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Soil Baiting, Rapid PCR Assay and Quantitative Real Time PCR to Diagnose Late Blight of Potato in Quarantine Programs

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ABSTRACT

Phytophthora infestans (mont) de Bary is a pathogen of great concern across the globe, and accurate detection is an important component in responding to the outbreaks of potential disease. Although the molecular diagnostic protocol used in regulatory programs has been evaluated but till date methods implying direct comparison has rarely used. In this study, a known area soil samples from potato fields where light blight appear every year (both A1 and A2 mating type) was assayed by soil bait method, PCR assay detection and quantification of the inoculums. Suspected disease symptoms appeared on bait tubers were further confirmed by rapid PCR, inoculums were quantified through Real Time PCR, which confirms presence of *P. infestans*. These diagnostic methods can be highly correlated with one another. Potato tuber baiting increased the sensitivity of the assay compared with direct extraction of DNA from tuber and soil samples. Our study determines diagnostic sensitivity and specificity of the assays to determine the performance of each method. Overall, molecular techniques based on different types of PCR amplification and Real-time PCR can lead to high throughput, faster and more accurate detection method which can be used in quarantine programmes in potato industry and diagnostic laboratory.

Keywords: Detection, Late blight, PCR, Potato, *P. infestans*, Real time PCR, Soil

सारांश

आलु बालीको पछौटे डढुवा रोग लगाउने ढुसी *Phytopthora infestans* को पहिचान रोग लाग्नू अगावै हुन सक्नु संसारभर नै महत्वपुर्ण विषय हो । बिभिन्न Molecular प्रबिधीहरुको प्रयोगबाट यस्को नियमन कार्यक्रमहरुको मुल्याङ्कन गरीए पनि कमै उपयोग भएको देखिन्छ । यो अध्ययनमा डढुवा रोग लागेको क्षेत्रको माटोको पाँसो प्रयोग गरी ढुसीको दुबै प्रजनन (ए र बि) अवस्थाको अध्ययन Molecular प्रबिधीका तरीकाहरु PCR Amplification र Real Time PCR ले भरपर्दो र प्रभावकारी रुपमा छिटो पहिचान गर्न सकिने देखियो जुन प्रबिधी आलुको रोग पहिचान गर्ने प्रयोगशाला तथा क्वारेन्टाइन कार्यहरुमा प्रयोग गर्न सकिने देखिन्छ ।

INTRODUCTION

Potato (*Solanum tuberosum* L.), in India, is cultivated in almost all the states under very diverse conditions and second largest producer in the world after China (Scott and Suarez 2011, 2012). United Nation Food and Agriculture Organization declared year 2008 as the International Year of the Potato, to raise its profile in developing nations, calling the crop a "hidden treasure" (Hussain et al 2016a). Potato is a universal crop; however, one of the diseases known as late blight caused by *P. infestans* remains the quintessential example that has survived despite being collective concerted intervention and efforts for control and management programmes (Gary and Viviana 2004). Diagnosis of this pathogen in both planting material and soil, still a question (Gracia-Pedrajas 1999, Mascarello et al 2001, Volossoiuk 1995, Hussain et al 2013, 2013a) caused by the biological properties of the pathogen, the character of infection as well as multi-dimensional environmental and biochemical interactions (Hu et al 1993; Robb et al 1994). Since the appearance of A2 type strain (Singh et al 1994) in India, the chance of genetic variation of the pathogen is getting higher with more diversity.

P. infestans requires a living host to survive between seasons, usually it remains in infected tuber seeds or in the soil and after harvest or anywhere potatoes might be discarded. Late blight of potato appears throughout the year in Northern Western Hills (Himachal Pradesh), Western hills (Shillong), Nilgiri Hills (Karnataka), and Plateau (Uttarakhand) as well as in plains (Uttar Pradesh, Punjab, Bihar) where potato crop is grown leading to almost 30-40% loss annually (Singh et al 1994). Both A1 and A2 mating type population co-exist at HP, Punjab,

Karnataka, Shillong as in some parts of U.P. Tubers that have been discarded at any stage of crop production or handling (harvest, storage, shipping, spring cleanup, or planting) are known as culls, culls may survive if they are not destroyed (frozen, crushed, composted, or buried at least 2 feet beneath the soil surface) (www.toxipedia.org/2010).

