propagules to select mutants. Therefore, we developed defined conditions for chemical mutagenesis and enrichment of auxotrophic mutants. We describe the changes in degree of pathogenicity and expression of symptoms of singly- and multiply-marked auxotrophic mutants obtained by chemical mutagenesis under defined conditions.

* Present address: Faculty of Agronomy, University of Chile, Santiago.

METHODS

Strains and media. The wild-type isolate of the *Fusarium* crown-rot organism was obtained from infected tomato seed and subsequently passed through the susceptible tomato variety, Bonny Best. The clones of the 'purple variant' used were obtained as single-spore isolates of microconidia from the wild type. The culture of the Race 1 isolate of the *Fusarium* wilt organism used throughout these studies was obtained from the culture collection within this department.

Media utilized routinely were potato-dextrose agar (PDA), water agar (WA) and Schizophyllum migration complete medium (SC) (Snider & Raper, 1958), and Schizophyllum minimal medium (SM) (Raper & Miles, 1958).

Mutagenesis procedures

Production of spores. Whenever possible, only microconidia were used for mutagenesis. To obtain microconidia of the wild-type *Fusarium* crown-rot organism and of the *Fusarium* wilt organism, the fungi were grown in SC broth at 25 °C on a reciprocal shaker for four days. The microconidia were separated from mycelium and macroconidia by filtration, centrifuged at 196 g (average) in a clinical centrifuge for 5 min, and washed twice by resuspension in sterile distilled water. The final concentration of spores treated with the mutagen was adjusted routinely to 10⁶ microconidia/ml.

To obtain a large number of microconidia of the 'purple variant', culture conditions had to be altered. At optimal temperatures for growth (24 to 27 °C), primarily macroconidia are produced; however, at the extremes (15 to 18 °C or 33 to 35 °C) in still culture, microconidia developed almost exclusively. Therefore, the latter conditions were used routinely with the 'purple variant'.

Mutagens. The chemical mutagens utilized were nitrous acid (HNO₂) ethylmethanesulphonate (EMS), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Conditions of mutagenesis yielding 10 % survival were determined for each mutagen and used throughout these studies.

Mutagenesis. The precise conditions for mutagenesis were as follows:

(i) Nitrous acid. Washed conidia were resuspended in 0.1 M-acetic acid-sodium acetate buffer, pH 4.4. A freshly prepared solution of NaNO₂ (0.5 M) was added to give a final concentration of 0.05 M, and 2 × 10⁷ conidia were treated at 25 °C in 20 ml of a solution containing 0.66 ml NaNO₂ (0.5 M) and 19.33 ml acetate buffer (0.1 M, pH 4.4). The treatment was stopped at previously determined times by transferring portions to phosphate buffer pH 8.0 and washing twice by centrifuging at 196 g (average). Finally, the washed conidia were resuspended in sterile saline for further manipulation.

(ii) Ethylmethanesulphonate. An equal volume of EMS solution in phosphate buffer (pH 8.0) was mixed with washed conidia suspended in distilled water to give final concentrations of 3 % (w/v) EMS and 10⁶ conidia/ml. Mutagenesis was terminated at previously determined times by diluting the cell suspension with two volumes of sterile phosphate buffer. The conidia were washed twice with sterile phosphate buffer and the final pellet was resuspended in phosphate buffer.

(iii) Nitrosoguanidine. We treated 10⁶ conidia with NTG at a concentration of 30 to 45 µg/ml at 25 °C. Mutagenesis was terminated by dilution with twice the volume of sterile distilled water. The conidia were concentrated by centrifuging at 196 g (average), washed twice with sterile water, and resuspended in sterile water.

