



Early Detection of *Phytophthora colocasiae* causing Leaf Blight Disease of Taro using Nucleic Acid Spot Hybridization Technique

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Abstract

Taro (*Colocasia esculenta*), is the most important edible species of the monocotyledonous family Araceae. Leaf blight caused by *Phytophthora colocasiae*, a soil born oomycetes, has become a major limiting factor in taro production worldwide including India, resulting in heavy yield losses. Currently available methods for the detection of *P. colocasiae* includes conventional culture-based morphological approaches and PCR based diagnosis, which entirely depends on laboratory equipment. This study presents an alternative nucleic acid spot hybridization technique with the development of highly specific nucleic acid probes. The developed probes showed good quality, high specificity and optimum sensitivity, thus could be used as an effective diagnostic tools for detection of *P. colocasiae* species, due to the sequence specificity of the nucleic acid molecule.

Key words: *Phytophthora colocasiae*, taro leaf blight, diagnosis, nucleic acid spot hybridization

Introduction

Taro serves as an important staple crop for millions of people in developing countries, with nearly 1000 cultivars. The fungus-like oomycete *Phytophthora colocasiae* Raciborski (*P. colocasiae*), causing taro leaf blight (TLB) disease is of prime importance among various taro diseases. In susceptible varieties, it can reduce corm yield by up to 50% (Jackson., 2001; Singh et al., 2006) and leaf yield by 95% (Trujillo., 1967). The pathogen causes circular, water soaked, necrotic spots on the surface of leaves, followed by the collapse of the plant.

An accurate disease diagnosis and precise identification of the pathogens involved is an essential prerequisite to apply more effective and adequate management strategies, for instance determining the need for fungicide applications to control disease frequency (Ward et al., 2004; Lee et al., 1993). The conventional detection techniques are so time consuming, and allow only a limited number of samples processing at a time (Ward et al., 2004; Winton and Hansen, 2001).

A range of molecular (Panabieres et al., 1989; Liew et al., 1991; Lee and Taylor, 1992; Whisson et al., 1992; Tyler et al., 1995) as well as serological methods (Förster et al., 1989; Nygaard et al., 1989; Oudemans and Coffey, 1991a,b) have been developed in order to improve accuracy and efficiency of the diagnostic methods used to distinguish different *Phytophthora* species. (Dobrowolski, 1998; Meng et al., 1999; Schubert et al., 1999).

Currently, PCR based diagnosis are available for most of the *Phytophthora* species including *P. colocasiae* based on the conserved regions within the genome (Mishra et al., 2010; Nath et al., 2014). Even though PCR methods are the standard in diagnosis, these techniques are comparatively expensive, depends on the availability of equipment and need stringent practical conditions that require expertise to standardize a reliable and robust PCR prior to evaluation (Malorny et al., 2004). In addition, PCR amplification also demands pure DNA's as samples, in order to avoid false negative results due to the presence of PCR inhibitory substances in crude DNA

samples. Above all, in PCR assays specificity mainly rely on 20–25 bases primer and the stringent practical conditions that require expertise to standardize a reliable and robust PCR prior to evaluation (Pravi et al., 2015).

Nucleic acid spot hybridization (NASH) assay is one of the more attractive alternative to PCR based diagnosis. DNA probe was considered as the first molecular tool used in the identification, detection and phylogenetic analysis of phytopathogenic microorganisms, which was enhanced by the development of protocols for extracting DNA from different sources like pure fungal cultures, diseased plant parts, plant viruses, bacteria, infected soil or air samples. (Yogendra Singh et al., 2013; Miller and Martin, 1988). To the best of our knowledge, this is a first time approach that focused on the development of a *P. colocasiae* specific probe for detection of which in infected plant tissue and soil samples, with the potential application of DNA hybridization based approaches.

