Brazilian Journal of Microbiology (2008) 39:554-560
ISSN 1517-8382

A NEW PCR APPROACH FOR THE IDENTIFICATION OF *Fusarium graminearum*

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Submitted: October 17, 2007; Returned to authors for corrections: December 24, 2007; Approved: July 13, 2008.

ABSTRACT

The main objective of this work was to develop a PCR protocol for the identification of *Fusarium graminearum*, based on a pair of primers targeted to a segment of the 3´coding region of the *gao*A gene that codes for the enzyme galactose oxidase (GO). This region has low homology with the same region of GO genes from other fungi. Genomic DNA from 17 strains of *Fusarium* spp. isolated from diseased cereals, from several other *Fusarium* species, and from other fungi genera was analyzed in a PCR assay using this primer set. The 17 strains of *Fusarium* spp. were also analyzed for the GO enzyme production in submerse fermentation in a new formulated liquid medium. All strains that were morphologically and molecularly identified as *F. graminearum* were able to secrete the enzyme and had a positive result in the used PCR protocol. No DNA fragment was amplified using genomic DNA from other *Fusarium* species and species of other fungi genera. The results suggest that the proposed PCR protocol is specific and can be considered as a new molecular tool for the identification of *F. graminearum*. In addition, the new formulated medium is a cheap alternative for screening for GO screening production by *F. graminearum*.

Key words: *Fusarium graminearum*, galactose oxidase, *gao*A gene, molecular identification.

INTRODUCTION

F. graminearum has been described as the major causal agent of the scab or fusarium head blight on wheat and barley in several regions of the world, including Brazil (9,17,22). This disease is recognized as one of the most destructive pathology of small cereal crops, reducing grain yield and quality. *F. graminearum* is also a known producer of mycotoxins, mainly of deoxynivalenol and zearalenone, what poses threats to human and animal health and food safety (11,22).

Traditional diagnostic methods for detection and identification of *F. graminearum* in culture or in infected grains are based on micro and macro morphological features. This process is time consuming, requires training, and it can often be difficult to distinguish between similar species. Molecular methods, more sensitive and faster are also employed to the specific identification of *Fusarium* species. There are reports of the use of PCR with primers targeted to the internal transcribed sequence (ITS) of the ribosomal DNA (24) for the detection and identification of *F. graminearum*. However, sequences in the ITS regions have shown to be highly variable in fusaria (20). There also is a report of PCR primers targeted to the junction promoter/5´-coding region of the *gao*A gene, which codes for the enzyme galactose oxidase (GO), for the identification of *F. graminearum* (19). But, the authors of that primer set have reported that it failed to identify one *F. graminearum* strain that was a GO producer (19).

The monomeric enzyme galactose oxidase (D-galactose: O_2 oxidorredutase, EC. 1.1.3.9) is a copper enzyme that catalyzes the oxidation of primary alcohols to its respective aldehydes, with concomitant reduction of O_2 to H_2O_2 (23,30). This enzyme has several biotechnological applications including: lactose and galactose assays in dairy industry and in clinical laboratory (1,13), carbohydrate synthesis (16), biotransformation of

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glycobiopolymers (6), histochemistry studies (25), and colon cancer diagnosis (26).

Extra cellular production of GO has been shown for *F. graminearum* (3,5,7), *Gibberella fujikuroi* (2), *Fusarium moniliforme* f. sp. *subglutinans* (5), and *Fusarium acuminatum* (5). However, most of the studies and the industrial production of GO use the isolate *F. graminearum* NRRL 2903, that was initially classified as *Dactylium dendroides* (3,19,21). The *gao*A gene has no intron and has been cloned for this strain (18).

Problems associated with the use of GO regards the availability of sufficiently pure enzyme. The purification procedures are laborious and the commercial preparations are very expensive (16). Because of these reasons, the applications of GO would be beneficial by the finding of new producer isolates that could be able to produce high levels of the enzyme. Traditional methods for screening GO production are based on submerse fermentation and search for enzyme activity in the liquid medium (5,7).

