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A Simple, Economical and Rapid Method to Isolate High Quality DNA from Oomycetes

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Abstract

Several approaches have been advocated to diagnose and control the devastating diseases caused by the oomycetes, most of which are done using molecular techniques that require DNA with high quantity and quality. An attempt has been made to develop simple, economical and rapid method to isolate high quality DNA from 10 different oomycetes at Central Tuber Crops Research Institute, Thiruvananthapuram, India. This protocol was based on the sodium dodecyl sulphate (SDS) method, which produced high quality DNA, either from liquid or plate cultures, without maceration in liquid nitrogen, devoid of harmful chemicals like β–mercaptoethanol, phenol, chloroform, etc., and does not require any incubation steps. The total time to complete the whole procedure was less than 30 minutes. The high quantity and purity of the isolated genomic DNA was confirmed by restriction digestion analysis which demonstrated that the developed method provided DNA of sufficient quality for molecular analysis. The method developed is safe, convenient, economical and time saving like commercial DNA extraction kits. It is useful for laboratories with minimum financial resources for handling large number of samples .

Key words: Oomycetes, DNA extraction, YPT amplification, restriction analysis, RAPD

Introduction

Oomycetes pathogens cause several devastating diseases on many plants, throughout the world, which may lead to heavy yield loss (Erwin and Ribeiro, 1996). Abad et al. (2008) and Duran et al. (2009) reported that conventional culture techniques were time consuming and the percentage of miroflora identified was less. This leads to the introduction of molecular techniques. Molecular diagnosis helps to handle large number of samples rapidly in a short time using small quantities of DNA and are far more reliable and accurate than the conventional methods. One of these cultivation-independent methods is real-time PCR, which can rapidly detect and quantify microbial nucleic acid sequences even from very small amount of DNA. The ultimate sensitivity of such techniques only depends on the DNA that is free of PCR inhibitors. DNA of good quality play a vital role in the success of genetic diversity studies and molecular mapping techniques such as RAPD, CAP, RFLP, AFLP, etc. (Cooke et al., 2000).

DNA isolation kits usually provide a good result, but it is not cost effective. Several procedures for extracting DNA from oomycetes have been described, but most of them use toxic and hazardous chemicals, which require special effort to minimize exposure (Niu, et al., 2008; Zhang et al., 1996). Most of the established DNA isolation methods require specific controlled conditions as well as specific equipments, which are costly and may not be available in all laboratories. Moreover poor sensitivity and long turnaround times create trouble. Adequate and intact DNA with maximum purity, in a cheap and rapid way, is the outcome of a good extraction procedure for the isolation. Hence an attempt has been made to develop simple, economical and rapid method

to isolate high quality DNA from 10 different oomycetes in the present study.

Materials and Methods

Biological material

Ten different species of oomycetes which included *Phytophthora* as well as *Pythium* species (Table 1) were selected to isolate high quality DNA at Central Tuber Crops Research Institute, Thiruvananthapuram, India. Cultures were stored in carrot agar medium at 24°C.

Table 1. Details of oomycetes used for the study

S.l No.	Isolates	Host plant	Infection
1	*Phytophthora colocasiae*	Taro	Leaf blight
2	*Phytophthora megakarya*	Cocoa	Black pod
3	*Phytophthora* sojae	Soybean	Root rot
4	*Phytophthora parasitica*	Tomato	Root rot
5	*Phytophthora palmivora*	Cassava	Tuber rot
6	*Phytophthora capsici*	Pepper	Leaf blight
7	*Phytophthora meadii*	Periwinkle	Collar rot
8	*Pythium aphanidermatum*	Grasses	Leaf blights
9	*Pythium ultimum*	Tomato	Damping-off seed
10	*Pythium debaryanum*	Chilli	Damping-off seedlings

Growing the mycelia

Cultures were grown in potato dextrose broth in the 90 mm diameter plates. Two to three plugs of mycelia were used to inoculate each plate and allowed to grow until the plate were ~50% full, which took about one week. The mycelia was harvested, dried on a filter and washed with water. The mycelia can be used for downstream processing or stored at -20°C until use.

Extraction of DNA

Hundred mg of mycelia was macerated thoroughly in a pre-chilled mortar without buffer and transferred to an eppendorf tube containing 500 μl of pre warmed extraction buffer and kept in a water bath at 65°C for 1 min. The content was again transferred to a mortar and ground well until it had the consistency of a fine cream with 1 ml of extraction buffer (0.2 M Tris (pH 8.5), 0.25 M NaCl, 25 mM EDTA, 0.5% SDS, PVP) and then transferred to 2 ml centrifuge tubes for a brief centrifugation at 13,000 g for 5 min. To the supernatant, 0.1volume of guanidine hydrochloride was added and mixed vigorously with 10 mg ml^{-1} proteinase K. It was then centrifuged for 5 min at 13,000 g and the supernatant was transferred to a fresh tube. Equal volume of isopropanol, was added to it and mixed gently for 8-10 times. It was again centrifuged for 5 min at 13,000 g. Finally the supernatant was removed and the pellet was collected. The DNA pellet was washed with 0.2 volume 70% ethanol and centrifuged for 5 min at 13,000 g. The supernatant was discarded. After air drying the pellet was resuspended in 100 μl of TE buffer for further downstream processing or stored in -20°C.

