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Duplex PCR for detection of early and late blight coinfecting potato

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ABSTRACT

The disease identification at initial level is utmost factor for management of any disease. The detection of early and late blight at initial level is more important for its management due to fast spreading behaviour of these diseases as late blight devastates entire crop within a week. Although, morphological and serological methods are available, but due to their time consuming nature, sometimes there is failure in diagnosing latent infection with very low level inoculums. Moreover, diagnosis of both the diseases one by one is laborious. Therefore in the present investigation, a duplex PCR method was developed for simultaneous detection of *Alternaria solani* and *Phytophthora infestans*. PCR conditions and annealing temperature of both the primers [PINFTH2 (F)-GGGGGTCTTACTTGGCGGCG, PINFTH2(R)-CAAACCGGTCGCCAACTCGC for *P. infestans* and BIAMASbt (F)-GCTCCCACTCCTTCCGCGC, NBAIMASbt(R)-GGAGGTGGAGTTACCGACAA for *A. solani*] were standardized separately. Then the optimization of the PCR reactions and condition of duplex PCR was also standardized. The results revealed detection of single amplicon of 524bp with *P. infestans* while with *A. solani* 289bp amplicon was detected in genomic DNA from mycelium as well as in plants infected with both the diseases. This duplex PCR enabled the simultaneous detection of both the pathogens. In early stages of disease development, suspected samples (plants & tubers) were tested with this duplex PCR, which could diagnose both the pathogens correctly. The protocol will help in diagnosis of latent infection in potato tissues; and it will also be used as a diagnostic tool for researchers engaged in potato seed production and multiplication reducing cost and time thereby.

Key words: *Solanum tuberosum*, *Alternaria solani*, *Phytophthora infestans*, duplex PCR, diagnostic.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important non grain food crop in the world and occupies third place after rice and wheat (Rajiv and Prashant, 14). *Phytophthora infestans* (Mont.) de Bary, is the oomycete that caused the historic Irish potato famine during 1845 and it still is the single most important impediment worldwide for the potato and tomato growers/industries. Moreover, this disease is considered as re-emerging in the forms of different genotypes (Fry *et al*., 5). The pathogen has been the subject of intense research pursuit for more than a century due to its global economic importance. This pathogen produces many cycles in a crop season and passes crop free periods in the infected tubers or as oospores in soil (Darsow, 1). In India, late blight appears in most of the potato growing regions in varying degree causing losses up to 90% depending upon the variety and control measures adopted (Singh *et al*., 17). Recently, Lal *et al*. (11) reported that on overall basis 10-15% potato tubers were lost due to late blight in India during 2013-14. It was observed that A2 mating was more aggressive than A1 mating thereby more losses may be occurred in future due to late blight (Sharma and Singh, 16). In India, early blight disease was first reported by Butler (1903) on potato leaves at Farrukhabad in Uttar Pradesh (Dutt, 2). It causes up to 20% loss in *Kharif* crops in Ranchi and adjoining plateau region (Sharma, 15). The early blight caused by *Alternaria solani*, results in defoliation of potato plant, reducing the photosynthetic area and ultimately the yield of plant. The incidence of this disease is more, now-a-days, due to changes in climatic conditions and adopted agronomic practices. Moreover, it is more serious particularly in sandy soil along with high rainfall conditions and cause significant loss of the potato yield. There are numerous methods available for diagnosis of *Phytophthora* and *Alternaria* species complex. These conventional methods largely rely on morphological characteristics, such as the presence or absence of sporangia/spores, shape and size of sporangia, colony morphology and pigmentation etc. Hence, these require isolation of the fungus from infected tissues and plating on selective media, which may take several weeks, so the identification of fungi using morphological characteristics is not only difficult and time consuming but also requires expertise. This difficulty has been reflected in frequent misidentifications of both *Phytophthora* and *Alternaria* species in fungal collections (Yoder and

