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Evaluation of the Efficiency of the Products of a Non-Phenol-Chloroform Dna Extraction Method as Templates for Pcr Analysis of Some *Fusarium oxysporum* Isolates

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Abstract: Four *Fusarium oxysporum* isolates, f.sp *lycopersici* Race 1 (Kis-1), Race 2 (880621a-1), Race 3 (Tomino-1c) and f.sp *radicis lycopersici* (MAFF 103044) were obtained from the stock of the Laboratory of Plant Pathology, Tokyo University of Agriculture and Technology, Japan. They were cultured on Potato Sucrose Agar (PSA) at 26.3°C and genomic DNAs were successfully extracted using a novel non -Phenol-Chloroform DNA extraction method. The DNA extracts were efficient as templates for polymerase chain reaction analysis using primer sets based on the *Fusarium* ribosomal DNA intergenic spacer region (IGS), as well as race-specific primers sp13 and sp23 primers, based on the endo- and exo-polygalacturonase gene sequences. The results showed that the method recently described for the extraction of DNA from *Magnaporthe grisea* could also be used for fusarial DNA extraction useful in widespread diagnostics and molecular analyses.

Key words: Fusarial DNA · race-specific primers · IGS region · PCR · novel non-phenol-chloroform method

INTRODUCTION

The vascular wilt fungus *Fusarium oxysporum* is a filamentous Ascomycete whose various forms attack different plant species. The species of *Fusarium* have traditionally been differentiated by their morphological characteristics on selective media [1, 2]. It is almost impossible, however, to identify pathogenic types, or forma speciales and races of *Fusarium oxysporum* using morphological features.

Immunoassays was initially proposed as alternative methods to use of morphological characterization [3, 4] while recently, molecular markers have become popular for identifying species and subspecies in fungi. Although the intergenic spacer region (IGS) sequence is useful for the general diagnosis of the *Fusarium oxysporum* genome regardless of race, race-specific identification should prove more useful. Different races are known to infect and induce diseases differentially on different cultivars of the same host species [5].

The successful extraction of DNA in sufficient quantity and quality is an essential step for molecular analyses of fungi [6]. Ability to do this at relative speed and at the micro level may be all the more desirable considering cost implications.

The objective of this study was to evaluate the efficiency, in the extraction of the fusarial genomic DNA, of the novel non phenol-chloroform method described by Saitoh *et al.* [6] and the use of the DNAs as templates in polymerase chain reaction analysis based on the intergenic spacer region IGS primers and the specific primers- sp13 and sp23.

MATERIALS AND METHODS

Extraction and purification of genomic DNAs from fungal mycelia by non- phenol-chloroform method: The method employed was essentially the mini-preparation protocol reported by Saitoh *et al.* [6]. About 10 mm diameter mycelia block from each of the four fungal isolates was cut from a 5-day old fungal culture and placed in separate 1.5 ml Eppendorf tubes. Five hundred (500) µl of lysis buffer (200 mM Tris HCl, 50 mM Ethylene diamine tetra acetic acid (EDTA), 200 mM NaCl, 1% n-Lauroyl Sarcosine-Na; pH 8.0) was added. The mixture was kept for 10 min at room temperature before being dispersed with tooth pick. Potassium Acetate (150 µl) was then added and the mixture vortexed for 10 seconds.

Centrifugation was carried out thereafter at ca.18000 g (15000 rpm) for 5 min at 4°C using a Tomy micro refrigerated centrifuge. About 300 µl supernatant was pipetted from each tube and transferred to fresh tubes while 750 µl of 99.5% ethanol was added and mixed by inverting the tubes several times. The mixture was

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centrifuged once again at 15000 rpm at 4°C for 5 min to pellet the DNAs.

The supernatant was decanted and the DNA pellet was washed by adding 1000 µl of 70% ethanol and re-pelleting by centrifugation as before. The isolated DNA was air-dried for about 15 min and then re-suspended in 50µl TE (Tris- HCl, EDTA, pH 8.0) buffer or MilliQ water.

Parts of the preparations were analyzed by agarose gel electrophoresis to confirm successful extraction on each occasion. The remaining parts were kept at -20°C until used. The use of air drying or evaporator as well as use of TE or water did not affect the stability of the DNA preparation for at least 4 weeks in storage.