To make sure that the planting materials in pathogen free soil-fast, the sensitive tests for easy pathogen diagnosis are certainly required (Vidic et al 2017).Various detection techniques, from baiting using susceptible host material, wet sieving and direct microscopic examination, incubating immersion tubers in soil and plate profiling are known both for qualitatively and quantitatively investigations (Hussain et al 2013, Hussian and Singh 2016). All these methods require only basic laboratory facilities but are labour-intensive and time consuming. The development of new molecular based assays has significantly advanced the detection of this fungus. Since early 80s, PCR-based molecular techniques have been introduced for the detection of this pathogen within a short time (Henson and French, 1993). For many pathogens molecular detection is fully successful (Johanson 1994), whereas the methodology for other pathogenic organisms still needs more validation and special developments taking into account biology of host-pathogen interactions (Heinrich et al 2001). Internal Transcribed Spacer (ITS) are most commonly used targets regions for diagnostics assays as they are highly conserved than other regions and are present in multiple copies in pathogen genome, which improve assay sensitivities (White et al 1990, Nazar et al 1991, O Donnell 1992, Bryan et al 1995, Bonantsetal 1997, Hussain et al 2013). Quantative Real-Time PCR is a new advance in PCR methodology. Several studies have demonstrated that the sensitivity of Real-Time PCRcan also exceed that of conventional PCR by up to 1000 times (Hu et al 1993, Mumford et al 2004, Ratti et al 2004, Hussain et al 2013b, 2014). In addition, Real-time PCR can also be used to quantify target nucleic acid in a range of sample types. For example, Real-Time PCR assays could be used to inform potato growers industries about the level of *P. infestans* in seed tubers at planting, a factor known to affect disease levels on daughter tubers (Simons and Gilligan 1997). These approaches were used to investigate the distribution of *P. infestans* inoculum in field soil samples before the planting of a potato seed tubers and to determine threshold levels of inoculum, to investigate the relative importance of seed and soil borne inoculums that's why this study was conducted so that it provide us information on behalf of which disease management decisions can be made and to reduce the risk of spread of *P. infestans* that was the main goal of this work by using these methods. A very few studies on direct comparison of these methods has been analysed within a naturally infested diseased area/location.

MATERIALS AND METHODS

Soil Baiting Method

Natural field soils where late blight appears every year were collected (during crop season 2013-2014) from CPRI, Shimla, HP, CPRS, Shillong, Meghalaya, Agriculture Research Station, (Hassan) Karnataka and CPRS, Jalandhar, Punjab. Major rocks and hard material were removed through standard size sieve. Baiting method was followed as per Hussain et al 2015 protocol conducted at Plant Pathology lab. Central Potato Research Institute campus, Modipuram. Soil samples were moistened, (Potato tuber slices where dipped for 5 min. in 200ml of distilled water containing dissolved 1mg (each Nystatin, Rifampicin and Benlat), dried for 5 min. on filter paper, tuber slice were inserted in pots, pre-incubated at 18ºC for four days, and then taken out from plastic pots, washed under running tap water and incubated at 18±ºC in Bio-oxygen demand incubator dark chamber (**Figure** 1). Sterile soil (autoclaved for 30 minutes, 121 psi) having no inoculum was served as negative water control. This protocol has been already standardized and validated with same filed soil samples.For further confirmation of *P. infestans*, gDNA was extracted from the baited tubers/artificially infected tubers were amplified through rapid PCR assay using *P. infestans* specific primers PIn6F/PIn6R (Hussain et al 2013c).

Genomic DNA Extraction

Healthy potato tubers of *cv*. Kufri Bahar were used as bait. This experiment was conducted in Plant pathology section (Molecular lab) CPRI campus, Modipuram. Few tubers were artificially infected with *P. infestans* sprongiasphore, kept at ±18˚C bio-oxygen demand incubator dark in plastic try. Genomic DNA from all the baited tubers/artificially infected tubers, were isolated using alkaline lysis method following the procedure of Wang et al (1993). The Alkaline lysis procedure consisted of grinding potato peels in 0.5NNaOH, then immediately transferring 20µl of the extract to a sterile 1.5 ml tube. The tubes were microcentrifuged (17,000 xg) for 5 min to pellet the tissue, and 5µl of supernatant was removed and immediately diluted with 450µl100mMTris (pH 8.0). The samples were then either used immediately for rapid PCR assay or kept at -20˚C for later study. gDNA from *P. infestans* mycelium (maintained/available in the lab) was extracted using Qiagen Kit following manufacturers protocol. The DNA quantification was performed using Nanodrop 2000 (Thermoscientific) and was analysed on 0.8% Agarose gel in 1X TAE buffer. All the gDNA samples were stored at -20°C.