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Christianson, 18; O'Donnell *et al*., 13; Kulik *et al*., 11). Later, ELISA (Enzyme Linked Immunosorbent Assay) based identification was also tried for diagnostic of these pathogens with low level of sensitivity as compared to DNA based techniques (El Komy *et al*., 4). After introduction of PCR (Polymerase Chain Reaction) by Kary Mullis in 1983 it took only about a decade to revolutionise the area of disease diagnostics as it offered explicit advantages over traditional tests like organisms do not needed to be cultured prior to their detection and protocols are highly sensitive and rapid. Consequently, the researchers quickly adopted DNA-based techniques. The PCR method is considered to be the most sensitive diagnostic technique, wherein, extremely low amounts of inoculum of the target pathogen could result in a positive signal. Hence, at field level also, the pathogen detection using PCR is highly desirable to estimate the level of latent infection to plan cost-effective spray schedules for the control of both the blights. Owing to these situations, duplex PCR assays are needed for quick detection of pathogens in a single reaction (Hu *et al*., 7). Both the diseases *i.e.*, early blight and late blight hamper potato production by reducing the yield, quality of tubers and results in financial loss to both farmers as well as the potato seed industry. Keeping in view the importance of the diseases and economic value of the crop, the present studies were undertaken to develop duplex PCR to quickly detect both *A. solani* and *P. infestans* in a single test.

MATERIALS AND METHODS

The pure cultures of the *A. solani* and *P. infestans* were maintained at Plant Pathology lab in ICAR-Central Potato Research Institute, Regional Station, Modipuram, Meerut, Uttar Pradesh, India. The potato leaves containing dual infection of pathogens (*A. solani* and *P. infestans*) were brought in polythene bag to the lab and ground in liquid nitrogen to a fine powder using sterilized and dried pre-chilled mortar and pestle. Total genomic DNA was extracted from the infected leaves as well from pure cultures of both the pathogens by using Qiagen DNA easy Mini Kit. The pure cultures of the pathogen were grown in sterilized Potato Broth Medium (*A. solani*) and Pea Broth medium (*P. infestans*) at 25°C and 18°C, respectively and after one week mycelia were harvested for extraction of genomic DNA. The extraction protocols were followed as per the Qiagen Mini Kit manufacturers' instructions.

A species specific SCAR (Sequence Characterized Amplified Region) marker for detection of *P. infestans* (Hussain *et al*., 6) was used in the present study. Similarly, beta tubeline based sequence of *A. solani* was used for detection of *A. solani* (IIHR, 8).

The purified DNA was quantified in a Spectrophotometer (Biofuture, China) and diluted to a uniform concentration of 20ng. PCR reactions were carried out in 25 µl of reaction volume containing 2.5µl of 2x Taq Buffer, 2.5µl of 2mM dNTPs, 2.0 µl of 10 Picomol of Forward and Reverse primers both, 0.2µl of Taq DNA Polymerase enzyme and double distilled water for volume make up. PCR reaction was carried out in Eppendorf Mastercycler (Germany).

The sensitivity of the primer PINFTH2 (F)-GGGGGTCTTACTTGGCGGCG, PINFTH2(R)-CAAACCGGTCGCCAACT CGC was tested with *P. infestans* isolates at 10 ng, 1ng, 1000 pg, 100 pg, 50 pg, 10 pg and 1pg.

