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Full Length Article

Serological and Molecular Detection of Late Blight Pathogen and Disease Development in Potato

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ABSTRACT

In the present work, we investigated the specificity and sensitivity of serological and molecular tools for the detection of *Phytophthora infestans* in infected tissues of susceptible and resistant potato cultivars and to study disease development among these cultivars. Serological tools [enzyme-linked immunosorbent assay (ELISA) and dot blot immunoassay (DIA)] were performed using antiserum raised from soluble mycelial protein of *P. infestan*s, while molecular tool [Polymerase chain reaction (PCR)] was performed using specific primer to *P. infestans*, which designed on the basis of internally transcribed spacer1 ITS1 (GenBank accession no AY770739). Cross-reactivity of the antiserum was tested against 50 µg antigens of 9 isolates of *P. infestans* and antigens of *Fusarium* sp., *Pythium* sp., *R. solani, M. phaseolina, A. solani, E. carotovora* and *R. solanacearum.* Results showed that the antiserum was adequate specificity among different antigens of the tested isolates of *P. infestans*. On the other hand, PCR amplification indicated that, all the *P. infestans* isolates amplified a product of approximately 813bp with the primer. While no amplification products were obtained with the other tested genera. The detection sensitivity of the antiserum ranged from 5-50 µg of *P. infestans* antigen. The PCR assay was also very sensitive for detecting *P. infestans*, only 1pg of purified DNA or DNA isolated from 500 sporangia was needed to detect the pathogen. Applications of serological and molecular tools were effective to detect the pathogen in infected symptomatic, asymptomatic potato tissues (Leaves & tubers) and can also provide important information on *P. infestans*-potato interaction and disease development.

Key Words: Late blight; Potato; Serological and molecular tools; Detection; Disease development

INTRODUCTION

Accurate identification and early detection of pathogens is the cornerstone of disease management in many crops. Many plant pathogens are difficult to identify using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy. It is difficult to determine the extent of progression of *Phytophthora infestans* through blighted potato tissues. Although measurements of lesion diameter or area have been used, but these methods do not differentiated between zones of tissue, which are densely permeated by hyphae and those in which hyphae are sparse (Harrison *et al*., 1990). Alternatively a chemical method for estimating fungal material in infected plants based on a quantitative analysis of chitin has also been reported, but this method cannot be used for *Phytophthora* spp., because chitin is absent from their walls (Ride & Drysdale, 1972).

Technologies for the detection of plant pathogens have already undergone two major breakthroughs over the past 30 years. The first was the advent of antibody based detection; in particular monoclonal antibodies and enzyme-linked immunosorbent assay (ELISA) (Kohler & Milstein, 1975; Clark & Adams, 1977), which drastically increased the speed with which pathogen antigens could be detected *in vivo*. This approach was an important turning point in virology and bacteriology, because pathogens could be identified and detected much more rapidly (Miller & Martin, 1988). Also, ELISA has been shown to be capable of detecting a wide variety of fungi in plant tissues (Harrison *et al*., 1991; Gabler, 1998; Skottrup *et al.*, 2006). DNA-based technologies, polymerase chain reaction (PCR), with the capability to amplify the original target DNA several million folds thus increasing the sensitivity of pathogen detection was used (Mullis & Faloona, 1987). Using these techniques it is now possible to detect and quantify the presence of such pathogens in the crop environment and within infected tissues. These methods can also provide important information on host pathogen interaction and disease development (Harrison *et al*., 1990; Tooley *et al*., 1997 & 1998; Lévesque, 2001).

In the present work, we investigated the specificity and sensitivity of serological and molecular tools for the detection of *P. infestans* in infected tissues of susceptible and resistant potato cultivars and to study disease development among these cultivars.

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MATERIALS AND METHODS

Potato: Two potato cultivars (*Solanum tuberosum*) one being resistant (Hanna) and the other susceptible (Lady-Rosetta) to late blight disease were used in this study (El_Komy, 2007) obtained from International Potato Center (CIP) Kafr El-zayat, Egypt. Potato plants were generated from high quality potato seed tubers and grown in clay pots containing mixtures of soil: sand: peat moth at a ratio of 4:4:1, respectively. Potato plants were kept in a growth chamber at 18ºC for 16 h photoperiod for 6 weeks prior to inoculation.

Isolation of *Phytophthora infestans*: Nine different isolates of *P. infestans* (P01, P03, P12, P14, P16, P18, P19, P20 & P32), were isolated from samples of potato plants showing symptoms of late blight collected from different locations in Alexandria and El-Behera Governorates during the period 2003-2005 according to Malcolmson (1979). Isolates were grown on Rye agar medium (60 g of rye grain, 20 g sucrose and 20 g Bacto agar then adjusted volume to 1 L) and incubated at 18°C in the dark for two weeks to allow growth of the pathogen (Peters *et al*., 1998).

Inoculation with *P. infestans*: At the four leaf stage, plants of Hanna and Lady-Rosetta potato cultivars were inoculated with 15 mL plant^{-1} of sporangia suspension (4000 sporangia mL^{-1}) of aggressive *P. infestans* isolate P14 (El_Komy, 2007). Sporangia for inoculation were taken from infected potato tuber slices (Lady-Rosetta cv.). Inoculated plants were placed in moist chamber in a greenhouse at 18°C and 100% of relative humidity (RH). Control treatment was treated with sterilized distilled water (Niderman *et al*., 1995). Potato tuber-discs (2 cm diameter & 0.3 mm thickness) were prepared from resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars and inoculated with *P. infestans* as described previously and then incubated in moist chamber at 18^{o}C and 100% RH.

Serological Studies

Preparation of antigen: The antigen was prepared from total protein extracted from mycelium of *P. infestans* isolate P14. The mycelial mat was filtered through muslin, washed twice with phosphate buffer (pH 7) and lyophilized. Lyophilized mycelium (1 g) was ground with liquid N. Phosphate buffer saline (PBS; 10 mL) containing 0.1% cystein, 0.1% ascorbic acid, and 17% sucrose was added to the ground mycelium (El-Nashaar *et al*., 1986) and the suspension was centrifuged at 10000×*g* for 30 min at 4^{o}C. The supernatant was concentrated over polyethylene glycol 6000 and then dialyzed against PBS (0.1 M, pH 7) for 24 h. Pellet was re-suspended in 1 mL of PBS. The protein content was determined (Bradford, 1976) and adjusted to 1 mg mL^{-1} in PSB and stored at -20^{o}C as antigen.

