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Full Length Research Paper

Influence of DNA treatments on Southern blot hybridization analysis of *Fusarium oxysporum* F. sp *lycopersici* and F. sp *radicis-lycopersici* genomic DNAs

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DNA samples obtained by a non-phenol/chloroform isolation method, from three races of *Fusarium oxysporum* f. sp. *lycopersici* and f. sp. *radicis-lycopersici* were treated in different ways with a view to evaluating the effect of three pre-electrophoresis DNA treatments on the outcome of Southern blot hybridization analysis using a Digoxigenin (DIG)-IGS fragment probe. Results showed that where DNA material is scarce, the use of undigested (native) fungal DNA not only saved time but it also gave better hybridization signal than predigestion treatments with EcoRV restriction enzyme. Hot water digestion of DNA prior electrophoresis and hybridization gave the least satisfactory result.

Key words: *Fusarium oxysporum*, DIG-IGS Probe, Southern hybridization.

INTRODUCTION

Basically, genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis though agarose gel. The DNA is then denatured *in situ* and transferred to a solid support such as a nylon filter membrane and hybridized to a radiolabeled DNA or RNA, and auto radiography is used to locate the positions of bands complementary to the probe (Sambrook et al., 1989). Nowadays, other methods of detection of the filter-bound DNA exist, which preclude the use of autoradiography that has potential hazards associated with the use of radioactive materials. Such methods include the use of digoxigenin-conjugated probes that are then assayed by immunoblotting methods.

The species of *Fusarium*, which is the pathogen model being used in this study, have traditionally been differentiated by their morphological characteristics on selective media (Nelson et al., 1983; Burgess et al., 1994). It is almost impossible, however, to identify pathogenic types, or forma speciales and races of *Fusarium oxysporum* using morphological features.

Arie et al. (1995, 1997) have proposed immunoassays as alternative methods for differentiating them while recently; molecular markers have become popular for identifying species and subspecies in fungi. Some of the molecular techniques that have been reported include amplified fragment length polymorphisms (AFLP) (Vos et al., 1995), random amplified polymorphic DNA (RAPD) (Kalc et al., 1996), restriction fragment length polymorphisms (RFLP) (Baayen et al., 1997) and direct amplification of length polymorphism (Desmarais et al., 1998) among others.

Earlier sequence analysis of the Intergenic spacer region (IGS) fragment of the *Fusarium oxysporum* f. sp. *lycopersici* race 1, 2, and 3 as well as the f. sp. *radicis-lycopersici* genomic DNA (Balogun, 2007), revealed more than 95% similarity in the sequences of the four fungal isolates under study here. It was therefore the objective of this present study to test the efficiency of PCR-amplified IGS fragment labeled with Digoxigenin (DIG), as a probe, in the detection of the *Fusarium* genomic DNAs in samples and to compare the effect of pre-electrophoretic DNA treatments on the outcome of the blot analysis.

MATERIALS AND METHODS

Preparation of DIG –IGS probe

DIG-PCR was carried out to amplify the IGS fragment from fungal genomic DNA templates. A 20 µl PCR mix comprised of 2 µl dNTP-DIG conjugate (Boerhinger Manneheim, Germany), 2 µl of 10X standard Taq polymerase buffer (New England Biolabs, USA), 0.15 µl Taq DNA polymerase, 0.5 µl each of 20 pMol/µl i.e. 10 pmol of

Primers- FIGS 11 and 12 (Forward and Reverse), and 1 µl (10 ng) of each DNA template. MilliQ H_2O completed the rest. The PCR thermal condition was 94°C - 30 s, 58°C - 30 s, and 72°C -1 min for 35 cycles. A final extension at 72°C was for 7 min. The PCR products were analyzed on 2% TAE-Agarose gel to confirm success or otherwise of the reaction.

Preparation of DNA samples for southern hybridization analysis

The DNA samples obtained by the novel non-Phenol-chloroform method described by Saitoh et al. (2006) were subjected to thee different types of treatments before agarose gel electrophoresis to determine their influence on blotting and subsequent hybridization and detection.

One part of the genomic DNA was subjected to the traditional restriction enzyme digestion using EcoRV (New England Biolabs Inc, USA), which according to search using the GENETYX program has no restriction site within the IGS sequence of the DNA templates. It was expected therefore that a single band would be produced if hybridization was successful. The treatment was done by adding 2 µl each of NE Buffer 3 (50 mM Tris –HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1.0 mM DTT, pH 7.9 at 25°C), Bovine Serum Albumin (BSA) (10 mg/ml), and EcoRV in that order to 14 µl of genomic DNAs (ca. 2.8 µg) in Eppendorf tubes. The content was mixed briefly and then incubated at 37°C overnight (about 16 h).

