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IDENTIFICATION OF *FUSARIUM* SPECIES BY ISOZYME ANALYSIS

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Cellulose-acetate electrophoresis (CAE) was used to investigate isozyme polymorphisms among different isolates of *Fusarium cerealis*, *F. culmorum*, *F. graminearum* and *F. pseudograminearum*. After initial testing of 18 enzymes in three buffer systems for activity and resolution of bands, 12 proved to be appropriate for analysis of the full sample set. Comparing the different electrophoretic types (ETs), adenylate kinase (AK), NADP dependent glutamate dehydrogenase (NADP GDH), peptidase B (PEP B), peptidase D (PEP D) and phosphoglucomutase (PGM) proved to be diagnostic for at least one species examined. However, only PEP D was useful alone as a marker to distinguish the four taxa studied providing a rapid and simple CAE based diagnostic protocol.

Keywords: *Fusarium* spp., isozyme analysis

Introduction

Several *Fusarium* species are involved in seedling blight, foot rot and head blight (scab) diseases of small grain cereals and grasses as well as ear and stalk rot of maize. *F. graminearum* is generally regarded as the most important of the species causing head blight in the warmer cereal growing regions of the world (U.S.A., Central Europe, South America and Australia), whereas *F. culmorum* is often the predominant *Fusarium* species in cooler regions, such as Northwest Europe [1, 2].

Identification of *Fusarium* species based on morphological and cultural characteristics is not always simple. Considerable expertise is required to differentiate and identify *F. graminearum* Schwabe and closely related species, *F. cerealis* Cooke

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(syn: *F. crookwellense*; Burgess, Nelson and Toussoun) and *F. culmorum* W. G. Smith, because their traits exhibit variations on a continuous scale that may overlap between the species. Furthermore, two morphologically and culturally indistinguishable populations, designated Group 1 and Group 2, were distinguished within *F. graminearum* [3, 4]. The two groups differ in their ability to produce perithecia in cultures initiated from single macroconidia; with Group 1 being unable and Group 2 being able to produce perithecia. In spite of the high degree of morphological similarity, Láday et al. [5] found significant, geographically independent differences in the isozyme profiles between the isolates of Group 1 and Group 2. Recently, Aoki and O'Donnell [6] described the *F. graminearum* Group 1 as a new species under the name *F. pseudograminearum*, based on DNA sequence data from β-tubulin gene.

The use of molecular markers for species-specific detection assays has become widespread. Based on polymerase chain reaction (PCR), highly sensitive diagnostic assays have been implemented successfully for *F. graminearum* [7–12], *F. culmorum* [7, 8, 11, 12], *F. cerealis* [7, 12], and *F. pseudograminearum* [6]. Although several molecular markers have been developed to identify the above-mentioned species, no molecular marker has been found to be suitable for differentiating and identifying all four species.

The aim of the present study was to detect isozyme(s) that could be used to identify *F. cerealis, F. culmorum, F. graminearum* and *F. pseudograminearum* at the species level.

Materials and methods

The 70 *Fusarium* strains (13 *F. cerealis*, 16 *F. culmorum*, 28 *F. graminearum* and 13 *F. pseudograminearum*) used in this study are listed in Table I. All isolates were grown from single conidia. For morphological identification, sporulation was promoted by both carnation-leaf agar (CLA) [13] plates at ambient temperature under neonlight/UV_{360nm} with a photoperiod of 12 h until sporulation occurred. Perithecium production was determined using the method described by Windels et al. [14].

