TÜRK
TARIM ve DOĞA BİLİMLERİ
DERGİSİ

TTDB

www.dergipark.gov.tr/turkjans

TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

Immunological Diagnostic of *Phytophthora infestans* from Host Tissues (Potato) by ELISA Method

[1]Touseef HUSSAIN*, [2]Bir Pal SINGH, [3]Firoz ANWAR

[1]Dept. of Life Science, Uttarakhand Technical University, Dehradun, 248009, U.K, India
[2]ICAR-Central Potato Research Institute, Shimla-171001, H.P, India
[3]Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia-80200

*Corresponding author: hussaintouseef@yahoo.co.in

Received: 06.02.2017 Received in Revised: 11.08.2017 Accepted: 11.08.2017

Abstract

Oomycete pathogens of the genus *Phytophthora* are the most destructive plant pathogens known. They spread mainly through the movement of infested soil, water and infected plants and plant material. Especially damaging as a source of inoculum are those plants/seeds that are infected but do not show signs of symptoms either because the disease has not yet progressed to the stage where symptoms are evident, or due to suppression of symptom development by the use of fungicides. In our indirect ELISA method, *P.infestans* exhibited strong positive reaction with sporangia (2.256), mycelium (1.256) as well as oospores (2.286) whereas no reaction with other fungal pathogens of potato. *P.infestans* was detected by indirect ELISA in potato leaves (1.212) and tubers (1.201). Our study was to confirm the detection of *P.infestans* irrespective of inoculum present in the host tissues (0.435), by which planting material by quarantine dept., horticultural dept. from one state to another. It could be used as reliable routine diagnostic test to replace current identification and isolation, detection methods.

Key words: *Phytophthora infestans*, oospores, potato, ELISA

Introduction

Potato (*Solanum tuberosum* L.) is a member of the night shade family (Solanaceae) and is the third most important crop in the world after Rice Wheat and Maize which is consumed by more than a billion people world-wide (Haas *et al*., 2009;Hultberg, 2010). Potato late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is a major problem in potato and tomato production throughout the world. Substantial yield reduction in potato attributed to foliar and tuber late blight occurs on a yearly basis (Guenther et al., 2003). Potato has also a place in history since the failure of Irish potato in middle of the nineteenth century led to one of the most dreaded famines in the history of western world followed by an unparalleled migration (Elansky et al., 2001). Worldwide, healthy seed potato production normally relies on certified seed potato propagation schemes. Serological methods based on mono- and polyclonal antibodies, such as Enzyme-Linked Immunosorbent Assay (ELISA), offer rapid, affordable, easily accessible and adaptable approaches to pathogen detection and quantification. This serological method has been successfully used for detection of viral, bacterial and fungal pathogens in numerous pathosystems, and can also be developed for *P. infestans* sexual oospores detection (Clark and Adam, 1977; Walsh et al., 1996; Afouda et al., 2008; Ceasar et al., 2007). Late blight appears every year in epiphytotic form in the Indian hills, plains and the disease causes premature foliar damage due to which entire crop is killed before achieving full growth leading to heavy crop losses. Current management options for potato late blight are based primarily on fungicide applications (Kirk et al., 2005) along with some, yet limited, alternative controls (Nyankanga et al., 2008; Olanya et al., 2008). Oospores of *P. infestans* are routinely observed in culture, in soil and in plants when opposite mating types are present, and can also be formed through self-fertilization in the absence of both mating types (Turkensteen et al., 1996; Flier et al., 2006).

However, little is known regarding the occurrence and role of oospores in the development and spread of late blight, or on the effectiveness of current control measures on oospore production/viability. Oospores have the potential to increase the genetic diversity of *P. infestans* and can survive for many years under adverse conditions (Turkensteen et al., 2000). Previous research has noted the presence of oospores in potato fields of the Toluca Valley in Mexico (Fernandez-Pavia et al., 2004), as well as in potato soils of the Netherlands (Turkensteen et al., 2000). However, little effort has been made in the India to assess oospore presence in potato soils and plants, mostly due to the lack of specific and expedient diagnostic tests. Current detection methods are based on tissue clearing/staining techniques and wet sieving/centrifugation followed by visual microscopic observations, which are tedious, time-consuming, and extremely limited in scope and the number of samples that can be processed. Modern molecular methods such as Polymerase Chain Reaction (PCR), or rDNA techniques are available for detection of *P. infestans* genetic material (Hussain *et al*., 2016a:2016b).

