Isozyme characterisation of *Fusarium graminearum* isolates associated with head blight of irrigated wheat in South Africa

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Received 16 March 1999; revised 2 June 1999

Head blight, caused by certain species in the genus *Fusarium*, has become an economically important disease of wheat under centre pivot-irrigation along the Orange River region in South Africa. Isozyme analysis, by means of starch gel electrophoresis, was employed to compare 73 *Fusarium* isolates, obtained from three farms in this region, with isolates of *Fusarium acuminatum*, *F. chlamydosporum*, *F. compactum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum* Group I and Group II, *F. moniliforme*, *F. oxysporum*, *F. solani*, and *F. subglutinans*. For all 11 *Fusarium* species, nine loci were identified in eight enzyme systems and 19 electrophoretic phenotypes (EPs) were detected, each composed of isolates from one species only. The extent of isozyme polymorphisms found among these *Fusarium* spp. provides a potential method for delineating isolates at species level. Six EPs, of which the most common one comprised 36 isolates, were identified within isolates obtained from the three farms. These isolates clustered together with the *F. graminearum* Group II reference isolates. Based on perithecial production, their identity was confirmed as *F. graminearum* Group II.

Keywords: *Fusarium graminearum*, head blight, isozyme analysis, wheat.

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Introduction

Head blight or scab of wheat, caused by species in the genus *Fusarium*, is a major disease in the humid and semi-humid wheat (*Triticum aestivum* L.) growing areas of the world (Wiese 1987; Bai & Shaner 1994; Parry *et al.* 1995). *Fusarium graminearum* Schwabe, the anamorph (conidial stage) of *Gibberella zeae* (Schw.) Petch, is the principal *Fusarium* species responsible for head blight worldwide (Strausbaugh & Maloy 1986; Clear & Patrick 1990; Wong *et al.* 1992; Jiang *et al.* 1995). Other species such as *F. crookwellense* Burg., Nels. & Touss., *F. culmorum* (Smith) Sacc., *F. avenaceum* (Corda ex Fr.) Sacc., *Microdochium nivale* (Fries) Samuels & Hallett, and *F. poae* (Peck) Wollenw., may also contribute to the head blight complex but are of lesser importance and occur only under environmental conditions that are highly favourable to their development (Sutton 1982; Strausbaugh & Maloy 1986; Wiese 1987; Scott *et al.* 1988; Wilcoxson *et al.* 1988; Snijders 1990).

In South Africa, head blight has been associated with *F. graminearum* Group II and *F. crookwellense* (Marasas *et al.* 1988; Scott *et al.* 1988). More recently, the disease became epidemic on farms along the Orange River in the Northern Cape Province, where wheat is grown under centre pivot-irrigation (Scott & Smith 1995). Factors not usually associated with *F. graminearum* infection on irrigated wheat in South Africa, such as the emergence of completely blighted heads from boots, and lower temperatures during epidemics, were observed during these outbreaks. This raised the question whether *F. crookwellense*, known to cause head blight in other cooler regions of South Africa (Scott *et al.* 1988), could possibly be the principal pathogen.

Fusarium spp. have traditionally been identified using morphological and cultural characters (Nelson *et al.* 1983). Identification is complicated by subtle differences between some species and environmental influences on species morphology. Based solely on these parameters, it is thus difficult to differentiate between *F. graminearum* and *F. crookwellense*. Furthermore, characterisation of *formae speciales* and races within a specific *Fusarium* spp. is largely defined by nonmorphological characters as no morphological correlations have been discovered (Nelson *et al.* 1983).

In this study, the use of isozyme analysis, which has been shown to be successful in distinguishing fungal species when morphological characters are inconclusive, was examined for suitability as a quick and reliable technique to identify the causal organism(s) of head blight on irrigated wheat in the Prieska district of South Africa. A secondary objective was to investigate the extent of genetic variation within the causal organism(s).