For isozyme analysis isolates were grown initially in 15 ml CMC broth inoculated with three mycelium discs (5 mm diameter) from young colonies on potato dextrose agar [9]. Cultures were incubated for 3 days on an orbital shaker (120 rpm) at 25 °C, then the 15 ml of culture were transferred into 100 ml YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2.0% D-glucose) and were incubated for 2 days on an orbital shaker (120 rpm) at 25 °C. Young mycelia were vacuum-filtered on Whatman No. 1 filter paper, washed three times with 50 ml of distilled water and lyophilized. Dried mycelia were pulverized by mortar and pestle and stored at -20 °C

until needed for enzyme extraction. For protein extraction and isozyme analysis we used the method described by Láday and Szécsi [15]. Staining protocols followed have been described previously by Hebert and Beaton [16]. All samples were extracted and analysed twice in separate runs. Chemicals were purchased from Sigma Chemical Company.

The determination of the relative mobility of the bands was carried out with a ChemiImager 4000 Low Light Imaging System (Alpha Innotech Corporation, San Leandro, U.S.A.) using Bio-gene Version 97 computer software (Vilber Lourmat, Marne-la-Vallée, France).

The relative mobility (R_f value) of each isozyme band was calculated using the anodally moving band (R_f =100) of isolate NRRL 26939, which was arbitrarily chosen as the standard. For each enzyme assay, bands were designated by the abbreviations of enzymes and the percentage mobility of the band relative to the standard band.

Results

Eighteen enzymes were tested initially in three continuous buffer systems for activity and resolution of bands. Of those, 12 enzymes that showed clear, reproducible and resolvable banding patterns with at least one continuous buffer system were selected for the full sample set, from which 37 distinct bands were scored and used in the analysis (Table II, pp 328–329).

Of the 12 enzymes stained, G6PDH, GPI, IDH, MDH and 6PGDH were monomorphic for the four species. In the patterns of FUM and PEP A, polymorphisms were found only in some cases, while AK, NADP GDH, PEP B, PEP D and PGM proved to be diagnostic for at least one species examined.

The patterns of 2 polymorphic and 5 diagnostic enzymes were used to group the strains into electrophoretic types (ETs). With this grouping we obtained 13 different ETs (Table I, pp. 326–328). Of the 60 isolates studied 52 fell into five common ETs, ET I and II for *F. cerealis*, ET VI for *F. culmorum*, ET VIII for *F. graminearum* and ET XI for *F. pseudograminearum*. The remaining ETs were all represented by unique isolates. Isolates of *F. cerealis* were grouped in 5 ETs as a result of polymorphisms in FUM, PEP A, PEP B and PGM. This was the only species to display polymorphisms for FUM and PEP A; PGM patterns split the isolates into two main ETs, one with *F. culmorum* type band (R_f = 93), the second with *F. graminearum* type band (R_f = 100) (Figure 1/D). *F. graminearum* contained 3 ETs. Of the 18 isolates 16 belonged to one ET; the discrepancies were represented by unique isolates Fg 7.3 and Fg 7.17 differing in their PEP B (R_f = 92) and PGM (R_f = 112) bands, respectively. Three ETs were also detected for *F. pseudograminearum*, however polymorphisms were only due to 2

different electromorphs of PEP D, one for NRRL 28333 isolate from South Africa and the other for NRRL 28438 isolate from USA with R_f value pairs of 74:115 and 78:115, respectively. In the case of *F. culmorum* only 2 ETs were found, isolate NRRL 29139 from Canada differed from the other isolates of its species in its faster moving PEP D band with an R_f value of 117.

Considering the interspecific polymorphisms, all the 5 diagnostic enzymes proved to be appropriate to identify *F. pseudograminearum*; that is, for all the five enzymes *F. pseudograminearum* showed unique band patterns. Only PEP B and PEP D were diagnostic for *F. graminearum*; AK and PEP D were diagnostic for *F. culmorum*; and only PEP D was useful in the identification of *F. cerealis* (Figure 1). Thus, out of five diagnostic enzymes only PEP D was found to be suitable in the differentiation and identification of all the four species. In the patterns of the common isozyme phenotypes of PEP D an identical band (R_f = 89) was shared among *F. cerealis, F culmorum* and *F. graminearum*, the faster moving bands had different mobilities with R_f values of 100, 106, 112 for *F. graminearum, F. cerealis* and *F. culmorum*, respectively. For common isozyme phenotype of PEP D of *F. pseudograminearum* there was a band pair with R_f values of 82 and 115. As mentioned above polymorphisms were only found in a few isolates (Figure 1/A).