Quantification and quality check of DNA

Gel checking of isolated DNA samples

Two micrograms of each DNA sample was electrophoresed on a 0.7% agarose gel containing ethidium bromide (5 μg ml^{-1}) with TAE buffer at 60 V for 2-3 h. DNA was visualized and photographed with Image Analyser.

Quantity analysis

The purity of the DNA was estimated from the A260/A280 ratio, whereas the yield was obtained by measuring absorbance at 260 nm with a spectrophotometer (GeneQuant pro, Biochrom Ltd., England). DNA concentration was calculated.

YPT amplification

Each oomycete (Table 1) was amplified by performing PCR with equal amount of DNA, oomycetes specific primer based on Ras-protein region, *Taq* DNA polymerase ac-cording to manufacturer instructions (Invitrogen, CA, USA). To assess the suitability of isolated DNA, Ypt forward and reverse primers (5-CGACCATKGGTGTGGACTTT-3 and 5-ACGTTCTCMCAGGCGTATCT-3) were used. PCR was carried out with the 25-μl PCR mixture of 2.5 μl buffer (10 X *Taq* DNA polymerase buffer containing 15 mM $MgCl_2$, Merck Biosciences, Bangalore, India); 2.5 μM dNTPs (from 10-mM stock, Merck Biosciences); 15 ng primers (random decamer primer, Operon Technologies Inc. Alameda, California); 1 U *Taq* DNA polymerase (Merck Biosciences); and 20 ng of DNA, in the following protocol: An initial denaturation at 94°C for 5 min; 35 cycles of

denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 10 min. The PCR products were electrophoresed using a 1.5% (w/v) agarose gel, which was stained with EtBr and visualized under UV light and gel image was taken.

Restriction analysis

The DNA was restricted by *EcoR I* (Merck Biosciences) using 3 U/μg of DNA and the reaction mixture was incubated at 37°C overnight. The digested DNA was separated on 0.8% agarose gel, stained with ethidium bromide and observed under UV light.

RAPD amplification

RAPD analysis was done with OPT-04, using 20 ng of each genomic DNA, after standardization. The 25-μl PCR mixture contained 2.5 μl of buffer (10 X *Taq* DNA polymerase buffer containing 15 mM $MgCl_2$, Merck Biosciences, Bangalore, India); 2.5 μM dNTPs (from 10-mM stock, Merck Biosciences); 15 ng primers (random decamer primer, Operon Technologies Inc. Alameda, California); 1 U *Taq* DNA polymerase (Merck Biosciences, Bangalore, India); and 20 ng of DNA. The mixture was kept in the thermal cycler (Biorad) with the following cycles: 1 cycle at 95°C for 5 min followed by 42 cycles at 92°C for 1 min, 37°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 8 min. The amplified fragments were separated on 2% agarose gel containing ethidium bromide (0.5 μg ml^{-1}), observed under UV light and photographed using UV transilluminator (Alfa imager TM2200).

Results and Discussion

Isolation of genomic DNA using the described method was quite easy and did not take more than 15 min.The results of the separa-tion of the extracted DNA on a 0.7% agarose gel (Fig. 1), showed that the new method yielded high-quality DNA, which was transparent, non-viscous, free of smearing and poor visible contamination of RNA. The 1kb DNA marker fragment was clearly visible, indicating that DNA degradation had not occurred.

DNA yield from oomycetes mats using this SDS method ranged from 120 to 160 μg g^{-1} fresh weight mycelium with an A_{260}/A_{280} ratio close to 2.00, indicating very little contamination of the DNA fraction by proteins, polysac-charides or aromatic compounds (Hansen et al., 2007). Results obtained with the GeneQuant spectrophotometer for the purified DNA by the current method showed A_{260}/A_{280} ratio ranging from 1.70 to 1.95 (Table 2).

PCR amplification of a fragment of the RAS-related proteins (YPT) region resulted in an amplification of single band of ~ 470bp (Fig. 2). The band was high in quality, sharp and crispy without any dimers.

