Heterokaryosis in *Fusarium tricinctum* and *F. sporotrichioides*

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Heterokaryons were formed in intra- and interspecific crosses between *Fusarium sporotrichioides* and *F. tricinctum* auxotrophs. Segregant homokaryons were evaluated for trichothecene toxin production in culture. Results were consistent with nuclear control of toxin synthesis. The sexual compatibility of auxotrophs and 30 additional *F. tricinctum sensu* Snyder & Hansen strains was tested. Perithecial production was restricted to crosses between Florida isolates pathogenic to English ivy (*Hedera helix*). The linkage of several auxotrophic markers was determined by analysis of progeny of certain crosses. No T-2 toxin was produced by sexually compatible *F. tricinctum* isolates.

INTRODUCTION

Fusarium tricinctum (Corda) Sacc. and *Fusarium sporotrichioides* Sherb. are common cereal fungi capable of trichothecene (12,13-epoxytrichothecene) synthesis (Bamburg & Strong, 1971; Booth, 1971; Smalley & Strong, 1974; Mirocha *et al.*, 1977; Pathre & Mirocha, 1977). Ingestion of infested cereal products by farm animals may result in severe or lethal toxicoses (Hsu *et al.*, 1972). The magnitude of strain toxicity varies considerably (Cullen, 1981; Cullen *et al.*, 1982), and loss of toxicity during culture storage or with repeated transfer is common (Bamburg & Strong, 1971; Marasas, 1969; Cullen, 1981). Possible mechanisms of culture degeneration include spontaneous mutation, and segregation of heterokaryotic or extrachromosomal elements.

Little is known about genetic regulation of terpene synthesis in fungi. Genetic control of diterpene synthesis has been studied by analysing sexual crosses of *Gibberella fujikuroi* Sawada (= *F. moniliforme*) (Phinney & Spector, 1967). Several non-allelic genes regulating gibberellin production were identified. No information is available concerning the genetics of trichothecene biogenesis.

Gibberella tricincta, the perfect state of *F. tricinctum*, was recently identified among strains pathogenic on English ivy (*Hedera helix*) in Florida (El-Gholl *et al.*, 1978). The mating system involves bipolar heterothallism. The toxicity of these perithecial isolates and their sexual compatibility with strains from different regions were not reported.

Analyses of vegetation and soil samples from Southeast Asia have shown the presence of mixtures of *Fusarium* toxins (i.e. T-2 toxin, diacetoxyscirpenol, deoxynivalenol, nivalenol and zearalenone) (Mirocha *et al.*, 1982) which, on the basis of numerous laboratory investigations (Bamburg & Strong, 1971; Smalley & Strong, 1974; Suzuki *et al.*, 1980), cannot be attributed to any single isolate of *Fusarium*. It is of considerable interest to know whether new phenotypes, in terms of toxin production, can be generated by genetic recombination or heterokaryosis.

Abbreviations: CMC, carboxymethylcellulose medium; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; SC, Snider's complete medium; SCA, Snider's complete medium + agar; VM, Vogel's minimal medium; VMA, Vogel's minimal medium + agar

The objectives of the present study were to ascertain the toxicity and range of sexual compatibility of *G. tricineta* strains, and to synthesize heterokaryons between auxotrophs. Through analysis of selected crosses and heterokaryons we define linkage relationships of several genetic markers and present evidence that T-2 toxin production is under nuclear control.

METHODS

Sources of cultures. Cultural variants of *G. tricineta* pigmented red (R-P), white (W-P) and brown (B-P) were mating types +, - and -, respectively. These are the same strains used in the original description of the perfect state and were generously supplied by N. E. El-Gholl (Florida Department of Agriculture and Consumer Services, Gainesville, Fla., U.S.A.). *Fusarium sporotrichioides* strain T-2 (NRRL 3299) was isolated from corn in France and was used extensively in early trichothecene studies (Bamburg & Strong, 1971; Smalley & Strong, 1974).

