Defence Life Science Journal, Vol. 3, No. 1, January 2018, pp. 41-44, DOI : 10.14429/dlsj.3.11491

PCR-based Methods for Identification and Detection of *Phytophthora infestans* in Infected Leaves of Tomato

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ABSTRACT

Every year there is a huge amount of loss of tomato crop due to infection of late blight caused by *P. infestans*. To minimise this loss, it is important to visualise disease infection, so as the control measures can be taken up rapidly. The aim of this study was to develop a schematic protocol in order to examine the disease outbreak. *P. infestans* were isolated and identified based on morphological characteristics, serological and species-specific PCR assays. Ten samples were processed and examined morphologically and serologically (dipstick). However, on molecular examination only three isolates were confirmed as *P. infestans*. Four sets of PCR primers i.e., AE-7, O-8, INF, ITS 3 and ITS 4 were validated for accurate detection of *P. infestans* infection. All the sets of primers gave positive result by giving amplicons of expected size.

Keywords: Late blight; PCR; Dipstick; Sporangia

1. INTRODUCTION

Late blight is a major threat to global food security, accounting for huge losses of potato and tomato crops worldwide[1]. The causative agent, *Phytophthora infestans* is diploid, heterothallic and biotrophic. Typically, late blight is characterised by an infection phase, followed by a necrotrophic phase during which host cell death is induced[2]. In India, late blight occurs regularly in entire potato growing belt[3], though infection of tomato is generally reported first, which is subsequently carried over to potato. For management of the disease, early and rapid detection of pathogen in infected plants is required[4]. Identification of most fungi is principally based on morphological characteristics of the colony in the culture. However, such characteristics may not be unique to a species and closely related species may have similar morphologies. Immunological methods are preferred over morphological methods[5], as fungi are known to produce specific soluble exoantigens[6]. However, these methods too sometimes fail to differentiate up to the species level. DNA sequences provide sufficient resolution for detection not only up to the level of species, but also to the levels of clades, tribes, populations, etc. by targeted amplification of specific regions.

Considering the economic importance of *P. infestans*, rapid and accurate detection is all the most important. Even though, *P. infestans* is the principle phytophthora infection to solanaceae family members, other species like *P. parasitica*, *P. capsici* and *P. nicotianeae* may also infect potato and tomato. Here, we report a reliable procedure for confirmation of *P. infestans* at early stages.

Received : 12 June 2017, Revised : 08 November 2017
Accepted : 09 November 2017, Online published : 15 December 2017

2. MATERIALS AND METHODS

2.1 Collection and Isolation of *P. infestans*

Infected leaves of tomato plants were collected during a survey of late blight in Haldwani, Uttarakhand, India in December 2016. The collected samples were incubated for 48 h at 18 °C. The leaflet containing lesion was positioned upside down in a petri dish containing selective medium (Rye Agar A). Petri dishes were incubated for one week at 18 °C. Mycelia emerging from the leaflets were then co-cultured in ampicillin-contained Rye Agar A medium at 18 °C. A total of ten isolates were purified and processed.

2.2 Morphological and Serological Examination

Leaf discs containing the inocula were excised and examined by microscopy for identification of *P. infestans*. Lactophenol blue staining was performed and examined under microscope. Preliminary screening of *Phytophthora* species was done by using prepared Phytophthora Immunostrip (Agdia, USA). Infected plant tissues were taken and inserted between the mesh linings near the bottom of the sample extraction bag containing SEB1 buffer (Agdia, USA). Samples were extracted by thoroughly macerating with Agdia tissue homogeniser until homogenous green or light brown coloured solutions were obtained. Immunostrip was inserted in each of the samples and were allowed to remain in the sample extract for 30 min. Test strips were removed to interpret the result.

2.3 Genomic DNA Isolation

For molecular validation, to a 1.5-mL microcentrifuge tube containing 750 µL of lysis buffer (200 mM Tris-HCl, pH 8.0; 25 mM ethylene diaminetetra acetic acid, EDTA, pH 8.0; 250 mM NaCl, 0.5 per cent sodium dodecyl sulfate), a small

lump of mycelia from young culture collected from infected tomato field samples was added by using a sterile toothpick, The tissue was homogenised properly and spinned at 13,000 rpm for 5 m. The supernatant was transferred to another 1.5 mL micro centrifuge tube and centrifuged again as described above. After transferring the supernatant to a new 1.5-mL micro centrifuge tube, 500 µl of phenol:chloroform:alcohol (25:24:1) was added and again centrifuged. Upper layer was taken and an equal volume of isopropyl alcohol was added. The tube was mixed by inversion briefly. The tube was spinned for 13,000 rpm for 10 m, and the supernatant was discarded. The resultant DNA pellet was washed in 300 µL of 70 per cent ethanol. The DNA pellet was air dried and dissolved in 50 µL of deionised water.