Agarose gel electrophoresis analysis of the DNA preparations: The electrophoretic patterns of the fresh fungal DNA preparations were analyzed on 1% agarose gel. Five (5) µl of each of the preparations was mixed with 2 µl of 6x loading buffer and loaded into the wells of the gel. The gel was run at 100V for 45 min. Visualization of the DNA bands was achieved by staining the gel in 0.05% Ethidium bromide (EtBr) (Merck) solution for 15 min and then placing in an Ultraviolet (UV) lamp chamber for viewing at 254nm wavelength. Gel patterns was recorded at 1/2 s or 1s exposure as may be desired.

Polymerase chain reaction using isolated DNAs as templates

Use of the IGS primer: Polymerase chain reaction was performed to test the quality of the extracted DNAs with regard to their usability as templates to amplify the Intergenic Spacer region (IGS) fragment of the *Fusarium* genome. Specific primers, FIGS11 forward (GTAAGCCGTCCTTCGCCTCG) and FIGS12 reverse (GCCATACTATTGAATTTTGC) sequences were used.

A normal 10 µl PCR mix per tube contained 1µl of 10x Taq polymerase buffer, 0.2 µl of 10 mM dNTP mix (New England Biolabs, USA), 0.05 µl of Taq DNA Polymerase, 0.2 µl each of the 2 primers (FIGS11 and FIGS12) and 7.35 µl milliQ H_2O. An amount needed for the number of tubes (samples) were pre-mixed in an Eppendorf tube and later dispensed at 9 µl to individual PCR reaction tubes. This made it easier to pipette the small amounts of reagents needed. Finally, 1 µl of each of the template DNA (set at different concentrations) was added and briefly vortexed using a capsufuge. All the while the tubes were kept on ice.

The PCR reaction was carried out using the Gene Amp PCR system 9700 series (Applied Biosystems, Foster City, USA). The PCR condition was an initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C, 30s; annealing at 59°C, 30s and elongation at 72°C, 1 min. A further extension was carried out for 7 min after the last cycle, while the system was kept at 4°C until the PCR product was retrieved for electrophoretic analysis.

Use of the sp13 and sp23 primers: Two specific primers viz; sp13, sp23 and a universal one Uni, which were described by Hirano and Arie [7] were used. DNA templates were prepared as usual and the normal PCR reaction mixtures were prepared as follows: A 30 µl mixture contained 3 µl of 10X standard Taq polymerase buffer, 0.9 µl 10 mM dNTP mix, 0.15µl Taq DNA polymerase, 3 µl of each of the primers (forward and reverse sequences), 1 µl of template DNA (ca. 10ng); and milliQ H_2O to make 30 µl.

The PCR conditions necessitated different annealing temperature for the different primers. The Takara Gradient PCR cycler (Takara, Shiga Japan) was used to graduate the annealing temperature from 58°C to 65°C to allow choice of desired temperature. Eventually, the tubes containing the different primers were arranged such that the cycling condition for the Uni primer was 94°C, 1 min; 61°C, 1 min; and 72°C, 2 min. For the Sp13 it was 94°C, 1 min; 59.4°C, 1 min; and 72°C, 2 min. For the Sp23 primer it was 94°C, 1 min; 61.9 °C, 1 min; and 72°C, 2 min. This was carried out over 50 cycles. The first denaturation temperature was at 94°C for 5 min and a final extension at 72°C was for 7 min. At the end of the reaction samples were analyzed on 2% Agarose gels to confirm success or failure of the PCR process.