Material and Methods

Isolation

Seventy-three *Fusarium* isolates were established from wheat plants sown under centre pivot-irrigation near Prieska, Northern Cape in 1994 (Table 1). A seed sample from the same field was also obtained after harvest. Infected rachis internodes, spikelets and immature and mature seed were plated on a selective medium for *Fusarium* isolation (van Wyk *et al.* 1986). Segments from resulting colonies were transferred to carnation leaf agar (CLA) and incubated under near-ultraviolet (UV) light to induce sporulation (Fisher *et al.* 1982). Conidia obtained from sporulating colonies were suspended in sterile water and dispersed on water agar. The plates were incubated at 25°C for 6 h after which single germinating conidia were transferred to potato-dextrose agar (PDA). Monoconidial isolates were subsequently transferred to PDA and stock numbers were allocated to isolates. All isolates were tested for perithecial production by growing single-conidium isolates on 2% CLA in petri dishes. Cultures were examined for perithecia and ascospores after 3 to 5 weeks incubation under near-UV and fluorescent lights.

Electrophoresis

Monoconidial isolates were grown on PDA (Difco) on which sterile cellophane (visking tubing) had been placed to facilitate harvesting of the mycelium and prevent agar contamination for electrophoresis. After incubation at 25°C for 10 days, the mycelium was scraped off with a scalpel and ground to a fine powder in liquid nitrogen using a mortar and pestle. Samples were transferred to 2 ml Eppendorf tubes containing 0.5 ml extraction buffer (1.21 g Trizma Base, 0.292 g

Table 1 Isolate number, host (plant part), geographic origin and electrophoretic phenotype (EP)[a] of *Fusarium* isolates studied

Isolate[b]	Host (plant part)	Origin (farm)	EP
F. acuminatum			
MRC 6270	Wheat debris	Free State	XVIII
F. chlamydosporum			
MRC 6243	Wheat	Free State	XIV
F. compactum			
MRC 6278	Wheat	Free State	XII
F. crookwellense			
MRC 6273, 6274	Wheat	Free State	X
MRC 3928	Plant debris	Petrus Steyn	X
F. culmorum			
MRC 6277	Wheat	Free State	IV
MRC 3298	Wheat (seed)	Bloemfontein	V
F. equiseti			
MRC 3432	Wheat debris	Free State	XV
F. graminearum Group I)			
MRC 6251	Wheat	Free State	II
MRC 4899	Barley (seed)	Caledon	XI
F. graminearum (Group II)			
CCP 42	Wheat (rachis)	Prieska (Remhoogte)	VII
MRC 4927	Wheat (seed)	Swellendam	IX
CCP 132	Wheat	Greytown	IX
CCP 150	Wheat	Greytown	VI
CCP 153	Wheat	Greytown	VIII
CCP 177, 178, 182	Wheat (rachis)	Prieska (Remhoogte)	I
CCP 179, 183	Wheat (rachis)	Prieska (Remhoogte)	III
CCP 180, 181	Wheat (rachis)	Prieska (Remhoogte)	VI
CCP 184, 185	Wheat (spikelets)	Prieska (Remhoogte)	VIII
CCP 186	Wheat (spikelets)	Prieska (Remhoogte)	VI
CCP 187–189	Wheat (spikelets)	Prieska (Remhoogte)	VII
CCP 190, 191	Wheat (seed)[c]	Prieska (Remhoogte)	VIII
CCP 192–194	Wheat (seed)[c]	Prieska (Remhoogte)	VII
CCP 195–202	Wheat (rachis)	Prieska (Muishoek)	VIII
CCP 203–209	Wheat (seed)[c]	Prieska (Muishoek)	VIII
CCP 210–214	Wheat (spikelets)	Prieska (Muishoek)	VIII
CCP 215, 217, 220, 221	Wheat (rachis)	Prieska (De Hoek)	VIII
CCP 216, 218, 219, 222	Wheat (rachis)	Prieska (De Hoek)	VI
CCP 223–227, 229	Wheat (seed)[c]	Prieska (De Hoek)	VI
CCP 228	Wheat (seed)[c]	Prieska (De Hoek)	VIII
CCP 230, 235–238	Wheat (spikelets)	Prieska (De Hoek)	VI
CCP 231–234, 239	Wheat (spikelets)	Prieska (De Hoek)	VIII
CCP 240	Wheat (seed)	Prieska (Remhoogte)	VII
CCP 241	Wheat (seed)	Prieska (Remhoogte)	IX
CCP 242, 244, 357, 358, 360	Wheat (seed)	Prieska (Remhoogte)	VI
CCP 243	Wheat (seed)	Prieska (Remhoogte)	VIII
CCP 356	Wheat (seed)	Prieska (Remhoogte)	VII
F. moniliforme			
MRC 6265	Wheat debris	Free State	XIX
F. oxysporum			
MRC 6263	Wheat debris	Free State	XVII
F. solani			
MRC 6258	Wheat	Free State	XVI
F. subglutinans			
CCP 156	Wheat	Greytown	XIII

[a]Electrophoretic phenotypes were identified according to eight enzyme systems (Table 2).