PCR Assay

PCR study was carried out in 25µl reaction volumes. Each reaction consisted of approximately 20ng of the template DNA, 2.5µl1xTaq Buffer with 2.0MmMgCl$_2$, 2.5µl 200 mM dNTPs (Fermentas), 10pmol PIn6F (CTGCTGGCTTTATTGCTGGCGGCTA)/ PIn6R (ATGGTTCACCAGTCCATCACG) (Hussain et al 2013c) each, 0.2 units Taq DNA Ploymerase (Fermentas) and sterile distilled water was added to make up the final volume. Primer was screened for their optimum amplification in PCR (Eppendorf Master cycler). Thermal cycling parameters: initial denaturation at 94°C for 2 min, followed by 30cycles consisting of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec and extension at 72°C for 1 min. A final extension at 72°C for 10 min followed. PCR amplified products were separated on agarose gel electrophoreses (1.5% w/v) in 1x TBE running buffer and photographed under UVPro Silver (UK) after staining with Ethidium Bromide (0.5µg/ml). This methodology has been standardized and validated thrice will biological samples as well (Hussain et al 2013c). This molecular experiment was carried out in Plant Pathology section (Molecular lab) CPRI Campus, Modipuram.

Quantitative Real Time PCR

A Real Time qPCR study was carried out in StepOne™ Plus Real Time PCR System (Applied Biosystem) using Power SYBR™ Green PCR Master mixer. This molecular experiment was carried out in Plant Pathology section (Molecular lab) CPRI Campus, Modipuram.Each amplification trail was performed in a 96-well PCR plate covered with optical adhesive cover. The PCR reaction (25µl) contained 12.5µl 2X Power SYBR™ Green PCR Master Mix, 3µm of TaqF/TaqR primer set (Hussain et al 2005;Hussain et al 2013a;2013b;2014) based on ITS regions, genomic DNA (as per dilutions) with milli Q water for reaction volume make up. Three replicates for each DNA dilution was set up in 96 well plate for plotting the standard curve. PCR cycle included initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. the fluorescence resulting from the binding (intercalation) of SYBR™ Green into the amplicons was recorded at the end of the elongation step of every cycle. A melt curve analysis was done to evaluate the amplicons, which includes 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

Preparations of gDNA Standards

If not otherwise mentioned the genomic DNA was used for standard curve by using 5ng as the initial concentration and using the five dilutions by making 1:10 dilution series for genomic DNA. Each standard dilution was tested in triplicate to ensure repeatability. Absolute Real-time PCR data were analyzed with SDS 2.4 Software (Applied biosystem). All analyses were based on the Ct values of the PCR products, which is the PCR cycles at which the fluorescence measured between each cycle exceeds a threshold determined by background fluorescence at baseline and is placed in the exponential phase of the amplification curve. The baseline was set automatically by software. The SDS software calculated the standard curve for each run based on the Ct for the standard.

RESULTS

Soil Baiting

The currently performed bait method detects the *P. infestans* inoculum within 48hrs (irrespective of zoospores, sporangia and oospores), from naturally infested soils collected from different geographic locations (**Figure** 1).

PCR Assay

A single PCR amplified product of approximately 615bp in size was amplified from bait potato tubers, artificially infected tubers as well as from crushed mycelium of *P. infestans*. Entire ITS ribosomal DNA (rDNA) sequences from *P. infestans* isolate have been submitted to Genbank (KFl10980). No amplifications from healthy tubers (**Figure** 2). With PIn6F/PIn6R primer set, 10fg of *P. infestans* DNA was detectable (Figure not shown).