To the our knowledge, there is few report on the specific detection of *P. infestans* oospores with polyclonal antibodies. There is a need for a cost-effective test that can be done directly on dormant tuber samples at harvest and during storage to ensure early identification and detection of infected lots of seed tubers. In this study, mycelium and sporangia were studied besides oospores for this reason the purpose of our study was undertaken to evaluating the potential significance and control of *P. infestans* inoculums in potato production.

Materials and Methods

Isolation of *P. infestans* isolates

Late blight infected potato susceptible cv. Kufri Bahar leaves, stem, tubers collected during 2013 crop season (modipuram, Meerut city, U.P) and processed for isolating the blight pathogen. The selective media Rye A amended with antibiotics, Rifampicin (0.02 g/l), Polymixin B Sulphate (0.05 g/l), Ampicillin (0.10 g/l), Vancomycin (0.05 g/l) was used for the purpose of isolating *P. infestans*. The fungal isolates were maintained on Rye /Pea Agar media depending on the requirement (Caten and Jinks, 1968; Goodwin *et al.,* 1992a).

Preparation of *P. infestans* zoospore suspension

This was done following the method of Gupta, (2000). Tubers of susceptible cv. Kufri Bahar were surface sterilized with ethyl alcohol and cut into 8-10 mm thick slices using a sharp sterilized knife, placed in plastic petriplates (9mm) and inoculated with *P.infestans* by scrapping the fungal mycelium on slice surface with sterilized needle and incubated in air tight plastic boxes lined with moist foam at 18˚C for a week in the dark. A thick white growth of sporangiophore with plenty of zoosporangia covered the tuber slice surface within 5-6 days. Tuber slices containing *P.infestans* were gently dipped in sterile distilled water to dislodge the zoosporangia and incubated at 12˚C for one hour for release of zoospores.

Production of oospores

P. infestans oospores were developed on Rye agar media by pairing A_1 and A_2 mating types *in vitro*. The isolates belonging to A_1 and A_2 mating types were paired by placing them at 3-4 cm. apart on the Rye agar medium in a petridish (9mm). The perti dishes were incubated at 18±1^{o}C for 10 days under dark (Shattock et al., 1986, Singh et al., 1994; Gupta, 2000).

Extraction of oospores

Oospores formed at the interface of interacting A_1 and A_2 colonies were extracted by modifying the method of Forster et al., (1983). The mycelium at the interface of the paired isolates was extracted by scrapping with the help of a spatula. The agar scrappings containing oospores were grounded in distilled water using an electric grinder. Dispense the slurry containing oospores in 1.5ml micro centrifuge tube by adding a pinch or 0.5 mg/ml of lysing enzyme and vortex thoroughly. Kept these tubes at 18^{o}C. After 24 hours, the mycelial fragments were completely digested by lysing enzyme. Remove the lysing enzyme by washing 3 or more times with sterile distilled water *via*. Centrifugation for 10,000 rpm for 10 minutes. This experiment was carried out under aseptic conditions. Store the tubes in refrigerator till use.

Indirect ELISA method

Disposable polystyrene flat bottom micro-ELISA plates were used in the study. The double sand witched method was used with alkaline phosphate enzyme, following the test procedures described by Clark and Adam, (1977). Different tissue of late blight infected tuber and leaves were used along with sporangia, oospores and mycelium for indexing individually. Late blight infected samples were collected during 2013, crop season from Shimla, Himachal Pradesh, India. Extract of all source were diluted in 1:10 in extraction buffer.

