



Early detection of *Phytophthora infestans* in potato using carbohydrate binding module 1 protein (CBD1) based antiserum

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Abstract

Phytophthora infestans is a global challenging pathogen among the biotic stresses which affect healthy and sustainable potato production. Desirable and successful management strategies depend on understanding of its biology, disease cycle and more importantly rapid and accurate detection of the pathogen during early stage of infection. Here, we report production of recombinant antibodies against whole cellulose binding domain (CBD-1) protein of *P. infestans* and a synthetic signal peptide of CBD1 and their effectiveness in detection of *P. infestans* both in infected potato leaf and tuber. The recombinant whole CBD1 protein and synthetic signal peptide of CBD1 protein was used to immunize the rabbits and blood serum was collected, purified and measured by SDS-PAGE. Among the two antibodies, synthetic peptide based antibody showed high specificity for mycelia, sporangia and zoospores. It also showed strong signal intensity for *P. infestans* infected leaf and tuber tissues. The data indicated that the produced synthetic peptide antiserum was efficient and accurate in discrimination of negative and positive samples in ELISA. Reaction of antiserum with early stage of infected leaf and tuber tissue confirmed the expression of the CBD protein, which in turn confirmed the presence of the pathogen in the plant tissues. Therefore it will enable to detect the pathogen in the potato seed before planting as well as in the standing crop in the field thus, would be helpful to ensure production of healthy seed potatoes and for effective schedule for management practices.

Keywords *Phytophthora infestans* · Diagnosis · Seed potato · CBD1

Abbreviations

| | |
|--------|---|
| CBD1 | Carbohydrate binding module1 |
| ELISA | Enzyme linked immune sorbent assay |
| DAS | Double antibody sandwich |
| RT-PCR | Reverse transcriptase polymerase chain reaction |

Introduction

Phytophthora infestans (Mont.) de Bary is one of the most economically important oomycete pathogen of potato and tomato crops worldwide (Fry et al. 2015). The oomycetes have a typical cell wall feature distinguishable from several

other eukaryotic microorganisms. The β -1, 3-glucan polymers and cellulose are the main components of oomycetes; unlike fungal cell walls, they contain little chitin (Erwin et al. 1983; Erwin and Ribeiro 1996). Most stages in the oomycete life cycles are diploid (Hardham 2007). Infection generally starts under moist environment, when motile zoospores released from sporangia encyst, germinate and start infection from leaf to shoot and stem (Hardham 2001; Hardham and Mitchell 1998). The appressorium is the infective structure formed from germ tube, which facilitates its adherence and penetration to plant surfaces. Once established inside the cell, the pathogen starts secreting apoplastic and cytoplasmic effector proteins for establishment of infection process.

Detection of *P. infestans* inoculum in the standing crop and from the planting material is one of the challenging tasks in healthy crop production. The traditional techniques, such as culturing, include morphological character studies, which are time consuming and require extensive knowledge in taxonomy. It is difficult to determine visually the extent of progression of the pathogen during its biotrophic phase, (Harrison et al.

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1990). Hence, the development of quick, reliable and economical diagnostic techniques for early detection is a number one priority for the production of healthy seed tubers, and to prevent the spread of the disease in field with timely management practices. Polymerase chain reaction (PCR) assays using specific PINF primers were proven to be rapid in detection of *P. infestans* in potato and tomato fields (Trout et al. 1997; Hussain et al. 2013). They however involve high cost because they require testing large number of samples. Therefore for easy, accurate and rapid detection of pathogen at the early stage of host interactions, we targeted the cell wall based molecule, carbohydrate binding domain 1 (CBD1) for serology based *P. infestans* detection. CBD1 protein has been proven as a novel cell wall associated protein for easy and rapid detection of *P. infestans* (Jones and Ospina-Giraldo 2011). An efficient purified antigen is required for the production of good quality polyclonal antibodies. However, low titer and mixed oomycete infection can hinder the fidelity of the purification process (Abdel-Salam et al. 2004, 2005). This in turn necessitates the use of large amount of starting-infected materials and therefore increases the chance of having high background related to plant-host antigens in the induced antiserum. The presence of multiple oomycete species can lead to co-purification of other species and hence to the production of mixed antibodies, thereby affecting oomycete diagnosis. Hence, we have used recombinant CBD1 protein and synthetic signal peptide of CBD1 protein to produce antibodies for early detection of *P. infestans* in seed tubers and early infection in standing crop. The synthetic signal peptide based antiserum accurately discriminated negative and positive samples in ELISA as compared to whole recombinant protein.