Preparation of antiserum: Antiserum against *P. infestans* (isolate P14) was prepared using New Zealand white rabbit by four weekly intramuscular injections with purified preparation of the fungus at concentration of 1 mg mL^{-1}. For the first injection the purified fungus was emulsified with an equal volume of Freund´s complete adjuvant (Difco lab, USA) and for subsequent injections the purified fungus was emulsified with an equal volume of Freund´s incomplete adjuvant. The injections were administered at one week interval. Two weeks after the last injection, rabbits were bled and the blood was collected from the marginal ear vein then left for two hours at room temperature for clot formation then kept in refrigerator overnight. The antiserum was clarified by centrifugation at 3000*xg* for 30 min at 4^{o}C and stored at -20^{o}C until use (Srivastava & Arora, 1997).

Indirect ELISA: The indirect enzyme-linked immunosorbent assay (ELISA) was carried out as described by Fegla *et al*. (1997). Disposable polystyrene flat bottom micro-ELISA plates were used. The tested samples were immersed in liquid nitrogen, ground into a powder using a mortar and pestle and then suspended in 5 mL phosphate buffer pH 7 and centrifuged at 10000*xg* for 10 min. The protein content was determined and adjusted to 0.5 mg mL^{-1} in PSB (Bradford, 1976). Wells were coated with antigens by adding 100 µL of each sample to the bottom of the well and incubated for 3 h at 37^{o}C. The plates were rinsed thrice by flooding wells with phosphate buffer saline+tween 20 (PBST), 5 min each. Aliquots (100 µL) from the diluted antiserum (1:500 diluted in PBST) were added to each well, after which the plates were incubated at 37^{o}C for 2 h, then washed as mentioned earlier. Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (whole molecule, enzymatic activity 457 units mL^{-1}) obtained from Sigma Chem. Co St Louis, Mo (Production#A8025) was diluted 1:1000 in serum buffer and 100 µL was added to each well, followed by one hour incubation at 37^{o}C, then the plates washed as mentioned earlier. Enzyme substrate (100 µL), 0.5 mg mL^{-1} paranitrophenyl phosphate (Sigma#104) in 10% diethanolamine buffer, pH 9.8 were added to each well and incubated at room temperature (25^{o}C) for about 30 min. The enzyme activity was stopped by adding 50 µL of 3 M NaOH. The ELISA values measured by Universal automated microplate reader El $_{X}$ 800 and were expressed as absorbency at 405 nm. Absorbency values of at least double of that of the healthy control, were considered positive. In each set of test, wells lacking antigen (coating buffer only) were included as blanks.

Determination of antiserum titer: The titer of *P. infestans* antiserum was determined by using indirect ELISA as described previously. Extracts from total protein of *P. infestans* (isolate P14) or healthy potato cultivar (Lady-Rosetta) as control were prepared and the protein content was determined (Bradford, 1976) and adjusted to 0.5 mg mL^{-1} in PSB. Serial dilutions of double fold up to 1:5.12×10^{5} of antiserum were used.

Dot blot immunoassay (DIA): DIA was carried out as described by Fegla *et al*. (2001). A grid consisting of 1 cm squares was drawn on nitrocellulose membrane sheet (NCM, 0.45 nm Bio-Rad Laboratories, Richmond, CA) with a pencil. The sheet was then cut to a size that would accommodate the number of samples in an individual test.

The tested samples (leaves & tuber-discs) were immersed in liquid nitrogen, ground into a powder using a mortar and pestle and then suspended in 5 mL phosphate buffer pH 7 and centrifuged at 10000×*g* for 10 min. The protein content was determined and adjusted to 0.5 mg mL^{-1} in PSB (Bradford, 1976). The nitrocellulose membrane was dipped in phosphate buffer pH 7 and placed on filter paper for 5 min to dry. Each sample (2 µL) was spotted on the nitrocellulose membrane in the center of each grid square and dried for 5 min. The membrane was then placed in a Petri-dish containing 10 mL blocking solution (2% bovine serum albumin & 2% Triton x -100 solution in phosphate buffer pH 7), gently agitated for one hour (40 oscillations min^{-1}). The membrane was removed from the blocking solution, dipped in distilled water and washed twice by agitation for 10 min in phosphate buffer+tween 20, then the membrane was transferred to another Petri dish containing 10 mL of fungal-antiserum (1:500 diluted in PBST) and gently agitated for 2 h. The membrane was removed from the first antibody solution, dipped in distilled water, and washed twice by agitation for 10 min in phosphate buffer+tween 20. The membrane transferred to 1:1000 dilution of goat anti-rabbit *I gG* conjugate to alkaline phosphatase in PBST and gently agitated for one hour. Finally, the membrane was removed from the second antibody dilution, dipped in distilled water and washed twice by agitation for 10 min each in phosphate buffer+tween 20. The 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate solution was prepared for the final washing in which membrane was incubated for color development. After color development, the reaction was stopped by washing the membrane in 0.01 M Tris- HCL containing 0.05 M EDTA, pH 7.5.

Specificity of *P. infestans* antiserum: Using the indirect ELISA and Dot Blot Immunoassay (DIA) specificity of *P. infestans* antiserum was studied as described earlier. Samples of total protein (0.5 mg mL^{-1}) were prepared from 9 isolates of *P. infestans* and antigens of *Fusarium* sp., *Pythium* sp., *Rhizoctonia solani, Macrophomia phaseolina, Alternaria solani, Erwinia carotovora* and *Ralistonia solanacearum*. Total protein extract of healthy Lady-Rosetta leaves was served as control. Absorbance values of at least double of that of the control were considered positive.