Materials and Methods

Biological materials and culture conditions

P. colocasiae isolates were obtained from infected leaf and soil sampled from different locations in India. Conventional methods were used for the isolation of *P. colocasiae* isolates. To eliminate surface debris and contaminants, leaves were washed with dilute liquid detergent and rinsed with running tap water. Four small pieces were cut from the advancing margins of each leaf, surface sterilized in 0.6% sodium hypochlorate for 2 min, blotted on a sterile paper towel, and placed on 1.5% water agar. For mycelial development, plates were then incubated in 24°C under constant fluorescent light for 1 to 3 days. Two hyphal tips from each diseased leaf were transferred to V8 agar and incubated under continuous fluorescent light at 24°C. The isolates were further purified by growing single mycelia from each colony onto potato dextrose agar (PDA: 250 g potato l⁻¹, 20 g dextrose l⁻¹ and 20 g agar l⁻¹) plates. Cultures of *Phytophthora* species, other fungi and bacteria, required for specificity analysis of probe, were obtained

from ICAR-IISR (Calicut, India), ICAR-CTCRI (Thiruvananthapuram, India) and College of Agriculture (Thiruvananthapuram, India).

Extraction of Genomic DNA

Mycelial DNA was extracted from all oomycetes including *P. colocasiae* cultures according to the protocol of Archana et al., 2014. DNA from soil was extracted with some modifications of the mycelial DNA isolation method. Isolation of genomic DNA from other fungal cultures and plant parts done by previously standardized methods (Mishra et al., 2008a). Bacterial cultures were grown in Luria–Bertani broth overnight at 27°C and genomic DNA was extracted by the method of Sambrook et al., (1989). DNA quantification was done with Qubit Fluorometer (Invitrogen, California, USA) and visualized by 0.7 % agarose gel electrophoresis followed by ethidium bromide with a 1.5 kb DNA ladder (Fermentas Life Sciences, Burlington, Canada). The isolated genetic materials were air dried and re-suspended in 100 µl of TE buffer and stored in -20°C.

PCR amplification and sequencing

To confirm the identification of isolates to the species level, each oomycete (Table 1) was amplified by performing PCR with equal amount of DNA, oomycetes specific primer based on Ras-related protein region, Taq DNA polymerase according 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 MgCl₂, 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 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.

The amplification products were purified to remove excess primers and nucleotides using a QIAquick PCR purification kit (Qiagen, Limburg, the Netherlands), and the concentration of elute was confirmed by electrophoresis. The purified products were cloned into pTZ57R (Insta) T/A cloning vector

(MBI Fermentas, USA) as described in manufacturer's protocol and these recombinant plasmids were then transformed in competent *Escherichia coli* strain (DH α) competent cells (Promega Corp., Madison, WI, USA), by heat shock method (Sambrook et al. 14). Ampicillin-resistant (100 μ g ml⁻¹) clones (blue/white selection method), were confirmed by colony-PCR using primers M13F (5'-GTAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGACC-3'), after incubation at 37°C for 16 h in Luria-Bertani (LB) selection medium containing ampicillin, IPTG (isopropylthio- β -galactoside) and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Using GenElute Plasmid Miniprep Kit (Sigma, USA), the recombinant plasmids were extracted.

Recombinant clones from three different *P. colocasiae* were submitted for sequencing (Sygenome). The sequencing result was edited and subsequently evaluated using BLASTN (Altschul et al., 1990) to determine whether it had any potential homologies with any of the known sequences present in the GenBank database of the National Center for Biotechnology Information (NCBI) (Benson et al., 2013) and also aligned using the BioEdit sequence alignment clustal programme (Hall, 1999) with available sequences of *Phytophthora* and fungal species. The sequences have been later submitted to Genbank database under the accession numbers: KY178299, KY178301 and KY178302.

P. colocasiae specific probe development

To design *P. colocasiae* specific probe, the sequences of Ras-related protein region of *P. colocasiae* were aligned with the 100 other sequence collected from the GenBank database, that showed maximum similarity when assessed using BLASTN. All the sequences belong to *Phytophthora* species only and there were about 36 different species in that list (*P. taxon*, *P. citrophthora*, *P. citricola*, *P. plurivora*, *P. multivora*, *P. heveae*, *P. coccis*, *P. katsurae*, *P. pseudosyringae*, *P. castaneae*, *P. agathidicida*, *P. nemorosa*, *P. ilicis*, *P. tentaculata*, *P. tropicalis*, *P. iranica*, *P. quercina*, *P. agathidicida*, *P. megasperma*, *P. gallica*, *P. obscura*, *P. idaei*, *P. cactorum*, *P. pseudotsugae*, *P. hedraiaandra*, *P. insolita*, *P. palmivora*, *P. cinnamomi*, *P. hibernalis*, *P. niederhauserii*, *P. boehmeriae*, *P. vignae*, *P. uliginosa*, *P. pluvialis*). A multiple sequence alignment was made for these sequences using the Clustal W program in BioEdit software. The alignment was analyzed for divergences among the sequences, which