The second part (ca. 1400 ng) was heat-treated in boiling water for 6 min and then chilled immediately on ice to maintain the expected single stranded nature of the DNAs after untwining the helix. Two bands were expected if hybridization was successful. The third part (1400 ng), was left untreated and run on the gel in native form. Thus, only one band was expected if hybridization was successful.

Capillary transfer of DNA to Nytran Nylon membrane

DNA was transferred to Nytran Nylon membrane by Capillary Elution method with alkaline buffer (0.4 M NaOH, 1 M NaCl) (Sambrooks et al., 1989). The procedure involved denaturation of the DNAs in situ with a weak solution of HCl (1 ml/50 ml H_20) for 15 min, washing properly in water and transferring the DNA overnight for about 16 h on a transfer rack.

The principle involved here is that as the buffer is pulled upward by capillary forces, though the gel, it picks along the denatured DNA and deposits it on the surface. The membrane is positively charged and so retains the DNA molecules preventing them from escaping along with buffer though the filter membrane (Sambrook, 1989).

Prehybridization and Hybridization procedures

The transfer stack was dismantled and the filter membrane was carefully removed using forceps. The surface containing the DNA was marked with a pencil as a guide to prevent confusing the surfaces. The membrane was cleaned briefly in 2 x SSC buffer and thereafter put in between two sheets of paper towel and baked in an oven for 2 h at 80°C.

The membrane was transferred to a hybridization bag (ca.10 cm x 6 cm) and 8 ml of hybridization buffer (2% blocking reagent, 5 x SSC, 0.02% SDS 0.1% Sarcosyl) was added. The bag was heat-sealed after making sure that as much air as possible has been eased out. The membrane was then incubated at 42°C in an EYELA hybridization oven for 1.5 h.

The membrane was removed and placed in a new hybridization bag. Meanwhile some 15 min to the end of the prehybridization step, 8 µl (2 µl of each of the four) of the IGS probes was placed in an Eppendorf tube and boiled at 100°C for 10 min. It was immediately transferred on ice. The denatured probe was added to the bag content and sealed. The membrane was incubated in a hybridization oven at 42°C overnight (ca. 16 h).

Washing procedure

The membrane was placed in a Tupper dish and washed first at low stringency in 60 ml of 2x SSC, 0.1% SDS (made from 6 ml 20 x SSC stock and 600 µl of 10% SDS stock) for 30 min divided into 2 washes of 15 min each with 30 ml buffer. While this was going on, 60 ml of a second buffer (0.1 x SSC, 0.1% SDS) was pre-warmed at 68°C in the hybridization oven. The content was split into two parts as before for two 15 min washes at 68°C. At room temperature the membrane was briefly transferred to a buffer (0.1 M Maleic acid, 0.15 M NaCl), before the detection process commenced.

Detection procedure

The process of detection started with addition of 30 ml of blocking buffer (6 ml of 10% Skim milk in 54 ml of buffer 1, i.e. Maleic acid and NaCl) to the gel in a Tupper dish. This was to further reduce chances of un-specific binding of probes to the membrane. The membrane was incubated for 45 min before the addition of anti – DIG Fab Fragment (Roche Diagnostics, Mannheim, Germany) at the rate of 1:5,000 (i.e. 6 µl/30 ml blocking buffer). The membrane was incubated for 45 min.

At the end of the incubation, the blocking buffer was removed while membrane was treated with washing buffer (0.1 M Maleic acid, 0.15 M NaCl, Tween 20 0.3%). Washing was for 15 min each with 30 ml buffer. The membrane was transferred briefly to substrate solution i.e. buffer 3 (100 mM Tris-HCl, pH 9.5 100 mM NaCl, 50 mM $MgCl_2$) before the visualization process with NBT/BCIP solution.

Vizualization of band

The membrane was transferred to the hybridization bag for color development. Vizualization of the hybridized bands was achieved by addition of NBT/BCIP from a stock solution to a final concentration of 2% in substrate solution. In this case, 160 µl of the dye solution was added to 8 ml of substrate solution and the mixture poured unto the hybridization bag containing the membrane. The bag was sealed and kept in the dark for about 30 min.

RESULTS AND DISCUSSION

Gel electrophoetic patterns were monitored for different stages of the experiments as a way of monitoring progress and to authenticate the various stages. Figure 1 shows the appearance of the gel after it was run on a 0.8% agarose gel and stained with ethidium bromide (0.5 µg/ml) prior to transfer. It shows that only the lanes that were loaded with native DNAs were visible while the other lanes that were digested with restriction enzyme and hot water remained invisible. This was expected as the digestion would have rendered the DNA invisible at this stage.