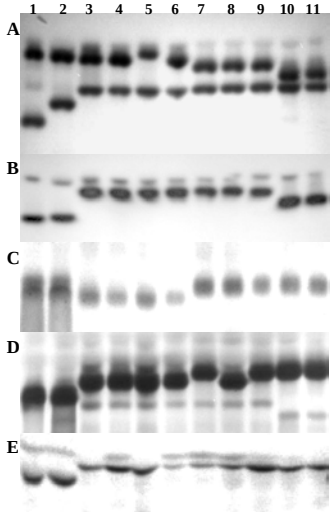

Figure 1. Cellulose acetate gels stained for peptidase D (A), peptidase B (B), adenylate kinase (C), Phosphoglucomutase (D) and NADP dependent glutamate dehydrogenase (E).
Lanes 1–2, *F. pseudograminearum* (NRRL 28333, Fg 7.11), lanes 3–6, F. culmorum (NRRL 25475, 022205, NRRL 29139, CBS 251.52), lanes 7–9, *F. cerealis* (NRRL 13721, NRRL 28442, Fckw 3), lanes 10–11, *F. graminearum* (NRRL 26939, CBS 389.62)

Discussion

In the present study CAE was used to investigate the isozyme banding patterns of 70 isolates of *F. cerealis, F. culmorum, F. graminearum* and *F. pseudograminearum* from various regions around the world. Given the good resolution of isozymes on precast gels, short run time and minimal equipment requirements, CAE has been used frequently and is an excellent diagnostic tool. Oudemans and Coffey [17] found CAE to be an efficient method to identify three morphologically distinct species of *Phytophthora* while in the work of Láday et al. [5] CAE proved to be appropriate for differentiation of *F. graminearum* and *F. pseudograminearum*. Rapid identification of 5 Australian *Ganoderma* spp. with CAE has been described by Smith and Sivasithamparam [18] using the patterns of the single locus of G6PDH. This work also provides isozyme markers for differentiation and identification of 4 *Fusarium* species. While all the 5 diagnostic enzymes (AK, NADP GDH, PEP B, PEP D and PGM) resulted in *F. pseudograminearum*-specific banding patterns, only PEP D was appropriate for the identification of *F. cerealis*. Both AK and PEP D were diagnostic for *F. culmorum*; while PEP B and D proved to be useful in the identification of *F. graminearum* (Figure 1). PEP D was the only enzyme which was diagnostic for all the 4 species therefore the PEP D patterns obtained by CAE provide a highly efficient diagnostic tool for differentiating and identifying *F. cerealis, F. culmorum, F. graminearum* and *F. pseudograminearum* (Figure 1/A).

Although only PEP D was found to be suitable for the differentiation and identification of all the above-mentioned species, different combinations of the 5 diagnostic enzymes could also be used for the diagnosis. For example, a combination of PEP B and AK provides ETs that are suitable for classification. Furthermore, with the number of different isozymes potentially available for use in isozyme analysis it is highly probable that an excellent molecular marker for the differentiation and practical diagnosis of different *Fusarium* species can be found. Moreover, when using PCR to determine which of the four species a given isolate belongs to, four separate reactions are required with different primer pairs and different conditions for each reaction. Our equipment capacity for the CAE diagnostic test allows two enzymes yield results to be obtained with one run, thus making it much faster than PCR-based methods. Additionally, after sample preparation about 300 isolates can be examined a day by CAE with one set of equipment. Based on these facts CAE provides a rapid and accurate method for classification of *Fusarium* isolates.