Reactivity of *P. infestans* antiserum to the purified antigen of *P. infestans*: Reactivity of *P. infestans* antiserum to the purified antigen of *P. infestans* (isolate P14) was determined using indirect ELISA. The antigen was diluted with phosphate buffer to adjust the given concentration (0.1-50 µg). The same concentrations of the total protein extract of healthy Lady-Rosetta leaves served as a control. Absorbance values of at least double of that of the control were considered positive.

Serological Detection of *P. infestans* in Inoculated Potato Tissues

In potato tissues inoculated with *P. infestans* alone: Total protein extracted from inoculated (*in vivo*) potato leaves (Hanna & Lady-Rosetta cvs.) and (*in-vitro*) potato tuber-discs (with *P. infestans* isolate P14 alone) as described earlier, were prepared after different periods of inoculation (12, 24, 48, 72 & 96 h). Total protein extracted from healthy potato leaves was used as control. Indirect ELISA and DIA were used as earlier described to detect late blight pathogen. *P. infestans* antiserum was used at dilution of 1:500 in serological tests. For ELISA test absorbance values of at least double of that of the control were considered positive.

In potato tissues with combined infection: Total protein extracted from inoculated (*in vivo*) potato leaves of resistant (Hanna cv.) and susceptible (Lady-Rosetta cv.) and (*in-vitro*) potato tuber-discs with *P. infestans* (isolate P14) and spore suspension of *A. solani*, after different periods of inoculation (12, 24, 48, 72 & 96 h). Indirect ELISA and DIA were used as previously described to detect late blight pathogen. Total protein extracted from potato tuber discs (Hanna & Lady-Rosetta) inoculated (*in vitro*) with *P. infestans* alone and suspension of 10^5 CFU mL^{-1} of *E. carotovora* or *R. solanacearum*, after different periods of inoculation (12, 24, 48, 72, 96 & 120 h) were prepared. Indirect ELISA and DIA were used as described earlier to detect late blight pathogen. *Phytophthora infestans* antiserum was used at dilution of 1:500 in serological tests. For ELISA test absorbance values of at least double of that of the control were considered positive.

Molecular Studies

Genomic DNA extraction: Total genomic DNA was extracted from nine isolates of *P. infestans* (P1, P3, P12, P14, P16, P18, P19, P20 & P32), in addition to *Fusarium* sp., *Pythium* sp., *R. solani, M. phaseolina, A. solani*. *E. carotovora, R. solanacearum* and of the potato cultivar Lady-Rosetta according to protocol of Griffith and Shaw (1998). Potato leaf tissue was processed by freezing with liquid nitrogen and was ground into a fine powder using a mortar and pestle. Approximately 100 mg of this powder was transferred to 1.5 mL micro-centrifuge tube and 600 µL of warm (65°C) modified CTAB extraction buffer (100 mM Tris-HCL [pH 8.0], 1.4 M NaCl, 2% CTAB [hexadecyltrimethylammonium bromide], 20 mM EDTA [sodium salt, pH 8.0]). Tubes were vortexed for 1-3 sec. and incubated for 60-90 min, in water bath at 65°C. After that, the sample was allowed to cool to room temperature for 5 min. A volume of 700 µL chloroform/octanol (24:1) was added, the solution was gently mixed for 5-10 min. The mixture was centrifuged for 10 min at 8000×*g*. Upper, aqueous layer (600 µL) was transferred to clean1.5 mL micro-centrifuge tube and a volume of 600 µL of cooled isopropanol was added to precipitate the DNA. The mixture was centrifuged at 5000×*g* for 2min at room temperature. The supernatant was decanted and 600 µL of 70% ethanol was added at room temperature and the tube was gently inverted the tube several times to wash the DNA. The mixture was centrifuged at 3000×*g* for 2 min at room temperature. Carefully the ethanol was aspirated using a pipette. The tube was inverted onto clean absorbent paper

and the pellet was air dried for 15 min. DNA pellet was re-suspended in 100 µL TE (10 mM Tris-HCL [pH 8.0], 1 mM EDTA [pH 8.0]) and stored at –20°C. DNA concentration was determined using spectrophotometer (Beckman DU-65) and was adjusted to 50 ng µ L^{-1}.

The bacterial DNA was isolated from the bacteria *E. carotovora* and *R. solanacearum* grown on nutrient agar medium. One colony of bacteria was re-suspended in 100 µL of sterile water, boiled for 10 min at 99°C, cooled for 10 min at 4°C and then centrifuged for 5 min at 10000×*g* at room temperature. The supernatant was used as a template for PCR. The nucleic acids prepared in such way were stored at -20°C.

Amplification of DNA: PCR amplification was carried out in 25 µL volumes containing:1 µL (50 pmol) of each primer, 0.3 µL *Taq* DNA polymerase (5 U μL^{-1}), 2.5 µL PCR buffer, 1 µL 10 mM Mg Cl_2, 1 µL 2.0 mM dNTPs (for each), 1 µL of template DNA (approximately 40 ng) and 17.2 µL sterilized distilled water. The PCR amplification conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec; annealing of primer for 40 sec at 55°C; extension for 1.5 min at 72°C and finally addition of 3' terminal A for 10 min at 72°C. Negative control (no template DNA) was used in every experiment for the presence of contamination in reagents (Shen *et al*., 2005). The primers were designed based on the basis of internally transcribed spacer1 ITS1 (GenBank accession no AY770739), the primers sequence: *Forward primer* (Pin1): 5' CAC CTA AAA ACT TTC CAC CGT 3'. *Reverse primer* (Pin2): 5' TGA GAT GCA TAC CGA AGT ACA C 3'. Amplified products were electrophoresed on 1.2% agrose gel containing 0.5 µL ethidium bromide with 1X TBE running buffer. 1 kb DNA Ladder (New England Biolabs) with size marker, ranged from 500 to 10000 bp was used as a molecular size standard.

Specificity of the primer: Genomic DNA isolated from *Fusarium* sp., *Pythium* sp., *R. solani*, *M. phaseolina*, *A. solani*, *E. carotovora*, *R. solanacearum* and the potato cultivar Lady-Rosetta were used to check the specificity of the primer. PCR conditions were as similar described above.