Methods and materials

Phytophthora infestans culture conditions and RNA isolation

Phytophthora infestans was maintained on Rye agar medium supplemented with sucrose as described by Sharma et al. (2016). Mycelia were grown for 7 days in solid Rye agar plates. Mycelium was removed and stored at -80°C until use (Division of Plant Protection, CPRI, Shimla). Total RNA from frozen mycelia was extracted using Nucleospin plant RNA kit (Machery Nagel, Germany) following the manufacturer's protocol. Integrity and yield of the RNA was tested by agarose gel electrophoresis.

Plant material and pathogen inoculum for late blight infection

Inoculum was prepared from 7 days old grown culture on rye agar medium in dark ($18 \pm 1^{\circ}\text{C}$), *P. infestans* mycelia were harvested in sterile water and stimulated to release

zoospores at 4°C . The sporangial suspension was observed under a haemocytometer and concentration was adjusted to 2×10^4 per mL to be used as inoculum. For whole plant assay, the plants were grown for 45 days in greenhouse and then shifted to *Phytophthora* screening chamber, where temperature ($18\text{--}20^{\circ}\text{C}$) and relative humidity ($>90\%$) were maintained. The plants were inoculated with hand sprayer to run off. Leaf samples were drawn after 5 days of inoculation, snap frozen and processed for ELISA analysis.

Potato tubers (cv. Kufri Bahar) were inoculated with zoospores (2×10^4 sporangia/mL) of *P. infestans* by syringe inoculation method near the eye as per the procedure of Appel et al. (2001) and incubated in humidity chamber at $18 \pm 1^{\circ}\text{C}$ till symptom development. Two peel strips (1–2 mm thick) were taken from rose to heel end using a hand potato peeler. The peels were kept in polythene bags and grinded using a sap extractor machine and processed for ELISA.

Gene identification and cloning

The carbohydrate binding module (CBM) family 1 motif (<http://www.cazy.org>) was used for tBLASTn searches of the genome of *P. infestans* (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans). The identified CBD1 gene sequence was used to design primers specific to CBD1 gene using the fast per programme. A cDNA pool was generated from total RNA using high capacity reverse transcriptase cDNA synthesis kit (Applied Biosystem USA). Complementary DNA (cDNA) was reverse-transcribed in a total reaction volume of 20 μL reaction mixture containing 10 μL of total RNA isolated, 0.8 μL of 100 mM dNTP mix, 2 μL 10 \times RT Buffer, 2 μL 10 \times random primer, 1 μL of reverse transcriptase enzyme and 4.2 μL RNase free water. Reactions were incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 s. DNA copies were generated from cDNA using CBD-Forward (5'-ATGACCTCGTTGCGACTCC-3') and CBD-Reverse (5'-CTAGAGCTCGAGTCGAATGAC-3') primers. PCR products were generated with cycle parameters of initial 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 56°C for 30 s, 72°C for 40 s and final cycle of 72°C for 5 min. Five micro litter aliquots of PCR products were analyzed on 1% agarose gels using 1 \times TAE with a 1 kb DNA ladder. Gels were stained with ethidium bromide and visualized by UV illumination. The products were cloned into pTZ57R/T vector, confirmed through restriction digestion and then sub-cloned into pETSUMO for protein expression in BL21 *E. coli* cells. Plasmids were isolated from overnight cultures of transformed *E. coli*, confirmed insertion through restriction digestion using *EcoRI* and *HindIII*. Subsequently, the undigested plasmid was sequenced using 3.2 pmol of M13 forward primer in

ABI3500 Genetic Analyzer, ABI, USA. Sequences were analysed and homology of CBD1 to known sequences were analysed using BLASTn (NCBI, <http://www.ncbi.nlm.nih.gov/blast/>).