led to the identification of species conserved sequence regions in *P. colocasiae*. An oligonucleotide probe, named PCSPB (CTTGGTGGT ATTCTCTAGAAAATCCGTACGATCGAGTTGGACGG CAAGACCATCAAGCTCCAGATTGTAAGCAACCAAAGTT TAAGACTCGTCATCCACCTGAGTACTCTGGACTAAT TAATGGTTGTGCCAACTCCCTTGTGCTCCT) was developed for the specific detection of *P. colocasiae*. The specificity of the designed probe was confirmed before synthesis following BLAST database searches of DNA sequences.

P. colocasiae detection based on Nucleic acid spot hybridization (NASH)

Preparation of non-radioactive biotin labelled probe

In a microcentrifuge tube, about 20 μ l (5–1,000 ng) of the probe for labelling was diluted to a final template volume of 34 μ l with nuclease free water followed by denaturation in boiling water for 5 min and rapidly placed on ice for next 5 min. In order to collect the liquid in the bottom, the tube was briefly centrifuged. To the denatured DNA, 10 μ l of 5X labeling mix (Biotinylated random octamers in 5X labeling buffer), 5 μ l of dNTP (1 mM dCTP, 1 mM dGTP, 1 mM TTP and 1 mM dATP/ Biotin-14-dATP) mix and 1 μ l of Klenow fragment (NEBlot Phototope kit, NEB, UK, NEB #N7550S) were added. The contents were then mixed by flicking the tube, centrifuged briefly to collect liquid in the bottom of the tube and incubated at 37°C for 2–3 h. The reaction was stopped by inactivating the enzyme by adding 5 μ l 0.2 M EDTA (pH-8). The probe thus developed was precipitated by adding 5 μ l of lithium chloride (4 M) and 150 μ l of ethanol and incubated at -20°C for 1 h 30 min. The tube was then centrifuged at 10,000g for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 70 % ethanol by centrifuging at 10,000g for 10 min at 4°C. The supernatant was carefully removed and the tube was air dried for 10 min. The pellet was then re-suspended in 20 μ l TE (10 Mm Tris, 1 mM EDTA, pH-8) and stored at -20°C for further procedures. The developed probe was named as PCSPB.

Spotting and probe quality analysis

Squares of 1 x 1 cm² were made with pencil impressions on Hybond N+ positively charged nylon membrane (Amersham-Hybond N+, GE Healthcare, UK) of desired size. One edge of the membrane was cut off to

specify the orientation of spotting. Both sides of the nylon membrane were then thoroughly wet using 6X SSC (prepared from 20X SSC stock solution) and dried on filter paper. By diluting the probe in 0.1 N NaOH, serial ten-fold dilutions of the nonradioactive biotinylated probe were prepared. Spotted 5 μ l of each probe dilution onto each square in the membrane and the spots were allowed to get absorbed into the membrane at room temperature, but complete drying was avoided. Cross-linking of spotted probe to the nylon membrane was achieved by exposing the membrane to UV using UV transilluminator for 66s. The membrane was then processed according to the user manual of Phototope-Star detection kit for nucleic acids (New England Biolabs, UK) and finally the probe quality was checked with Chemiluminescent Nucleic Acid Detection Module by exposing the membrane to X-ray film (Thermo Scientific).

Sensitivity and specificity analysis

As described before, the nylon membrane was spotted, rinsed in 6X SSC, UV cross linked for 66s. and dried on filter paper. The DNA of test samples for specificity analysis were denatured for 5 min in boiling water, spotted onto the membrane then were air-dried and DNA was cross-linked by UV exposure. Followed by crosslinking, the spotted membrane was placed in a hybridization bottle containing pre-hybridization solution (6X SSC, 59 Denhardt's reagent, 0.5% SDS and 100 μ g ml⁻¹ denatured salmon sperm DNA). The bottle containing the membrane was pre-hybridized in a hybridization oven, at 68°C for 1 h, with gentle rotation. While pre-hybridization was in progress, 10 μ l of appropriate biotinylated probe dilution was denatured by heating at 100°C for 10 min subsequently placed on ice for 5 min. The tube containing the diluted probe was centrifuged at 5,000 g for 5 min at 4°C. After pre-hybridization, the denatured biotinylated probe was added to the pre-hybridization solution containing the membrane and incubated at 68°C overnight in hybridization oven with gentle rotation. Thereafter the hybridization solution was discarded and the membrane was washed twice with 2X SSC and 0.1% SDS for 15 min each and final washes were given twice with 0.19 SSC and 0.1% SDS for 5 min each at 68°C with gentle rotation. Finally, the washed membrane was processed in accordance with the user manual of Phototope-Star