Because it would be interesting the development of a new molecular assay for the identification of *F. graminearum*, the main objective of this study was to develop a new PCR protocol using a primer set targeted to the internal 3´-coding region of the *F. graminearum gao*A gene. If proven specific, this method could be used for *F. graminearum* identification for fusarium head blight diagnosis and food quality assessment. Another objective of this work was to develop a new medium for GO screening production.

MATERIALS AND METHODS

Microorganisms and maintenance

Seventeen strains of *Fusarium* spp. (Table 1) were isolated from diseased wheat and triticale from Southern Brazil. The method for isolation, the geographic origin, the host, and the plant organ where they were isolated were described elsewhere (4,10,11). The isolates UEM 67 and UEM 68 were isolated in the city of Campo Mourão, Paraná state, from wheat seed. The *F. graminearum* original GO producer strain (GOPS) (3) and two other GO *Fusarium* spp. producers, *F. acuminatum* UnB 356 (5) and *F. moniliforme* f. sp. *subglutinans* UnB 379 (5), were used as references. All isolates are being maintained in potato dextrose agar (PDA) slants with trimestral transfer and in stocks under mineral oil.

Culture conditions for enzyme production

The liquid medium used for submerse fermentation was developed based on other media described in the literature (15,27,28). This medium was prepared in three separate solutions: solution A: 62 mM Na_2HPO_4, 62 mM KH_2PO_4, 13 mM $(NH_4)NO_3$, 15 mM $(NH_4)_2SO_4$, 15 mM NaOH, 14 mM KOH, 0.1% yeast extract, pH 7.0; solution B: 1.6 mM $MgSO_4{\cdot}7H_2O$, 11.8 µM $MnSO_4{\cdot}H_2O$, 10 µM $CuSO_4{\cdot}5H_2O$; and solution C: 1% glucose. The indicated concentrations are the ones in the final medium. Solutions A and B were sterilized by autoclaving, for 20 minutes. Solution C was sterilized in fluent vapor, for 20 minutes. Before use, the three solutions were aseptically combined: 22.5 ml of solution A, 0.5 ml of solution B, and 2.0 ml of solution C. A fragment of a fresh PDA culture (1 cm^3) was used as inoculum for 125 ml Erlenmeyer culture flasks containing 25 ml of the liquid medium. Culture involved an initial 4 day inoculum growth on a rotary shaker at 100 r.p.m., in dark, at 25ºC, followed by homogenization using passage first through a sterile sieve and then through a sterile 18-gauge needle attached to a sterile syringe. The homogenized was used as inoculum (2% v/v) to new 125 ml culture flasks, containing 25 ml of the same liquid medium, which were grown in the same conditions, for 72 hours, and then filtered through filter paper. The culture filtrates were used for the enzymatic analysis. The mycelia biomass was determined after drying the mycelia at 50ºC for at least 24 hours.

Enzyme assay

GO activity was assayed by the peroxidase/*o*-dianisidine colorimetric method (3,28), based on the estimation of the colored oxidized *o*-dianisidine. Filtrate (0.5 ml pure or diluted); 1.4 ml of the reactive mixture [50 mM phosphate buffer, pH 7.0; 0.2 mg/ml *o*-dianisidina (Sigma D-3252; previously dissolved in methanol 2 mg/ml); 0.04 mg/ml (6.0 U/ml) peroxidase (Sigma P-8125)]; and 0.1 ml of 0.5 M D-(+)-galactose were mixed and incubated for 10 minutes at 30ºC. The brown colored product was read at 460 nm. For these conditions, one enzymatic unit corresponded to an absorbance of 1.0. Culture medium filtrates that resulted in no activity at the 10 minutes reaction in the enzyme assay were incubated for extra 20 minutes and the absorbance was read again.