Initially, PCR conditions are standardized for both the primers according to their annealing temperatures. After selecting primer pair, PCR was performed at different annealing temperatures (50-65ºC) in a Gradient PCR (Genetic Asia Biotech). Based on the presence/absence of bands as well as band density the annealing temperature of 55ºC was finally selected for both the primers. PCR was carried out in 20µl reaction volumes. Each reaction consisted of 2.0µl template DNA, 2.0µl buffer 10X Green Taq buffer (with 1.5 mM $Mgcl_2$), 2.0 µl dNTPs (2 mM), 0.2µl Taq DNA polymerase (5U), 2.0 µl primer (10µM:F+R): PINFTH2 (F)-GGGGGTCTTACTTGGCGGCG, PINFTH2(R)-CAAACCGGTCGCCAACT CGC and *A. solani* primer based on beta tubeline gene NBIAMASbt (F)-GCTCCCACTCCTTCCGCGC, NBAIMASbt(R)-GGAGGTGGAGTTACCGACAA and remaining volume of double distilled water. The temperature profile of the PCR was pre-incubation at 94°C for 2 min leading to 30 cycles of melting at 94°C for 30 sec, annealing at 55°C for 30 sec, synthesis at 72°C for 1 min followed by final extension at 72°C for 10 mins. Negative controls (no template DNA) were used in every experiment to test for the presence of contamination in reagents. The amplified products were analyzed by electrophoresis in a 2.0% agarose gel in 1× TAE (0.04 M Tris–acetate, 1 mM EDTA, pH 8) buffer and visualized with UV light after ethidium bromide staining. The gel image was captured with gel doc system (Syngene, UK) for further analysis.

RESULTS AND DISCUSSION

Late blight and early blight are important diseases of potato. Both the diseases are primarily tuber borne in nature and their pathogens perpetuates from season to season through infected tubers. Hence, it

becomes more important to detect and eliminate such tubers which are possessing latent infections and have the potential to initiate epidemics under favourable environmental conditions. Similarly, the detection of these pathogens becomes even more important at initial stages of seed multiplication in hi-tech system of potato seed production. Generally, these diseases are managed after physical appearance in the fields through repeated sprays of fungicides starting with prophylactic sprays followed by sprays with systemic fungicides. Thus, cost of production is increased using fungicides sprays. Therefore, the various detection methods including serological were tried but were not found reliable in early detection of the infection. While, the PCR method is quick, reliable and conclusive and hence can help in selecting the source material at least in the initial generations of seed production programme. With this objective, initially, PCR conditions and annealing temperature of both the primers were standardized (Table 1) individually. The sensitivity of the primer PINFTH2 (F)-GGGGGTCTTACTTGGCGGCG, PINFTH2(R)-CAAACCGGTCGCCAACT CGC with *P. infestans* isolates reported up to 1.0 pg (Hussain *et al*., 6). This primer could not amplify the genomic DNA of closely related species of *Phytopthora* (*P. palmivora*, *P. cactorum*, *P. capsisci*, *P. colocasiae*) and other potato fungal pathogens (*Fusarium* spp and *Rhizoctonia solani*). Therefore, it was specific for *P. infestans*. Similarly, the species specific primer NBIAMASbt (F) and NBAIMASbt (R) was reported after testing with their closely related species *i.e. A. arborcence*, *A. brassicae*, *A. brassicola*, *A. alternata*, *A. cassiae A. gossypina*, *A. porri*, *A. polenderi*, *A. poneasis, A. triticola, A. zinnae, A. tennuissima and A. sesame* (IIHR, 8).

The PINFTH2 primer produced single band of size 524 bps from mycelial culture of *P. infestans* with best intensity at an annealing temperature of 55ºC while NBIAMASbt primer produced single band of size 289 bps from mycelial culture of *A. solani* at 55ºC (Fig. 1a & 1b). The next step was to standardize the conditions for a Duplex PCR protocol for simultaneous detection of early blight and late blight co-infecting the host plants. Therefore, series of gradient PCRs were set up using annealing temperatures ranging from 50ºC to 65ºC and a compromise could be reached with the annealing temperature of 55ºC where we could have good quality bands specific to both the pathogens and unspecific products were also not detected (data not shown).