detection kit for nucleic acids and chemiluminescence was documented by exposing the membrane to X-ray film.

Chemiluminescent Nucleic Acid Detection

The dot blot was placed in a hybridisation bottle containing 20 ml of blocking buffer and incubated for 15 min with gentle rotation. After discarding the blocking buffer, the membrane was incubated with stabilized Streptavidin-Horseradish Peroxidase Conjugate diluted to 1:300 in blocking buffer, with gentle rotation. After rinsing briefly with 1X wash solution in a sterile Petridish, the membrane was washed 4 times for 5 min each in 1X wash solution with gentle shaking. The blot was transferred to a new Petridish and 30 ml of Substrate Equilibration buffer (SEB) was added followed by incubation for 5 min with gentle shaking. Chemiluminescent Substrate Working Solution (SWS) was prepared by adding 6 ml Luminol/Enhancer solution to 6 ml Stable Peroxide Solution. The membrane was removed from the SEB and carefully blotted an edge of the membrane on a paper towel to remove excess buffer. The membrane was placed in a clean container and poured enough SWS to completely cover the surface and incubated without shaking in dark for 5 min at 4°C. The blot was removed from SWS and blotted an edge on a paper towel for 2-5s to remove excess buffer. The moist membrane was then placed in the X-ray cassette and a cling film was taped over it. Under dark conditions, an X-ray film was placed over the blot in the cassette and exposed for 5 min. Followed by the exposure, the X-ray film was washed in developer solution (Kodak) (Component A -13.25 g and Component B - 97.15 g dissolved in 1000 ml of sterile distilled water) for 3 min with continuous shaking, and washed in distilled water for 1 min with moderate shaking. The X-ray film was fixed in the fixer solution (Kodak) (Fixer - 267.7 g dissolved in 1000 ml of sterile distilled water) for 3 min with constant shaking followed by washing in distilled water for 1 min. The film was washed thoroughly under running tap water, dried, analysed for dark spots and photographed.

For sensitivity analysis, a tenfold serial dilution of *P. colocasiae* DNA starting from 10 ng μ l⁻¹ to 1 fg μ l⁻¹ was prepared. After denaturation, 1 μ l of each dilution of the samples were spotted directly onto the membrane and allowed to air dry. Followed by UV cross-linking,

hybridization was performed and finally detection process was done using Phototope-Star detection kit for nucleic acids.

Detection of pathogen from naturally infected samples by NASH

In order to confirm the efficacy of the newly designed probe in detecting *P. colocasiae* from naturally infected samples, healthy as well as infected samples were collected randomly from 38 plots, each plot with 25 plants, of ICAR-CTCRI and the hybridization experiments were performed thrice. Within that field, 28 healthy and 54 infected samples were considered during the first trial, followed by 40 and 58 in second trial and finally 50 and 82 in third trial. DNA isolation was done according to the previously described methods. DNA from pure culture of *P. colocasiae* and healthy plant tissues served as positive and negative control respectively.

Results and Discussion

Probe quality

The observation of quality analysis of probe (PCSPB) gave clear round spots upto 10^{-5} dilution of the probe that faint gradually with increase in dilution (Fig. 1).

Specificity

In specificity analysis, the developed probe gave positive spots for the target organism only, that is, no spots were there for any other tested microbes including other Oomycetes (Fig. 2).

Sensitivity

In sensitivity assay, the developed probe gave thick spots for *P. colocasiae* DNA upto 10^{-4} dilution followed by faint spots (Fig. 3).