DNA Extraction

Mycelia of the 17 isolates of *Fusarium* spp. and of the reference isolates were obtained in liquid culture, as described above. Mycelia of the other *Fusarium* species isolates and of the other fungi genera were obtained by transferring a fragment of a fresh PDA slant culture to 25 ml of the liquid medium described above or to 25 ml of liquid potato dextrose medium, respectively, in 125 ml Erlenmeyer flasks and culturing for three 3 days, in the conditions described above. Genomic DNA was extracted using the protocol described by Koenig *et al.* (14) and modified as follows. The mycelia mass obtained in liquid culture was collected by filtration in sterilized gauze, macerated in liquid nitrogen, and transferred to microcentrifuge tubes. The extraction buffer was added to the macerated mycelia in the proportion of 700 µl to each 300 µl of macerated mycelia. The extraction buffer contained, in the proportion of 1.0/1.0/0.4, nuclear lyses buffer (0.2 M Tris, pH 7.5; 50 mM EDTA; 2% (w/v) cetyltrimethylammonium bromide; pH 7.5), DNA isolation

Table 1. Used isolates with some culture characteristics and summary of the enzyme production and PCR results.

Species	Strain	Red pigment[c]	Spores in liquid culture	Galactose oxidase (U/g of dried mycelia)[d]	Specific PCR fragment with primers	
					GO	UBC85[g]
F. graminearum[a]	GOPS	+	–	4513 ± 471	+	+
F. graminearum	UEM 02	+	–	2018 ± 140	+	+
F. graminearum	UEM 08	+	–	1269 ± 323	+	+
F. graminearum	UEM 10	+	–	432 ± 92	+	+
F. graminearum	UEM 12	+	–	1351 ± 386	+	+
F. graminearum	UEM 13	+	–	1068 ± 145	+	+
F. graminearum	UEM 14	+	–	397 ± 3.7	+	+
F. graminearum	UEM 15	+	–	32 ± 5	+	+
F. graminearum	UEM 16	+	–	25 ± 10[e]	+	+
F. graminearum	UEM 18	+	–	29 ± 14	+	+
F. graminearum	UEM 29	+	–	751 ± 276	+	+
Fusarium sp.	UEM 32	–	+	–[e]	–	–
F. graminearum	UEM 35	+	–	372 ± 81	+	+
F. graminearum	UEM 41	+	–	1237 ± 42	+	+
F. graminearum	UEM 45	+	–	473 ± 156	+	+
Fusarium sp.	UEM 67	–	+	11 ± 3.6	–	–
Fusarium sp.	UEM 68	–	+	–[e]	–	–
Fusarium sp.	UEM 69	–	+	–[e]	–	–
F. acuminatum[b]	UnB 356	–	–	+[f]	–	–
F. moniliforme f. sp. *subglutinans*[b]	UnB 379	–	+	+[f]	–	–
F. moniliforme f. sp. *subglutinans*[b]	UnB 820	–	+	–[f]	–	–
F. solani[b]	UnB 622	–	+	–[f]	–	–
F. tricinctum[b]	UnB 1273	+	+	–[f]	–	–
F. decemcelulare[b]	UnB 133	–	+	–[f]	–	–
F. avenaceum[b]	UnB 1271	–	+	–[f]	–	–
G. fujikuroi[a]	NRRL 2278	–	+	–[f]	–	–
F. oxysporum f. sp. *vasinfectum*[b]	UnB 200	–	+	–[f]	–	–
Penicillium citrinum[a]		–	ND	ND	–	–
Penicillium wortmanii[a]		–	ND	ND	–	–
Ascochyta pisi[a]		–	ND	ND	–	–

[a] Strain kindly provided by Dr. C. Kemmelmeier (Universidade Estadual de Maringá, Brazil); [b] Strain kindly provided by Dr. J. C. Dianese (Universidade de Brasília, Brazil); [c] Culture in PDA, at 25ºC, in dark; [d] Values are means and standard deviation of the results obtained in the analyses performed independently in three culture flasks; [e] Determined after 30 minutes of reaction in the enzyme assay; [f] Determined by Barbosa-Tessmann *et al.* (5); [g] Primers described by Schiling *et al.* (24). ND – not determined.

