Journal of Food Legumes 29(1): 60-63, 2016

Efficient DNA extraction method from soil infested with *Fusarium* sp. for metagenomics study

PRIYANKA GANGWAR[1], PR SABALE[2], NAIMUDDIN[2] and KHELA RAM SOREN[1*]

[1]Division of Plant Biotechnology, [2]Division of Plant Protection, Indian Institute of Pulses Research, Kanpur - 208 024, India

E-mail: sorenars@gmail.com

(Received : 13 April, 2016; Accepted : 23 June, 2016)

ABSTRACT

Simple and rapid identification of pathogenic fungi from infested soil is pre-requisite to study the fungal dynamics in soil. The traditional methods of fungal characterization are more cumbersome and non-specific. Therefore, a rapid and specific method of fungal characterization through genomic DNA is more precise. Hence, in the present study, samples from infested soil were collected randomly for fungal DNA isolation. The DNA concentration ranged between 240 to 410 ng/ilwith A260/280 values of 1.48 to 1.69. The quality of DNA was confirmed by restriction digestion with *Hind III* and *Hae III* enzymes and further confirmed by PCR amplification with ITS specific primers. Sequencing and NCBI-BLAST analysis of 420bp amplicon confirmed presence of *Fusarium* spp. and sequence was submitted to NCBI database (Accession no.: KP153547 to KP153550). Homology search in NCBI-BLAST, found 94-96% homologues with *Fusarium* spp. Thus, the protocol used in present study helps in rapid detection of soil borne fungi and also gives information about fungal dynamics and facilitates in proper disease management.

Keywords: *Fusarium*, Soil samples, Restriction digestion, PCR, NCBI-BLAST

Molecular tools are useful for quantitative and qualitative evaluation of microbial communities in the soil ecosystem. Polymerase chain reaction has become a common biotechnological tool to access microbial communities. Recent molecular tools have been popularly used for community analysis (Mishra et al, 2012; Kumar and Anandaraj, 2006). In earlier studies, various microbial communities in soil were assessed by a number of morphological and biochemical tests (Fatima et al., 2011). But these techniques are very time consuming and laborious. However, microbe specific oligonucleotide probes to detect the pathogen have proved to be fast and reliable approaches (Miller, 2001). The filamentous fungi *Fusarium* is one of the member of microbial community in the soil ecosystem. However, few pathogenic species of *Fusarium* causes wilt diseases in wide variety of crop plants and is widely distributed in subtropical and temperate regions of the world (Saremi and Burgess, 2000). Fungus starts infection from the roots by penetrating vascular tissue; cause wilting, discoloration and death of the plant. Early detection of pathogens inoculums found in soil is crucial to avoid disease epidemics. However, the isolation of fungal DNA from infested soils is a very challenging task due to inhibitory substances like mineral salts, proteins and humic substances co-extracted with DNA and subsequently inhibit polymerase chain reaction (Damm and Fourie, 2005). The procedures should provide pure and sufficient quantities of DNA by removing inhibitory materials. Several procedures have been reported by various researchers for the extraction of microbial DNA from soil with large number of purification steps to make DNA suitable for PCR amplification and diversity study (Roose-Amsaleg et al., 2001; Soren et al., 2014, 2016). To reduce the cost, time and equipments involved in isolation and culturing of filamentous fungi, it is imperative to isolate fungal DNA from soil for early detection. Thus, the aim of the present study is to rapid detection of pathogen directly from soil using molecular tools.

MATERIALS AND METHODS

Sample collection and maintenance

A wilt sick field is located in Kanpur at latitude of 26.4^0 north and longitude 80.2^0 east with average rainfall 885mm/year. The soil is alluvial sandy loam with pH 8.0. For standardization of DNA extraction protocol from soil fungi, 4 soil samples were collected randomly from different location of *Fusarium* wilt sick plot, at IIPR(Table 1) before the onset of rains. Each soil sample was individually collected in a sterile plastic sample bags and directly used for DNA extraction. Remaining soil samples were stored at 4ºC.

Extraction of DNA from sick soil samples

Genomic DNA was extracted according to the procedure of Ogram et al. (1987) with some modifications as described herein. One gram of dry soil sample were taken and grounded with sterilized mortar-pestle using liquid nitrogen. The samples were collected in sterile eppendorf tubes. Subsequently 1% extraction buffer (100 mMTris-HCl (pH7.5), 1.4M NaCl, 20 mM EDTA (pH 8), 3% CTAB, 40

mg PVP (Polyvinylpyrrolidone) and 1ìl â-mercaptoethanol) was added and incubated at 65 ° C for 30 min. After incubation, equal quantity of C:I (24 ml chloroform: 1 ml isoamyl-alcohol) was added, vortexes and centrifuged at 4 ° C, 12000 rpm for 10 min. The supernatant containing DNA was precipitated using 2.5 volumes isopropanol. The DNA pellet was washed with 70% ethanol and finally dissolved in 200ìl of sterilized distilled water.