Expression and purification of whole CBD1 recombinant protein

To express the CBD1 protein, an *E. coli* BL21 colony containing the desired recombinant plasmid was used to inoculate liquid Luria–Bertani (LB) medium containing 100 μg /mL kanamycin and incubated overnight at 37°C with shaking at 250 rpm. One milliliter of overnight culture was inoculated into 250 mL of LB medium containing 100 μg /mL kanamycin and incubated at 37°C with vigorous shaking to reach an OD_{600} of approximately 0.5. One milliliter of culture was centrifuged at 12,000 rpm for 1 min at room temperature and the pellet was stored at -20°C . Expression of the recombinant protein was induced in the remaining culture by adding 0.5 mM IPTG for 5 h. Bacterial cells were harvested from 250 mL culture by centrifugation at $5000 \times g$ for 10 min. Protein was purified with Hook™ 6 \times His protein purification kit (Bacteria) from G-Biosciences. The purified protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide and stained with Coomassie Blue. Briefly, the purified protein was mixed in 20 μL of 1 \times SDS gel loading dye and heated to 100°C for 5 min prior to separation through a 12% SDS–PAGE.

Production and purification of antisera

Production of antisera work was outsourced to M/s Merck Biosciences (GeNei), Bangalore. The immunization of a rabbit involved injection of recombinant protein prepared under denaturing purification conditions. The animal received five weekly consecutive intramuscular injections. Each injection was composed of 500 μg fusion CBD mixed (1:1, v/v) with Freund's complete adjuvant in the first-week injection. Subsequent booster injections after second week used incomplete adjuvant instead. The rabbit was bled 1 week after the last injection. The serum fraction of the blood was separated through centrifugation at 1300 rpm for 15 min at 4°C , and then stored at -20°C . The IgG fraction was purified using 2 mL of Protein A Agarose beads from 5 mL of rabbit serum. The concentration of IgG was measured at 280 nm with 1 \times PBS. The measured IgG was frozen at -20°C . Alkaline phosphatase (AP)-IgG conjugate was prepared following the one-step glutaraldehyde method. The AP-IgG conjugate was preserved in 50% glycerol, 0.02% NaN_3 and stored at 4°C .

Synthetic peptide based antisera

The amino terminal portion of the protein was chosen as the antigenic target. The nucleotide sequence was translated into protein sequence to identify the peptide sequence that is responsible for the CBD1 domain. The identified peptide (SNLRNGDSSVPVRT) was synthesized from Merck Biosciences (GeNei). The synthetic peptide was labelled with KLH by EDC method and rabbits were immunized up to 75 days (New Zealand White) (Merck Biosciences GeNei™) as described above, and the final antibody titer was analyzed by DAS ELISA. The antibody purity was analyzed by SDS–PAGE and concentration was estimated by absorbance methods.

Determination of antiserum titre

Double Antibody Sandwich (DAS)-ELISA was used to measure titre of the CBD1-recombinant protein and synthetic peptide based antiserum and this test was also used to measure the specificity of the antiserum against *P. infestans*. To determine titre of the antiserum, the IgG was tested at three different dilutions (1:350, 1:400 and 1:450) against different dilutions of recombinant CBD-IgG/synthetic peptide-IgG labeled AP (1/250, 1/500, 1/1000).

