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An Easy Method to Isolate the High Quality DNA from Plant Pathogen *Alternaria* spp

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ABSTRACT

A simple and easy protocol for isolation of DNA from major plant pathogen *Alternaria* spp has been developed in which DNA extracted using CTAB extraction method from fresh mycelium of fungi crushed with sterile sand particles and liquid nitrogen. The DNA was precipitated first with equal volume of phenol-chloroform-isoamylalcohol (25:24:1) and further with equal volume of chloroform-isoamylalcohol (24:1). Repeated centrifugation was done at 10000 g for 10 minutes at room temperature. DNA was pooled by incubating the supernatant with pre-chilled isopropanol at -20°C for 1 hour. After that the samples were centrifuged for 15 minutes at 13000 g to pellet the DNA. The DNA pellet was washed with 200 µL of 70% ethanol, air dried and dissolved in 200 µL of TE buffer, thereafter 5 µL of RNase was added to each DNA Samples, mixed and incubated at 37°C for 1 hour. After RNase treatment, DNA samples were precipitated with chloroform-isoamylalcohol (24:1) again incubated with pre-chilled isopropanol and then DNA pellet which was finally dissolved in 20 µL of TE buffer and stored at -20°C till further use. Inter-simple sequence repeats (ISSRs) amplification reaction was carried out using 20 µL of reaction volume containing 2 µL of DNA.

Introduction

Alternaria species are known as major plant pathogens. It is a genus of ascomycete fungi, causing approximately 20% of agricultural spoilage. They are also common allergens in humans. They have been an important crop pathogen and owing to their agronomic importance and economic bearing on crop production, have been studied extensively. However, for genetic studies at molecular level, high quality pure genomic DNA of high molecular weight is basic requirement. A simple and easy protocol for isolation of DNA from *Alternaria* spp. was developed for its use in genetic studies.

Materials and Methods

Source of biological material

Five samples of *Alternaria* viz. *A. triticina, A. solani, A. brassiceae, A. burnsii and A. alternata* were collected from wheat, eggplant, cauliflower, cumin and hollyhock respectively and grown in potato dextrose agar medium (4 gm Potato dextrose agar dissolved in 100ml of distilled water) of under control temperature to obtain the mycelium for the isolation of DNA.

Pure culture of fungus

Alternaria were isolated from plant source and cultured on potato dextrose agar medium. The plates were incubated at 28°C for 72 hour. Subculture was done to get pure culture of *Alternaria*. The mycelium from pure fungal culture was used for DNA isolation.

Isolation of fungal DNA

Fungal DNA was isolated by the method described by Gontia-Mishra *et al.,* (2014) with some modification.

Molecular marker

Inter simple sequence repeat (ISSR) marker was used for amplification of fungal DNA. Primer 812 (5'GAGAGAGAGAGAG AGAA3') was used for PCR amplification.

PCR amplification

ISSR amplification reaction was carried out in 20 µl reaction volume for each 2 µl DNA sample. The reaction volume for five DNA samples was as follows: 54.5 µl of sterile water (MQ), 10 µl of buffer, 12 µl of $MgCl_2$, 2 µl of dNTPs, 10 µl of primer and 1.5 µl of Taq DNA polymerase. Amplification was performed in Eppendorf Master Cycler Gradient. Amplification conditions were maintained at 1 cycle at 94°C for 30 sec (denaturation); 55°C for 45 sec (annealing) with a step-wise reduction of 1°C per cycle, 72°C for 2 min (elongation) and 35 cycles of 94°C for 30 sec; 50°C for 45 sec; 72°C for 2 min, followed by 7 min at 72°C for final extension.

Amplified products were loaded on 1.5% agarose gel and electrophoresed using 1XTBE buffer at 80 volts. The gels were stained with 0.5 mg/ml ethidium bromide solution and visualized under UV light in gel documentation system.

Results and Discussion

Well grown fresh fungal mycelium was hooked from broth culture and wrapped with foil paper after washing with sterilized distilled water and kept at -20°C for overnight. 200 mg of fungal mycelium was used to crush with the help of mortar and pestle using liquid nitrogen with 2 gm of sterile sand particles (2-3 mm^2 size) in place of glass beads as per method adopted by Gontia-Mishra *et al.,* 2014. Fungal mycelium was transferred to a sterilized 1.5 mL micro centrifuge tube with the help of sterilized scalpel and added with 800 mL of DNA extraction buffer(0.1M Tris-HCl8 pH, 10mM EDTA 8 pH, 2.5M NaCl, 3.5% CTAB, 1.5 µL of 20 mg/mL proteinase K).