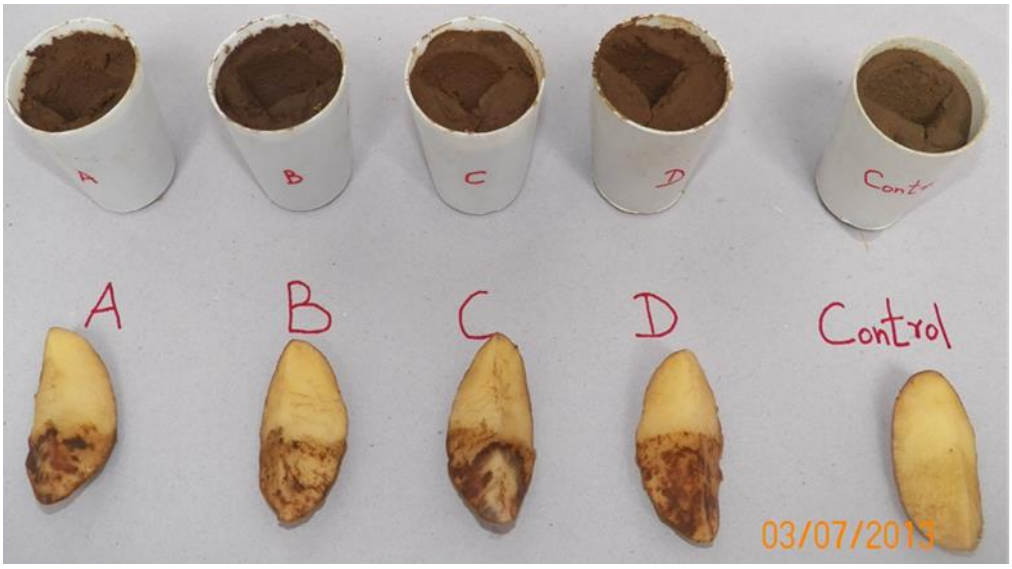

Figure 1. Baiting method by using potato tuber slice. A=Central Potato Research Institute, Shimla soil, B=Central Potato Research Station, Shillong soil, C=Agricultural Research Station, Hassan, Karnataka soil, D=Central Potato Research Station, Jalandhar, Punjab soil, C=control sterile soil.

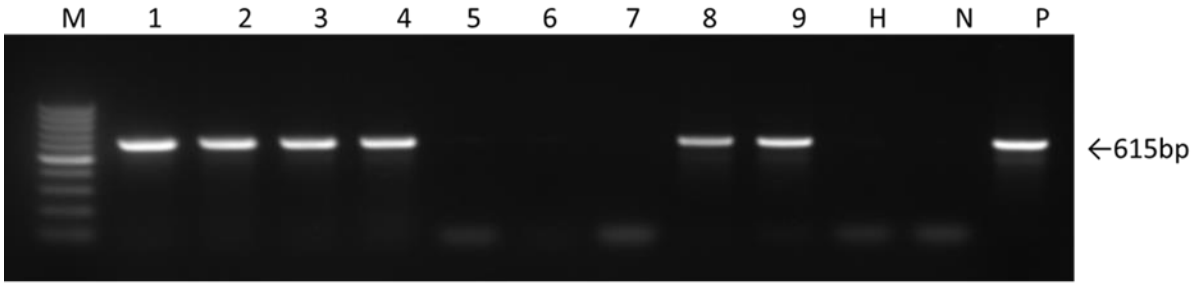

Figure 2. PCR amplification of gDNA obtained from soil bait tubers, amplified with PIn6F/PIn6R primer. Lane 1 to 4= tubers detecting *P. infestans* from soil, 5, 6 and 7=potato tubers obtained after harvesting, 8 and 9=artificially infected tubers, H=healthy tubers (gDNA), N=Negative control, P=Positive control (gDNA of *P. infestans* mycelium), M=100bp DNA marker (Fermentas).

Quantitative Real Time PCR

A standard curve was constructed by plotting known concentrations of *P. infestans g*DNA against the Ct values obtained from Real-Time PCR. In order to validate the reproducibility of the Real-Time PCR methodology was conducted with triplicate samples of pathogen gDNA. Highly reproducible Ct values with very small standard deviations were observed, the linear regression coefficient of the standard curve being R = 0.952. Within this standard curve, each 10-fold difference in initial DNA amounts was represented by approximately four-cycle differences in Ct. Under the protocol carried out, the minimum starting quantity of pathogen DNA that could be accurately quantified in our assays was 30ng, which corresponded to a Ct value of 18±1 (**Figure** 3). A Ct of 33±1 was considered to be the threshold value suitable for quantification, because PCR efficiency at a higher number of cycles was far from the value 1 (99.900% efficiency) (**Figure** 4). Using Real-time PCR, it was observed that, overall, there was no statistically significant difference (P < 0·05) in the amount of *P. infestans* gDNA detectable in each tuber baited sample.