Selection procedures. These were as follows:

(i) Filtration enrichment. Washed spores were incubated in 50 ml SM broth. Spores that did not germinate were separated from prototrophic germings by filtration at 24, 12 and 12 h intervals after inoculation. After the first and second filtrations, the spores were concentrated by centrifugation, washed twice in sterile distilled water, and resuspended in 50 ml fresh SM broth. Following the third filtration, 1 ml of the appropriate dilution of conidia was mixed with 1 ml molten 1 % SC agar and the entire mixture overlayed on SC agar in standard Petri plates. Normally, the dilution used yielded 30 conidia/plate. The overlayed plates were incubated at 25 °C for 48 h, after which time the survivors had produced visible colonies 2 to 4 mm in diameter with abundant conidia.

(ii) Inositolless starvation technique. The isolation of inositol-requiring mutants of the wild-type crown-rot strain permitted the use of inositolless-death selection (Lester & Gross, 1959; Shatkin & Tatum, 1961) to obtain multiple-auxotrophs. The inositol-requiring isolates were treated with mutagen and processed through filtration-enrichment in minimal medium containing inositol. After the third filtration, the remaining ungerminated conidia were concentrated, washed, overlayed on SM agar plates lacking inositol, and incubated at 25 °C for 4 days. At the end of this period, the plates were overlayed with SC agar, and the surviving conidia which produced visible colonies were isolated.

(iii) Characterization of auxotrophic mutants. Conidia of colonies which developed in SC agar after filtration and/or inositolless-death-enrichment were transferred to SC and SM agar. If no growth was visible after three transfers to minimal medium, conidia of the presumptive auxotrophs were transferred to minimal medium containing nutrient pools (Holliday, 1956). Single, double, and triple mutants were all characterized in this way.

Pathogenicity tests

The auxotrophic mutants were tested for pathogenicity as follows:

(i) Mutants of the wild-type crown-rot strain (CR) and the crown-rot variant (PV) were tested by a seedling-agar plate technique (Sanchez, 1974; Sanchez, Endo & Leary, 1974).

(ii) Mutants of the crown-rot wild-type and the crown-rot purple variant were tested under greenhouse conditions by attaching 0.5 cm plugs of SM agar containing mycelium and conidia to the stems of Bonny Best tomato plants with latex bandages (Sealtex). Symptom development was assayed after 7 to 14 days.

(iii) Pathogenicity of mutants of the *Fusarium* wilt strain was tested by dipping the roots of 7-day-old seedlings into a 2×10^6 /ml suspension of conidia and transplanting three infested seedlings (3 seedlings/pot) into steamed greenhouse planting mix. These plants were assayed for disease symptoms 2 weeks after transplanting.

RESULTS

Auxotrophic mutants from the wild-type *Fusarium* crown-rot strain and from the wild-type *Fusarium* wilt strain were isolated and characterized. The individual mutants required one, two or three exogenously-supplied nutrients for growth. Only one multiple mutant of the 'purple variant' of the crown-rot isolate has been isolated, possibly because the mutants appear to revert to prototrophy at a higher frequency than expected.

Each of the auxotrophic mutants was tested for pathogenicity by the seedling test or by greenhouse procedures. Mutants exhibiting the same requirement differed in their pathogenicity (Tables 1 and 2). For example, within groups of double mutants of the wild-type *Fusarium* crown-rot strain, i.e. *inos-met*, *inos-cys*, *inos-leu*, and *inos-arg* (Table 1), patho-

Table 1. *Auxotrophic mutants of the Fusarium crown-rot wild-type isolate and their pathogenicity*

The pathogenicity was tested in WA plates supplemented with the respective growth factors. The designation of mutation, e.g. *met-1*, *met-6*, refers to different isolates and does not imply different loci.