DNA was digested completely with restriction enzyme *Eco*RI and the restricted DNA produced a good banding pattern (Fig. 3) on 0.8% agarose gel, indicating complete digestion of DNA samples.Thus we can conclude that the purity and quality of the isolated genomic DNA was sufficient to get an efficient digestion by restriction enzymes. When another important molecular technique

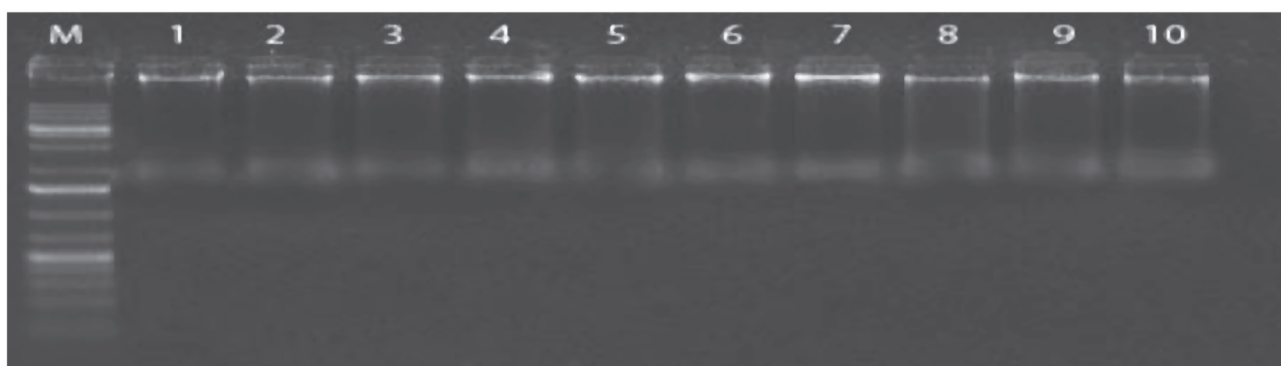

Fig. 1. Agarose gel (0.7%) electrophoresis of undigested genomic DNA isolated from 10 different fungal isolates. For each isolate 10 ng μl^{-1} genomic DNA was loaded (lanes 1-10); M = 1kb plus Fermentas DNA molecular weight ladder; Lane 1 –*Phytophthora colocasiae*; Lane 2-*Phytophthora megakarya*; Lane 3-*Phytophthora sojae*; Lane 4-*Phytophthora parasitica*; Lane 5-*Phytophthora palmivora*; Lane 6-*Phytophthora. capsici*; Lane 7-*Phytophthora meadii*; Lane 8-*Pythium apahnidermatum*; Lane 9-*Pythium ultimum* and Lane 10- *Pythium debaryanum.*

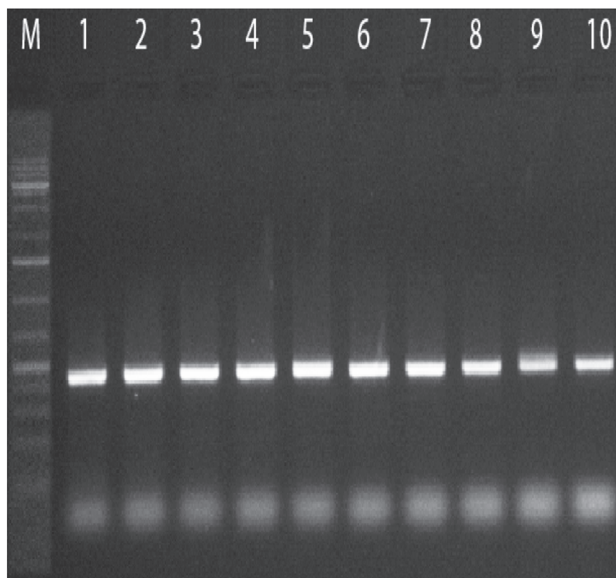

Fig. 2. Agarose gel (0.7%) electrophoresis of the amplified RAS-related proteins (YPT) products obtained with the Yph1F-Yph2R primer pair. M=Fermentas 1kb plus DNA molecular weight ladder. Lane positions are same as in Fig.1.

Table 2. DNA yield and quality from *Phytophthora and Pythium* species as determined by GeneQuant spectrophotometer measurements

Strain number	Samples	ng μl^{-1}	A260/ A280
1	*Phytophthora colocasiae*	1500.8	1.91
2	*Phytophthora megakarya*	1524.9	1.95
3	*Phytophthora* sojae	1457.4	1.82
4	*Phytophthora parasitica*	1511.1	1.89
5	*Phytophthora palmivora*	1177.2	1.80
6	*Phytophthora capsici*	1182.1	1.74
7	*Phytophthora meadii*	1523.5	1.75
8	*Pythium apahnidermatum*	1502.2	1.79
9	*Pythium ultimum*	1440.7	1.70
10	*Pythium debaryanum*	1313.3	1.72

RAPD was performed with OPT-04 primer, adequate amplification with good bands (06-10) for score was observed in all the isolates (Fig. 4), indicating the DNA quality obtained by this protocol can be considered good for RAPD reactions.