DNA purification and quantification

About 10 mg RNase enzyme was added to crude DNA samples (200 ìl) and incubated in waterbath at 37 ° C for 1-2 hours. After that DNA samples were purified by treating with equal volume of P:C:I (25 ml phenol: 24 ml chloroform: 1 ml isoamyl-alcohol) and C:I (24 ml chloroform: 1 ml isoamyl-alcohol) and centrifuged at 4 ° C for 10 min at 12000 rpm. The supernatant containing DNA was collected in new centrifuge tubes and precipitated by 1.5 volumes isopropanol. The tubes were incubated overnight in a -20^{0}C refrigerator and centrifuged at 4 ° C for 10 min at 12000 rpm. The pellets were washed twice with 70% ethanol; air dried and finally dissolved in 50 ìl of sterilized TE buffer (10 mMTris–HCl and 1 mM EDTA, pH 8.0). The subsequent DNA yields and quality were checked it on 0.8% standard gel electrophoresis. The purity of DNA was checked by restriction digestion using *Hind III* and *Hae III*. Restriction digestion was performed in 20ìl total volume containing 2 ìl of 10X restriction enzyme Buffer, a total 5-10 U of restriction enzymes (New England Biolabs Inc.) with 1 ìg of template DNA.

DNA concentration was quantified by using Biophotometer plus (Eppendorf). The ratio of the readings at 260 nm and 280 nm provides an estimate of the purity of DNA (Table 1).

PCR amplification of ITS region for detection

Detection of *Fusarium*spp. was performed using the DNA isolated from four infected soil samples as template. The fungal internal transcribed spacer (ITS) regions were amplified using primers ITS-F (5′AACTCCCAAACCCCTGTGAACATA 3′) and ITS-R (3′ TTTAACGGCGTGGCCGC 5′) proposed by Yazeed et al., 2011. PCR amplifications was performed in 10 ìl total volume containing 1X *Taq* Buffer, 0.2 mM of dNTPs mix (Fermentas) (100 mM of each dNTPs), 2.5pmole each forward and reversed primer (25 pmole each primer), 0.06 U of *Taq* polymerase (G Biosciences) with 25 ng of template DNA. PCR conditions were; initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, elongation for 2 min. The thermal cycles were terminated by a final extension of 10 min at 72°C. Amplified products were resolved in 1.5% agarose gel using 0.5X TAE buffer. The PCR products amplified from soil samples were sequenced (Integrated DNA Technologies) and homology search was carried out using NCBI-BLAST analysis.

RESULTS AND DISCUSSION

The protocol employed presently eliminates the time consuming and laborious steps of other protocols (Van Burik et al., 1998; Al- Samarrai et al., 2000). The DNA quality and quantity is affected by adsorption of DNA to soil particles or degradation and co-extraction inhibitors. Direct isolation of DNA from soil samples accumulates impurities, which are the potential inhibitors of polymerases and restriction endonucleases (Ghany and Zaki, 2002).Garcia-Pedrajaset al. (1999) developed DNA isolation protocol from soil by using skimmed milk to prevent loss of DNA by adsorption to soil particles. Amount and quality of DNA obtained in the study was suitable for PCR amplification and other molecular studies. The quantity of DNA obtained in this study was reasonably high compared with other literatures due to existence of high microbial load in the sick field and sandy loam soil. DNA yield was influenced by type of the soil type and microbial population in the soil. Sandy soils act as natural abrasive results in higher yield than clay soil (Gracia-Pedrajas et al.1999).

