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A RAPID, EASY AND HIGH YIELD PROTOCOL FOR TOTAL GENOMIC DNA ISOLATION OF *Colletotrichum gloeosporioides* AND *Fusarium oxysporum*

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ABSTRACT. A rapid, easy and simple protocol for total genomic DNA isolation was developted for *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. The total genomic DNA yield was very high and suitable to RAPD (random amplified polymorphic DNA), and restriction endonuclease reactions. DNA obtained by this protocol was not contaminated by protein or carbohydrate, and proteases, cesium chloride or phenol were also not necessary.

Key words: DNA isolation, plant pathogen, RAPD molecular marker.

PROTOCOLO RÁPIDO, FÁCIL E DE ALTO RENDIMENTO DE ISOLAMENTO DE DNA GENÔMICO TOTAL DE *Colletotrichum gloeosporioides* E *Fusarium oxysporum*

RESUMO. Um protocolo simples, fácil e rápido de isolamento de DNA genômico total de *Colletotrichum gloeosporioides* e *Fusarium oxysporum* foi desenvolvido. O rendimento de DNA total foi alto suficiente e apropriado para reações de RAPD (Polimorfismo de DNA Ampliado ao Acaso) e de restrição de endonuclease. O DNA obtido por este protocolo não apresentou contaminações por proteínas e nem por carboidratos e, não foram utilizados proteases, cloreto de césio e nem fenol.

Palavras-chave: isolamento de DNA, patógeno de planta, marcador molecular.

INTRODUCTION

Several DNA isolation methods have been developed since the introduction and application of molecular markers to study plant

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fungal genetics. Many protocols require the use of enzymes like proteases and/or phenol to obtain a suitable protein-free preparation. Such protocols have been used to isolate the DNA of *Fusarium oxysporum* f. sp. *vasinfectum* (Assigbetse *et al*., 1994), *Sclerotium rolfsii* (Nalim *et al.*, 1995), *Leptosphaeria maculans* (Melayah *et al.*, 1995), *Rhizoctonia solani* (Brisbane *et al*., 1994), *Monosporascus* spp. (Lovic *et al*., 1995), *Phytophthora* species (Parnabières *et al*., 1989), *Colletotrichum graminicola* (Guthrie *et al*., 1992) for PCR reactions. Centrifugation in gradient cesium chloride has also been applied in many procedures for DNA isolation, but this methodology is very laborious and time-consuming (Hayden *et al*., 1994).

Small quantity of DNA is required for RAPD reactions, but to digest with restriction endonucleases (Kuramae-Izioka *et al.*, 1997), high amount of DNA is usually necessary. We have improved a simplified, rapid and easy protocol for isolation of high quality DNA of *Colletotrichum gloeosporioides* and *Fusarium oxysporum* that can be used in RAPD assay.

METHODS AND RESULTS

DNA isolation. Isolates of *C. gloeosporioides* (01,02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15 and 16) were maintained in tubes containing PDA (potato-dextrose-agar) medium and mineral oil, and isolates of *Fusarium oxysporum* (A, B, C, D e E) in tubes containing soil until DNA extraction. One disc (0.5 mm diameter) from the tube containing isolates of *C. gloeosporioides* and one aliquot of soil containing *Fusarium oxysporum* were placed in a Petri dish containing 25 ml of PDA medium and maintained in an incubator at 24ºC, in darkness for 5 days. Three 0.5 mm diameter mycelium discs from the previous Petri dish were replaced into 500 ml Erlenmeyer flask containing 200 ml PD broth. The flasks were maintained in 12/12 (light/dark) photoperiod, during seven days at 24ºC, on a rotary shaker set to 150 rpm. Cultures were harvested by 8,000 x g centrifugation, rinsed three times with sterile distilled water and stored frozen at -20ºC until DNA isolation. The frozen mycelia were ground in mortar under liquid nitrogen. The resulting powder was suspended in two volumes of extraction buffer (100mM Tris pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl, 10mM β-mercaptoethanol, 1% sodium dodecyl sulphate) and transferred into