Isolate	Pathogenicity	Mutants	Pathogenicity
Wild-type	++++	CR (<i>inos-1 leu-1</i>)	++++
CR (<i>met-1</i>)	++++	CR (<i>inos-1 leu-2</i>)	+++
CR (<i>pan-1</i>)	++++	CR (<i>inos-1 leu-3</i>)	++
CR (<i>inos-1</i>)	++++	CR (<i>inos-1 leu-4</i>)	-
CR (<i>inos-1 met-2</i>)	+++	CR (<i>inos-1 arg-1</i>)	++++
CR (<i>inos-1 met-3</i>)	+++	CR (<i>inos-1 arg-2</i>)	++
CR (<i>inos-1 met-4</i>)	+++	CR (<i>inos-1 arg-3</i>)	+
CR (<i>inos-1 met-5</i>)	++	CR (<i>inos-1 arg-4</i>)	-
CR (<i>inos-1 met-6</i>)	+	CR (<i>inos-1 ade-1</i>)	+++
CR (<i>inos-1 met-7</i>)	+	CR (<i>inos-1 try-1</i>)	+++
CR (<i>inos-1 cys-1</i>)	++++	CR (<i>inos-1 nic-1</i>)	+++
CR (<i>inos-1 cys-2</i>)	++++	CR (<i>inos-1 cys-2 trp-2</i>)	++++
CR (<i>inos-1 cys-3</i>)	++++	CR (<i>inos-1 cys-2 leu-5</i>)	++++
CR (<i>inos-1 cys-4</i>)	++++	CR (<i>inos-1 cys-2 leu-6</i>)	++
CR (<i>inos-1 cys-5</i>)	++++	CR (<i>inos-1 cys-2 leu-7</i>)	++++
CR (<i>inos-1 cys-6</i>)	++++	CR (<i>inos-1 cys-2 arg-5</i>)	++++
CR (<i>inos-1 cys-7</i>)	++++	CR (<i>inos-1 cys-2 arg-6</i>)	++++
CR (<i>inos-1 cys-8</i>)	+++	CR (<i>inos-1 cys-2 hypox-1</i>)	++
CR (<i>inos-1 cys-9</i>)	+	CR (<i>inos-1 cys-2 hypox-2</i>)	++
CR (<i>inos-1 cys-10</i>)	+		

CR, *Fusarium crown-rot* wild-type; *inos*, requires inositol; *hypox*, requires hypoxanthine.

Table 2. *List of mutants of the Fusarium wilt isolate and their pathogenicity on Bonny Best tomato seedlings*

The designation of mutations, e.g. *met-1*, *met-6*, refers to different isolates and does not imply different loci. Wilt symptoms were produced after inoculation.

Single mutants	Pathogenicity	Multiple mutants	Pathogenicity
Wild-type	++++		
W (<i>arg-1</i>)	++++	W (<i>arg-3 met-4</i>)	-
W (<i>arg-2</i>)	-	W (<i>arg-3 met-5</i>)	+++
W (<i>arg-3</i>)	+++	W (<i>arg-3 met-6</i>)	+++
W (<i>met-1</i>)	-	W (<i>hypox-1 cys-1</i>)	-
W (<i>met-2</i>)	Pith rot	W (<i>arg-3 met-5 leu-1</i>)	-
W (<i>met-3</i>)	+++		
W (<i>trp-1</i>)	-		
W (<i>inos-1</i>)	-		
W (<i>cys-1</i>)	-		

W, *Fusarium wilt* race 1 isolate; for other abbreviations see Table 1.

genicity ranged through the entire rating system of highly pathogenic (++++) to no visible symptoms (-). The same pattern was seen in the triple mutants where CR (*inos-1 cys-2*) was used as the parent. Also, of the three independently-isolated leucine mutations, only one changed in pathogenicity.

None of the mutants of the *Fusarium wilt* strain (Table 2) had intermediate pathogenicity. Again, however, no correlation between loss of pathogenicity and the particular nutritional requirement was observed. For example, two of the three arginine-requiring mutants were able to infect and to become systemic in the plant under greenhouse conditions without arginine supplementation of the spore suspension or the soil mixture.