The detection of *Fusarium* spp. by conventional methods were very time consuming and laborious leading to delay in the process of disease management decisions. In the present study, DNA was isolated from 4 sick field soil samples randomly. The concentration of DNA was ranged from 240ng to 410 ng/ìl with A260/280 ratio around 1.48 to 1.69 (Table 1).The results of DNA electrophoresis in agarose gel are demonstratedin Fig.1 and visualized as sharp and distinct bands for all the soil samples. A clear and distinct restriction pattern upon restriction digestion of DNA using enzymes *Hind III* and *Hae III* confirmed the purity of DNA (Fig.2). The PCR amplification of DNA was carried out using ITS primer (Table 1). All four DNA samples produced the amplicon size of 420bp and the amplified bands were of the expected size (Fig. 3). This coincided in

Table 1. DNA quantification of four wilt infested soil samples

Soil samples	Location	DNA concentration (ng/ l)	Ratio readings at 260 nm and 280 nm
Sample 1	Wilt sick plot, IIPR	318	1.64
Sample 2	Wilt sick plot, IIPR	410	1.48
Sample 3	Wilt sick plot, IIPR	370	1.56
Sample 4	Wilt sick plot, IIPR	240	1.69

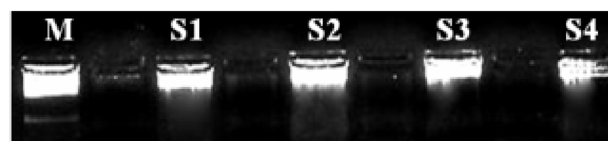

Figure 1. DNA extracted from four wilt infested soil samples, Lane M: ë DNA (600 ng).

length with the positive control DNA extracted from mycelium of *Fusarium oxysporum* f.sp. *ciceris* (*Foc*) culture. A healthy chickpea plant extracted DNA was used as negative control and these controls never produced visible bands. The PCR amplification of ITS regions represented, the protocol of DNA extraction is efficient and can be employed for soil microbial genomic studies.

The sequence homology of the amplified sequenced products by using NCBI-BLAST analysis was confirmed to be *Fusarium* spp with 94-96% similarity (Table 2). The sequences were submitted to NCBI database and GenBank accession numbers were obtained (Table 2). Several researchers have developed PCR based detection tests for several pathogens like *Phytophthora*species (Grote et al., 2002; Hussain et al., 2005), *Ralstoniasolanacearum* (Kumar and Anandaraj, 2006), *Phytophthoracinnamomi* (O'Brien, 2008) and *Agrobacterium tumefaciens* (Yang et al., 2011). SCAR markers developed against race 5 of *Foc* was obtained by RAPD analysis and were utilized for detection of pathogen from soil samples (Garcia-Pedrajaset al.1999). Detection of pathogen from soil helps in quantification of inoculums load in the field and helps indecision making system.

Table 2. Sequence homology result using NCBI-BLAST

Samples	Query length (bp)	Homologues sequence ID	% Identities	Scores (bits)	GenBank accession no.
S1	350	KJ720611	96%	560	KP153547
S2	316	KJ720612	95%	481	KP153548
S3	229	KJ720609	94%	327	KP153549
S4	344	KJ720610	96%	555	KP153550

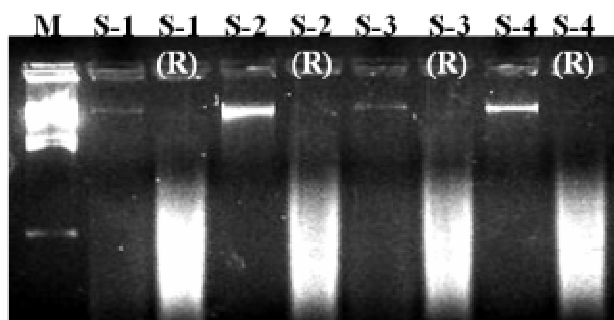

Figure 2. Lane M: ë DNA (Hind III digested), Lanes S-1 to S-4 unpurified soil DNA samples, Lanes S-l(R) to S-4(R) restricted soil DNA samples and both are digested by *Hind III* and *Hae III*.

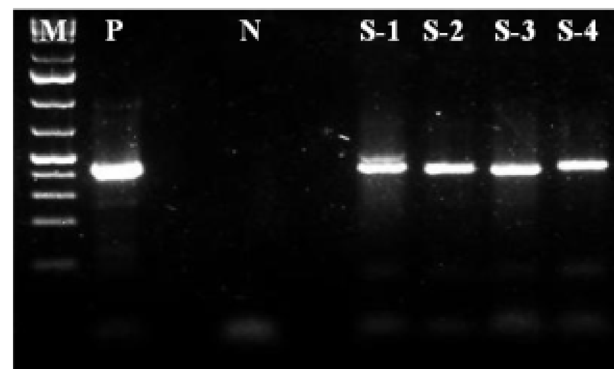

Figure 3. PCR amplification of *Fusarium* specific rDNA ITS region. Lane M: 1kb plus DNA Ladder (Fermentas), Lanes S1 to S4: Soil samples, Lane P: Positive control (mycelia sample), Lane N: Negative control.