tubes. The homogenate was incubated for 40 minutes at 65ºC. One-half volume of 5M potassium acetate was added to samples, gently mixed and incubated on ice for 30 minutes. The samples were centrifuged at 10,000 x g for 10 minutes and the supernatant of each sample was transferred into a clean tube, and extracted once more with equal volume of chloroform-isoamyl alcohol (25:1). The samples were manually, well mixed and centrifuged at 10,000 x g for 10 minutes and the supernatant transferred to another tube. One volume of isopropanol was added to precipitate the DNA, that was dissolved in 1/10 TE (10 mM Tris, pH 8.0, 1mM EDTA), and treated with 40 µg/ml of DNase-free RNase (Sigma Chemical Co., St. Louis, MO) at 37ºC, for 3 hours. The DNA was quantified in GenQuant spectrophotometer (Pharmacia). The A_{260nm}/A_{280nm} ratio of DNA preparations were between 1.7 and 1.9 indicating that they were essentially free of proteins and carbohydrates (Table 1). The final concentrations of DNA are summarized in Table 1. All DNAs were kept at -20ºC until RAPD reactions.

Table 1. DNA concentration (ng/µl) of *Colletotrichum gloeosporioides* and *Fusarium oxysporum* isolates and ratio (A_{260nm}/A_{280nm}) values calculated by GeneQuant spectrophotometer.

Isolate	DNA concentration (ng/µl)	Ratio values (A_{260nm}/A_{280nm})
01 (*C. gloeosporioides)*	729.1	1.693
02 (*C. gloeosporioides)*	809.7	1.791
03 (*C. gloeosporioides)*	640.9	1.900
04 (*C. gloeosporioides)*	786.6	1.845
05 (*C. gloeosporioides)*	903.9	1.809
06 (*C. gloeosporioides)*	1,131.3	1.752
07 (*C. gloeosporioides)*	609.4	1.699
08 (*C. gloeosporioides)*	743.7	1.789
09 (*C. gloeosporioides)*	1,032,3	1.823
10 (*C. gloeosporioides)*	800.8	1.780
11 (*C. gloeosporioides)*	936.9	1.731
12 (*C. gloeosporioides)*	698.4	1.865
13 (*C. gloeosporioides)*	1,211.3	1.811
14 (*C. gloeosporioides)*	940.5	1.788
15 (*C. gloeosporioides)*	726.4	1.742
16 (*C. gloeosporioides)*	1,110.8	1.822
A (*F. oxysporum*)	978.2	1.799
B (*F. oxysporum*)	965.4	1.801
C (*F. oxysporum*)	991.4	1.800
D (*F. oxysporum*)	867.5	1.761
E (*F. oxysporum*)	885.9	1.782

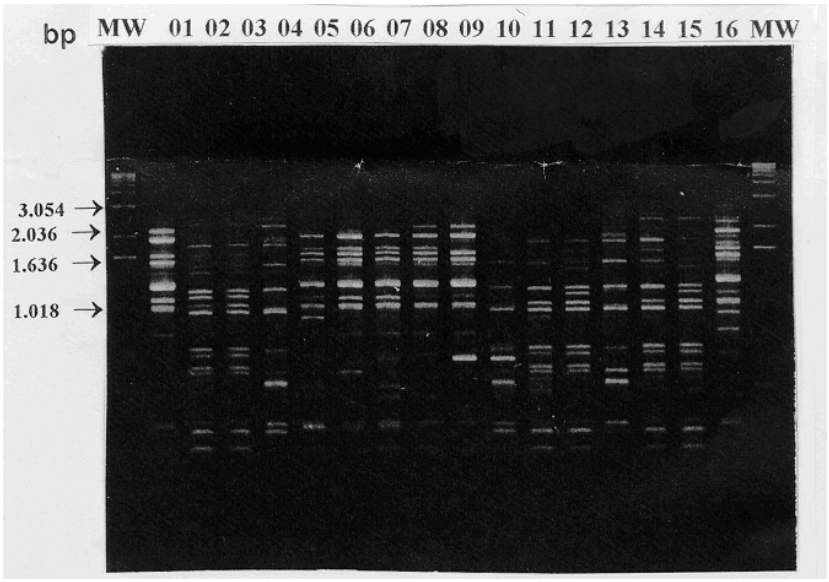

Figure 1. RAPD patterns generated from 16 different isolates of *Colletotrichum gloeosporioides* using primer OPG- 02. MW = Molecular weight marker.