Table 3. *List of auxotrophic mutants of the Fusarium crown-rot 'purple variant' isolate and their pathogenicity*

The pathogenicity was tested in WA plates supplemented with the respective growth factors. The designation of mutations, e.g. *trp-1*, *trp-2*, refers to different isolates and does not imply different loci.

Single mutations	Pathogenicity	Double mutations	Pathogenicity
Wild-type	++	PV (<i>inos-1 met-3</i>)	++
PV (<i>trp-1</i>)	-		
PV (<i>trp-2</i>)	++		
PV (<i>ade-1</i>)	++		
PV (<i>met-1</i>)	++++		
PV (<i>met-2</i>)	++++		

PV, *Fusarium* crown-rot 'purple variant'; for other abbreviations see Table 1.

There was no correlation between the number of nutritional requirements and the pathogenicity of an isolate.

Changes in pathogenicity were in both directions. Some mutants of the highly pathogenic *Fusarium* crown-rot strain (Table 1) and *Fusarium* wilt strain (Table 2) lost pathogenicity, whereas some mutants of the *Fusarium* crown-rot 'purple variant' were as pathogenic as the highly pathogenic strains (Table 3). Where mutants of the 'purple variant' showed increased pathogenicity, the morphology was also altered to that of the wild-type crown-rot strain.

One mutant of the wilt strain was unable to produce wilt symptoms but produced a generalized rot of all the tissues of the stem. The symptoms were different from those produced by the crown-rot strains under similar root-dip inoculation procedures. When this mutant was tested by placing an agar plug containing mycelium on the stem of susceptible tomato plants, no crown-rot-type symptoms were produced. Revertants of this mutant which still had the methionine requirement produced typical wilt symptoms.

DISCUSSION

Previous research on the influence of auxotrophic mutations on pathogenicity have indicated a relationship between failure to produce symptoms (loss of pathogenicity) and the inability of the mutant to grow and infect because of the lack of the required nutrient (Garber, 1954, 1956). Attempts have been made to correlate specific nutritional requirements with loss of pathogenicity (Garber, 1954; Tuveson & Garber, 1959; Lippincott & Lippincott, 1966; Coplin *et al.* 1974). This nutrition-inhibition hypothesis (Garber, 1956) does not apply to the auxotrophic mutants of the tomato-*Fusarium oxysporum lycopersici* host-parasite system studied.

In all the pathogenicity tests on mutants, minimum concentrations of the required nutrients were added to the agar to ensure germination and growth. Even when the required nutrients were added at concentrations which permitted optimum growth, mutants that were poorly pathogenic or non-pathogenic failed to demonstrate increased pathogenicity.

Mutants with the same single or multiple requirements (e.g. *inos* or *inos-met*) had different requirements for optimum growth. In several instances, the mutants that required a low concentration of the nutrient to achieve growth similar to the wild-type, were the least pathogenic even when the required nutrients were present.

Series of mutants having a common requirement manifested differing degrees of pathogenicity under all test conditions. Each of these mutants must have been the result of an independent mutational event because of the selection procedures employed. The filtration times used permitted the removal of germinated surviving conidia before any new conidia were produced. Therefore, the ungerminated conidia which were enriched were primarily auxotrophs.

The mutagenesis resulted in changes in genetic loci other than those involved in the biosynthetic pathway for which the requirement was imposed. The number and function of these loci are at present unknown but we feel reasonably certain they are not concerned with the nutrition of the organism. The procedure for mutagenesis and selection of mutants described should offer several advantages to future studies: (i) By controlled selection, mutants of the same species which exhibit distinct pathogenic symptoms produced and/or the degree of virulence can be specifically marked. Thus, any changes in pathogenicity will be identified and specifically correlated with the particular mutants under investigation. (ii) The availability of several multiply marked mutants, each with a common 'first' requirement but different 'second' and 'third' requirements, facilitates studies of the effect of nutritional requirements on the virulence of pathogens. (iii) Once a strain is suitably marked with distinct nutritional requirements which can be selected for, the effects of subsequent mutagenesis on such characteristics as virulence can be studied directly.