Sensitivity of the primer: In order to check the sensitivity of the *P. infestans* specific primer, the concentration of purified DNA of *P. infestans* (isolate P14) was determined spectrophotometrically as described above and then a dilution series of DNA (10, 1, 0.1, 0.01 ng, 1, 0.1 & 0.01 pg) were prepared. Additional sensitivity tests were performed using sporangia-spore PCR. Sporangia-spore PCR was performed of 2000, 1000, 800, 500, 300, 100 and 10 sporangia per reaction. A one-step boiling method was used for DNA extraction. One mL of sporangia-spore suspension transferred to a 1.5 mL micro-tube and centrifuged for 10 min at 5000×g. 500 µL of 10 mM Tris–HCl (pH 7.5) were added to the pellet containing sporangia-spore. DNA was released by boiling suspension for 10 min at 99°C, cooled for 10 min at 4°C and then centrifuged for 5 min at 10000×g at room temperature. The supernatant was used as a template for PCR. The nucleic acids prepared in such way were stored at -20°C (Kong *et al*., 2003). The sensitivity of the primer was also assessed using DNA extracted from infected symptomatic (96 hpi) and asymptomatic (48 hpi) potato tissues (leaves & tubers). PCR conditions were similar as described above.

Molecular Detection of *P. infestans* in Inoculated Potato Tissues

In potato tissues inoculated with *P. infestans* alone: Total genomic DNA extracted from inoculated (*in vivo*) potato leaves (Hanna & Lady-Rosetta) and (*in-vitro*) potato tuber-discs with *P. infestans* (isolate P14) alone as described previously, were prepared after different periods of inoculation (12, 24, 48, 72 & 96 h). Genomic DNA extracted from healthy potato was used as a control. Polymerase chain reaction (PCR), using the specific primer was applied as previously described to detect late blight pathogen.

In potato tissues with combined infection: Total genomic DNA extracted from inoculated (*in vivo*) potato leaves (Hanna & Lady-Rosetta) and (*in-vitro*) potato tuber-discs with *P. infestans* (isolate P14) and spore suspension of *A. solani*, were prepared after different periods of inoculation (12, 24, 48, 72 & 96 h) as described previously. Total protein extracted from inoculated (*in vitro*) potato tuber discs (Hanna & Lady-Rosetta) with *P. infestans* (isolate P14) and suspension of 10^5 CFU mL^1 of *E. carotovora* or *R. solanacearum*, were prepared after different periods of inoculation (12, 24, 48, 72 & 96 h) as described earlier. Genomic DNA extracted from healthy potato was used as control. Polymerase chain reaction (PCR), using the specific primer was applied as previously described to detect late blight pathogen.

RESULTS

Serological studies: The antiserum raised from soluble mycelial protein of *P. infestan*s (isolate P14) was used in serological studies [using indirect ELISA & dot blot immunoassay (DIA)], to detect the late blight pathogen and study the disease development in resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars.

Determination of antiserum titer: The titer of *P. infestans* (isolate P14) antiserum was determined by using indirect ELISA. Extracts (50 µg) from total protein of *P. infestans* or healthy potato cultivar (Lady-Rosetta) as control were used. Serial dilutions up to 1:5.12×10^5 of antiserum were used. Absorbance values of at least double of that of the control were considered positive. Results showed that positive ELISA values for the antiserum were up to 1:1.28×10^5 (Table I).

Reactivity of *P. infestans* antiserum to the purified antigen of *P. infestans*: Reactivity of *P. infestans* antiserum to the purified antigen of *P. infestans* (isolate P14) was

Table I: *Phytophthora infestans* antiserum titer (dilution end point) determined by indirect ELISA as absorbance values at 405 nm

Antiserum dilution	Healthy Potato antigen (Lady-Rosetta cv.)	*P. infestans* antigen (Isolate P14)
$1:5\times10^{2}$	0.073	0.595
$1:10^{3}$	0.065	0.544
$1:2\times10^{3}$	0.054	0.532
$1:4\times10^{3}$	0.051	0.478
$1:8\times10^{3}$	0.047	0.454
$1:1.6\times10^{4}$	0.038	0.146
$1:3.2\times10^{4}$	0.032	0.080
$1:6.4\times10^{4}$	0.018	0.049
$1:1.28\times10^{5}$	0.013	0.031
$1:2.56\times10^{5}$	0.009	0.012
$1:5.12\times10^{5}$	0.005	0.007

-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
- One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

Table II: Reactivity of *P. infestans* antiserum to the purified antigen of *P. infestans* (isolate P14) determined by indirect ELISA as absorbance values at 405 nm

Antigen concentration (µg)	*P. infestans* antigen (Isolate P14)	Healthy Potato antigen (Lady-Rosetta cv.)
50	0.542	0.037
25	0.442	0.030
10	0.125	0.025
5	0.099	0.016
1	0.025	0.014
0.1	0.011	0.007

-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

determined using indirect ELISA. The antigen was diluted with phosphate buffer to adjust the given concentration (0.1-50 µg). The same concentrations of the total protein extract of healthy Lady-Rosetta leaves served as control. Absorbance values of at least double of that of the control were considered positive. Data showed that, the antigens at concentrations of 5-50 µg were detected using indirect ELISA (positive ELISA values) (Table II). In contrast, antigens of 1 and 0.1 µg were not detected (negative ELISA values).