Test crosses were prepared between all pairwise combinations of the four strains described above and 30 additional strains in the sections *Sporotrichiella* and *Arthrosporiella* (Booth, 1971). The 30 originated from various hosts and geographical locations and included ten *F. tricineta*, seven *F. poae*, eight *F. sporotrichioides*, and five *F. fusarioides* strains. The strains chosen had been the subject of previous toxicological studies (Cullen, 1981) and many were morphologically similar to Florida isolates R-P, W-P, and B-P.

Contributors of strains included J. Andrews (Department of Plant Pathology, University of Wisconsin, Madison, Wis., U.S.A.), C. Booth (Commonwealth Mycological Institute, Kew, Surrey, U.K.), J. J. Ellis (Northern Regional Research Laboratories, Peoria, Ill., U.S.A.), W. Marasas (National Research Institute for Nutritional Diseases of South Africa, Tygerberg) and P. E. Nelson (Fusarium Research Center, Pennsylvania State University, University Park, Pa., U.S.A.). All cultures were maintained as single spore clones on heat-sterilized soil (Booth, 1971).

Media. All crosses were performed as described by El-Gholl *et al.* (1978) on Coastal Bermuda hay (*Cynodon dactylon* Pers.) stem segments sterilized with propylene oxide and incubated under continuous fluorescent lighting for approximately 3 months.

Snider's complete medium (SC) (Snider & Raper, 1958) and Vogel's minimal medium (VM) (Vogel, 1956) were supplemented with 1% (v/v) glucose and used throughout these studies. Nutritional requirements of auxotrophs were routinely met by supplementing VM medium with appropriate nucleic acid precursors or amino acids at a concentration of $100 \mu\text{g ml}^{-1}$. Sporulation was induced in shake cultures of carboxymethylcellulose medium (CMC) as described by Capellini & Peterson (1965). Resulting conidial suspensions were filtered through cheese-cloth, centrifuged at $10000g$ for 10 min, washed by centrifugation in sterile distilled water, and resuspended in water at approximately 1×10^6 spores ml^{-1} . Unless otherwise specified, all cultures were incubated at room temperature (about 22°C) for 12 h under fluorescent lighting.

Toxin production was evaluated on nutritionally complex media [2% (w/v) soya meal, 0.5% (v/v) cornsteep liquor, 10% (w/v) glucose] absorbed on to vermiculite as previously described (Cullen *et al.*, 1982). T-2 toxin was the only trichothecene detected by gas-liquid chromatography. Duplicate cultures were analysed and results are expressed in mean mg T-2 toxin per litre of fermentation medium.

Auxotroph isolation. UV-induced mutants were recovered following irradiation with a 254 nm UV-light source producing 5 J m^{-2} . Conidial suspensions (20 ml) containing 1×10^6 spores ml^{-1} were exposed with continual stirring until 80 to 90% of the spores were killed (4 to 8 min). Auxotrophic strains R-P-1 and T-2-2 were isolated by 'total isolation' techniques (Fincham *et al.*, 1979).

Mutant strains B-P-1, W-P-1 and T-2-1 were isolated by filtration enrichment. Following UV exposure, spores were diluted to 1×10^5 spores ml^{-1} in flasks containing 50 ml VM broth, and aerated on a rotary shaker (100 r.p.m.). Prototrophs were removed by filtration through Whatman no. 1 filter paper at 12 h intervals for 48 h. Final filtrates were diluted and plated on SC medium. Resulting colonies were tested for auxotrophy by transfer to VM plates.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis involved suspending CMC-derived conidia ($1 \times 10^6 \text{ ml}^{-1}$) in VM broth for 3 h prior to administering MNNG at a final concentration of $600 \mu\text{g ml}^{-1}$. Continual stirring was maintained for 30 min (50% survival) and then terminated by diluting with sterile distilled water. 'Total isolation' techniques were then used to isolate auxotroph R-P-2.