Thereafter, we validated the results by performing a series of Duplex PCRs using genomic DNAs from mycelial culture of both the pathogens (Fig. 2a). Similarly, duplex PCRs were performed using genomic DNAs from diseased leaves infected with both the disease (Fig.2b). As expected, amplicon size of 524bps with *P. infestans* and 289bps with *A. solani* were detected from both sources i.e., mycelial cultures as well as in infected leaves. In this study, we have developed a highly sensitive duplex PCR assay based on multi-copy rDNA sequences for the differential detection of *P. infestans* and *A. solani*. In diagnostic PCR, the quantity of the template is the crucial factor influencing amplification efficiency and

Table 1. Primer sequences, annealing temperature and expected amplicon size.

Primers	Sequences 5'to 3'	Annealing temperature (°C)	Amplicon size (bp)
PINFTH2 (F)	GGGGGTCTTACTTGGCGGCG	55	524
PINFTH2 (R)	CAAACCGGTCGCCAACTCGC		
NBIAMASbt (F)	GCTCCCACTCCTTCCGCGC	55	289
NBIAMASbt (F)	GGAGGTGGAGTTACCGACAA		

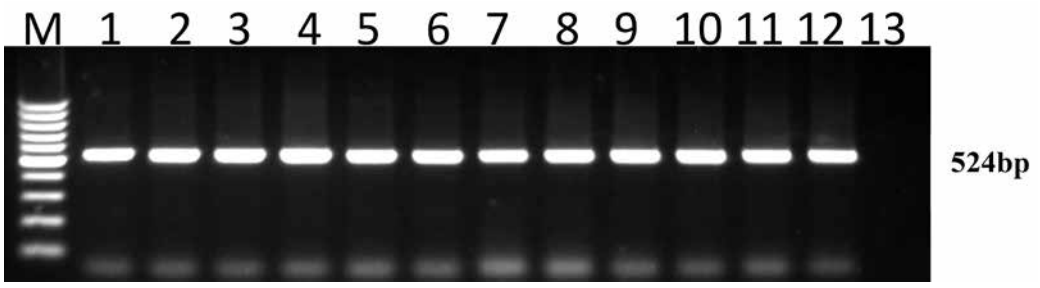

Fig. 1a. PCR amplification of *P. infestans* isolates with PINFTH2 (F) & PINFTH2 (R) primer Lane 1-12: *P. infestans* gDNA with PINFTH2 (F) & PINFTH2 (R) primer, 13: Negative control, M: 100 bp DNA ladder.

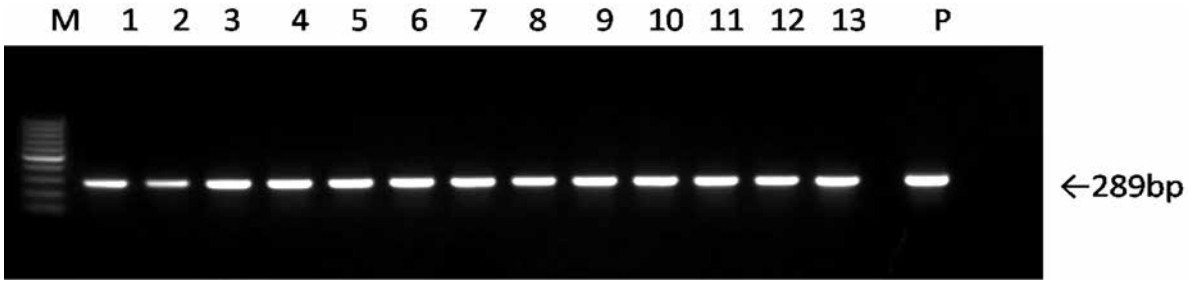

Fig. 1b. PCR amplification of *A. solani* isolates with NBAIMSbt primer Lane 1-13: *A. solani* gDNA with NBAIMSbt primer P: Negative control, M: 100 bp DNA ladder.