The mixture was vortexed at high speed for 5 min. The samples were placed in a water-bath at 65°C for 30 min after that centrifuged at 10000 g for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform- isoamylalcohol (25:24:1) was added. Thereafter, centrifuged at 10000 g for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform-isoamylalcohol (24:1) was added. Centrifuged at 10000 g for 10 min at room temperature. Supernatant was collected and 500 µL of pre-chilled

isopropanol was added. Precipitation of DNA occurs and for proper precipitation it was incubated at -20°C for 1 hour. Centrifuged for 15 min at 13000g to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 200 µL of 70% ethanol and kept at -20°C for overnight. DNA pellet was air dried and dissolved in 200 µL of TE buffer. 5 µL of RNase was added to samples, mixed and incubated at 37°C for 1 hour. Samples were washed with chloroform-isoamylalcohol (24:1) and centrifuged at 10000 g for 10 min.

Supernatant was collected and 500 µL of pre-chilled isopropanol was added. Samples were incubated at -20°C for 1 hour. Then the samples were centrifuged for 15 min. at 13000 g to pellet the DNA. DNA pellet was air-dried and 20 µL of TE buffer (10mM Tris-HCl 8pH, 1mM EDTA) was added and stored at -20°C till further use. The similar result was found as Gontia-Mishra *et al.,* (2014) for method of DNA isolation from filamentous fungi *Aspergillus* spp. In the present method, DNA was isolated from the *Alternaria* spp. and fresh fungal mycelium was collected on broth. One extra step was added the mycelium after keeping overnight at -20°C samples was crushed with sterile sand using liquid nitrogen. The quality DNA was found from all the samples of *Alternaria* spp.

Quality of the extracted DNA was observed by electrophoresis in 0.8% agarose gel, followed by staining with ethidium bromide (0.5 mg/L). DNA obtained is shown in Fig. 1. All the DNA samples were free from protein/ RNA impurities. The amount and quality of DNA samples were suitable for PCR amplification. PCR – based assays using ISSR molecular marker was done to check the suitability of extracted DNA for downstream analysis.

All the DNA samples were amplified using ISSR primer (5'GAGAGAGAGAGAGAG AA3') has been shown in Fig. 2. ISSR amplified product of DNA was observed by electrophoresis in 2% agarose gel followed by staining with ethidium bromide (0.5 mg/L).

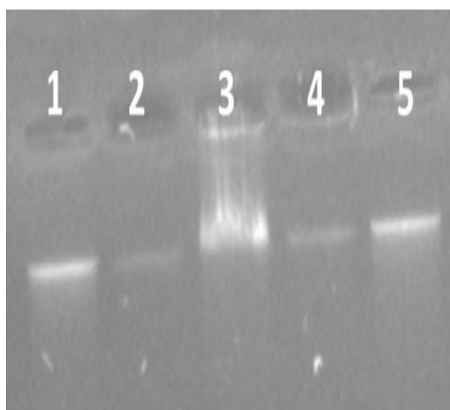

Fig.1 Agarose gel electrophoresis DNA of filamentous fungi *Alternaria* spp. 1. *A. triticina,* 2. *A. solani,* 3. *A.brassiceae,* 4. *A. burnsii* 5. *A. alternata*

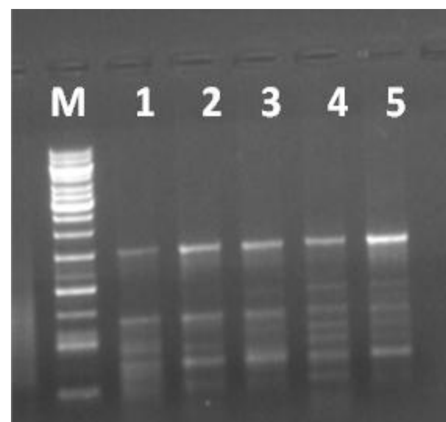

Fig.2 ISSR for fungal isolates Lane M - 1kb DNA Ladder, Lane 1-5 isolates of *Alternaria* spp.

It was found that the developed protocol is very easy method to isolate the quality DNA from *Alternaria* spp. Quality DNA can be used for further downstream molecular work.

The present protocol has been successfully extended to recover the DNA from other species of *Alternaria* and it may also helpful for many other filamentous fungi. Thus, the present method for DNA isolation from filamentous fungi could be used for various PCR based molecular assays.

References

Gontia-Mishra I, Tripathi N and Tiwari S. (2014).A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. *Indian Journal of Biotechnology*13:536-539.

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