Attempts to obtain auxotrophs by nystatin enrichment (MacDonald, 1968) were unsuccessful.

Heterokaryon formation and analysis. Conidia of auxotrophic strains were harvested from CMC flasks and adjusted to 1×10^6 spores ml^{-1} . All pairwise combinations of auxotrophs were tested for heterokaryon formation by transferring 0.1 ml of each spore suspension to the bottom of VM agar (VMA) slants. Duplicate slants were maintained in a vertical position for 14 d.

Before determining the presence and relative proportion of component nuclei in prototrophic mycelium, i.e. presumed heterokaryons, three successive mycelial transfers were made on VMA slants. With each transfer, small blocks (about 3 mm^2) of mycelium and agar were removed from the upper portion of slants. This procedure assured no carry-over of the initial inoculum.

Auxotrophic pairs exhibiting prototrophic growth were tested for cross-feeding as described by Sanchez *et al.* (1976).

Segments of mycelium and agar (approximately 5×5 mm) were removed from the upper portion of fourth transfer VMA slants and macerated in sterile distilled water. Spores, if present, and hyphal fragments were streaked on to VMA plates supplemented with the appropriate individual growth factors. After incubating 36 to 48 h, advancing hyphal tips (about 1 mm long) were transferred to VMA slants supplemented with the same growth requirement. After 5 d growth, hyphal tip transfers from these supplemented VMA slants were tested for growth on VMA and VMA supplemented with individual growth requirements.

To estimate the relative proportion of component nuclei, fourth transfer VMA slants were again macerated and instead of streaking, samples (0.1 ml) were spread on VM plates supplemented with individual growth requirements. In theory, resulting colonies should have been either prototrophic (heterokaryons), or, if the heterokaryon was unstable, auxotrophic for the particular growth factor. Hyphal tips from margins of rapidly growing colonies were transferred to SC agar (SCA) slants and after 3 d growth, were tested for auxotrophic requirement by transfer to VMA and VMA supplemented slants. Since all such cultures were determined to be auxotrophic, colony counts were presumed to reflect nuclear proportions in heterokaryons.

RESULTS

The range of sexual compatibility of the three Florida strains and strain T-2 was determined by selfing and pairwise crosses in all four possible combinations, and by additional pairwise crosses with 19 morphologically similar *Fusarium* isolates. Sexually compatible crosses were observed only among certain combinations of *F. tricinctum* strains from Florida. In agreement with the results of El-Gholl *et al.* (1978), fertile perithecia were produced when strain R-P was crossed with B-P or W-P. B-P $\times$ W-P crosses were incompatible.

Although proven pathogenic to English ivy by El-Gholl *et al.* (1978), the toxicity of Florida *F. tricinctum* isolates had not been previously tested. The extreme phytotoxicity of trichothecenes (Bamburg & Strong, 1971) and the symptoms occurring on leaves, i.e. dark lesions, suggested the possibility of trichothecene involvement in pathogenesis. However, the Florida isolates produced no detectable trichothecenes in vermiculite cultures. The detection limit for the procedure was approximately 0.5 mg T-2 toxin per litre of medium.

Because most isolates were sexually incompatible, we attempted to isolate auxotrophic mutants to facilitate parasexual genetic analysis. UV- and MNNG-induced auxotrophs are listed in Table 1. Two classes of auxotrophs were isolated twice. R-P-2 and T-2-1 required both valine and isoleucine suggesting dysfunction of an enzyme involved in later stages of valine and isoleucine synthesis, a phenomenon also observed in mutants of *Neurospora crassa* and *Saccharomyces cerevisiae* (Fincham *et al.*, 1979). Requirement for either adenine or hypoxanthine by R-P-1 and T-2-2 suggests enzyme deficiencies in purine synthesis prior to hypoxanthine formation.