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Table I

List of *Fusarium* isolates used in this study

***Fusarium* species**	**Isolate code**	**Geographical origin**	**Host**	**Source***	**Original code**	**ETs**
F. cerealis						
	NRRL 13721	Poznan, Poland		1	KF-748[a]	I
	Fckw 1	Australia	cereal debris	2	BBA 63558[b]	I
	Fckw 3	Germany	durum wheat	2	BBA 64545	I
	Item 619	Yugoslavia	wheat kernel	3		I
	Item 662	Poland	corn kernel	3	KF 967	I
	Item 667	Italy	potato tuber	3		I
	NRRL 28442	Valdivia, Chile	*Eucalyptus nitens* roots	1		II
	Fckw 2	Finland	potato tuber	2	BBA 64483	II
	Item 664	Germany	wheat kernel	3	BBA 64320	II
	Item 1097	Poland	potato tuber	3	NRRL A-28100	II
	NRRL 25805	Colombia	burnt páramo soil	1	CBS 195.80[c]	III
	NRRL 25491	Netherlands	*Iris hollandica*, bulb	1	CBS 589.93	IV
	SUF 570	Japan	wheat root	4		V
F. culmorum						
	NRRL 25475	Denmark	barley kernel	1	CBS 417.86	VI
	NRRL 29138			1	CBS 171.28	VI
	NRRL 29140	MN, USA		1	CBS 176.32	VI
	NRRL 29141	Netherlands	soil	1	CBS 256.51	VI
	CBS 251.52	Netherlands	wheat			VI
	72186	Finland	barley	5		VI
	72187	Finland	barley	5		VI
	72202	Finland	wheat	5		VI
	72305	Finland	wheat	5		VI
	022205	Israel	wheat seed	6		VI
	SUF 995	Washington, USA	wheat root	4		VI
	Item 345	France	corn kernel	3		VI
	Item 354	Basilicata, Italy	corn, stalk rot	3		VI
	Item 627	Yugoslavia	wheat kernel	3		VI
	Item 741	Peru	corn kernel	3		VI
	NRRL 29139	Canada	Avena sativa	1	CBS 173.31	VII

Table I (continued)

Fusarium species	Isolate code	Geographical origin	Host	Source*	Original code	ETs
F. graminearum						
	CBS 166.57	Netherlands	corn			VIII
	CBS 389.62	Netherlands	wheat			VIII
	70106	Finland	barley	5		VIII
	7137	Finland	feed mix	5		VIII
	72235	Finland	oat	5		VIII
	72323	Finland	wheat	5		VIII
	022016	Israel	corn seed	6		VIII
	SUF 555	Japan	corn root	4		VIII
	SUF 1021	Japan	wheat ear	4		VIII
	NRRL 26155	Ottawa, Canada	maize caryopsis	1		VIII
	NRRL 26939	North Dakota, USA	barley	1		VIII
	Fg 7.20	NSW, Australia	*Paspalum*, root	2	F11133	VIII
	Fg 7.9	Germany	maize	2	DSM 4527[d]	VIII
	Fg 11	Germany	wheat	2		VIII
	ITEM 644	Italy, Basilicata	*Panicus crusgalli*, stalk	3		VIII
	FG01	Hungary	Orobanche sp.	7		VIII
	FGK1	Hungary	maize kernel	7		VIII
	FGK2	Hungary	maize kernel	7		VIII
	FGÁ1	Hungary	barley seed	7		VIII
	FGÁ2	Hungary	barley seed	7		VIII
	FGB5	Hungary	wheat seed	7		VIII
	FGB6	Hungary	wheat seed	7		VIII
	F50	Yugoslavia	wheat seed	7		VIII
	ASK II	Yugoslavia	maize stalk	7		VIII
	1D	Yugoslavia	wheat stalk	7		VIII
	Pazova 1	Yugoslavia	wheat stalk	7		VIII
	Fg 7.17	NSW, Australia	maize stalk	2	F1402[e]	IX
	Fg 7.3	South Africa	maize	2	CBS 316.73	X
F. pseudograminearum						
	1250	Australia	wheat stem base	8		XI
	NRRL 28065	South Africa	*Medicaco* sp.	1		XI
	NRRL 28069	Settat, Morocco	wheat root	1		XI
	NRRL 28334	Swellendam, South Africa	*Medicaco truncatula*	1		XI
	NRRL 13821	Australia	wheat crown	1		XI