The mutants described here and the procedures for obtaining them offer advantages for future studies of such phenomena as (i) the importance of heterokaryosis versus heteroplasmosis in the determination and/or maintenance of pathogenicity of fungi, (ii) the importance of mutation in producing observed changes in pathogenicity characteristics of particular races of an organism, and (iii) the frequency of change in the symptoms produced by a pathogen due to mutation.

This work forms part of the Ph.D. thesis submitted to the University of California by L.S.

REFERENCES

- BROWN, W. (1926). Studies in the genus *Fusarium*. IV. On the occurrence of saltations. *Annals of Botany* **40**, 203–221.
- COPLIN, D. L., SEQUEIRA, L. & HANSON, R. S. (1974). *Pseudomonas solanacearum*: virulence of biochemical mutants. *Canadian Journal of Microbiology* **20**, 519–529.
- GARBER, E. D. (1954). The role of nutrition in the host–parasite relationship. *Proceedings of the National Academy of Sciences of the United States of America* **40**, 1112–1116.
- GARBER, E. D. (1956). A nutrition inhibition hypothesis of pathogenicity. *American Naturalist* **90**, 183–194.
- GARBER, E. D., SHAEFFER, S. G. & GOLDMAN, M. (1956). The virulence of biochemical mutants of *Erwinia aroidea* for varieties of radish and turnip. *Journal of General Microbiology* **14**, 261–267.
- HAYMAKER, H. H. (1928). Pathogenicity of two strains of the tomato wilt fungus, *Fusarium lycopersici* Sacc. *Journal of Agricultural Research* **36**, 675–695.
- HOLLIDAY, R. (1956). A new method for the identification of biochemical mutants of microorganisms. *Nature, London* **178**, 1987.
- LEARY, J. V. (1972). Heterokaryosis of *Fusarium oxysporum* Schlecht causing crown rot of tomato. *Phytopathology* **62**, 771.
- LEARY, J. V. & ENDO, R. M. (1971). A *Fusarium* induced root rot of staked tomatoes. *Phytopathology* **61**, 900.
- LESTER, H. E. & GROSS, S. R. (1959). Efficient method for selection of auxotrophic mutants of *Neurospora crassa*. *Science, New York* **127**, 572.
- LIPPINCOTT, B. B. & LIPPINCOTT, J. A. (1966). Characteristics of *Agrobacterium tumefaciens* auxotrophic mutant infectivity. *Journal of Bacteriology* **92**, 937–945.

- RAPER, J. R. & MILES, P. G. (1958). The genetics of *Schizophyllum commune*. *Genetics* **43**, 532-546.
- SANCHEZ, L. E. (1974). Heterokaryosis and genetic variability of a *Fusarium oxysporum* causing stem and root rot of tomato. Ph.D. thesis, University of California, U.S.A.
- SANCHEZ, L. E., ENDO, R. M. & LEARY, J. V. (1974). A rapid technique for identifying the *Fusarium* crown rot of tomato. *Phytopathology* (in the Press).
- SHATKIN, A. J. & TATUM, E. L. (1961). The relationship of *m*-inositol to morphology in *Neurospora crassa*. *American Journal of Botany* **48**, 760-771.
- SNIDER, P. J. & RAPER, J. R. (1958). Nuclear migration in the basidiomycete *Schizophyllum commune*. *American Journal of Botany* **45**, 538-546.
- TUVESON, R. W. & GARBER, E. D. (1959). Genetics of phytopathogenic fungi. I. Virulence of biochemical mutants of *Fusarium oxysporum* f. *lisi*. *Botanical Gazette* **121**, 69-74.
- WELLMAN, F. L. (1943). Increase of pathogenicity in tomato-wilt *Fusarium*. *Phytopathology* **33**, 175-193