Immunoassay tests

Indirect (I)-ELISA i.e. direct antigen coated ELISA was used to test the presence of CBD1 in *P. infestans* samples. Freshly grown *P. infestans* mycelium as well as sporulated mycelium was used for harvesting sporangia and zoospores. Zoospores of *P. infestans* were isolated by flooding sporulating mycelium on agar plates with sterile water at 4°C for 4 h. Zoospores released from sporangia were collected by decanting the suspension through 10 μm nylon mesh and collected in 50 mL polypropylene centrifuge tubes, centrifuging at 4000 rpm for 10 min at 20°C . Sporangia were collected by flooding the sporulating mycelia with sterile water and passed through 30 μm nylon mesh to avoid mycelia contamination. Samples of *P. infestans* mycelia, sporangia and zoospores were crushed 1/100 (w/v) in protein coating buffer (disodium hydrogen phosphate). The extract was dispensed into each well of Microtitre plates and incubated at 37°C for 1 h. The plates were washed with PBST between each step and a final detection was made via addition of 1.0 mg/mL para nitrophenyl phosphate (PNPP) substrate, dissolved in diethanolamine buffer (pH 9.8) and measurement of colour development. After coating the plates with the different IgG concentrations, the plates were blocked for 1 h at 37°C using 5% non-fat dry milk (w/v) and 1% (w/v) BSA.

Validation of the antiserum for early detection

Twenty-four hours post inoculated potato leaf and tuber tissues were used for analysis. The late blight susceptible potato cultivar Kufri Bahar was inoculated with *P. infestans*. The inoculum was prepared from 7 day-old cultures grown on rye agar medium in the dark (18 ± 1 °C). *Phytophthora infestans* mycelia were harvested in sterile water, and stimulated to release zoospores at 4 °C. The sporangia suspension was observed under a haemocytometer and the concentration adjusted to 4×10^4 per mL. Kufri Bahar plants were first grown for 30 days in a greenhouse, then transferred to a *Phytophthora* inoculation chamber maintained at 18–20 °C and 85–90% relative humidity. The plants were inoculated (50 plants) with the sporangial suspension and samples were collected after 24 h for ELISA detection. Similarly, 50 tubers of Kufri Bahar were also inoculated with *P. infestans* for late blight assay. Besides, 25 samples of mycelia, sporangia and zoospores of *P. infestans* were used in the study. ELISA tests were performed as follows. Crude protein extract was prepared in coating buffer (disodium hydrogen phosphate (Na_2HPO_4) 2.38 g, potassium dihydrogen phosphate (KH_2PO_4) 0.40 g, potassium chloride (KCl) 0.40 g, sodium chloride (NaCl), 16.00 g, Distilled water 2.00 l, pH 7.40) using dilution of 1:100 (100 mg of leaf, sporangium and zoospore sample/1 mL buffer). The filtered extract was dispensed into each well of ELISA plate at the rate of 100 μL using a micropipette and the plate was incubated at 37 °C for 1 h. The contents of the plate was poured off and rinsed with PBS-Tween. Washing was carried out for three times allowing 3 min time interval for each wash. The unoccupied spaces in the wells were blocked with 4% skimmed milk protein and 2.8 g/100 mL of polyvinyl pyrrolidone (PVP). 200 μL of this solution was dispensed into each well. The plate was then incubated for 1 h at 37 °C. The contents of the plate was poured off and rinsed with PBS-Tween. Washing was carried out three times allowing 3 min time interval between each wash. CBD1 primary antibody was dispensed into each well at the rate of 100 μL and the plate was incubated at 37 °C for 1 h. The contents of the plate was poured off and rinsed with PBS-Tween. Washing was carried out for three times allowing 3 min time interval for each wash. Alkaline phosphatase labelled anti-goat IgG at specific dilutions (1:10,000) was (supplied by Genei, at Bangalore) added to the wells at the rate of 100 μL and the plate was incubated at 37 °C for 1 h. The contents of the plate was poured off and rinsed with washing buffer. Washing was carried out for three times allowing 3 min time interval for each wash. Substrate p-nitrophenyl phosphate (PNP) was prepared in 10% diethanolamine. 0.05 mM of magnesium chloride was added to this solution. 100 μL of the substrate was added to each well and incubated for 30 min–1 h at

37 °C. Colour reaction was recorded at 408 nm using ELISA reader.