Specificity of the *P. infestans* antiserum: Cross-reactivity of *P. infestans* antiserum was tested against 50 µg antigens of 9 isolates of *P. infestans* (P01, P03, P12, P14, P16, P18, P19, P20 & P32) and antigens of *Fusarium* sp., *Pythium* sp., *Rhizoctonia solani, Macrophomia phaseolina*, *Alternaria solani*, *Erwinia carotovora* and *Ralistonia solanacearum.* Total protein extract of healthy Lady-Rosetta leaves was served as a control. Absorbance values of at least double of that of the control were considered positive. Maximum ELISA values (0.453-0.563) were detected with different

Table III: Specificity of *P. infestans* antiserum as indirect ELISA absorbance values (E405 nm) of total protein prepared from various sources

Source of antigen	Absorbance values (E405nm)
P. infestans (isolate P01)	0.477
P. infestans (isolate P03)	0.453
P. infestans (isolate P12)	0.499
P. infestans (isolate P14)	0.563
P. infestans (isolate P16)	0.475
P. infestans (isolate P18)	0.497
P. infestans (isolate P19)	0.502
P. infestans (isolate P20)	0.543
P. infestans (isolate P24)	0.479
P. infestans (isolate P32)	0.459
R. solani	0.027
Pythium sp.	0.126
M. phaseolina	0.028
Fusarium sp.	0.023
A. solani	0.018
R. solanacearum	0.032
E. carotovora	0.030
Healthy Potato (Lady-Rosetta)	0.038

-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

Table IV: Sensitivity test for *P. infestans* antiserum as indirect ELISA absorbance values (E405 nm) of total protein extracted from inoculated potato leaves after different periods of inoculation with *P. infestans*

Potato cultivar	Time post-inoculation (h)	Absorbance values (E405 nm)
Hanna	*Control	0.013
(Resistant)	12	0.013
	24	0.021
	48	0.031
	72	0.018
	96	0.010
Lady-Rosetta	Control	0.039
(Susceptible)	12	0.066
	24	0.075
	48	0.162
	72	0.264
	96	0.380

Control=un-inoculated tuber-discs (treated with sterilized water and measured immediately)
-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

isolates of *P. infestans*. Other than antigens of *P. infestans*, antiserum showed cross-reacted only with *Pythium* sp. (0.126). On the other hand, very week ELISA values (negative response) were noticed with the other tested organisms (Table III). Similar results were also obtained using Dot blot immunoassay (DIA), high dusky purple color was observed with all the tested *P. infestans* isolates. On the other hand, a slight purple color was showed with *Pythium* (Fig. 1).

Table V: Sensitivity test for *P. infestans* antiserum as indirect ELISA absorbance values (E405 nm) of total protein extracted from inoculated potato tuber-discs after different periods of inoculation with *P. infestans*

Potato cultivar	Time post-inoculation (h)	Absorbance values (E405 nm)
Hanna	*Control	0.064
(Resistant)	12	0.088
	24	0.112
	48	0.128
	72	0.126
	96	0.126
	120	0.101
Lady-Rosetta	Control	0.085
(Susceptible)	12	0.105
	24	0.148
	48	0.192
	72	0.350
	96	0.282
	120	0.418

Control=un-inoculated tuber-discs (treated with sterilized water and measured immediately)
-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

Table VI: Sensitivity test for *P. infestans* antiserum as indirect ELISA absorbance values (E405 nm) of total protein extracted from inoculated potato leaves after different periods of combined inoculation with *P. infestans* and *A. solani*

Time post-inoculation (h)	Absorbance values (E405 nm)	
	Hanna (Resistant cv.)	Lady-Rosetta (Susceptible cv.)
*Control	0.013	0.039
12	0.019	0.058
24	0.022	0.070
48	0.025	0.132
72	0.017	0.237
96	0.019	0.312

*Control=healthy leaves (treated with sterilized water and measured immediately)
-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

Serological Detection of *P. infestans* in Inoculated Potato Tissues

In potato tissues inoculated with *P. infestans* alone: The amount of mycelium of *P. infestans* was estimated by indirect ELISA after different periods of inoculation with *P. infestans* (isolate P14) using polyclonal antiserum specific to *P. infestans*, in leaves and tubers of resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars, by comparing absorbance values obtained with those given by un-inoculated samples for each cultivar. Absorbance values of at least double of that of the healthy control were considered positive. Compared with healthy control, pathogen was not positively detected in the susceptible potato (Lady-Rosetta cv.) until after 48 h post-inoculation in inoculated leaves (0.162) and tuber-discs (0.192). After that, a sharp increase in ELISA values was recorded with the time (Table IV & V). Low levels of ELISA value were obtained in case of resistant potato cultivar (Hanna). The amount of antigen in leaves and tuber tissues was sufficient to cause a positive ELISA reaction (0.031 & 0.128, respectively) only after 48 h post-inoculation (Table IV & V). Results of dot blot immunoassay were in agreement with those obtained by the indirect ELISA test (Fig. 2). Also, results indicated that among the inoculated potato (96-120 h post-inoculation), there was large amount of mycelium in leaves and tuber-discs of the susceptible potato cultivar, while the resistant potato cultivar contained less.

Table VII: Sensitivity test for *P. infestans* antiserum as indirect ELISA absorbance values (E405 nm) of total protein extracted from inoculated potato tuber-discs after different periods of combined inoculation with *P. infestans* and *E. carotovora* or *R. solancearum*

Time post-inoculation (h)	Absorbance values (E405 nm)			
	Hanna (Resistant cv.)		Lady-Rosetta (Susceptible cv.)	
	**E	***R	E	R
*Control	0.068	0.069	0.088	0.088
24	0.093	0.083	0.112	0.131
48	0.066	0.085	0.085	0.098
72	0.060	0.058	0.052	0.076

*Control=un-infected tuber-discs (treated with sterilized water and measured immediately)
**E=tuber-discs inoculated with *P. infestans* and *E. carotovora*; R=tuber-discs inoculated with *P. infestans* and *R. solanacearum*
-The experiment was repeated twice and ELISA absorbance values at 405nm are average of two replicates each
-One hundred µl from the diluted *P. infestans* antiserum (1:500 diluted in PBST) were used for each ELISA reaction
-Absorbance values of at least double of that of the healthy control were considered positive

In potato tissues with combined infection: Indirect ELISA was used to determine the presence of *P. infestans* in potato leaves inoculated with *P. infestans* and *A. solani*, or potato tuber-discs inoculated with *P. infestans* and *E. carotovora* or *R. solanacearum* (*in vitro* tuber-discs inoculation). Results revealed that, indirect ELISA values revealed the ability of the antiserum to detect the late bight pathogen at 48hpi in leaves of susceptible potato cultivar (Lady-Rosetta) inoculated with *P. infestans* and *A. solani* (Table VI). Whereas no positive ELISA values were recorded in case of resistant potato cultivar (Hanna). These results were in agreement with those obtained by dot blot immunoassay (Fig. 3). Potato tuber-discs of resistant and susceptible cultivars inoculated with *P. infestans* and *E. carotovora* or *R. solanacearum*, showed no positive ELISA values compared with healthy control (Table VII).