RAPD assay. RAPD assay was carried out to test the DNA quality. The procedures were essentially the same as described by Williams *et al.*, (1990). Reactions were performed in 500 µl Eppendorf tubes containing *Taq* polymerase buffer (1X), 1.5 mM $MgCl_2$; 200µM each dNTPs, 30 pmoles 10-nucleotide primer (Operon Technologies Inc., Alameda, CA), 9 ng of genomic DNA and 1.0 unit *Taq* polymerase (Gibco BRL Life Technologies, Inc.). The 20µl reaction solution was overlayed with 50µl of mineral oil. OPG-02, OPG-05, OPG-07, OPG-08, OPG-09, OPG-12, OPG-13, OPG-15, OPR-07, OPX-04 and OPX-08 primers were used for DNA amplification of *C. gloeosporioides* isolates and OPX-02, OPX-03, OPX-04, OPX-07, OPX-08, OPR-08, POR-09 and OPR-10 primers were used for *Fusarium oxysporum* isolates. The thermocycler (MJ Research) was programmed for one cycle at 94ºC for 2 minutes, followed by 35 cycles at 94ºC for 1 minute, 35ºC for 1 minute, 72ºC for 1.5 minutes, and one cycle at 72ºC for 5 minutes. Negative controls, in which DNA template solution was replaced by water, were included in all experiments to test for contamination. After the reaction 3.0µl of a solution containing 40% sucrose and 0.25% blue bromophenol was

added to each tube (Sambrook *et al*., 1989). The samples and 200ng of Ladder 1Kb molecular marker (Gibco BRL Life Technologies, Inc.) were loaded on a 1.7% agarose gel containing 0.5 mg/ml ethidium bromide. Gels were run in TBE (0.1M Tris-HCl, 0.1M boric acid, 0.02 mM EDTA, pH 8,3) buffer at approximately 5V/cm of gel. The gels were photographed under UV lights.

Adequate amplification with good bands for score was observed in both *C. gloeosporioides* and *F. oxysporum* species (Figures 1 and 2, respectively).

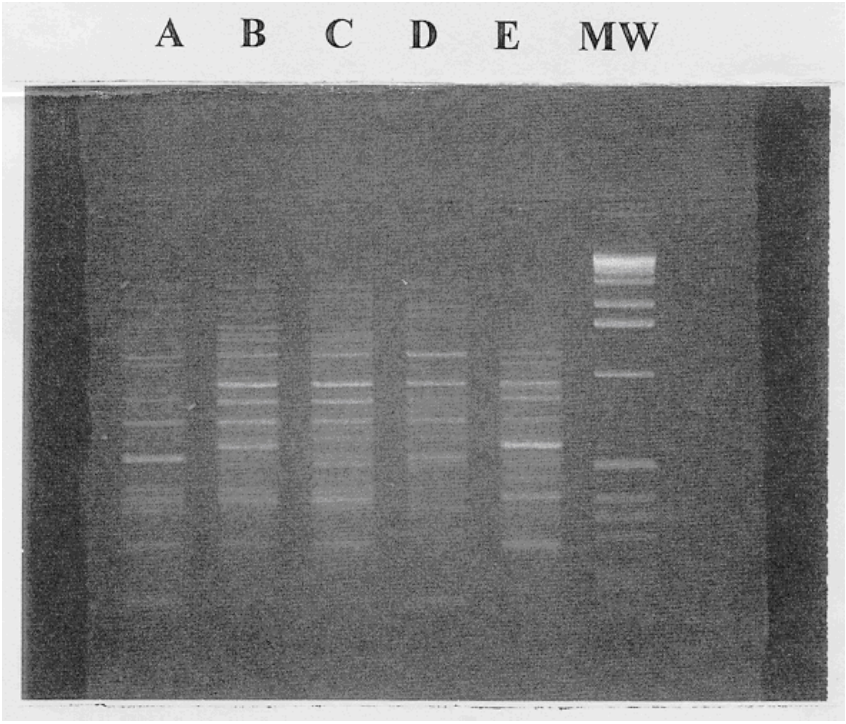

Figure 2. RAPD patterns generated from 5 different isolates of *Fusarium oxysporum* using primer OPX- 08. MW = Molecular weight marker.

The protocol for total DNA extraction showed to be very easy and rapid. This protocol did not use extraction with phenol, CsCl or addition of proteases. Lyophilization of the mycelium was also not required because it was possible to keep frozen mycelia at -20ºC until

DNA extraction. The DNA quality obtained by this protocol was considered good for RAPD reactions, without high contamination of proteins or carbohydrates by 260nm/280nm ratio value (Table 1). DNA yield was considered high enough for RAPD. Total genomic DNA extraction and RAPD assay can be carried out in just one day.

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