Specificity of *P. infestans* antiserum

The specificity of *P. infestans* CBD1 peptide based antiserum was studied as described earlier using the indirect ELISA. Samples of total protein (0.5 mg/mL) were prepared from *P. infestans*, *P. cinnamomi* and *P. capsici*. Total protein extract of healthy Kufri Bahar leaves served as control. An absorbance value of at least double of the value of that healthy control was considered positive.

Results

CBD1 gene cloning

RT-PCR assay using CBD1 specific primers successfully amplified the 393 bp CBD1 gene of *P. infestans*. The amplicon was cloned into the pETSUMO vector. Colony PCR and restriction digestion analysis confirmed the insertion of CBD1 amplicon in the pETSUMO vector. Further, sequencing of the plasmid confirmed the orientation of insertion. The obtained nucleotide sequence has been submitted to NCBI (Accession No. KF860832).

Expression of recombinant CBD1 protein and synthesis of signal peptide

The CBD1 gene of *P. infestans* was expressed in *E. coli* and purified under native and denaturing conditions. The non-induced culture produced a lower amount of protein than induced ones as expected. The purified fusion CBD1 measured 12 kDa in SDS-PAGE (Fig. 1a). The total yield of CBD1 fusion protein fractions was 7.2 mg/mL. The nucleotide sequence was translated into protein sequence to identify the peptide sequence that is responsible for the CBD1 domain. The identified peptide (SNLRNGDSSVPVRT) was synthesized (Merck Biosciences (GeNei)). The CBD1 Peptide synthesis and Hapten conjugation was analyzed by HPLC method. The peaks indicated the presence of the synthesized peptide CBD1 (Fig. 1b, c).

Production of antiserum and titre analysis

The synthesized peptide (SNLRNGDSSVPVRT) concentration measured by absorbance method was 7.9 mg/mL, which was used to raise the antibody. The antibody was purified using 2 mL of protein A Agarose beads from 5 mL of rabbit serum. Purified antibody was dialyzed against 1 \times PBS and the purity of the antibody was 90% as analyzed by SDS-PAGE (Fig. 1d). The titer was determined through