Preparation of antigen from *P. infestans* strain

The antigen was prepared from total protein extracted from mycelium of *P. infestans* isolate H.P1 (highly virulent isolate). The mycelial mat was filtered through muslin, washed twice with phosphate buffer (pH: 7), and lyophilized. Lyophilized mycelium (2 gm) was ground with liquid nitrogen. 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°C. The supernatant was concentrated over polyethylene glycol 6000 and then dialyzed against Phosphate Buffer (0.1 M, pH 7) for 24hours. The pellet was re-suspended in 1ml of Phosphate buffer. The protein content was determined (Bradford, 1976) and adjusted to 1mg/ ml in Phosphate buffer and stored at - 20°C as antigen.

Preparation of Antiserum against *P. infestans*

Antiserum against *P. infestans* (HP1) was prepared using New Zealand white rabbit (maintained at CPRI, Shimla animal house) by four weekly intramuscular injections with purified preparation of the fungus at concentration of 2mg/ml. For the first injection the purified fungus was emulsified with an equal volume of Freund´s complete adjuvant (Difco lab, U.S.A.) 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×*g* for 30 min at 4˚C and stored at - 20˚C until use (Srivastava and Arora, 1997).

Determination the Cross-reactivity of Antibodies

Culture of *P.palmivora*, *P.capsici*, *P.colocasia*, *P.cactorum* (obtained from IISR, Calicut), *Rhizoctonia solani* AG-3, *Fusarium* sp., *Alterneria solani* (maintained at CPRI, Shimla). Mycelium extracts of each culture will be diluted in PBS-Tween at a desired concentration and tested for cross-reactivity by ELISA on microtiter plates in comparison with *P. infestans* sexual oospores. The detection limits of antibodies will also be tested by preparing several concentrations of the antigen.

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 (that were previously found positive with PCR reaction) were immersed in liquid nitrogen, ground into a powder using a mortar and pestle, and then suspended in 5mL phosphate buffer pH7 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 hours at 37˚C or overnight at 4˚C. The plates were rinsed thrice by flooding wells with phosphate buffer saline + tween 20 (PBST), 5 minutes each. Aliquots (100 µl) from the diluted antiserum (1:200 diluted in PBST) were added to each well, after which the plates were incubated at 37˚C for 2 hours or at 4˚C overnight, 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 100Cl was added to each well, followed by one hour incubation at 37˚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˚C) for about 30 min. The enzyme activity was stopped by adding 50 µl of 3M NaOH. Intensity of colour was measured at 405nm using a micro plate titre reader (TECAN A-5082, Sun Rise, Austria). Absorbance of test samples compared with the healthy control. Samples showing absorbance (A_{405}) values more than two times the healthy control was considered as positive. In each set of test, wells lacking antigen (coating buffer only) were included as blanks. The cross-reactivity between tested potato antigens and *P. infestans* antiserum were detected. Cross-reactivity with phosphate buffer was served as control.

Result and Discussion

Antiserum production

Rabbits were immunized by using 2 mg/ml of the powdered mycelium. Reactions could be read 30 min after application of the substrate. Incubation for 60 min did not change readings for the negative control appreciably, but more *P.infestans* culture samples were off-scale.

Determination of antiserum titer

The antiserum titre was checked with microprecipitin test and was found to be 1:32(vol/vol), providing optimal reactivity of *P.infestans* in ELISA. The titer of *P.infestans* antiserum was determined by using indirect ELISA.

Extracts (50 µg) from total protein of *P.infestans* was used as positive control while healthy potato cultivar (cv. Kufri Bahar leaves) served as negative control. Different serial dilutions up to 1:32 of antiserum were used and tested. Absorbance values of at least double of that of the control were considered positive. Results showed positive ELISA values for the antiserum were up to 1:64 but not considered for further study, because the antiserum was showing improper readings as mentioned in Table 1.