[b]MRC numbers are accession numbers in the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (Promec) of the Medical Research Council (MRC), Tygerhoek, South Africa and CCP numbers refer to the culture collection maintained at the Department of Plant Pathology, University of the Orange Free State.

[c]Immature seeds.

EDTA, 38 mg NADP, and 10 mg PVP per 1L distilled H_2O) (Petrunak & Christ 1992).

The mycelial homogenate was centrifuged at 7000 rpm for 2 min. and the resulting supernatant was absorbed onto thick filter paper wicks (4 × 10 mm). Loaded wicks were placed at the cathodal origin of 12% (w/v) potato starch gels (Sigma Chemical Co., St Louis, Mo). Horizontal electrophoresis was carried out for approximately 3 to 4 h as described by Micales *et al.* (1986) using buffer systems of Ridgway *et al.* (1970) and Clayton and Tretiak (1972). After completion of electrophoresis, each gel was cut horizontally into six slices of which the top slice was discarded. To examine isozyme variation, the remaining slices were then individually assayed for twenty enzymes using the buffer systems mentioned previously (O'Malley *et al.* 1980; Micales *et al.* 1986; Selander *et al.* 1986). Gels were assayed for acid phosphatase (ACP), adenylate kinase (AK), aspartate aminotransferase (AAT), alcohol dehydrogenase (ADH), catalase (CAT), diaphorase (DIA), esterase (EST), glucose-6-phosphate dehydrogenase (G6P), glutamate dehydrogenase (GDH), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), peptidase (PEP; using leucyl-glycyl-glycine [LGG], leucyl-tyrosine [LT], and glycyl-leucine [GL] as substrates), peroxidase (PRX), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), and sorbitol dehydrogenase (SDH). All electrophoretic patterns were confirmed at least once depending on the resolution of the bands. For the eight enzymes which gave the clearest resolution (Table 2), each unique combination of electromorphs, representing an electrophoretic phenotype (EP), was assigned a roman numeral.

Isolates included as taxonomic markers were provided by the Medical Research Council of South Africa (MRC) and allocated with CCP stock numbers (Culture Collection of the Dept. Plant Pathology, University of the Orange Free State). These isolates were *F. acuminatum* Ell. & Ev. [CCP 253 (MRC 6270)], *F. chlamydosporum* Wollenw. & Reinking [CCP 247 (MRC 6243)], *F. compactum* (Wr.) Gordon [CCP 259 (MRC 6278)], *F. crookwellense* [CCP 256 (MRC 3928), CCP 254 (MRC 6273) and CCP 255 (MRC 6274)], *F. culmorum* [CCP 257 (MRC 6277) and CCP 258 (MRC 3298)], *F. equiseti* (Corda) Sacc. [CCP 260 (MRC 3432)], *F. graminearum* Group I [CCP 249 (MRC 4899) and CCP 248 (MRC 6251)], *F. graminearum* Group II [CCP 59 (MRC 4927), CCP 132, 150 and 153], *F. moniliforme* Sheldon [CCP 252 (MRC 6265)], *F. oxysporum* Schlecht. emend. Snyd. & Hans. [CCP 251 (MRC 6263)], *F. solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans. [CCP 250 (MRC 6258)], and *F. subglutinans* (Wollenw. & Reinking) Nels., Touss. & Marasas comb. Nov. (CCP 156). All *Fusarium* isolates used in the present study are maintained in the CCP culture collection of the Department of Plant Pathology, University of the Orange Free State.