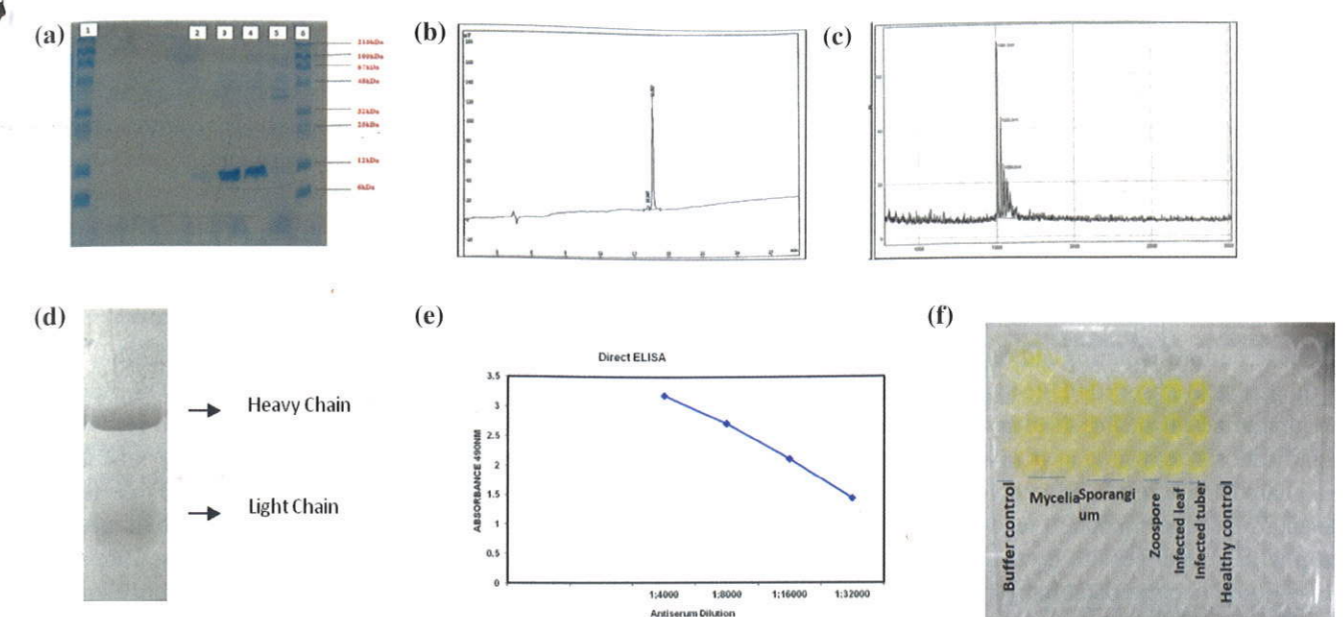


Fig. 1 Recombinant CBD protein expression and detection. **a** Analysis of recombinant CBD by SDS-PAGE gel stained with Coomassie staining solution (Lane 1: protein marker, Lane 2–4: Elution 3, 2 and 1, respectively of recombinant CBD1 after Ni-NTA chromatography; lane 5: Marker), **b** HPLC report for peptide synthesis, peak indicated that presence of CBD1 signal peptide, **c** HPLC report for

Peptide synthesis and hapten conjugation, peak indicates presence of CBD:hapten conjugate, **d** purified antibody detection using SDS-PAGE (25 μg), **e** determination titer of antibody by spectrophotometric analysis, **f** detection of CBD1 expression in *P. infestans* mycelia, sporangium, zoospores and infected potato leaf and tuber tissue using ELISA

Direct ELISA using purified peptide and the titre of the peptide antiserum was 1:32,000 (Fig. 1e). Similarly, the recombinant CBD1 antiserum was purified and evaluated by measuring its titer and efficacy in detecting *P. infestans* CBD1 using: I-ELISA and DAS-ELISA. DAS-ELISA was used to test the expression of CBD in *P. infestans* using its recombinant antiserum and peptide antiserum, and results showed that the peptide antiserum was more efficient than the recombinant antiserum (data not shown). Hence, the peptide antiserum was used for validation and specificity analysis. Several concentrations of IgG and IgG-AP conjugate were tested for DAS-ELISA optimization. Dilutions 1/350 and 1/400 of the peptide antiserum were the optimal dilutions for the IgG and IgG-AP conjugate, respectively.

Validation of the antiserum for early detection

The expression of CBD1 was detected using antiserum generated from the synthesized peptide of CBD1, in total protein isolated from *P. infestans* mycelium, sporangium, zoospores and early stage of infected potato leaf and tuber tissues using the indirect ELISA method. Results revealed that, compared with the healthy control sample, CBD1 expression was detected at strong signal density in mycelium, sporangium, infected leaf and tuber tissues, while in zoospores a weak signal (Table 1, Fig. 1f) was observed.

The amount of antigen (CBD1) in mycelium, sporangium, zoospores and infected leaf and tuber tissue was sufficient to discriminate between positive and negative samples in ELISA (Fig. 1f). Results indicated that a high amount of CBD1 protein expression was observed in mycelium followed by infected leaf and tuber tissues as compared to the sporangia and zoospores.