buffer (0.35 M sorbitol; 0.1 M Tris, pH 7.5; 5 mM EDTA; pH 7.5), and 5% Sarkosyl. The extraction buffer was combined right before use and was then added of 3.8 mg/ml of sodium bisulfite. The tubes were incubated in a dry bath at 65ºC for 60 minutes. After that, 500 µl of a mixture of chloroform:isoamylic alcohol (24:1) were added. The tubes were centrifuged at room temperature (12.000*g*, 10 minutes) and the supernatant fraction was transferred to clean tubes. Samples were treated with five µl of RNAse A (20 mg/ml), for 30 minutes, at 37ºC, and after with 5 µl of proteinase K (20 mg/ml), for 30 minutes, at 56ºC. The DNA was then precipitated with equal volume of isopropanol and overnight incubation at -20ºC. The precipitated DNA was collected by centrifugation at room temperature (12.000*g*, 10 minutes) and the DNA pellet was washed three times with cold 70% ethanol. The final DNA pellet was dried at room temperature and ressuspended with 50 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). The whole process was carried out under sterile conditions. The DNA was quantified in a spectrophotometer at 260 nm. The DNA final concentration was adjusted to 100 ng/µl in TE buffer.

PCR Analysis

For primer designing, GO coding genes from *F. graminearum* (*gao*A gene) (8,18) (GenBank AN M86819), from *Fusarium venenatum* (12), from *Aspergillus oryzae* (GenBank AN BD160924), and from *Fusarium sporotrichioides* (GenBank AN BI201662 and GenBank AN BI190798) were aligned using the ClustalW program (Fig. 1a). A region of low homology was chosen for primer designing. The primer set (GOFW 5´-ACCTCTGTTGTTCTTCCAGACGG-3´and GORV 5´-CTGGTCAGTATTAACCGTGTGTG-3´) were designed to amplify a 435 pb DNA fragment of a internal 3´-coding region of the gene (Fig. 1b), based on the sequence published by McPherson *et al*. (18) and Firbank *et al*. (8). Primers used for *F. graminearum* specific identification ($UBC85F_{410}$-$UBC85R_{410}$) were the ones described by Schiling *et al*. (24). All genomic DNAs used in this work were tested for PCR amplification suitability using the primers ITS4 and ITS5 targeted to the ITS-5.8 S rRNA region as described by White *et al*. (29). A DNA fragment of approximately 550 bp was amplified from all genomic DNAs using the ITS4 and ITS5 primers (not shown). The amplification reactions were performed in a termocycler Techne TC-312 (England) in PCR tubes containing 25 µL of the following reaction mixture: 50 mM KCl; 10 mM Tris, pH 7.5; 1.5 mM $MgCl_2$, 1.5 U of Platinum Taq DNA polymerase (Invitrogen); 0.2 mM of each dNTP (Invitrogen); 25 pmol of each primer (forward and reverse), and 20-400 ng of the DNA sample. The PCR reaction consisted of 25 cycles of 1 minute and 30 seconds at 94ºC, 1 minute and 30 seconds at 55ºC for the GO and UBC85 primers and at 50ºC for the ITS primers, and 2 minutes at 72ºC. Previously to the cycles, the samples were heated for 5 minute at 94ºC, and after the cycles the samples were incubated for 10 minutes at 72ºC and frozen at -20ºC until use. Positive control was the DNA from the isolate *F. graminearum* GOPS. Negative controls (no DNA template) were used in each experiment to test for the presence of DNA contamination of reagents and reaction mixtures. Ten microliters of the PCR reaction were analyzed in an agarose gel containing ethidium bromide (0.25 µg/ml). Molecular weight markers were the 100 bp markers from Promega or Invitrogen, USA. The PCR products were visualized and photographed under UV light.

a)

Primer GOFW (*F. graminearum*)	5´-ACCTCTGTTGTTCTTCCAGACGG-3´
F. venenatum	ACTTCTGTTGTTCTCCCCGATGG
A. oryzae	ACTTCGGTCGTCCTTCCTGATGG
F. sporotrichioides[a]	ACTTCTGCGGCTCCC---GAGGG
	** ** * * * ** **
Primer GORV (*F. graminearum*)	5´-CTGGTCAGTATTAACCGTGTGTG-3´
F. venenatum	TTGATCAGTGTTGACGGTATGCG
A. oryzae	CTGATCGGTGTTGACGGTGTGAG
F. sporotrichioides[b]	CCAG--GATACTGGATGCTTTTC
	* * * *

b)