NASH based detection of pathogen from naturally infected samples

High to low hybridization spots were observed from the naturally infected samples. On the other hand, no

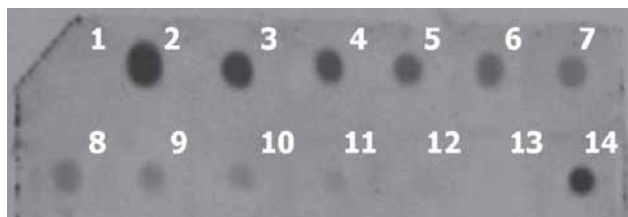


Fig. 1. Analysis of quality of the developed probe (PCSPB). Lane 1—negative control (Buffer), Lane 2–13—probe dilutions (10^{-1} to 10^{-12}), Lane 14—positive control

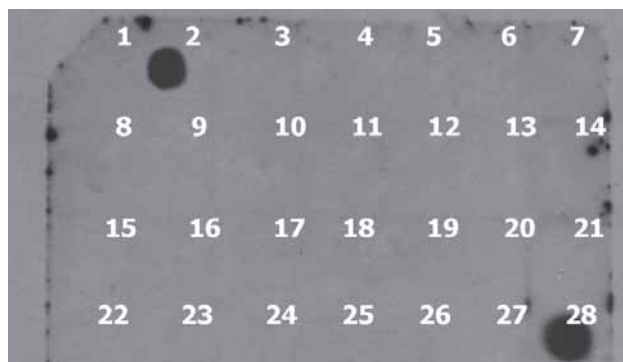


Fig. 2. Specificity analysis of probe. Lane 1—negative control (Buffer), Lane 2—*Phytophthora colocasiae*, Lane 3—*Fusarium solani*, Lane 4—*P. parasitica*, Lane 5—*P. capsici*, Lane 6—*P. palmivora*, Lane 7—*Pythium debaryanum*, Lane 8—*Pythium ultimum*, Lane 9—*P. boehmeriae*, Lane 10—*Rhizoctonia solani*, Lane 11—*Trichoderma viride*, Lane 12—*Trichoderma harzianum*, Lane 13—*P. meadii*, Lane 14—*Fusarium oxysporum*, Lane 15—*Mucor spp.*, Lane 16—*Colletotrichum falcatum*, Lane 17—*Colletotrichum dematium*, Lane 18—*Colletotrichum capsici*, Lane 19—*Colletotrichum truncatum*, Lane 20—*Rhizopus oryzae*, Lane 21—*Sclerotium fumigatus*, Lane 22—*Sclerotium delphinii*, Lane 23—*Sclerotium oryzae*, Lane 24—*Sclerotium hydrophilum*, Lane 25—*Erwinia carotovora*, Lane 26—*Macrophomina phaseolina*, Lane 27—*Helminthosporium oryzae*, Lane 28—positive control (*Phytophthora colocasiae*)

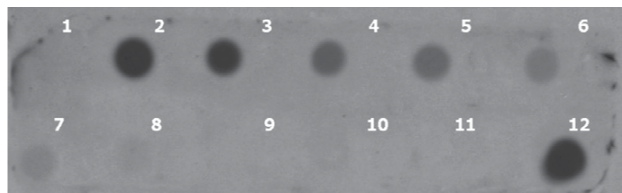


Fig. 3. Analysis of sensitivity of the developed probe (PCSPB). Lane 1—negative control (Buffer) : Lane 2–11—*P. colocasiae* DNA dilution (10^{-1} to 10^{-10}), Lane 12—positive control

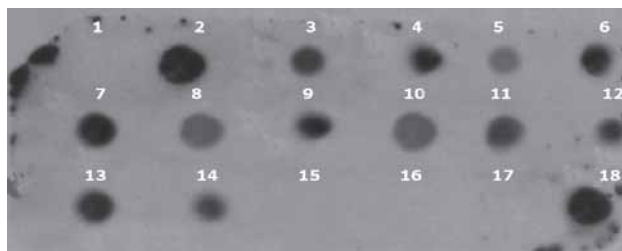


Fig. 4. NASH based detection *P. colocasiae* in naturally infected samples. Lane 1—negative control, Lane 2–6—infected leaf DNA, Lane 7–10—infected tuber DNA, Lane 11–14—infected soil DNA, Lane 15—healthy leaf DNA, Lane 16—healthy tuber DNA, Lane 17—uninfected soil DNA, Lane 18—positive control (*Phytophthora colocasiae* pure culture DNA)

hybridization spot was seen from the healthy sample used as negative control, while thick spots were developed in the positive control (Fig. 4).

Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches. However, there are certain drawbacks, which include, the success of which reliance on the ability of the organism to be cultured and allow only a limited number of samples processing at a time. These limitations have led to the development of molecular approaches with improved accuracy and reliability. Until the mid-1980s, prior to the introduction of PCR, nucleic acid based diagnostics commonly demanded the use of probes, and they continued to be widely used as a substitute to PCR for detection of pathogen (Mutasa et al., 1993; Rasmussen and Reeves, 1992; Ward and Gray, 1992). However, nowadays, for sensitive diagnostic protocols, probes are largely used in conjunction with PCR to yield more specific and accurate results than when using PCR alone (Knoll et al., 2002; Williams et al., 2001).

Above all, most laboratory diagnostic procedures have so far proved inapt for field use, as they are neither sufficiently flexible nor portable. By making recognition easier, these techniques play complementary roles in expanding our understanding of the biology, taxonomy and ecology of soil-borne pathogens (Fox., 1990). Nucleic acid spot hybridization is a one of the basic technique in molecular biology that takes advantage of the capability of individual single-stranded nucleic acid molecules to develop double-stranded molecules. As per Watson-Crick base pairing, hydrogen-bonding mediated binding of adenine with thymine and guanine with cytosine. The isolation of species-specific gene sequences is readily achieved by the development of recombinant DNA methodology and DNA-DNA and RNA-DNA hybridization procedures (Brooker et al., 1990, Stahl and Kane., 1992). Probes may be radioactive or non-radioactive in nature. Even though radioactive probes are more sensitive, non-radioactive probes more popular due to of many advantages like stability, safety and *in situ* detection. Nucleic acid probes can be used to distinguish unculturable microorganisms and pathogens in the environs or simply provide rapid detection and diagnosis of species and group levels and thus the practice of probes

has predominantly been evident in microbial ecology (Brooker et al., 1990).

However, to be effective in these applications, a hybridization assay must be able to detect an organism in its natural environment at population levels below those causing yield losses (Gilbertson et al., 1989). In this context an attempt was made to assess the potential of RAS-related protein (Ypt1) region based probes for species identification.

The quality analysis showed that, the probe dilutions upto 1–0.01 pg is capable of detecting *P. colocasiae* for hybridization assays, indicating the good quality of developed probe. At the same time, the sensitivity analysis implies that the probe seems to have a detection limit of 10–15 pg which is an important factor when considering the detection of plant pathogens from field and soil samples. The specificity assay confirms that, the newly designed RAS-related protein (Ypt1) region based probe is conserved within the *P. colocasiae* species only. Conditions that allow the probe to bind only to the homologous sequences in a DNA sample prevent cross-reaction with DNA from unrelated organisms. Thus, the analysis of quality, specificity and sensitivity of the designed probe clearly shows that NASH using the developed probe allows the detection of minimum amount of the pathogen and can therefore be used for the detection of the presence of pathogen in a field soil or planting material before plantation as an early warning detection and diagnostic tool. Hybridization assay using the specific probe was effective in detecting the pathogen from naturally infected samples including plant tissues as well as soil. The intensity of the spots were varied according to the pathogen concentration, that is, samples with higher pathogen concentration gave thick spots whereas those with lower pathogen concentration gave moderate to faint spots. Exposure of chemiluminescence emitting membrane to X-ray film needs to be prolonged for getting better results, especially in the case of field samples, to capture weak signals from the samples with minimum amount of pathogens.

Designing of oligonucleotide probes with good specificity and sensitivity is the crucial step for development of a DNA hybridization method. The target region selected for the designing probe, which is unique as well as

conserved across the entire population of organism of interest, determine the success of probe design.

Conclusion

Detection and hybridization of the probe to a specific nucleic acid sequence of a particular microorganism on the membrane indicates the presence of a unique or complementary fragment in the DNA sequence and, perhaps, confirm an infectious disease. This hybridization based diagnostic method gave a good result in detection and diagnosis of *P. colocasiae* in plant tissue and soil, which was reliable, economical, especially in laboratories that lack the facilities to work with expensive equipment and above all this technique is not restricted to the laboratory, thereby forming a vital part of disease risk analysis.

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