Table 1. Standardization of concentration antisera titer of *P. infestans*

Sr.No.	Antisera Titer	Tissue leaf	Tuber Mycelium	Sporangia	Zoospore
1	1:1	++++	++++	++++++	++++++
2	1:2	++++	++++	++++++	++++++
3	1:4	++++	++++	+++++	++++
4	1:8	++++	++++	++++	+++++
5	1:16	+++	+++	++++	++++
6	**1:32**	**+++**	**+++**	**+++**	**+++**
7	1:64	++	++	+++	+++
8	1:128	+	+	+++	+++
9	1:256	-	-	++	++
10	1:512	-	-	+	+

Where ++++++ Higher interaction +++++ good interaction ++++ interaction +++ fair interaction ++ poor interaction + very poor interaction - No reaction

Purification of IgG and estimation of its concentration

IgG was purified as per procedure described in the materials and methods and its concentration was estimated 10.6mg/ml estimated by the standard protocol.

Standardization of ELISA

ELISA protocol was standardized by checker board with the dilutions 1:200, 1:400 and 1:800 both for IgG and Enzyme Conjugate. Infected and healthy plant sap dilution used was 1:10 and 1:50 respectively. The optimal dilution was found to be 1:200 both for IgG and EC (Table 2).

Table 2. ELISA reading at 405nm

Sr. No	Antigen Titre	Healthy Control	Buffer Control	Oospores	Sporangia	Mycelium	Tissue Leaf	Tissue Tuber
1	1:200	0.079	0.071	2.286	2.256	1.256	1.212	1.201
2	1:400	0.062	0.052	2.001	1.520	1.702	0.684	0.744
3	1:800	0.046	0.036	0.861	0.513	0.191	0.171	0.156

Table 3. Cross-reactivity check of IGg

Healthy control	Buffer control	*P.infestans*	*Fusarium* sp.	*R.solani*	*A.solani*	*P.capsici*	*P.cactourm*	*P.colocasiea*	*P.pamlivora*
0.129	0.117	0.435	0.124	0.123	0.124	0.322	0.332	0.330	0.332
0.130	0.116	0.433	0.123	0.122	0.123	0.330	0.331	0.335	0.334
0.131	0.117	0.436	0.125	0.124	0.125	0.332	0.332	0.333	0.336

Data in Table 2 revealed that, indirect ELISA absorbance values (E405nm) were higher in the case of oospores (2.286) and sporangia (2.256), followed by mycelium (1.256), as compared with infected leaf (1.212) and tuber (1.201). These results indicates that higher reaction and more specific antigens were detected in the sexual oospores and asexual sporangia than in the artificially infected potato leaf and tuber.

The cross-reactivity between tested antigens and *P. infestans* antiserum were detected. Data in Table (3) revealed that, indirect ELISA absorbance values (E405nm) were higher in case of other *Phytophthora* species, *P.capsici* (0.322) and *P.cactorum* (0.322), *P.colocasiea* (0.330) and *P.pamlivora* (0.332) compared with other potato fungal pathogens *Fusarium* sp. (0.124), *Rhizoctonia solani* AG-3(0.123) and *Alterneria solani* (0.124).

These results indicating that, higher reaction and more specific antigens were detected in the other *Phytophthora* species than in the other fungal pathogens of potato. Such results are in agreement with those reported by Abd-El-Rehim and Hashem (1970); Abd-El-Rehim and El- Meleigi (1973); Abd-El-Rehim *et al*., (1974); Palmerly and Callow (1978); Alba and DeVay (1985); Tohamy, (1985) and El-Kaffash, (1990), who indicated the presence of more specific antigens between the susceptible host and the pathogen than the resistant host. However, there are some reports which contradict these findings which support the common antigen. Barna *et al*., (1978) reported that the protein sharing between hosts and pathogens was poor if any. Likewise, Alba *et al*., (1973) did not detect any common antigens between uredospores antiserum of *Hemileia vastatrix* and their corresponding resistant and susceptible Coffea Arabica.

Assay parameters, sensitivity, and specificity:

Polyclonal antibody IgG used at 2 µg/ml for coating plates used at a dilution of 1:32 (vol/vol) as the detection antibody provided optimal reactivity with *P.infestans* in the ELISA. Reactions could be read 30 min after application of the substrate. Incubation for 60 min did not change readings for the negative control appreciably, but more *P.infestans* culture samples were off-scale (Table 1). The ELISA was very sensitive and could detect *P.infestans* in culture extracts of HP-1 diluted to 1:5,000,000 (wt/vol; Table 1). Extracts of other potato fungal isolates exhibited no positive reactions in the tests. The ELISA was specific to *P.infestans* only. All other isolates of *Phytophthora* species reacted strongly with the antibodies in the ELISA, with mean OD values exceeding 0.200 (Table 3). All of these reactions were considered to be negative compared to the buffer control.