RESULTS AND DISCUSSION

Electrophoretic patterns of the genomic DNAs: The electrophoretic patterns on 1% agarose gel of the genomic DNAs extracted from the 4 fungal isolates are as shown in Plate 1. As observed, the quantity of DNA extracted from the isolates showed that the method was efficient in the isolation of Fusarial DNAs. Saitoh *et al.* [6] had also obtained enough quantity of DNA isolates from *Magnaporthe grisea.*

Electrophoretic patterns of the IGS amplicons: The electrophoretic patterns of the amplicons under varying PCR conditions are as shown in Plate 2. The desired band was that between 600 and 650 bp using the 100 bp DNA marker as benchmark. Saitoh *et al.* [6] had also reported

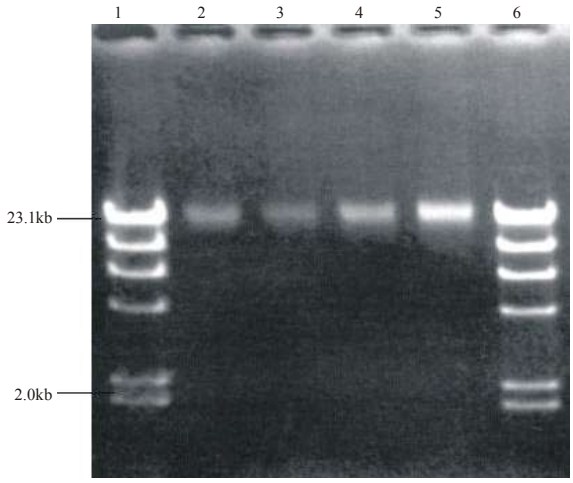

Plate 1: Electrophoretic pattern on 1%Agarose gel., of the genomic DNAs from 4 isolates of *Fusarium oxysporum* extracted by the novel non-phenol chloroform method
Lanes 1 and 6 are Hind III λ DNA markers. Lanes 2, 3, 4, and 5 are *F. oxysporum* fsp *lycopersici* race 1(Kis-1), Race 2 (888601-a), race 3 (tomino-1c) and fsp *radicis lycopersici* respectively.

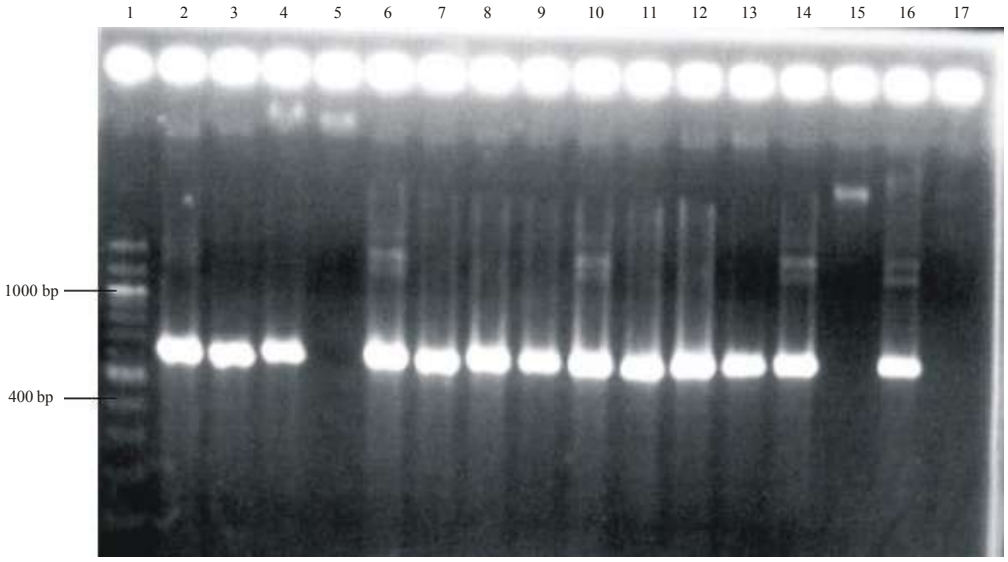

Plate 2: Agarose gel electrophoretic analysis of the PCR products in which the ribosomal intergenic spacer region primers (IGS) were used, while the *Fusarium oxysporum* isolates served as templates
FIGS forward and reverse primers were used to amplify the rIGS region of *Fusarium oxysporum* genomic DNAs. PCR mix in each case was 10 µl/tube
Lane 1: 100bp DNA marker. Lanes 2, 3, 4 and 5 = 1µl of 100% DNA extract from f.sp *lycopersici* races 1,2, 3 and f.sp *radicis lycopersici*, from 10- day old culture used as templates. Lanes 6, 7, 8, 9 = 1 µl of 10% concentration of the fore going extracts used as templates. Lanes 10, 11, 12, and 13 = 1µl of 100% DNA extracts from 4 -day old fungal mycelial culture of races 1, 2, 3, and rly. Lanes 14 and 15 are from 10ng/µl concentration of conventionally purified Kis-1 and *Phytophtora infestans* DNAs used as positive and negative controls. Lanes 16 and 17 = 1ng/µl of the controls.

that the DNAs obtained from *Magnaporthe grisea* were pure enough to be used as templates in PCR reactions using primers that amplified the internal transcribed spacer (ITS) region of the fungus.