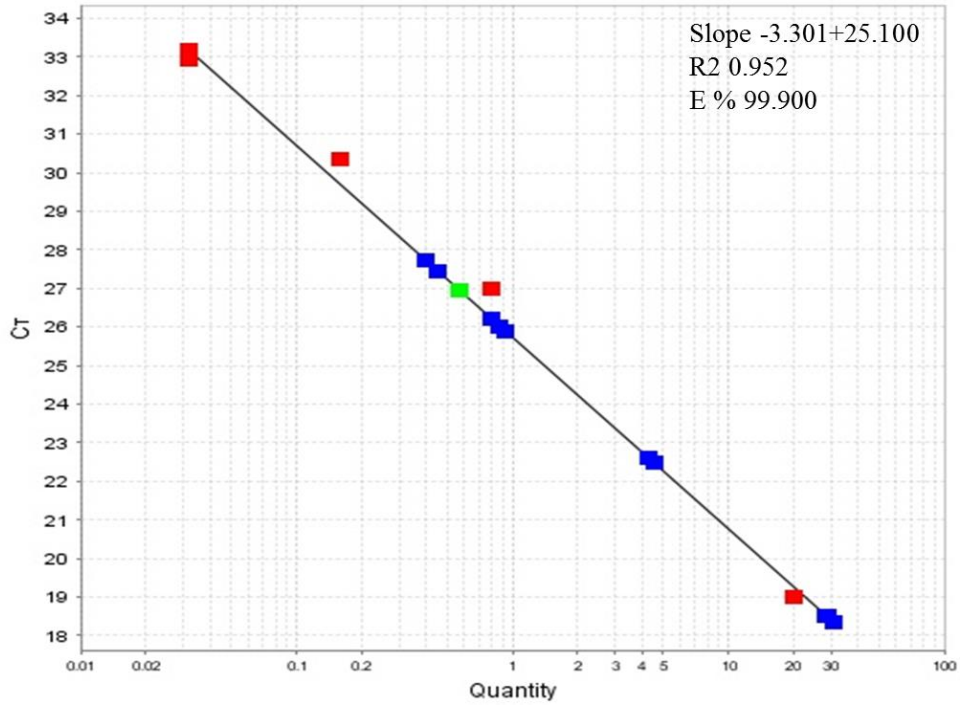

Figure 3. Standard curve for the Real time PCR of the amplification products using Taqf / Taqr primer.

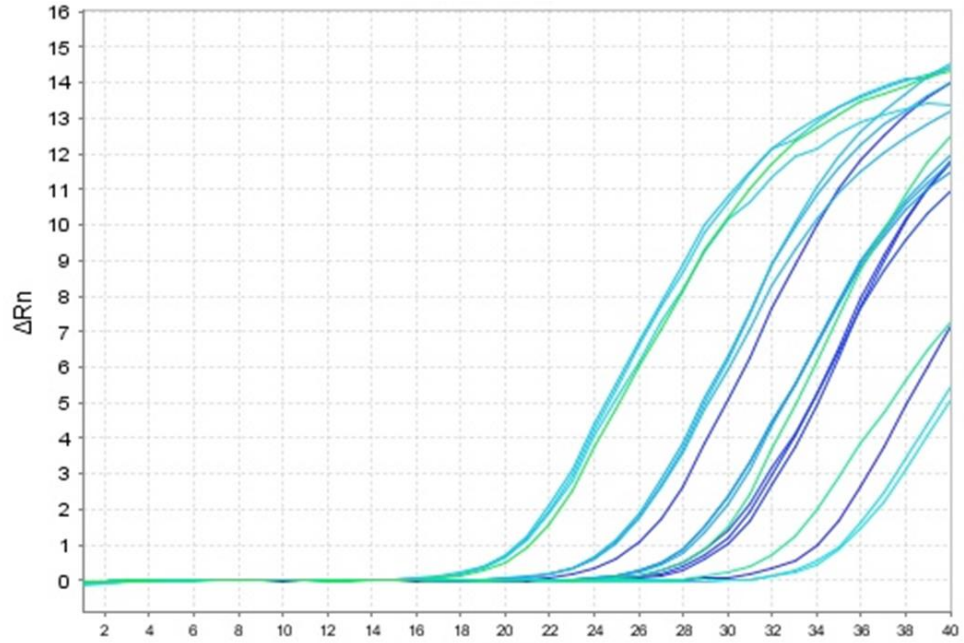

Figure 4. Quantitative Real timePCR through SYBR Green technology for the detection and quantification of *P. infestans* from soil baited tubers.