Detection of *P.infestans* in host tissues

P.infestans was detected by ELISA in leaves, tubers that were collected during potato crop season (Table 3). Both samples of *P.infestans* infested, OD readings exceeded 1.2. Both *P.infestans*-infected potato samples yielded positive OD readings ranging from 1.200 to over 1.212. These values were in range with those of oospores and sporangia having similar infection levels. There were very high correlations ($r > 0.99$) between OD readings and theoretical infection levels.

Conclusion

Potato cultivated in different regions of the world is severely affected by a number of diseases infected by fungi, bacteria, viruses, nematodes and phytoplasmas which reduce the production in both quality and quantity through their adverse effects (Qamar *et al*., 2003).Visual identification procedure also requires placement of infected tissue on culture medium or in moist chambers to observe growth and /or sporulation characteristics of *P.infestans*, which may take 6-7 days for conformation. Similarly quantification of diseased tissue is based on visual observation. The sensitive detection and quantification of *P. infestans* in tubers, other host plant and field soil samples are important steps in the development of a strategic programmes for the early diagnosis and management of late blight diseases. The specificity and sensitivity of this ELISA system makes this method a precise and rapid tool for identification, detection of *P.infestans* inoculum in host tissues and for diagnosis of disease caused by the pathogen. Furthermore, the system has excellent potential for direct quantification of the fungus in plant tissues and, therefore, has immediate applications in research on the pathogen's ecology, epidemiology, and host interactions.

The presence of specific common antigen between a pathogen and host has been discussed by several authors (Abd-el-rehim and Hashem, 1970; Abd-el-rehim and El-melegi, 1973 and Abd-el-rehim *et al*., 1974). Earlier studies stated the use of Polylconal antibody for the detection of *P.infestans* from the host tissues (Beckman *et al*., 1994). During our study Anti-*P.infestans i.e.* IgG reacted strongly with an extract of *P.cactorum*, *P.colocasiea, P.palmivora* and *P.capsici* in indirect-ELISA method. Thus although polyclonal antibodies may be useful for detecting a range of *Phytophthora* species, it is likely that monoclonal antibodies will be required to identify individual species (Hardham *et al.,*1986) which supports our study also. In previous studies also (Hardham *et al.,* 1986; Estrada-Gracia *et al.,* 1989; Hardham *et al*., 1994) species specific antibodies that react with components on the surface of zoospores and cyst have been obtained. PAbs that react with cyst or hyphal walls, on the other hands are not species specific as reported by Hardham *et al.,* 1986; Ali-shtayeh *et al.,* 1991; Miller *et al*., 1997). This is due to the fact that hyphal walls contain many components that are common wall constituents. These components may dominate the immune response and thus reduces the chances of obtaining the species specific antibodies (Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981; Hardham and Mitchell, 1998). PAbs raised by using mycelium or hyphal cell wall extracts against *Phytophthora* species have not proved to be species-specific. These studies are in agreement with our results

also (Malajczuk *et al.,* 1975; White *et al.,* 1976; Mohan, 1988).