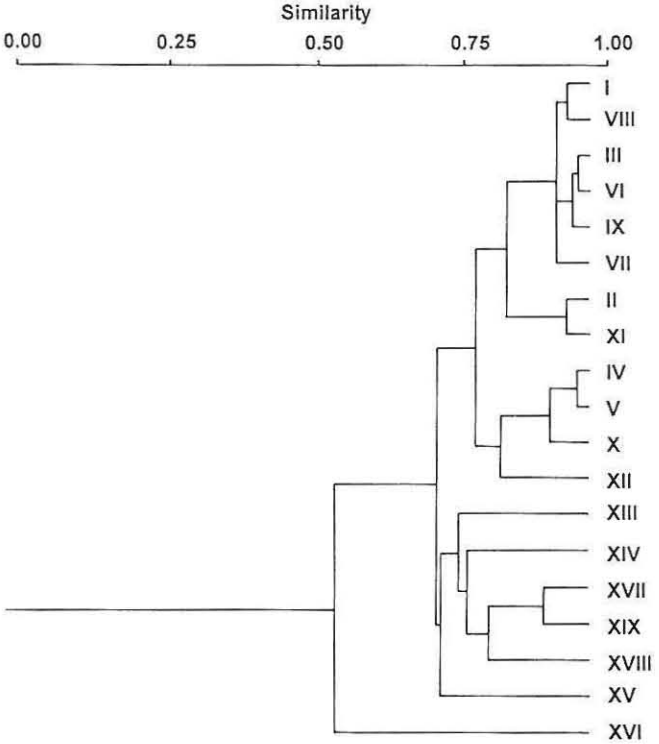

Figure 1 A UPGMA phenogram illustrating similarity among electrophoretic phenotypes of two *Fusarium graminearum* Group I (EPs II and XI) isolates, 77 *F. graminearum* Group II (I, II, VI-VIII, and IX) isolates, three *F. crookwellense* (EP X) isolates, two *F. culmorum* (EPs IV and V) isolates, and one isolate each of *F. compactum* (EP XII), *F. subglutinans* (EP XIII), *F. chlamydosporum* (EP XIV), *F. equiseti* (EP XV), *F. solani* (EP XVI), *F. oxysporum* (EP XVII), *F. acuminatum* (EP XVIII), and *F. moniliforme* (EP XIX).

Data analysis

Allozymes were scored as present or absent for each isolate and a table of simple matching coefficients (Ssm) for each pair of isolates was generated using the formula described by Sneath and Snokal (1973): $Ssm = m/(m+u)$, where m is the number of bands found in common between two isolates and u is the total number of bands unique to each isolate. A dendrogram was constructed for each set of coefficients using the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) (Sneath & Snokal, 1973). All data analyses were performed using the programs SIMQUAL and SAHN of the software package NTSYS-pc version 1.80 (Exeter Software, Setauket, NY).

Table 2 Enzymes and buffer systems which gave clear resolution of bands in isozyme comparisons of *Fusarium* species

Enzyme	Abbreviation	E.C. number	Buffer system
Acid phosphatase	ACP	3.1.3.2	RW
Adenylate kinase	AK	2.7.4.3	AC[b]
Esterase	EST	3.1.1.1	RW
Isocitrate dehydrogenase	IDH	1.1.1.42	AC
Peptidase (glycyl-leucine)	PEP(GL)	3.4.x.x	RW
Peptidase (leucyl-glycyl-glycine)	PEP(LGG)	3.4.x.x	RW
Peptidase (leucyl-tyrosine)	PEP(LT)	3.4.x.x	RW
Phosphoglucose isomerase	PGI	5.3.1.9	RW[a]

[a]Discontinuous buffer according to Ridgway *et al.* (1970), pH 8.5, 75A, 4h.

[b]Continuous buffer according to Clayton and Tretiak (1972), pH 6.1, 90A, 3h.

Table 3 Isozyme electromorphs[a] for 19 electrophoretic phenotypes in 92 isolates of 11 *Fusarium* species

Species	EP[c]	Number of isolates	Enzymes[b]							
			EST	PEP(GL)	PEP(LGG)	PEP(LT)	ACP	AK	PGI	IDH
F. acuminatum	XVIII	1	A	E	B	C	C	A	C	B
F. chlamydosporum	XIV	1	A	D	G	C	E	B	A	C
F. compactum	XII	1	A	F	F	D	E	C	C	E
F. crookwellense	X	3	F	F	I	F	E	C	C	E
F. culmorum	IV	1	J	F	J	F	E	C	C	E
	V	1	E	F	J	F	E	C	C	E
F. equiseti	XV	1	K	B	A	A	E	C	B	B
F. graminearum (Gr. I)	II	1	C	E	E	H	E	C	C	E
	XI	1	B	E	E	H	E	C	C	E
F. graminearum (Gr. II)	I	3	D	C	K	G	E	C	C	E
	III	2	G	C	K	G	E	C	C	E
	VI	24	H	C	K	G	E	C	C	E
	VII	9	L	C	K	G	E	C	C	E
	VIII	36	M	C	K	G	E	C	C	E
	IX	3	I	C	K	G	E	C	C	E
F. moniliforme	XIX	1	K	C	G	C	A	C	C	B
F. oxysporum	XVII	1	K	C	C	C	D	C	C	B
F. solani	XVI	1	N	A	D	B	B	B	B	A
F. subglutinans	XIII	1	A	E	H	E	D	C	A	D

[a]Individual electromorphs are indicated by capital letters.