540 591 Primer GOFW 1917 2507
*gao*A gene
2351 Primer GORV
Pro-sequence Mature protein

Figure 1. The *gao*A gene targeted set of primers. a) Correlation among the sequence of the GO primers targeted to the *F. graminearum gao*A gene (18) (GenBank AN M86819), and the sequences of the GO genes from *F. venenatum* (12), *A. oryzae* (GenBank AN BD160924), and *F. sporotrichioides* ([a]GenBank AN BI201662 and [b]GenBank AN BI190798) obtained by ClustalW alignment of the respective genes. Stars indicate conserved nucleotides. b) A schematic representation of the *gao*A gene as reported by McPherson *et al*. (18) and Firbank *et al*. (8), indicating the positions of the GO primers. The mature protein and the pro-sequence present in the immature protein (8) are indicated.

RESULTS

The results of the enzyme production by the isolates are summarized in Table 1. No enzyme activity was detected in the filtrates of the isolates UEM 32, UEM 68, and UEM 69, even in the 30 min enzyme assay. Some isolates produced a good level of the enzyme, but no isolate could produce levels of GO higher than the ones produced by the reference isolate (GOPS).

PCR analysis with the GOFW and GORV primers revealed amplification of a single DNA fragment from purified genomic DNA of all *F. graminearum* strains (Fig. 2a, Table 1). The obtained fragment size was in good agreement with the theoretical size of the fragment (435 bp) expected from the *gao*A gene sequence data (18). The GO primers had the same specificity as the *F. graminearum* specific primers UBC85 (Fig. 2b, Table 1), which could amplify a 332 bp fragment from the same isolates. The used PCR protocol and primers could not amplify any fragment, specific and non specific, using DNA template from other species of *Fusarium* and from species of other fungi genera (Table 1)

To test the method sensitivity, the DNA of the GOPS isolate was ten-fold serially diluted and tested in the PCR protocol (Fig. 3). The PCR method could amplify a DNA fragment with concentrations of DNA as low as 4 ng.

DISCUSSION

Markus *et al*. (15) optimized the production of galactose oxidase by *F. graminearum* in shake culture flasks. They described a liquid culture medium composed by nitrate and ammonia as the nitrogen source, yeast extract, glucose as the carbon source, and mineral ions. They also reported that thiamine could substitute the yeast extract with no loss in enzyme production, and that sorbose could replace glucose with an increment in enzyme production. Subsequently, Shatzman and Kosman (27) reported the influence of medium