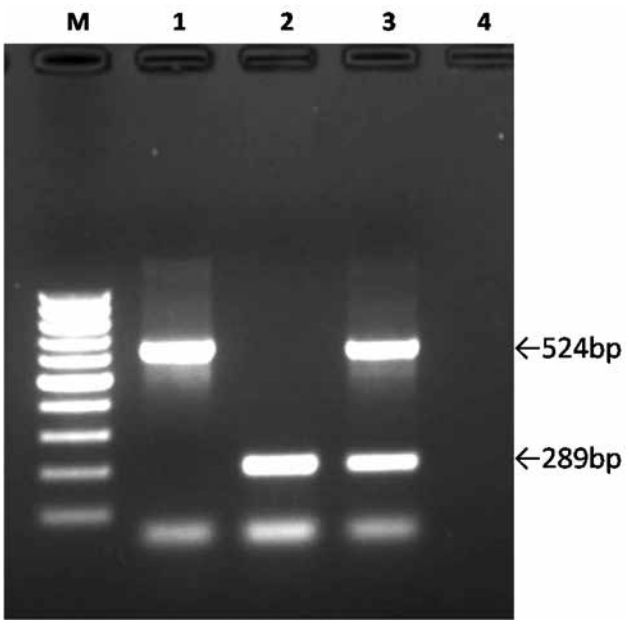

Fig. 2a. Agarose gel of PCR amplified products. Lane 1: *P. infestans* gDNA with PINFTH2 (F) & PINFTH2 (R) primer 2: *A. solani* gDNA with NBAIMSbt primer 3: duplex PCR products of *P. infestans* and *A. solani* 4: Negative control, M: 100bp DNA ladder.

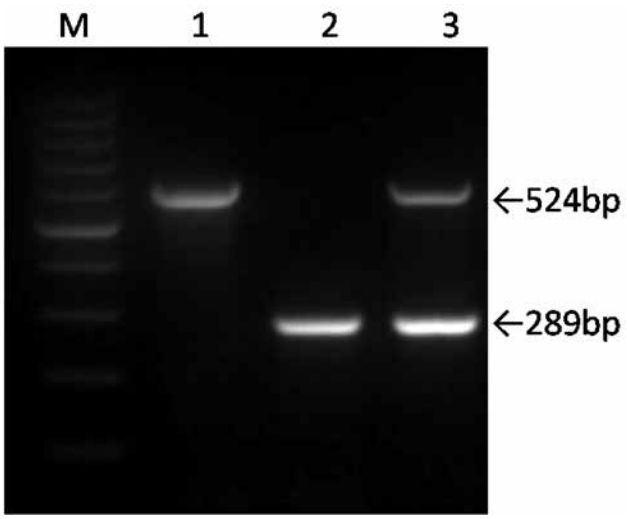

Fig. 2b. Agarose gel of PCR amplified products of symptomatic tissue. Lane 1: *P. infestans* infected leaves with PINFTH2 (F) & PINFTH2 (R) primer 2: *A. solani* infected leaves with NBAIMSbt primer 3: duplex PCR products of *P. infestans* and *A. solani* M: 100bp DNA ladder.

thus the detection level (Knoll *et al*., 10). Several PCR diagnostic protocols for Phytophthora species have been developed on the basis of ITS2 (Kulik *et al*., 11), 28S (Edel *et al*., 3) and IGS rDNA (Jurado *et al*., 9); however, these protocols have their own limitations.

It is also well established that the sensitivity of the procedure can be increased by the use of primers targeting multi-copy regions of rDNA. These primers targeting rDNA sequences have been estimated to detect the pathogens even if the concentration of template DNA is as low as 1 to 10 pg. The ability to use two primer pairs in one reaction enabled the detection of two target species simultaneously. The use of such a protocol will definitely reduce the time and cost of analysis. This development will definitely help potato seed growers as well as agencies involved in breeders and certified seed production programmes as well as in the exchange of plant genetic resources. Moreover, the incipient seed can be easily diagnosed and subsequent plant protection measures could be meticulously planned thereby saving a lot of costs which are now incurred as prophylactic sprays. The study concludes that this duplex PCR based diagnostic method will precisely detect the latent infection and will be a valuable diagnostics tool for the researchers engaged in multiplication and production of potato seed.

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