[b]Abbreviations as in Table 2.

[c]Electrophoretic phenotypes were identified according to eight enzyme systems (Table 2).

Results

On carnation leaf agar all 73 isolates produced perithecia containing ascospores which resembled *G. zeae*, the sexual stage of *F. graminearum* Group II. Enzyme-staining protocols that resulted in poor resolution or no activity were AAT, ADH, CAT, DIA, GOP, GDH, LDH, LAP, MDH, PRX, PGM, and SDH. Eight enzymes provided consistent results with adequate isozyme resolution (Table 2). Clear resolution of bands was indicative of nine genetic loci that were all polymorphic. The number of alleles per locus ranged from three for AK and PGI to nine for PEP (LT) and PEP (LGG). Isolates of the eleven *Fusarium* spp. included in this study separated into 19 electrophoretic phenotypes. Each EP was composed of isolates from only one species (Table 1). A UPGMA phenogram, illustrating percentage similarity among EPs of the 11 *Fusarium* spp., is presented in Figure 1.

Sixty-three of the 73 *Fusarium* isolates collected from the farms Remhoogte, Muishoek, and De Hoek produced banding patterns similar to the four reference isolates of *F. graminearum* Group II. Six EPs were found within the *Fusarium* isolates from Prieska. Electrophoretic phenotype VIII was most common and comprised 36 isolates, including six isolates from Remhoogte, nine isolates from De Hoek, all 20 isolates from Muishoek, and reference isolate CCP 153. Electrophoretic phenotype VI included eight isolates from Remhoogte, 15 isolates from De Hoek, and a reference isolate CCP 150. Electrophoretic phenotypes I, III, VII, and IX respectively, included three, two, nine, and one isolate from the farm Rernhoogte. Electrophoretic phenotype IX also included reference isolates CCP 59 and CCP 132. Allozymes identified in each of the 11 *Fusarium* spp. are recorded in Table 3. Comparisons within Table 3 show that the *F. graminearum* Group II isolates differed only in their EST banding patterns. Considerable differences were observed among the eleven *Fusarium* spp. (Table 3, Figure 2). Bands shared between the species varied from two for PEP (LOG) to nine for AK. The two *F. graminearum* Group I isolates, two *F. culmorum* isolates, and three *F. crookwellense* isolates differed from the *F. graminearum* Group II isolates in their EST, PEP (GL), PEP (LT), and PEP (LOG) banding patterns (Figure 2).

Discussion

Electrophoretic patterns of soluble enzymes are representative of the genome of an organism and can be used in the differentiation of fungal species (Burdon & Marshall 1981; Bonde *et al.* 1984; Bonde *et al.* 1993) and to determine the extent of genetic variation within and among related fungal populations (Burdon & Roelfs 1985; Micales *et al.* 1992; Petrunak & Christ 1992; Stanosz *et al.* [in press]). Furthermore, Huss *et al.* (1996) determined isozyme phenotypes for 101 strains of *Gibberella fujikuroi* (Sawada) Ito, that represent six mating populations, from a variety of plant hosts in dispersed geographic locations. Although a close genetic relationship was found among the mating populations, they could distinguish five of the six mating populations from one another using isozyme polymorphisms. By means of morphological criteria and isozyme analyses, the present study confirmed that *F. graminearum* Group II is the causal organism of head blight in the Prieska region of South Africa. This corresponds with a previous study where *F. graminearum* was identified as the pathogen responsible for head blight in irrigated wheat in Northwest and KwaZulu-Natal (Scott *et al.* 1988). During the latter survey no head blight was observed on irrigated wheat in the Northern Cape, whereas *F. crookwellense* was restricted to the cooler regions of the eastern Free State (Scott *et al.* 1988).