Harrison *et al*., 1990 developed a polyclonal antiserum, which reacted with mycelial extracts of two *Phytophthora* spp. but not with those of 10 other pathogens of potato. *P.infestans* was readily detected by ELISA using either the plate trapped antigen or F(ab`) antibody fragment techniques. The amount of mycelium was estimated by comparing the values obtained in ELISA with those for known concentrations of *P.infestans* mycelium. Earlier in similar type of tests Hardham *et al*., 1986 found that anti *P. fragariae* γ-globulin reacted strongly with extracts of *P.infestans*, *P.cryptogea* and *P.nicotianae* but not with those of four other unrelated fungi. Thus, although polyclonal antibodies may be useful for detecting a range of *Phytophthora* spp. it is likely that monoclonal antibodies will be required to identify individual species. Commercially available ELISA tests for *Phytophthora* detection based on antibodies to a generic *Phytophthora* antigen have proved useful as a quick means of identifying infected plants although they can only identify to the genus level (McDonald *et al.,* 1990; Ali-Shatayeh *et al.,* 1991). Knapova *et al.,* (1992, 1993) report on the successful use of the ELISA to detect this fungus like pathogen at a very low concentration after it manifested itself in the potato plant. However, problems may occur regarding the specificity of the antiserum used resulting in cross-reactions. But for a routine testing of samples this protocol can be used for the detection of *P.infestans*. As since there is no any significant reports that other *Phytophthora* sp. infecting potato and hampering in healthy seed production. Easy to-use-test-kit can help to determine whether or not certain plant pathogens are present so we can make an informed decision. ELISA test kits reliably determine whether target pathogen is present. The test kit is only one tool in an overall diseases monitoring progress in potato production.

Acknowledgements:

This study was made possible through the financial support and fellowship to the first author from the ICAR-New Delhi-Phytofura Project.

References

Abd El-Rehim, M.A., Hashem, M.A. 1970. A serodiagnosic method for the differentiation between resistance and susceptible Egyptian cotton varieties to infection with *Fusarium oxysporum*. *Phytopathology*, 67: 180-182.

Abd El-Rehim, M.A., El-Melegi, M.A. 1973. Serological and immunoelectrophoretical differences between *Fusarium* species. *Biochemical Systematics and Ecology,* 8: 7-9.

Abd El-Rehim, M.A., Ibrahim, I.A., Michail, S.H., El-Melegi, M.A. 1974. Serological relationships between *Pythium irreglare* buisman and certain Tomato cultivars. Zb1. Bokt. Abt. H, Bd., 129: 179-182.

Alba, A.P.C., Devay J.E. 1985. Detection of cross reactive antigens between *Phytophthora infestans* (Mont.) de Barry and *Solanum* species by indirect enzyme linked immunosorbent assay. *Phytopathol. Z.* 112: 97-104.

Ali-Shtayeh, M.S., MacDonald, J.D., Kabashima J., 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water.*Plant Disease,* 75: 305-311.

Afouda, L., Wolf, G., Wydra, K. 2008. Development of a sensitive serological method for specific detection of Latent infection of *Macrophomina phaseolina* in cowpea. *J. Phytopathology.*

Bartnicki-Garcia, S. 1968.Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Review of Microbiology*, 22: 87-108.

Barna, B., Balazs, E., Kiraly, Z. 1978. Common antigens and rust diseases. *Phytopathol. Z.,* 93: 336-341.

Beckman, K.B., Harrison, J.G, Ingram, D.S. 1994. *Physilology Mol. Pathol.*44, 19.

Bradford, M.M. 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72: 248-254.

Caesar, T., Lartey, R.T., Shelver, W.L. 2007. Enzyme-linked immunosorbent assay for *Cercospora beticola* in soil. *Journal of Sugarbeet Research*, 44: 51-70.

Clark, M.F., Adam, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, 34: 475-483.

Caten, C.E., Jinks, J.L. 1968. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural Variation. *Canadian Journal of Botany,* 46: 329-348.

El-Kaffash, W.M.I. 1990. Specificity of host lectins to the corresponding pathogens. M. Sc. Thesis, Faculty of Agriculture, Alexandria University, Egypt, pp: 84

El-Nashaar, H.M., Moor, L.W., George, R.A. 1986. Enzyme linked immunosorbent assay

quantification of initial infection of wheat by *Gaeumannomyces graminis* var. *tritici* as moderated by biocontrol agents. *Phytopathology,* 76: 35-47.

Elansky, S.N., Smirnov, A.N., Dyakov, Y., Dolgova, A., Filippov, A., Kozlovsky, B., Kozlovskaya, I., Russo, P., Smart, C., Fry, W.E. 2001. Genotypic analysis of Russian isolates of *Phytophtora infestans* from the Moscow region, Siberia and Far East*. J. Phytopath*., 149: 605-611.