All mutants isolated had absolute requirements for growth factors and could only grow with appropriate nutrient supplements at $10 \mu\text{g ml}^{-1}$ in Vogel's minimal agar. W-P-1 would grow with 0.1 to $1 \mu\text{g}$ methionine (ml VMA^{-1}). During the screening of mutants, many morphological and 'leaky' mutants were obtained and discarded. One such morphological mutant had a leathery texture and lacked aerial mycelium similar to the 'button' mutants described by Prasad (1949). This mutant was auxotrophic inasmuch as it grew slowly on SCA and not on VMA, but the specific growth requirement was not identified.

In sexual pairing of all auxotrophs and auxotroph-wild-type combinations, three crosses yielded fertile perithecia: W-P-1 $\times$ R-P, W-P-1 $\times$ R-P-2 and W-P $\times$ R-P-2. Analysis of single ascospore progeny indicated that Met⁻ and Ilv⁻ phenotypes involved single unlinked genes and that the Ilv⁻ phenotype was linked to pigmentation (data not shown). None of the sexually compatible auxotrophs were toxin producers, eliminating the possibility of genetic analysis of toxin produced by sexual crosses.

As an alternative approach, all possible pairs of auxotrophic mutants were tested for heterokaryon formation on minimal medium. Of 15 possible pairwise combinations of auxotrophs, three yielded growth after four successive transfers on VMA: T-2-1 + T-2-2, R-P-1 + R-P-2, and T-2-1 and B-P-1 (Table 2). Relative to the wild type, heterokaryon T-2-1 + B-P-1 had

Table 1. *Fusarium tricinctum* and *F. sporotrichioides* auxotrophs and their origins

Strain designation		<i>Fusarium</i> species*	Nutritional requirement	Mating type	T-2 toxin† (mg l ⁻¹)	Mutagen‡
Auxotroph	Parent					
R-P-1	R-P	<i>tricinctum</i>	Adenine or hypoxanthine (Ade ⁻)	+	ND	UV
R-P-2	R-P	<i>tricinctum</i>	Valine and isoleucine (Ilv ⁻)	+	ND	UV
B-P-1	B-P	<i>tricinctum</i>	Lysine (Lys ⁻)	—	ND	UV
W-P-1	W-P	<i>tricinctum</i>	Cysteine or methionine (Met ⁻)	—	ND	UV
T-2-1	T-2	<i>sporotrichioides</i>	Valine and isoleucine (Ilv ⁻)	?	8.9	UV
T-2-2	T-2	<i>sporotrichioides</i>	Adenine or hypoxanthine (Ade ⁻)	?	20.3	MNNG

* Taxonomic distinctions based upon Booth's species concept for *Fusarium* (Booth, 1971).

† Toxicity determinations represent mean of duplicate auxotroph cultures. ND, none detected (<0.5 mg l⁻¹).

‡ A 254 nm UV-light source was used. MNNG was used at a concentration of 600 µg ml⁻¹.

Table 2. Analysis of segregant homokaryons from heterokaryons

Parental strains and T-2 toxin yield		Reisolated homokaryon:					
		A			B		
		Nutritional phenotype	No. isolated	T-2 toxin* (mg l ⁻¹)	Nutritional phenotype	No. isolated	T-2 toxin* (mg l ⁻¹)
T-2-1 <i>ilv</i> 8.9 mg l ⁻¹	+	T-2-2 <i>ade</i> 20.3 mg l ⁻¹					
		Ilv ⁻	16	11.7 ± 2.0	Ade ⁻	64	20.0 ± 6.8
T-2-1 <i>ilv</i> 8.9 mg l ⁻¹	+	B-P-1 <i>lys</i> ND	22	9.1 ± 3.5	Lys ⁻	12	ND
			•				
R-P-1 <i>ade</i> ND	+	R-P-2 <i>ilv</i> ND	52	ND	Ilv ⁻	24	ND

* The values for T-2 toxin concentration represent means of duplicate cultures ± one standard deviation. ND, none detected (<0.5 mg l⁻¹).

significantly reduced sporulation. Enlarged conidia, often associated with diploidization (Fincham *et al.*, 1979), were not observed. Heterokaryons T-2-1 + T-2-2 and R-P-1 + R-P-2 produced no spores.