While the prehybridization treatment of the membrane was on going, the gel was re-immersed in EtBr solution

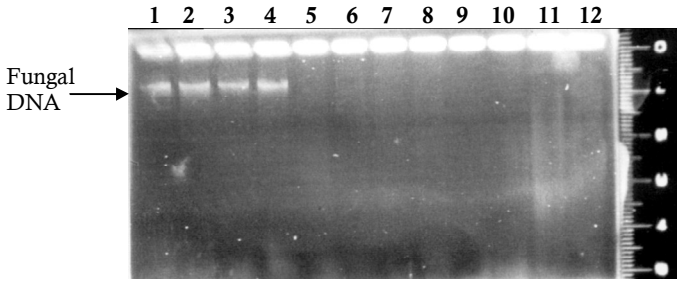

Figure 1. Ethidium bromide staining of 1% agarose gel before capillary transfer of DNA to Nytran nylon Membrane for Southern blot hybridization analysis. Lanes 1 - 4 are native DNAs from F.ol races 1, 2, 3 and f.sp rly respectively. Lanes 5 to 8 are DNAs denatured in boiling water for 6 min; Lanes 9 - 12 are genomic DNAs digested overnight with EcoRV.

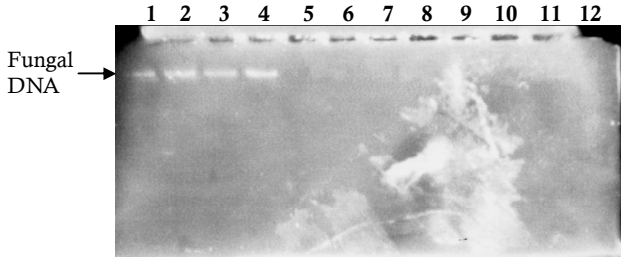

Figure 2. Ethidium bromide staining of gel after 16 h of DNA transfer in alkaline buffer. Lanes 1 - 4 are native DNAs from F.OL races 1, 2, 3 and rly respectively. Lanes 5 to 8 are DNAs denatured in boiling water for 6 min; Lanes 9 - 12 are genomic DNAs digested with EcoRV overnight.

for about 30 min. This was to confirm how far the transfer process went after 16 h. Figure 2 shows the gel after staining for 30 min. Substantial part of the native DNAs was still visible. This was expected in the sense that it is normally very difficult to move all the DNAs within 16 h; more so when the size is above 20 kb (Sambrook, 1989).

After hybridization, washing and upon addition of BCIP/NBT dye, color development was noticeable as from about 5 min. By 30 min, the gel was photographed. Figure 3 shows the hybridization signals after 30 min of exposure to the NBT/BCIP dye.

The import of the results is that in the process of diagnosis, as may be necessary from time to time, native *Fusarium* DNA was reasonably hybridizable without going though all the rigors of digestion with restriction enzymes and so on. Moreover, where resources and DNA materials are scarce, as evident in the appearance of the bands, hybridizing native fusarial DNAs may be more efficient than using digested materials. Besides, it could save time needed for the whole process. Detection in cases where the pre electrophoresis treatment was with hot water may need more DNA quantity. The same was probably true of pre-digestion with EcoRV, hence, the unsatisfactory nature of their hybridization signals in this

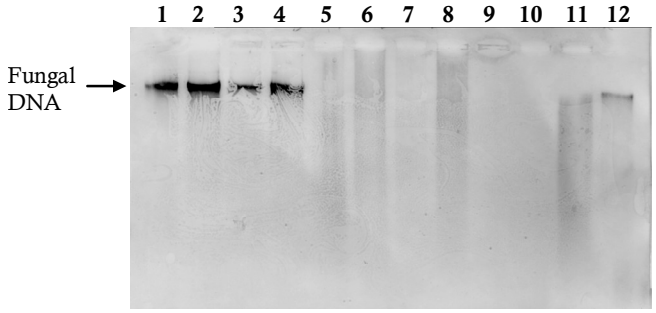

Figure 3. Southern hybridization analysis of genomic DNA from four isolates of Fusarium oxysporum exposed to different treatments before gel electrophoresis. Lanes 1 - 4 are native DNAs from F.ol races 1, 2, 3, and f.sp. rly. Lanes 5 – 8 are samples denatured in hot water. Lanes 9 – 12 are samples digested with EcoRV overnight.

study. Other experiments, using any one of the probes alone, produced identical results indicating that all the four probes are not necessary at once. This may further reduce cost of diagnosis especially under tight research budget.

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