DISCUSSION

P. infestans is the most challenged pathogen to study till date globally because the pathogen continues to appear in surprising new locations or with surprising new intensity (Fry et al 2015), that behaves as a biotroph in nature but it can also be maintained on artificial Rye Agar media. Plant diseases epidemics can cause famines, eliminates a thriving industry that adversely affect the growth, physiological functioning and productivity of a crop. Depending on variety used, especially in susceptible varieties, potato yield loss caused by *P. infestans* ranges between 29–100% (Hussain and Singh 2016). Pathogen actively seeks infection sites on its host plant both above and below ground. It can form thick-walled resting spores called Oospores (sexual) that can overwinter in the soil. Diseased tubers can bring inoculum that spreads to other parts of the plant and to other plants in the fields. Our results concludes to detect, identify and quantify *P. infestans* presence in infested field soil samples comparing the more improved, standardized soil bait method with rapid PCR assay and Real Time

PCR for saving time and accuracy in diagnostic programmes. This bating method could be very useful for detecting and identification of *P. infestans* in potato seed industry/private seed growers, our results are in agreements with previous findings by Lees et al (2012), Hussain et al (2015). Baiting has several advantages over isolation method. Soil bait method in practical is, rapid and low-cost, and efficient for the identification and discrimination of *P. infestans*. O'Brien et al (1996) recognized that future the diagnostics methods should be simple, rapid, specific, sensitive and quantifiable. By global trade, the inoculum can cross continents and oceans (Widmark, 2010). Because of the reliance of humans on plants for food, fibers and other resources, understanding plant diseases and their control is of vital importance to our survival.

The NaOH based lysis is less laborious than CTAB extraction but is not as well suited for long term maintenance of DNA samples. In previous studies by Tooley1997; Wangsomboondee and Ristanio 2000; Hussain et al 2005; Lees et al 2012; Hussain et al 2013 reports that detection of the pathogen in soil is challenging and molecular detection may prove a valuable alternative to baiting methods, which are in agreements with our study also (**Figure** 2). Nuclear rDNA, including the small and large subunits, 5.8 S, and the Internal Transcribed Spacer (ITS) region, proved an ideal target for specific PCR primers, as each sequence is variable at the family, genus, or species level (Lee and Taylor 1992). Identification of the causal agent and prevalence of a disease is very essential for adequate and timely management of disease, which in turns depends on accurate examination and early detection of the pathogen in soil. In our present investigation, PIn6F/PIn6R primers were found highly specific to *P. infestans* only, as observed in previous findings by Hussain et al 2013c, which confirms our results also in right directions. This developed assay will also be beneficial for determining the population's biology and monitoring of pathogen from one state to another. DNA-based detection and identification methods described here can be used to supplement or confirm soil baiting method (**Figure** 1). In our present investigation, the newly designed primer proved to be an efficient and species-specific discrimination which would be useful in developing a rapid and sensitive diagnostic PCR based assay. The quantitative Real-Time PCR methods have been shown previously to be sufficiently sensitive to detect such latent infection (Suarez et al 2005; Hussain et al 2013). This has a number of uses, including clarifying pathways of infection, and early detection of low levels of pathogen inoculum. In our investigation, it is in agreements with previous findings of Hussain et al 2013a; 2013b.This study will also useful in the development of more improved methodology to detect and quantify *P. infestans* inoculum in unknown soil conditions. Real Time PCR signals were linearly related to the concentration of *P. infestans* sporangia material spiked into soil and compost (Hussain et al 2014).

In previous findings by Martin et al 2000; McCartney et al 2003; Thortan et al 1992 reported that the potential of molecular detection in plant pathology has been shown in several studies which confirms our findings also. To reduce the risk of incorrect identification (*eg* in regulatory applications), confirmation of morphometric properties/expertise trained personal (from culture isolation) should be included in the diagnostic programme at every district level. There are numerous applications that will greatly improve our understanding of *P. infestans* biology, for studying the epidemiology of pathogen in soils, including how geography, temperature, moisture, and cropping systems affect the species composition. This will prove useful for disease management perspective for the evaluation of various control measures, including fungicide sensitivity, quantitative host resistance, and various cultural management strategies. All the developed methods together in combination can facilitate detection, confirm genus *P. infestans* identity, quantity and be applied during control and monitoring activities.

CONCLUSION

The development of such methods would facilitate the study of early events in the infection process and could be used to compare rates of pathogen multiplication and movement. With this information, potato growers can make an informed decision as to whether it would be prudent to cultivate resistant/susceptible variety. Given the development of these specific, sensitive and quantitative assays for the detection of Late blight of potato, in future, work can now be carried out to study the epidemiology of the diseases it causes and to answer questions concerning and the relative roles of seed and soil born inoculum in causing disease.

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