2.4 Molecular Validation by PCR

Templates were amplified by PCR using primer pairs described in Table 1. PCR amplification reactions were done in 10 µL reaction mixtures, containing 100 ng of template DNA, 100 pm of each forward and reverse primers, 2.5 mM dNTPs, 1x PCR buffer, 0.2 U *Taq* polymerase and nuclease free water to make up the volume. Amplifications were carried in a thermal cycler (Sure Cycler 8000, Agilent technologies) following: one cycle at 94 °C for 30 s; 35 cycles of denaturation at 94 °C for 30 s annealing as computed according to [2(A or T) + 4(G or C)] – 5 °C for 30 s (Table 1) and 45 s extension at 72 °C, followed by one extension cycle at 72 °C for 10 min. All amplifications were separated on 4 per cent agarose gel, along with 300 and 700 bp DNA ladders (MBI Fermentas). The gel was stained in ethidium bromide (0.5 µg/ml) for 30 min, and photographed under Typhoon Trio^{+} Variable Mode Imager.

3. RESULT AND DISCUSSION

Accurate detection and identification of causal pathogens of crop plants is indispensible for proper crop and pest/pathogen management strategies. Detection of *P. infestans* (causing late blight) process begins with visual assessment of symptoms, which are generally brownish-black lesions on the margins of the leaf and white growth visible on the abaxial surface of the leaf lesions (Fig. 1).

Under microscopic examination distinctively semi-papillate and lemon shaped (Fig. 2) sporangia were seen with compound and sympodial sporangiophores (Fig. 2). All the infected leaves were identified as *Phytophthora* species. The

Figure 1. Visual brownish-black lesions, as indicated by red arrow, on the margin of the leaf and on the abaxial surface of the infected leaf.

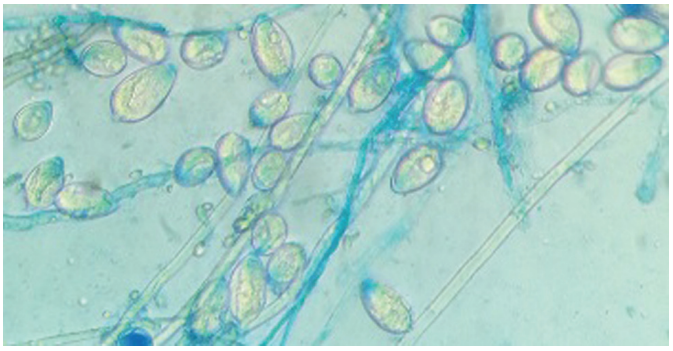

Figure 2. Isolated strain of *Phytophthora infestans* visualised under microscope after straining with Lacto phenol blue sporangia with sporangiophores. Lemon shaped spores are marked with red arrow.

macroscopic and microscopic morphology of the pathogen including the shapes of the spores produced are also the basis of identification and classification up to genus[10]. However, microscopic examination for identification of *P. infestans* is not straightforward due to similarities with closely related species.

To validate, serological examination was carried out. Clear differences were detected between control (C) and positive (P) pre-wetted strips of membrane (dipsticks). However the intensity of bands was low, this could be due to the low pathogen titre in the sample. All pathogen infected leaves tested positively by this method (Fig. 3). The dipstick, presumably had sensitivity and specificity for *Phytophthora* and in the present context, it is believed that closely-related species produce similar mycotoxins which could not be easily resolved. Thus, results of micro-and macro-scopic examination only

Table 1. List of primer pairs used for molecular validation of *Phytophthora infestans*

Primer ID	Primer Sequence (5'-3')	Amplicon size (bp)	T_a (°C)	Reference
AE-7-1 AE-7-2	GCC GCC GAC ATA TTG AAT CAA ATC TGC GAA CGA GAC AT	171	50	Judelson and Tooley[7]
O-8-1 O-8-2	AAG ATG ATG TTG GAT GAT TG TGC CTG ATT TCT ACC TTC T	245	58	Judelson and Tooley[7]
INF-F INF-R	TGG GCG AGC CCT ATC AAA A CCG ATT CAA ATG CCA AGC TAA	613	50	Hussain[8], *et al.*
ITS3 ITS4	GCA TCG ATG AAG AAC GCA GC TCC TCC GCT TAT TGA TAT GC	612	50	Ristaino[9], *et al.*

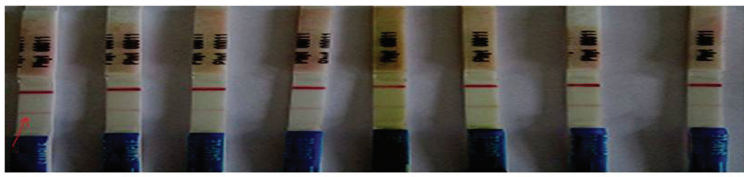

Figure 3. Dip sticks help detection of *Phytophthora* (red coloured arrow), against the upper C band.