Using randomly amplified polymorphic DNA (RAPD) markers, Schilling *et al.* (1996) found a high degree of genetic variability among *F. graminearum* isolates from different geographic

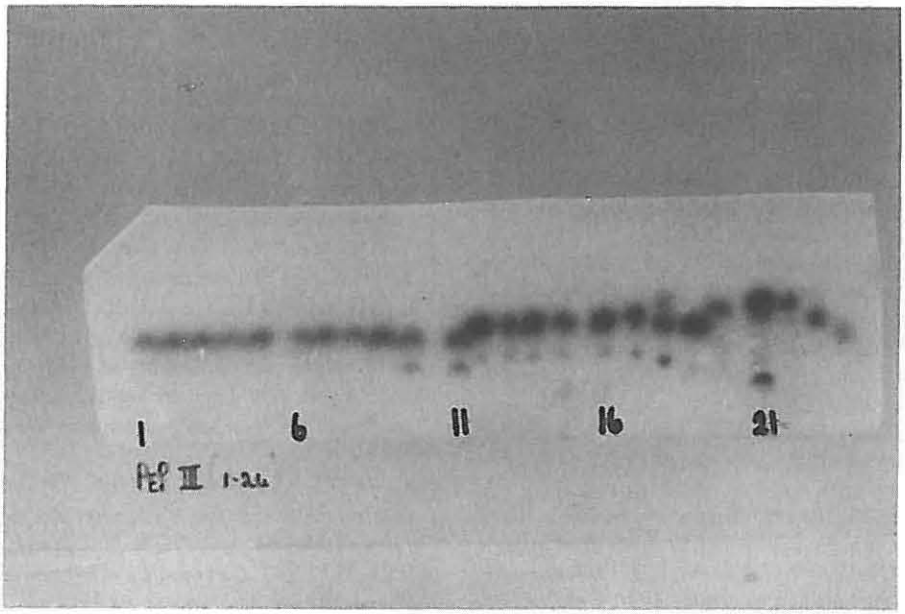

Figure 2 Electrophoretic banding patterns for peptidase (leucyl-glycyl-glycine) [PEP (LGG)] showing from left to right, nine *Fusarium graminearum* Group II isolates, two *F. graminearum* Group I isolates, three *F. crookwellense* isolates, two *F. culmorum* isolates, and one isolate each of *F. compactum, F. subglutinans, F. chlamydosporum, F. acuminatum, F. solani, F. oxysporum, F. moniliforme*, and *F. equiseti*.

regions. In contrast, a 94% similarity was found in the present study among the *F. graminearum* Group II isolates collected from farms in the Prieska region, with all isolates obtained from the farm Muishoek being homogeneous. This high degree of genetic homogeneity for *F. graminearum* is consistent with that observed within wheat and corn isolates from Canada (Ontario mostly) using RAPD and PCR amplification (Ouellet & Seifert 1993) and is comparable to that observed within the mating population of *F. solani* (Crowhurst *et al.* 1991). Leung and Williams (1986) suggested that homogeneity refers to the origin of a few founder isolates from a common ancestral population, carrying only a fraction of the genetic variability existing within the species. The recent introduction of head blight to the Prieska region from a common geographic origin of inoculum, probably through infected seed, may thus have contributed to the observed homogeneity of the *F. graminearum* population.

Our isozyme data suggested a close genetic relationship (similarity level > 80%) among morphologically similar species such as *F. graminearum, F. crookwellense, F. culmorum*, and *F. compactum*. Yli-Mattila *et al.* (1996) studied RAPD patterns of *F. avenaceum, F. graminearum, F. culmorum, F. equiseti, F. oxysporum*, and *F. redolens* Gerlach & Nirenberg isolates. They found that the morphologically related species, *F. graminearum* and *F. culmorum*, clustered together at a similarity level of > 63% . In the present study isozyme analyses provided consistent results, allowing clear differentiation of the different *Fusarium* spp. included as outgroups as well as among isolates of *F. graminearum* Group I and Group II.

Enzymes differentiating between Groups I and II were EST, PEP (GL), PEP (LGG), and PEP (LT). This finding is consistent with that of Schilling *et al.* (1996) who found that isolates of *F. graminearum* split into two main clusters corresponding to Groups I and II. However, an additional study incorporating a larger and geographically diverse population of Group I and II isolates is necessary to confirm whether isozyme analysis can consistently distinguish between the two groups.

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