It proved impossible to determine the levels of toxin produced by heterokaryons. On complete media suitable for toxicity evaluations, heterokaryons segregated into component homokaryons. On synthetic, toxin-supporting medium (Cullen *et al.*, 1982), extremely slow growth precluded determinations of toxin production.

Newly formed hyphal tips obtained from macerated heterokaryons on supplemented VMA were almost exclusively homokaryotic. In contrast, hyphal tips transferred from supplemented VMA to VMA prior to significant hyphal elongation were still capable of prototrophic growth, i.e. heterokaryotic. These results demonstrate that prototrophic growth is most likely due to the formation of heterokaryons that are unstable unless grown under selective conditions.

Using the test of Sanchez *et al.* (1976), no 'cross-feeding' was detected between auxotrophic components of heterokaryons. This procedure involved inoculating two different auxotrophs at opposite sides of a VMA plate which was supplemented with the nutritional requirement(s) of one strain. If cross-feeding occurred, the non-supplemented auxotroph would grow as required nutrient(s) diffused through the agar.

The nuclear contribution of each parental strain to a heterokaryon was assessed as described in Methods. Homokaryons were isolated following growth of heterokaryons on a non-selective medium and scored for parental markers. Nuclear proportions and toxin production of segregant homokaryons are presented in Table 2. The results are consistent with nuclear control

of trichothecene synthesis and pigmentation. For example, all *Ilv*⁻ homokaryons derived from heterokaryon T-2-1 + B-P-1 produced toxin and were pigmented reddish-white, whereas *Lys*⁻ homokaryons from the same heterokaryon produced no trichothecenes and were pigmented brown. In the case of heterokaryon T-2-1 + T-2-2, the magnitude of toxin production appeared to be under the control of nuclear genes, since homokaryon T-2 toxin levels were similar to parental strains and correlated with the segregation of auxotrophic markers.

Unequal numbers of component nuclei were isolated from heterokaryons (Table 2). For example, *Ade*⁻ colonies were recovered four times more frequently than *Ilv*⁻ colonies in analysis of heterokaryon T-2-1 + T-2-2. Similar results have been reported for diauxotrophic heterokaryons of *F. oxysporum* (Sanchez *et al.*, 1976) and other fungi (Fincham *et al.*, 1979).

DISCUSSION

Our results are the first description of heterokaryosis within and between *F. sporotrichioides* and *F. tricinctum*. Because prototrophs or other recombinant phenotypes could not be isolated from heterokaryons, parasexuality was not demonstrated. Intrastrain heterokaryons (i.e. T-2-1 + T-2-2 and R-P-1 + R-P-2) and, depending upon taxonomic perspective, either an interstrain or an interspecific heterokaryon (i.e. T-2-1 + B-P-1) were isolated.

The two taxonomic points of view are represented by the systems devised by Booth and by Snyder & Hansen. According to Snyder & Hansen (Snyder & Hansen, 1945; Toussoun & Nelson, 1976), *F. tricinctum* (Corda) Sacc. and *F. sporotrichioides* Sherb. are equivalent to *F. tricinctum*. Booth (1971) however, makes narrower distinctions based on conidiophore morphology and separates *F. tricinctum sensu* Snyder & Hansen into *F. tricinctum*, *F. poae*, and *F. sporotrichioides*.

Heterokaryosis between *F. sporotrichioides* and *F. tricinctum sensu* Booth is suggestive of genetic homology but does not warrant favouring either taxonomic system. The heterokaryons were only obtainable through the use of strong selective pressure and were extremely unstable. Moreover, their growth rates and morphologies were clearly aberrant. If vegetative compatibility is to be used as a taxonomic criterion, many more interstrain heterokaryons, especially neutral heterokaryons (Parmeter *et al.*, 1963), are needed.