Molecular studies: Specific primer to *P. infestans*, which was designed on the basis of internally transcribed spacer1 ITS1 (GenBank accession no AY770739) was used in

Fig. 1: Specificity of *P. infestans* antiserum to detect *P. infestans* isolates using dot blot immunoassay (DIA)
1 and 9 = Phosphate buffer; 2 – 8 = antigens of *P. infestans* isolates (P1, P3, P12, P14, P18, P20 and P32 respectively); 10 = antigen of *Fusarium* sp.; 11= antigen of *Pythium* sp.; 12 = antigen of *R. solani*.; 13 = antigen of *M. phaseolina*; 14 = antigen of *A. solani*; 15 = antigen of *E. carotovora*; 16 = antigen of *R. solanacearum*

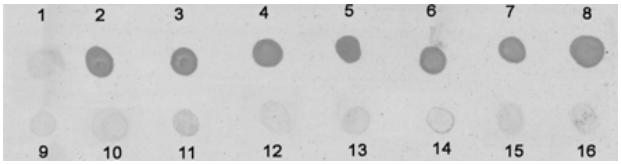


Fig. 2: Sensitivity of *P. infestans* antiserum to detect *P. infestans* in artificially inoculated tissues of resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars after different periods of inoculation with *P. infestans* using dot blot immunoassay (DIA)

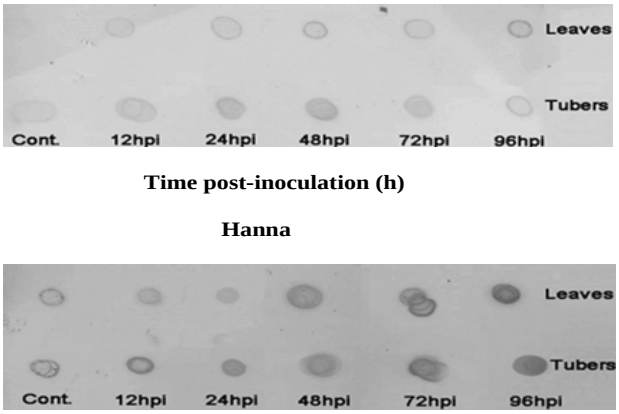
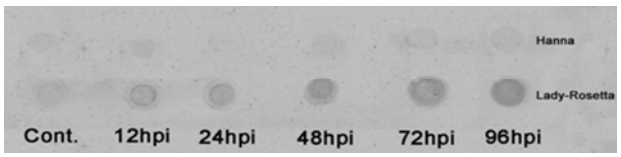


Fig. 3: Sensitivity of *P. infestans* antiserum to detect *P. infestans* in artificially inoculated leaves of resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars after different periods of combined inoculation with *P. infestans* and *A. solani* using dot blot immunoassay (DIA)



molecular studies to detect the late blight pathogen and study the disease development in resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars.

Specificity of the primer: Genomic DNA isolated from nine isolates of *P. infestans* (P1, P3, P12, P14, P16, P18, P19, P20 & P32), in addition to *Fusarium* sp., *Pythium* sp., *R. solani*, *M. phaseolina*, *A. solani*, *E. carotovora*, *R. solanacearum* and of the potato cultivar Lady-Rosetta were examined by PCR with the *P. infestans* specific primer. PCR amplification indicated that, all the *P. infestans* isolates amplified a product of approximately 813bp with the primer. On the other hand, no amplification products were obtained with the other tested genera (Fig. 4).

Fig. 4: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing specificity of molecular detection using *P. infestans*-specific primer
M= DNA marker; Lanes 1-9 = Template DNA of *P.infestans* (P1, P3, P12, P14, P16, P18, P19, P20 and P32 respectively); Lean 10 = Template DNA of *R. solani*; Lean 11 = Template DNA of *Pythium* sp.; Lean 12 = Template DNA of *M. phaseolina*.; Lean 13 = Template DNA of *Fusarium* sp.; Lean 14 = Template DNA of *A.solani*.; Lean 15 = Template DNA of *R. solanacearum*.; Lean 16 = Template DNA of *E. carotovora*; Lean 17 = Template DNA of potato cultivar (Lady-Rosetta)

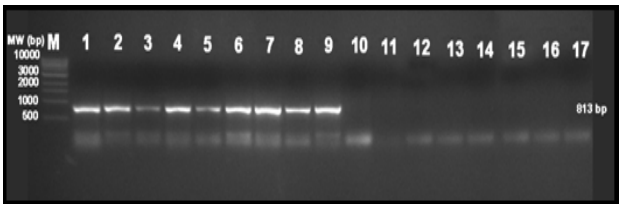


Fig. 5: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of molecular detection of serial concentration of DNA of *P. infestans* using *P .infestans*-specific primer
M= DNA Marker

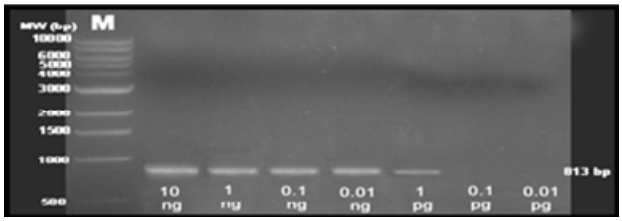
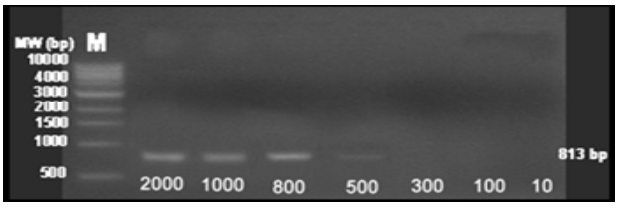