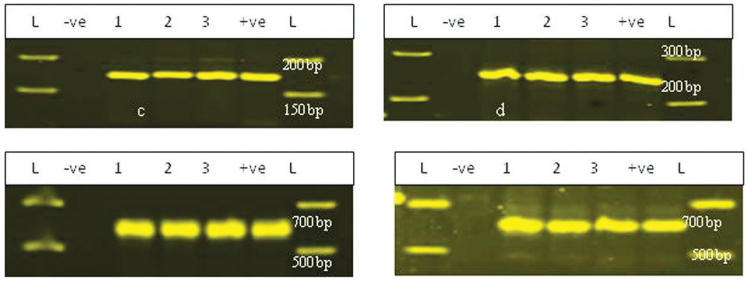

Figure 4. Amplicons obtained selectively on amplification using the AE-7 (a), O 8 (b), INF (c) and ITS (d) set of primers with either Lane – indicate negative control, Lanes 1-3 (samples collected from field, while lane + indicate positive control. Indicated in the margins are size standards from a ladder.

were corroborated, without conclusively testing the species by dipsticks.

Finally, PCR amplification using primers given in Table 1 were carried out. On electrophoresis of the amplified product, band in expected size ranges were obtained in only three lanes (Fig. 4) implying that only three of the isolates were *P. infestans* while other were possibly closely related species within the *Phytophthora* genus.

The above data suggest that priming sites are presumably conserved in *P. infestans* as all the primer, as listed in Table 1 was developed for exotic strains of *P. infestans.* PCR is an important tool for identification of species. The above set of experiments has been repeated several times, without affecting the reproducibility, and consequently accuracy of the experiments. In some of the experiments, template DNA was used without purification, and in amount less than 50 ng. Still, the accuracy remained unaffected, implying higher sensitivity of the experiments. The fact that genomic DNA can be used even without purification also makes the present procedure fast.

4. CONCLUSION

We present a sequential set of tests that can ensure error-free identification of *P. infestans.* Occurrence of lemon shaped spore is an early indication of *P. infestans.* Microscopic examination coupled with serological assessment is a validation experiment. However none of these is Fool proof to discriminate up to species level. Choice of primer in present study for molecular identification has proved to be unambiguous procedure for identification of *P. infestans*, and shall be used with confidence for identification of pathogen. PCR being a time tested and reliable technique ensures elimination of false positives.

ACKNOWLEDGEMENTS

The research work including fellowship to H.K. was funded by DRDO. Authors thank Director DIBER for providing research facilities required in the present study.

REFERENCES

1. Pacilly, F.C.A.; Groot, J.C.J.; Hofstede, G.J.; et al. Analysing potato late blight control as a social-ecological system using fuzzy cognitive mapping. *Agron. Sustain. Dev*., 2016,**36**, 35. doi:10.1007/s13593-016-0370-1
2. Dodds, P.N. & Rathjen, J.P. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.*, 2010, **11**, 539–548. doi: 10.1038/nrg2812x.
3. Chowdappa, P.; Kumar B.J.N. & Madhura, S. Severe outbreaks of late blight on potato and tomato in South India caused by recent changes in the *Phytophthora infestans* population. *Plant Pathol*., 2015, **64**, 191-199. doi: 10.1111/ppa.12228
4. Zwankhusizen, M.J.; Govers, F. & Zakoks, J.C. Development of potato late blight epidemics: disease foci, disease gradients, and infection sources. *Phytopathol.*, 1998, **88**, 754-63. doi: 10.1094/phyto.1998.88.8.754.
5. Dewey, F.M.; Twiddy, D.R.; Phillips, S.I.; Grose, M.J. & Wareing, P.W. Development of a quantitative monoclonal antibody based immunoassay for *Humicola lanuginose* in rice grains and comparison with conventional assays. *Food Agric. Immunol*., 1992, **4**, 153–67. doi: 10.1080/09540109209354764.
6. Kaufman, L. & Standard, P.G. Specific and rapid identification of medicinally important fungi by exoantigen detection. *Ann. Rev. Microbiol.*, 1987, **41**, 209–25. doi: 10.1146/annurev.mi.41.100187.001233.
7. Judelson, H.S. & Tooley, P.W. Enhanced polymerase chain reaction methods for detecting and quantifying *P. infestans* in plants. *Phytopathol.*, 2000, **90**, 1112-19. doi: 10.1094/phyto.2000.90.10.1112.
8. Hussain, S.; Lees, A.K.; Duncan, J.M. & Cooke D.E.L. Development of a species-specific and sensitive detection assay for *Phytophthora infestans* and its application for monitoring of inoculums in tubers and soil. *Plant Pathol.*, 2005, **54**, 373-382. doi: 10.1111/j.1365-3059.2005.01175.x
9. Ristaino, J.B.; Madritch, M.; Trout, C.L & Parra, G. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora. App. Env. Microbiol.*, 1998, **64**, 948-954.
10. Narayanasamy, P. Microbial plant pathogens detection and disease diagnosis. *Fungal Pathogens.,* 2011, **1**, 948-958. doi:10.1007/978-90-481-9769-9.

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