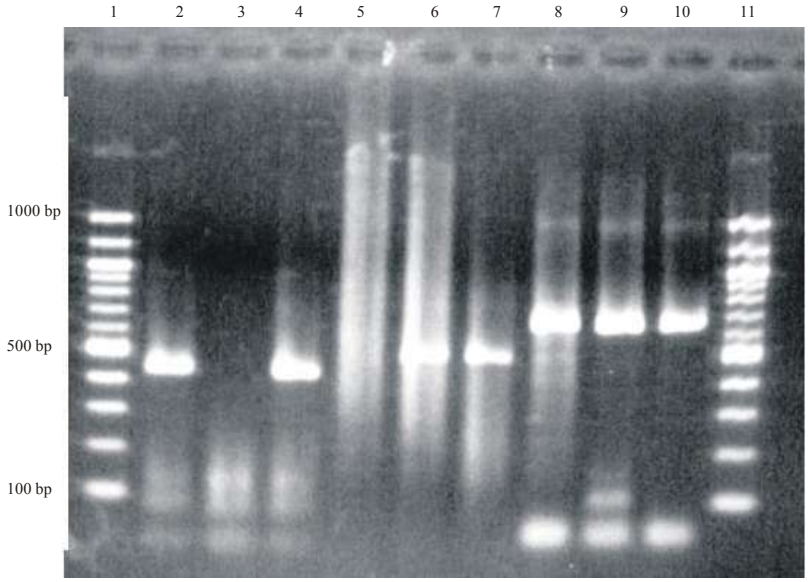

Plate 3: Agarose gel electrophoretic analysis of the PCR products in which race specific primers sp13, sp23 and the uni primer were used, while the *Fusarium oxysporum* isolates served as templates.
Lanes 1 and 11= 100bp DNA marker; lanes 2, 3, 4 = f.sp *lycopersici* races 1, 2 and 3 with the sp13 primer respectively. Lanes 5, 6, 7= races 1, 2 and 3 with the sp23 primer. Lanes 8,9,10 = races 1, 2, 3 with the uni Primer.

Further consideration of the band patterns reveals that the IGS fragments was amplified from both the DNA templates obtained from the novel method (e.g. Race 1 in Lanes 2, 6 and 10) as well as the templates obtained from the conventional phenol-chloroform method (Race 1 in Lanes 14 and 16). This is a confirmation that fusarial analysis using the IGS primer can be done successfully using DNA extracts from the novel method. This can be time -saving and cost- effective while saving one the problem of phenol-chloroform disposal, especially in low budget laboratories of the developing world.

Electrophoretic patterns of the sp13 and sp23 amplicons: As can be observed in Plate 3, the sp13 primer amplified a fragment between 400 and 500 bp in races 1 and 3 but not in race 2 while the sp23 primer amplified a fragment just above 500 bp in race 2 and 3 but not in race 1. The uni-primer, however, amplified a fragment just below 700 bp in all of the races. The result was consistent with that reported by Hirano and Arie [7] in which the sp13 primer specifically amplified a 458 bp fragment in race 1 and race 3 while the sp23 primer amplified a 518 bp fragment in race 2 and race 3. In that report, the fragment from f.sp *radicis lycopersici* primed by the sprl primer banded at a higher level (ca. 900bp). This result was a further confirmation of the efficiency of the DNA isolates obtained by the mini preparation method in PCR- based Fusarial race differentiation analysis.

The results in this study, put together, showed that the techniques herein described can be quick diagnostic methods for fusarial DNAs as had hitherto been found out in respect of *Magnaporthe grisea* by Saitoh *et al* [6].

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