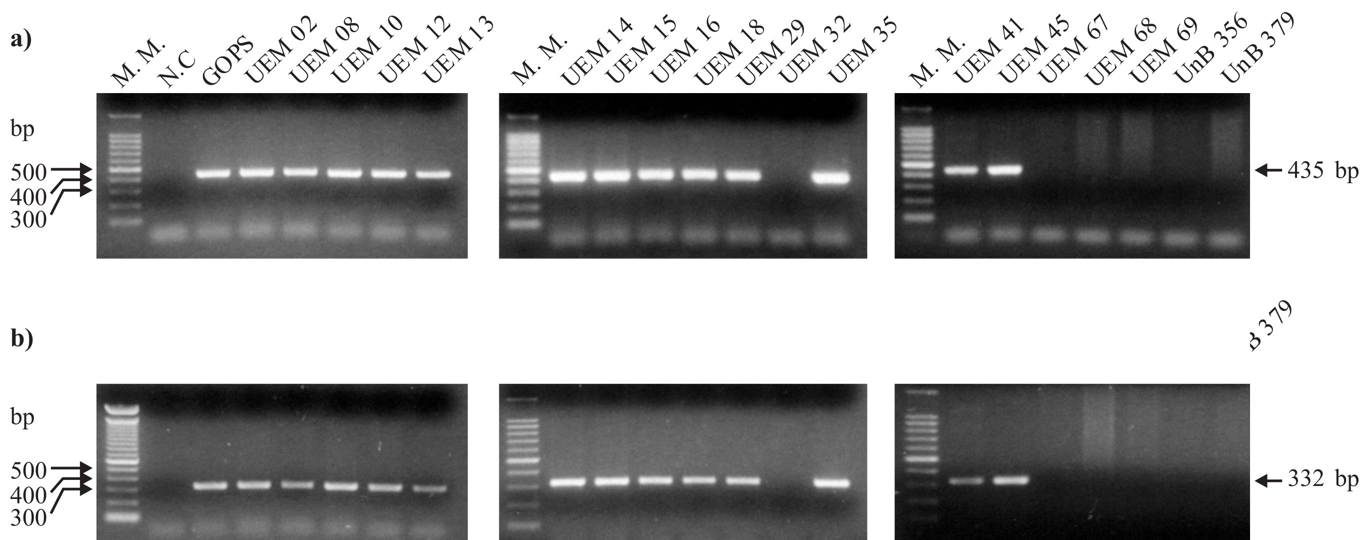

Figure 2. Specificity of the PCR protocol with the GOFW-GORV primer set. a) PCR reactions with the GOFW-GORV primer set using 400 ng of genomic DNA from the isolates. b) PCR reactions using the primers $UBC85F_{410}$-$UBC85R_{410}$ (24). Reactions were analyzed in 1.5% agarose gel stained with ethidium bromide. M.M. indicates the 100 bp Molecular Marker lane. N.C. indicates the Negative Control PCR reaction. GOPS indicates the PCR reaction in which the DNA of the reference galactose oxidase producer strain of *F. graminearum* was used. UEM 02, UEM 08, UEM 10, UEM 12, UEM 13, UEM 14, UEM 15, UEM 16, UEM 18, UEM 29, UEM 32, UEM 35, UEM 41,UEM 45, UEM 67, UEM 68, and UEM 69 indicates the PCR reactions in which the DNA from the respective isolate of *Fusarium* spp. was used. UnB 356 indicates the PCR reaction in which the DNA of the reference strain of *F. acuminatum* was used. UnB 379 indicates the PCR reaction in which the DNA of the reference strain of *F. moniliforme* f. sp. s*ubglutinans* was used. The size of the amplified fragments is indicated on the right side.

pH on enzyme production. More recently, Tressel and Kosman (28) described a medium containing nitrate and ammonia as the nitrogen source, thiamine as the only vitamin, sorbose as the carbon source, mineral ions, and having a better buffering system for pH 7.0. Sorbose is an expensive carbohydrate and some of the strains used in this work could not grow well in the medium reported by Tressel and Kosman (28). Because of this, a combined and new medium was formulated. This new medium has the buffering system of the medium developed by Tressel and Kosman (28) and the other components used in the medium described by Markus *et al*. (15). All used microorganisms could grow well in this new medium and it represents a cheap alternative for *F. graminearum* GO screening production in submerse fermentation.

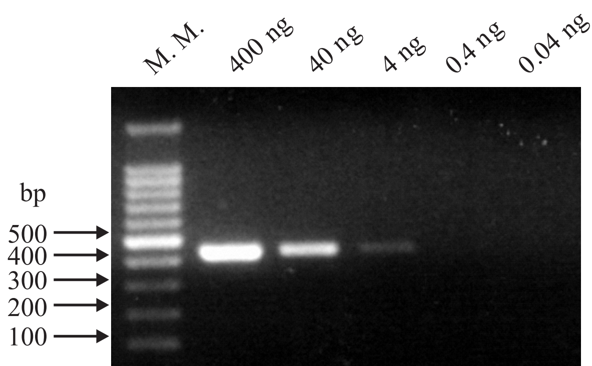

Figure 3. Sensitivity of the PCR method with the GOFW-GORV primer set. PCR using a ten-fold serial dilution of genomic DNA from *F. graminearum* reference strain GOPS as template. Agarose gel (1.5%) stained with ethidium bromide showing the amplification products. M.M. indicates the 100 bp Molecular Marker lane.