Table I (continued)

Fusarium species	Isolate code	Geographical origin	Host	Source*	Original code	ETs
	NRRL 28388	Breeza, Australia	soil	1		XI
	NRRL 28060	California, USA1	oat stem base	1		XI
	NRRL 28061	Darling Downs, Australia	wheat stem base	1		XI
	Fg 7.11	NSW, Australia	wheat crown	2	F5647	XI
	Fg 7.15	NSW, Australia	wheat crown	2	F11133	XI
	Ispave 218	Italy, Foggia	durum wheat crown	9		XI
	NRRL 28333	Oudtsboorn, South Africa	*Medicaco* pasture	1		XII
	NRRL 28438	Wyoming, USA	wheat crown	1		XIII

*1 = Northern Regional Research Laboratory, ARS/USDA, Peoria, Illinois, USA; K. O'Donnell;
2 = University of Hohenheim, Institute of Plant Breeding, Seed Science, and Population Genetics, Stuttgart, Germany; E. M. Möller;
3 = Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; A. Logrieco;
4 = Shinshu University, Ueda, Nagano-ken, Japan; T. Matuo;
5 = Agricultural Research Centre, Institute of Plant Pathology, Vantaa, Finland; E. A. Jamalainen;
6 = The Hebrew University of Jerusalem, Department of Botany, Jerusalem, Israel; A. Z. Joffe;
7 = Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary; Á. Szécsi;
8 = Queensland Wheat Research Institute, Toowoomba, Australia; R. L. Dodman;
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e L. W. Burgess, Sydney, Australia

Table II

Enzyme systems tested in this study and their Enzyme Commission (E.C.) numbers

Enzyme	Abbreviation	E.C. No.	Activity	No. of bands in the patterns	Electromorphs	Optional buffer system(s)
Aconitase	ACN	4.2.1.3	–	–	–	–
Adenylate kinase	AK	2.7.4.3	+	1	3	TG 8.5
Fumarate hydratase	FUM	4.2.1.2	+	1	2	TG 8.5
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.4 9	+	3	1	TG 8.5; TGC 7.5

Table II (continued9

Enzyme	Abbreviation	E.C. No.	Activity	No. of bands in the patterns	Electromorphs	Optional buffer system(s)
Glutamate dehydrogenase (NAD)	GDH	1.4.1.2	–	–	–	–
Glutamate dehydrogenase (NADP)	GDH	1.4.1.4	+	1	2	TG 8.5
Glucose-6-phosphate isomerase	GPI	5.3.1.9	+	1	1	TG 8.5; TGC 7.5
Hexokinase	HEX	2.7.1.1	–	–	–	–
Isocitrate dehydrogenase (NADP)	IDH	1.1.1.42	+	1	1	CAAPM 7.0
Malate dehydrogenase	MDH	1.1.1.37	+	3	1	CAAPM 7.0; TGC 7.5
Malic enzyme	ME	1.1.1.40	–	–	–	–
Peptidase A (Gly-Leu)	PEP A	3.4.11/13	+	1	3	TG 8.5
Peptidase B (Leu-Gly-Gly)	PEP B	3.4.11/13	+	1	5	TG 8.5
Peptidase D (Phe-Pro)	PEP D	3.4.13.9	+	2	7	TG 8.5
Phosphoglucomutase	PGM	5.4.2.2	+	1	4	TG 8.5
6-Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	+	1	1	CAAPM 7.0
Peroxidase	PRX	1.11.1.7	–	–	–	–
Succinate dehydrogenase	SUD	1.3.99.1	–	–	–	–

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