Estrada-Garcia, M.T., Green, J.R., Booth, J.M., White, J.G., Callow, J.A. 1989. Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens, *Exeprimental Mycology*, 13: 348-355.

Fegla, G.I., El-Samra, I.A., Noaman, K.A., Younes, H.A. 1997. Host range, transmission and serology of an isolate of tomato yellow leaf curl virus from tomato of plastic houses in northern Egypt. Proc.1st scien. Conf. of Agric. Assiut Univ., Assiut, December 13-14: 549-568.

Fernandez-Pavia, S.P., Grunwald, N.J., Diaz-Valasis, M., Caldena_Hinojosa, M., Fry, W.E. 2004. Soilborne sopores of *Phytophthora infestans* in Central Mexico survive winter fallow and infect potato plants in the field. *Plant Disease*, 88: 29-33.

Forster, H., Ribeiro, O.K., Erwin, D.C. 1983.Factors affecting oospore germination of *Phytophthora megasperma* f. sp. *medicaginis. Phytopathology,* 73: 442-448.

Flier, W.G., Kessel, G.J.T., Scheppers, H.T.A.M., Turkensteen, L.J. 2006. The impact of oospores of *Phytophthora infestans* on late blight epidemics. Working papers - In: Plant Research International, Wageningen, Netherlands, pp. 18-22.

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N., Fry, W.E. 1992b.Clonal diversity and genetic differentiation of *Phytophthora infestans* population in northern and central Mexico. *Phytopathology,* 82: 955-961.

Guenthner, F.F., Michael, K.C., Nolte, P. 2001. The economic impact of potato late blight on US growers. *Potato Research,* 44: 121-125.

Gupta, J. 2000. Variability and epidemiology of *Phytophthora infestans* affecting potatoes in India. Ph.D thesis. Rafi Ahmad Kidwai Institute of Agricultural Sciences. Aligarh Muslim University. Aligarh. pp.172.

Hardham, A.R., Mitchell, H.J. 1998. Use of molecular cytology to study the structure and biology of phytopathogenic and mycorrhizal fungi. *Fungal Genetics and Biology*, 24: 252-284.

Hardham, A.R., Cahill, D.M., Cope, M., Gabor, B.K., Gubler, F., Hyde, G.J. 1994. Cell surface antigens of *Phytophthora* spores: biological and taxanomic characterization, *Protoplasma*, 181: 231-232.

Harrison, J.G, Barker, H, Lowe, R, Rees, E.A. 1990. Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by Enzyme linked immunosorbent assay. *Plant Pathol*, 39: 274-277.

Hardham, A.R, Suzaki, E, Perkin, J.L., 1986. Monoclonal antibodies to isolates species and genus specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamon*.*Can. J. Bot*., 64: 311-21.

Haas, B.J, Kamoun, S., Zody, M.C., Jiang, R.H.Y., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D, Raffaele, S. and Torto-Alalibo, T. 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature*, 461: 393-398.

Hultberg, M., Bengtsso, T., Liljeroth, E. 2010. Late blight on potato is suppressed by the Biosurfactant producing strains *Pseudomonas koreensis* 2.74 and it's Biosurfactant. *BioControl,* 55: 543-550.

Hussain, T. 2016a.Detection and quantification of *Phytophthora infestans* from host tissue and infested soil.Ph.D.Thesis. Dept. of Life Science,Uttarakhand Technical University, Dehradun, U.K.p150.

Hussain, T.2016b.Diagnostic of most important pathogen on potato through PCR techniques: A systematic Review.*Trends in Biosciences.9(4):203-212.*

Knapova, G., Schober-Butin, G., Fehrmann, H. 1992. Nachweis von *P.infestans* (Mont.) de bary mittels ELISA. *Mitt. Biol. Bundesanst*. 283-328.

Knapova, G., Schober-Butin, B., Fehrmann, H. 1993.Nachweis von *P.infestans* in Gewebe. Abstract 12th Triennial Conference of the European Association for Potato Research, Paris, 467-468.