Based on the above discussion we conclude that the DNA isolated from soil samples in this study was suitable for molecular studies. Such a study can facilitate fungal dynamics, evolution and taxonomy studies at molecular level within a very short time span.

ACKNOWLEDGEMENTS

This work was financially supported by the Indian Council of Agriculture Research funded project on 'Outreach program on *Phytopthora*, *Fusarium* and *Ralstonia* disease in horticultural and field crops'.

REFERENCES

Al-Samarrai TH and Schmid J. 2000. A simple method for extraction of fungal genomic DNA.Lett. Appl. Microbiol. **30**: 53-56

Damm U and Fourie P. 2005. A cost-effective protocol for molecular detection of fungal pathogens in soil.South African Journal of Science.**101**:135–139.

Fatima F, Chaudhary I, Ali J, Rastogi S and Pathak N. 2011. Microbial DNA extraction from soil by different methods and its PCR amplification.Biochem Cell Arch **11**: 1

Garcia-Pedrajas MD, Bainbridge BW, Heale JB, Perez-Artes E, JimenezDiaz RM. 1999. A simple PCR based method for the detection of chickpea-wilt pathogen *Fusarium oxysporum* f. sp.*ciceris* in artificial and natural soil.Europ. J. Plant Pathol. **105**: 251-259.

Ghany AGAA and Zaki EA. 2002.Efficient direct purification method for high-quality DNA from soil. Arab J Biotech **6**(1): 49-56

Grote D, Olmos A, Kofoet A, Tuset J, Bertolini E and Cambra M. 2002.Specific and sensitive detection of *Phytophthoranicotianae* by simple and nested-PCR.Eur J Plant Pathol**108**: 197–207

Hussain S, Lees AK, Duncan JM and Cooke DEL. 2005. Development of a species-specific and sensitive detection assay for *Phytophthorainfestans* and its application for monitoring of inoculums in tubers and soil. Plant Pathol**54**: 373–382

Kumar A and Anandaraj M. 2006. Method for isolation of soil DNA and PCR based detection of ginger wilt pathogen *Ralstoniasolanacearum*. IndPhytopathol**59**: 154–160

Miller DN. 2001. Evaluation of gel filtration resins for the removal of PCR- inhibitory substances from soils and sediments. J of Microbiol Methods **44**: 49-58

Mishra RK, Pandey BK, Pandey A, MohdZeeshan and Pathak N. 2012. Specific detection of *Fusariumoxysporumf. sp.psidii*(*Fop*) causing wilt disease of *Psidiumguajava L* in India In: National Symposium on Microbes in Health and Agriculture JNU, New Delhi, India. 87 pp.

O'Brien PA. 2008. PCR primers for specific detection of *Phytophthoracinnamomi*. Aust Plant Pathol**37**: 69–71

Ogram A, Sayler GS and Barkay T. 1987.The extraction and purification of microbial DNA from sediments. J Microbiol Methods **7**: 43-51

Roose-Amsaleg CL, Garnier-Sillam E and Harry M. 2001.Extraction and purification of microbial DNA from soil and sediment samples.Appl Soil Ecol **18**: 47–60

Saremi H and Burgess LW. 2000. Effect of Soil Temperature on Distribution and Population Dynamics of *Fusarium* Species. J AgrSci Tech **2**: 119-125

Soren KR, Gangwar P, Chaudhary RG and Datta S. 2016. Population distribution and genetic relatedness in Indian *Fusarium udum* isolates based on RAPD marker system. Journal of Food legumes : 52-76.

Soren KR, Gangwar P, Chaudhary RG and Datta S. 2014. Population distribution and genetic relatedness in Indian *Fusarium udum* isolates based on ribosoml internal transcribe spacer and elongation factor**.** Journal of Environmental Biology.**36**:1063-1070.

Van Burik JA, Schreckhise RW, White TC, Bowden RA and Myerson D. 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Medical Mycology **36**: 299–303.

Yang, Wei, Lei, Ji, Li-Rong, Tan, Shi-Mo, Li, Yan, Wang, Hong-Xia, Liu, Yu-Ming and Luo. 2011. Sensitive and specific detection of *Agrobacteriumtumefaciens* in soil using a rapid polymerase chain reaction (PCR). Afr J Microbiol Res **5**: 708–713

Yazeed, HAE, Hassan A, Moghaieb REA, Hamed M and Refai M. 2011. Molecular Detection of Fumonisin-producing *Fusarium* species in animal feeds using polymerase chain reaction (PCR). Journal of Applied Sciences Research **7**(4): 420-427.