All isolates that could pigment the PDA medium with a red pigment and that did not produce spores in liquid culture, similar to the reference *F. graminearum* strain GOPS (Table 1), could produce the enzyme and had a DNA fragment of the *gao*A gene amplified in the PCR reaction. In addition, all of these isolates were molecularly identified as *F. graminearum* with the UBC85 primers (Table 1, Fig. 2b). These results are in agreement with previous screening studies, which evidenced that production of galactose oxidase is much more abundant among *F. graminearum* strains (5,7).

The primers targeted to the *gao*A gene used in this study appears to be specific for *F. graminearum*, since they did not

amplify any DNA fragment, specific or non specific, when the genomic DNA of other GO secreting *Fusarium* species was used in the PCR protocol, including the genomic DNA of the GO producing reference isolates *F. acuminatum* and *F. moniliforme* f. sp. s*ubglutinans* (Fig. 2a). This may indicate differences among the GO gene of those *Fusarium* species with the *gao*A gene from *F. graminearum*, within the primers localization. Considering this, the isolate UEM 67 that was not morphologically nor molecularly identified as *F. graminearum*, with the UBC85 primers and with the GO primers (Table 1; Fig. 2a), but produced the enzyme in a very low level could be one of these reference species, probably *F. moniliforme* f. sp. *subglutinans*, because of the spore production in the liquid culture medium.

The designed pair of primers targeted to the GO gene could not amplify any fragment of DNA, specific or non specific, when genomic DNA from other fungi genera was used in the established PCR protocol (Table1). This certifies the specificity of the generated method.

Taking into consideration the specificity of the GO primers PCR protocol, the generated methodology could be considered as a new molecular tool for *F. graminearum* identification. Its use for the molecular identification of *F. graminearum* represents an advance for head blight diagnosis and food safety assessment.

ACKNOWLEDGEMENTS

This research was supported by: the International Foundation for Science, Stockholm, Sweden, and The Organization for the Prohibition of Chemical Weapons (OPCW), THE HAGUE, Netherlands, through a grant to Dr Ione Parra Barbosa Tessmann. This research was also supported by the Fundação Araucária (Secretaria da Ciência e Tecnologia do Estado do Paraná, Brazil). The authors wish to thank Dr. Carlos Kemmelemeir and Dr. José Carmine Dianese for the kind donation of some of the isolates.

RESUMO

Um novo protocolo de PCR para a identificação de *Fusarium graminearum*

O principal objetivo deste trabalho foi desenvolver um novo protocolo de PCR para identificação de isolados de *Fusarium graminearum*, baseado no uso de um par de iniciadores direcionado para um segmento da região 3′ codificadora do gene *gao*A que codifica a enzima galactose oxidase (GO). Esta região possui baixa homologia com a mesma região de genes da GO de outros fungos. O DNA genômico de 17 cepas de *Fusarium* spp. isoladas de cereais infectados com sintomas, de vários outras espécies de *Fusarium* e de outros gêneros de fungos foi analisado em um protocolo de PCR utilizando os iniciadores desenhados. Os 17 isolados de *Fusarium* spp. também foram analisados para a produção da enzima GO em fermentação submersa em um novo meio líquido. Todas as cepas que foram morfologicamente e molecularmente identificadas como *F. graminearum* foram capazes de secretar a enzima e tiveram um resultado positivo no protocolo de PCR, utilizando os iniciadores direcionados para o gene *gao*A. Nenhum fragmento de DNA foi amplificado quando foi utilizado o DNA genômico de várias outras espécies de *Fusarium* e de espécies de outros gêneros de fungos. Os resultados sugerem que o protocolo de PCR gerado é específico e pode ser considerado como uma nova ferramenta molecular para a identificação de cepas de *F. graminearum*. Além disso, o meio líquido formulado é uma alternativa barata para a avaliação da produção de GO por *F. graminearum*.

Palavras-chave: *Fusarium graminearum*, galactose oxidase, gene *gao*A, identificação molecular.

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