Kirk, W.W., Abu-El Samen, F.M., Muhinyuza, J.B., Hammerschmidt, R., Douces, D.S., Thill, C.A., Groza, H., Thompson, A.L. 2005. Evaluation of potato late blight management utilizing host plant resistance and reduced rates and frequencies of fungicide applications. 24: 961-970.

Malajczuk, N., Mccomb, A.J., Parker, C.A. 1975. An immunofluorescence technique for

detecting *Phytophthora cinnamomi* Rands. *Phytopathology*, 85: 1057-1063.

McDonald, J.D., Stites, J., Kabashima, J. 1990. Comparison of serological, and culture plate methods for detecting species of *Phytophthora, Pythium*, and *Rhizoctonia* in ornamental plants. *Plant Disease,* 74: 655-659.

Mohan, S.B. 1988. Evaluation of antisera raised against *Phytophthora fragariae* for detecting the red core diseases of strawberries by enzyme-linked immunoasorbent assay (ELISA). *Plant Pathology*, 37: 206-216.

Miller, S.A., Madden, L.V., Schmitthenner, A.F. 1997. Distribution of *Phytophthora* spp.In field soils determined by immunoassay. *Phytopathology*, 87:101-107.

Nyankanga, R.O., Wien, H.C., Olanya, O.M. 2008. Effects of mulch and potato hilling on development of foliar blight (*Phytophthora infestans*) and the control of tuber blight infection. *Potato Research,* 51: 101-111.

Olanya, O.M., Plant, A.B., Larkin, R.P., Honeycutt, C.W. 2008. Infection potential of hairy nightshade by *Phytophthora infestans* and late blight implications of the alternate host. *J. Phytopathology.*

Palmerley, R.A., Callow, J.A. 1978. Common antigens in extracts of *Phytophthora infestans* and potatoes. *Physiol. Plant Pathol.* 12: 241-248.

Qamar, N., Khan, M.A., Rashid, A. 2003. Screening of potato germplasm against potato virus X (PVX) and potato virus Y (PVY). *Pakistan J. Phytopath*., Vol. 5(2): 181-184.

Shattock, R.C., Tooley, P.W., Fry, W.E. 1986. Genetics of *Phytophthora infestans*: Characterization of single-oospore cultures from A_1 isolates induced to self by intraspecific stimulation. *Phytopathology,* 76: 407-410.

Singh, B.P., Roy, S., Bhattacharyya, S.K., 1994**.** Occurrence of the A_2 mating type of *Phytophthora infestans* in India**.** *Potato Research,* 37: 227-231**.**

Srivastava, A.K., Arora, D.K. 1997. Evaluation of polyclonal antibody immunoassay for detection and quantification of *Macrophomina phaseolina. Plant Pathol*., 46: 785-794.

Tohamy, A.T.A., 1981. The roles of certain host biochemical components in pathogenesis. Ph.D. Thesis, Faculty of Agriculture, Alexandria University, Egypt, pp: 96.

Turkensteen, L.J., Flier, W.G., Mulder, A., Baarlen, V.P. 1996. Evidence for sexual reproduction of *Phytophthora infestans* in an allotment garden complex in the Netherlands. Pp.356-357 In: Abstracts of conference papers, posters and demonstrations. 13th Tiennial Conference of the European Association of Potato Research, Veldhoven, Netherlands, 14-19, July 1996.

Turkensteen, L.J., Flier, W.G., Wanningen, R., Mulder, A. 2000. Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* 49: 688-696.

Walsh, J.A., Merz, U., Harrison, J.G. 1996. Serological detection of spore balls of *Spongospora subterranea* and quantification in soil. *Plant Pathology,* 45: 884-895.

Wessels, J.G.H., Sietsma, J.H. 1981. Fungal cell walls a survey, in Encyclopedia of plant Physiology New Series Volume 13B. Plant Carbohydrates (Tanner, W. and Loewus, F.A. Eds), Springer-Verlag, Berlin, pp. 352-394.

White, D.G. 1976. The preparation and use of a fluorescent antibody reagent for the detection of *Pythium graminicola*, *Phytopathology*, 66:523-525.