Others have reported inter-specific *Fusarium* heterokaryons. Dhillon *et al.* (1961) and Coy & Tuveson (1961) reported forced heterokaryons between *G. fujikuroi* and *F. oxysporum*, and between *F. oxysporum* and *F. solani*, respectively. Parmeter *et al.* (1963) have disputed these findings because single hyphal tips or conidia of the diauxotrophs were not isolated. Singh & Hoffmann (1969) synthesized heterokaryons between auxotrophs of *F. oxysporum* and *F. redolens*. Genetic recombinants were isolated from sectors of heterokaryon cultures suggesting mitotic recombination, i.e. parasexual cycle. As in the present case, taxonomic distinctions between *F. oxysporum* and *F. redolens* are vague. Snyder & Hansen's scheme, for example, considers *F. redolens* synonymous with *F. oxysporum*, whereas Booth considers *F. redolens* as *F. oxysporum* var. *redolens*. Neither species is known to produce trichothecenes.

Most auxotroph combinations were unable to form heterokaryons. In the case of R-P-1 + T-2-2 and R-P-2 + T-2-1 combinations, the auxotrophic mutations may have involved the same locus and thus be unable to complement each other. In other cases, specific genetic barriers to vegetative compatibility and/or non-allelic failures to complement may have prevented heterokaryosis.

An increased range of toxin production might occur by natural or artificial synthesis of stable heterokaryons among certain species. In contrast, the lack of toxin production and restricted sexual compatibility of *G. tricincta* strains, suggest that variation by meiotic recombination is unlikely, although more comprehensive surveys of *G. tricincta* isolates are needed. The foregoing is not meant to imply that mixtures of *Fusarium* toxins, as found in Southeast Asian samples, must necessarily be explained by the activities of a single strain. In fact, combinations of toxins which suggest synthesis by multiple species have been detected in nature (Mirocha *et al.*, 1979). Assuming the desire or need, large-scale industrial production of trichothecene mixtures would certainly be facilitated by identifying or 'engineering' the appropriate strain, as opposed to

separate fermentations and subsequent mixing. Our results show that heterokaryons involving toxic fusaria, albeit unstable, are possible.

Following the reasoning of Jink's 'heterokaryon test' (reviewed in Fincham *et al.*, 1979), our results support the thesis of nuclear control of T-2 toxin synthesis. This conclusion is based on the consistent association of toxicity with parental auxotrophic requirements of segregant homokaryons. Except for rare diploidization and subsequent breakdown, recombinant phenotypes among single cell isolations would unequivocally disprove the hypothesis of nuclear control, whereas negative results provide equivocal evidence for nuclear control. In the present case, for example, extranuclear elements may be involved but obscured by incomplete mixing of cytoplasm within the heterokaryons and/or by complex nuclear-cytoplasmic interactions controlling toxin production. Further characterization of heterokaryons, isolation of diploid strains from heterokaryons, or segregation analysis from appropriate sexual crosses are needed to augment our conclusions.

The inability of the English ivy pathogens to produce trichothecenes in culture is not surprising since non-toxic strains of *F. tricinctum* are well known (Cullen, 1981; Suzuki *et al.*, 1980). These results do not exclude the possibility of toxin synthesis during pathogenesis and/or under different cultural conditions.

Sexual outbreeding within *G. tricincta* appears quite restricted. Except for the pathogenic isolates supplied by El-Gholl *et al.*, strains used in test crosses were from outside Florida and were either saprophytic or isolated from stored products. Consequently, it is not clear if incompatibility is limited to geographic and/or host factors. A precedent for such breeding barriers exists among *G. fujikuroi* (= *F. moniliforme*) and *Hypomyces solani* f. sp. *cucurbitae* (= *F. solani*) (Phinney & Spector, 1967; Snyder *et al.*, 1975). More *G. tricincta* strains need to be tested. Hopefully, sexually compatible, toxin-producing strains will be isolated, allowing genetic analysis of trichothecene biogenesis through sexual crosses.

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