Fig. 6: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of molecular detection of serial concentration of *P. infestans* sporangia using *P. infestans*-specific primer



Sensitivity of the primer. In order to check the sensitivity of the *P. infestans* specific primer, the concentration of purified DNA of *P. infestans* (isolate P14) was determined spectrophotometrically and then a dilution series of DNA (10, 1, 0.1, 0.01 ng & 1, 0.1 & 0.01 pg) were prepared. Data

Fig. 7: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of the *P.infestans*-specific primer to detect *P. infestans* in symptomatic and asymptomatic potato tissues

M = DNA Marker; Leans 1-2 = Template DNA from symptomatic (96hpi) and asymptomatic (48hpi) leaves respectively; Lean 3 = Template DNA from *P. infestans* isolate P14; Leans 4-5 = Template DNA from asymptomatic (48hpi) and symptomatic tubers-discs (96hpi) respectively

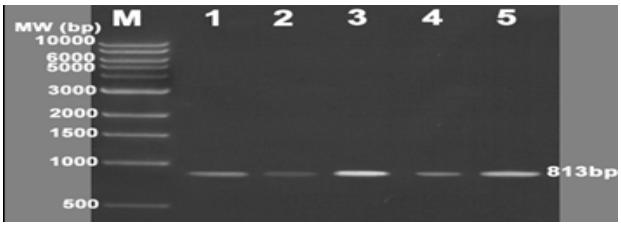
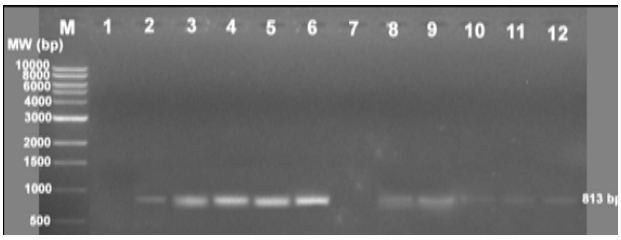


Fig. 8: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of the *P. infestans*-specific primer to detect *P. infestans* in artificially inoculated leaves of susceptible (Lady-Rosetta) and resistant (Hanna) potato cultivars after different periods of inoculation with *P. infestans*

M= DNA Marker; Leans 1-6= Template DNA from inoculated potato leaves (Lady-Rosetta cv.) after 0, 12, 24, 48, 72, and 96hpi respectively; Leans 7-12 = Template DNA from inoculated potato leaves (Hanna cv.) after 0, 12, 24, 48, 72, and 96hpi respectively



showed that, the specific primer yielded positive results at five concentrations of DNA ranging from 10 ng to 1 pg, but no positive signal at 0.1 and 0.001 pg DNA (Fig. 5). Further sensitivity tests were done using sporangia PCR. Sporangia PCR was performed of 2000, 1000, 800, 500,300, 100 and 10 sporangia per reaction. Specific primer consistently resulted in a positive signal for all numbers of sporangia except for 300, 100 and 10 sporangia per reaction (Fig. 6). The sensitivity of the *P. infestans* specific primer was also assessed using DNA extracted from infected symptomatic (96 hpi) and asymptomatic (48 hpi) potato tissues (leaves & tubers). The assay was effective with DNA extracted from infected symptomatic and asymptomatic potato tissues (leaves & tubers). The signal was strongest in symptomatic tissues indicating higher inoculum density (Fig. 7).

Molecular Detection of *P. infestans* in Inoculated Potato Tissues

In potato tissues inoculated with *P. infestans* alone: Sensitivity of the *P. infestans* specific primer for the detection of *P. infestans* (isolate P14) in infected potato after different periods of inoculation was studied. After a simple template preparation from potato leaves or tuber-discs artificially

Fig. 9: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of the *P.infestans*-specific primer to detect *P. infestans* in artificially inoculated tuber-discs of susceptible (Lady-Rosetta) and resistant (Hanna) potato cultivars after different periods of inoculation with *P. infestans*

M= DNA Marker; Leans 1-8= Template DNA from the inoculated potato tuber-discs (Lady-Rosetta cv.) after 0, 12, 24, 48, 72, 96, 120 and 144hpi respectively; Leans 9-16= Template DNA from the inoculated potato tuber-discs (Hanna cv.) after 0, 12, 24, 48, 72, 96, 120 and 144hpi respectively

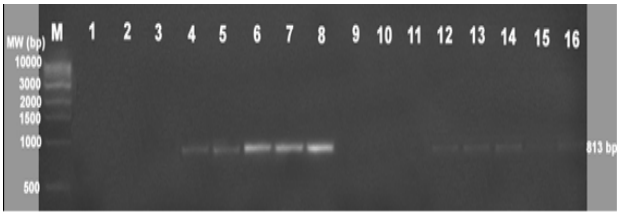


Fig. 10: Agrose gel electrophoresis of polymerase chain reaction (PCR) showing sensitivity of the *P. infestans*-specific primer to detect *P. infestans* in artificially inoculated potato tissues (Lady-Rosetta cv.) after different periods of inoculation with combined inoculation with *P. infestans* and *A. solani* or *E. carotovora* or *R. solancearum*

M= DNA Marker; Lean 1= Template DNA of *P. infestans*; Leans 2= Negative control (no template DNA); Leans 3-8= Template DNA from inoculated potato leaves (Lady-Rosetta cv.) with *P. infestans* and *A. solani* after 0, 12, 24, 48, 72, and 96hpi respectively; Lean 9= Template DNA from un-inoculated potato leaves (Lady-Rosetta cv.); Leans10-11= Template DNA from inoculated potato tuber-discs (Lady-Rosetta cv.) with *P. infestans* and *E. carotovora* after 48 and 96hpi respectively; Leans 12-13 =Template DNA from inoculated potato tuber-discs (Lady-Rosetta cv.) with *P. infestans* and *R. solanacearum* after 48 and 96hpi respectively; Lean 14 = Template DNA from inoculated potato tuber-discs (Lady-Rosetta cv.) at 0hpi

inoculated with *P. infestans* PCR amplification was carried out by using the specific primer. A single PCR product of approximately 813 bp was detected in infected potato cultivars (resistant & susceptible) starting from 12 hpi in leaf samples and 48 hpi in tuber-disc samples (Figs. 8 & 9). The efficacy of the assay was high in susceptible tissue exhibiting blighted symptoms, but lower in resistant tissues. In the Lady-Rosetta samples (susceptible cv.) PCR product gave a strong signal increasing with the time post inoculation period indicating a high inoculum density, whereas opposite trends were observed with Hanna (resistant cv.) samples.