The present method of DNA isolation is simple, rapid and cost effective than all the other available DNA isolation protocols. One of the main highlight of this method is that it doesn't require any toxic chemicals such as liquid nitrogen, phenol, chloroform etc. Most of the conventional DNA isolation methods start with the

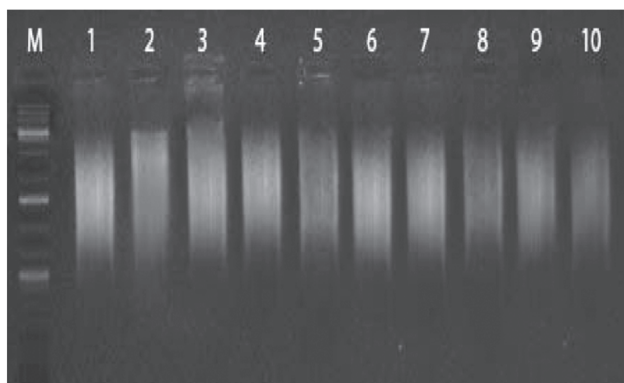

Fig. 3. Agarose gel (1.5%) electrophoresis of genomic DNA digested with the restriction enzyme *Eco* R-1. M = Fermentas 1kb plus DNA molecular weight ladder. Lane positions are same as in Fig. 1.

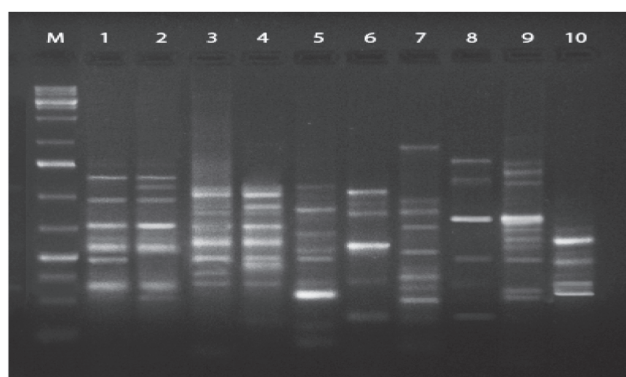

Fig. 4. Amplification with RAPD primer OPT-04.M =Ladder Fermentas 1kb plus. Agarose gel (1.5%) electrophoresis of RAPD products obtained by using the OPT-04 primer. 100-bp.Lane positions are same as in Fig. 1.

expensive and hazardous procedure of grinding mycelia with liquid nitrogen (Karakousis et al., 2006; Sharma et al., 2003). Here the same is replaced with alternate cold (0°C) and heat shock (65°C) treatment with proper grinding to a fine paste, which actually determines the extent of success of the DNA isolation. Some other chemicals such as sodium/potassium acetate, phenol, chloroform, etc. were also excluded. Another peculiarity of this method is that it does not demand any special or costly equipment in any part of the procedure as in many other procedures (Mahuku, 2004), so that can be performed in any laboratory with the basic requirements. The duration of each step is cut short in this altered method, with enhanced productivity, in both quality as well as quantity. Incubation steps were standardized to minimum without affecting the quality and quantity of DNA. Puchooa (2004) also reported that there were no

significant differences in the yield of DNA when incubated at 65ºC and 45ºC for 5 min. All the above qualities make this method an economical one with the production of high quality and quantity of DNA. Some of the DNA isolation kits can provide excellent result with small amount of mycelium, but in many cases it is not (Sharma et al., 2000; Li et al., 2001; Buldewo and Jaufeerally-Fakim, 2002; Keb-Llanes et al., 2002; Horne et al., 2004), moreover their applications are limited to a certain number of samples and so are not economical. The quality of the extracted DNA was proven to be high after its assessment by a number of sensitive downstream processes.

Conclusion

It was proved that the quality, quantity and integrity of DNA obtained were very good and comparable with traditional extraction methods. The extraction method gave good yield of high molecular weight, digestible DNA across a wide range of oomycete; which was reliable, non-hazardous, time saving, economical, especially in laboratories that lack the facilities to work with expensive chemicals and equipments and effective even in low oomycete biomass of 5 mg. This method was successfully used with mycelia cultured on different media such as oat meal agar, V-8 agar and potato-dextrose agar.

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Dear Dr. Suja,

My comments on the ms follows

The authors attempted a new DNA extraction method for Oomycetes. However the new method presumed to be superior and economical has no comparison with known / established methods. They may be requested to provide complete information on the experiments conducted to prove the worthiness of the study. Also many statements made by the authors seem not original ie reproduced as it is from other sources. Let them be modified to authors own narration or sentences.

For acceptance, the ms needs thorough changes and drastic modifications in the content, clarity and style.

The ms is attached with my comments in the text for your observations and further action.

Thank you.

sincerely,

Viswanathan