Specificity of CBD1 peptide antiserum

Cross reactivity of *P. infestans* CBD1 peptide antiserum was tested against 200 ng of total protein of *P. cinnamomi* and *P. capsici*. Absorbance value showed a very weak ELISA

Table 1 CBD1 expression in *P. infestans* infected leaf, tuber and in different stages of the pathogen (absorbance at 405 nm in ELISA)

| Sample | OD value @ 405 nm with SD |
|-----------------------------------|---------------------------|
| Mycelium | 2.15 \pm 0.03 |
| Sporangium | 1.89 \pm 0.04 |
| Zoospores | 1.32 \pm 0.04 |
| Kufri Bahar @ 24 hpi | 1.98 \pm 0.12 |
| Healthy Kufri Bahar | 0.09 \pm 0.19 |
| Kufri Bahar tuber sample @ 24 hpi | 1.69 \pm 0.14 |

signal for mycelium as well as for zoospores. This indicates that CBD1 domain has no cross reactivity with *P. cinnamomi* and nor *P. capsici*.

Discussion

Phytophthora infestans is difficult to identify when sporulation or other characteristic morphological structures are absent. The technique described herein eliminates the need for pure culture isolation and also complex extraction of pathogen DNA prior to PCR. Symptomless seed tubers are no guarantee for having healthy seed tubers, but bear the significant risk of bringing *P. infestans* to the field. The cell wall based antiserum ELISA could be used as a novel tool to detect *P. infestans* in potato seed-lots as well as in standing crop with greater sensitivity than was earlier possible and hence to reduce the potential for seed transmission of this destructive pathogen.

Serological and molecular detection tools can generate accurate results rapidly enough to be useful for management decisions. It is entirely depending on the use of molecular epitopes which are expressed during early phase of the pathogen infection and specificity of the pathogen. In the present work, we investigated the expression of cell wall localized cellulose binding module i.e. CBD1. The purified CBD1 generated in this study measured 12 kDa in SDS-PAGE which is consistent with that reported for CBD protein of *P. infestans* by Jones and Ospina-Giraldo (2011). The developed recombinant antiserum has the specificity for the specific epitopes coding for CBD1 signal domain. ELISA was performed using antiserum raised from synthesized CBD1 peptide and the assay was able to detect the presence and absence of the pathogen in terms of expression of pathogen CBD1.

As reported earlier, the presence of CBM1 may allow for interaction with cellulose outside of the cell wall. CBEL (carbohydrate binding elicitor lectin), is another domain which was associated with adhesion to cellulosic substrates, contribute to adhesion (Gaulin et al. 2002). The CBD1 synthetic peptide based antiserum produced in this study showed specificity to *P. infestans*. Both I-ELISA and DAS-ELISA, the antiserum successfully detected mycelia and sporangia of *P. infestans*. These results are in corroboration with the reports of Jones and Ospina-Giraldo (2011). Here, we reported the expression of CBD1 in zoospores at significant level compared to healthy control samples. The detection sensitivity of antiserum ranged from 100 to 200 ng of *P. infestans* mycelia, sporangium and zoospores total protein.

Protein purification problem encountered in oomycetes have been easily resolved by recombinant technology. One of the major advantages of this technology is high fidelity of the recombinant expressed protein and specific antibody

production without interfering with plant proteins. The recombinant bacterial clones carrying the plasmid insert of the cellulose binding protein genes can guarantee an endless supply of *P. infestans* antibodies. The CBD1 protein helps the overall integrity of the cell wall and protects it from attack of cellular enzyme glucanase secreted from plants and from saprophytic fungi (Picard et al. 2000). CBD1 antiserum, using ELISA technique detected the CBD1 expression range of 2.5 fold at early stage of the infected potato leaf and tuber tissues, compared with that of healthy tissues. These results are in agreement with those reported by earlier workers (Harrison et al. 1990; Kyneroova et al. 1998; Skottrup et al. 2006; Jones and Ospina-Giraldo 2011).

This is the first report showing the sensitivity of cell wall bound carbohydrate binding domain antiserum to detect the presence and absence of the pathogen in potato tissues. The present study has demonstrated that CBD1 based antiserum is effective in determining the presence of pathogen in host tissues and technique can be utilised by the farmers in the form of ELISA kit/lateral flow immune assay kit, after commercialisation. Endless supply of antigen from recombinant bacterial clones carrying the CBD genes and high sensitivity, feasibility are other advantages of this assay.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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