In potato tissues with combined infection: PCR amplification using the specific primer and templates DNA

extracted from leaves of Lady-Rosetta infected with both *P. infestans* and *A. solani* yielded only the predicted PCR product (813 bp) after 12 hpi. No PCR products were obtained from plants un-inoculated with *P. infestans* (Fig. 10). On the other hand, using DNA templates prepared from tuber-discs infected with a mixture of *P. infestans* and *E. carotovora* or *R. solanacearum*, the primer detected fungal DNA in samples inoculated with both *P. infestans* and *R. solanacearum* only 48 hpi (Fig. 10).

DISCUSSION

Phytophthora infestans is able to penetrate epidermal cells of many different plant species, including non-host plants, which indicated that, the defense responses mainly occur post-penetration (Vleeshouwers *et al*., 2000). In the early phases of interaction, there are only small differences between compatible and incompatible reactions (Cuypers & Hahlbrock, 1988; Freytag *et al*., 1994). Differences in the plant's reaction are discernible once the hyphae reach the mesophyll. In compatible interaction, some cells undergo hypersensitive response (HR), but the hypha can escape and continue to colonize the tissue. In the incompatible interaction, a larger number of cells undergo HR and the pathogen is contained and dies (Beyer *et al*., 2001).

Accurate identification and early detection of pathogens is the cornerstone of disease management in many crops. Many plant pathogens are difficult to identify using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy. It is difficult to determine the extent of progression of *P. infestans* through blighted potato tissues. Measurements of lesion diameter or area have been used, but these methods do not differentiate between zones of tissue, which are densely permeated by hyphae and those in which hyphae are sparse (Harrison *et al*., 1990). Serological and molecular detection tools can generate accurate results rapidly enough to be useful for disease management decisions. In the present work, we investigated the specificity and sensitivity of serological and molecular tools for the detection of *P. infestans* in infected tissues of susceptible and resistant potato cultivars and studied disease development among these cultivars. Serological tools [enzyme-linked immunosorbent assay (ELISA) and dot blot immunoassay (DIA)] were performed using antiserum raised from soluble mycelial protein of *P. infestan*s (isolate P14). While molecular tool [Polymerase chain reaction (PCR)] was performed using specific primer to *P. infestans*, which was designed on the basis of internally transcribed spacer1 ITS1 (GenBank accession no AY770739). Cross-reactivity of the antiserum was tested against 50 µg antigens of 9 isolates of *P. infestans* and antigens of *Fusarium* sp., *Pythium* sp., *R. solani, M. phaseolina, A. solani, E. carotovora* and *R. solanacearum*. Results showed that, maximum ELISA values (0.453-0.563) were detected with different isolates of *P. infestans*. Other than antigens of *P. infestans*, antiserum cross-reacted only with *Pythium* sp. (0.126). On the other hand, very week ELISA values (negative response) was noticed with the other tested organisms. Similar results were also obtained using DIA. Such results are in agreement with those reported by Harrison *et al*. (1990); Kynerova *et al*. (1998); and Skottrup *et al*., (2006). This lack of specificity in the antiserum with *Pythium* sp. may be attributed to the very close taxonomical relation between *Pythium* sp. and *P. infestans*. This indicated that, there are also many molecular epitopes on the fungal surface, which are common to a range between *Phytophthora* and *Pythium* species (Gautam *et al*., 1999). On the other hand, PCR amplification indicated that, all the *P. infestans* isolates amplified a product of approximately 813 bp with the primer. While, no amplification products were obtained with the other tested genera. The greater specificity may be due to the high copy number of ITS target sequences in the genome of *Phytophthora* spp. (Cooke *et al*., 2000).

The detection sensitivity of the antiserum is ranged from 5-50 µg of *P. infestans* antigen. The PCR assay was also very sensitive for detecting *P. infestans*, only 1 pg of purified DNA or DNA isolated from 500 sporangia was needed to detect the pathogen. Also the serological and molecular tools were effective to detect the pathogen in infected symptomatic and asymptomatic potato tissues (Leaves & tubers). In potato tissues inoculated with *P. infestans* (isolate P14) alone, results of ELISA and DIA showed that, the pathogen was positively detected in the resistant and susceptible potato after 48 h post-inoculation in inoculated leaves and tuber-discs. These results were in agreement with those of Harrison *et al*. (1991 & 1994); Beckman *et al*. (1994); Kynerova *et al*. (1998); Schlenzig *et al*. (1999); Skottrup *et al*. (2006), who developed an antiserum to detect *P. infestans* from infected potato. Also in potato tissues inoculated with *P. infestans* alone, single PCR product of approximately 813 bp was detected in infected potato cultivars (resistant & susceptible) starting from 12 hpi in leaf samples and 48 hpi in tuber-disc samples. These results indicated that the efficacy of the molecular tool to detect the late blight pathogen was higher than the serological tool. These results generally are in line with those reported by Niepold and Schober (1995); Tooley *et al*. (1997 & 1998); Trout *et al*. (1997); Niepold and Schober (1999); Judelson and Tooley (2000) and HuiShan and Wang (2004), who reported that, the molecular tool, which is often simpler to perform than other procedures for estimating *P. infestans* growth during the early stages of potato-tuber infection. The high ELISA values and the strongest PCR signal showed with susceptible cultivars compared with resistant ones indicated that, there were large amount of mycelium in leaves and tuber-discs of the susceptible potato cultivar, while the resistant potato cultivar contained less.

In a summary, using serological and molecular tools it is possible to detect and quantify the presence of *P.*

infestans in infected tissues even before the appearance of symptoms. These tools can also provide important information on *P. infestans*-potato interaction and disease development.

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