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**Research Paper**

# Development of enzyme immunoassay for quantitation of mycelial biomass in *Brassica* genotypes against Alternaria blight

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## Abstract

*Brassica* crops faces a major infestation of *Alternaria brassicae*, a causal agent of Alternaria blight. Adopted chemical methods for controlling *Alternaria* infestation are ineffective and environmentally challenging. To bypass these challenges, strategies of enhancing innate immunity of *Brassica* plant resistance to pathogen, or suppression of virulence seems promising. For suppression of virulence early detection of pathogen, before symptom appearance is the main limitation. Efficient disease management appropriately buffered with resistance disease programmme depends largely on the reliable identification and characterization of fungal isolate. Therefore, there is a need to develop a potential tool for immunological characterization of isolates of *A. brassicae*. Polyclonal antibody against total mycelia of *A. brassicae* was generated to develop ELISA based method for early detection and quantitation of mycelial biomass. Total protein was extracted from pathogen inoculated Brassica leaves (Varuna and PAB 9511) during different time interval of infection. The quantitative estimation of mycelial biomass of *A. brassicae* was estimated by indirect ELISA using polyclonal antiserum. When compared with healthy control, pathogen was not detected in the susceptible cultivar Varuna until after 6 h of post-inoculation. It was also observed that among the inoculated genotypes of *Brassica*, mycelium biomass was increased with disease progression in susceptible cultivar (Varuna), while the tolerant cultivar (PAB 9511) contained less. This demonstrated the efficacy of adopted immunological technique as a potent tool for detection, quantitation of mycelial biomass for characterization of differential tolerance against Alternaria blight in *Brassica* genotypes.

**Key words:** *Alternaria brassicae, Alternaria blight, ELISA*

## Introduction

Rape seed mustard is the second most edible oil seed crop in India. Compare to other edible oils, the rape seed mustard oil has the lowest amount of harmful saturated fatty acids and it also contains adequate amount of the two essential fatty acids linoleic and linolenic acid. Productivity of rapeseed mustard in India varies significantly from state to state, depending on the climatic conditions and other environmental factors prevailing in the respective areas. Low productivity of rape seed is attributed to its susceptibility of various diseases and the major one is Alternaria Blight (Meena *et al.*, 2010). A special pattern of development of several *Alternaria* species on leaf discs of host and non host is known. When pathogen attacks on leaf, firstly the germination of spore takes place followed by germ tube formation and formation of appressorium. It is found that in host and non host species pathogen penetration is not different. However, plant responses to attempt penetration, which includes the formation of callose-containing papillae, callose deposition in the walls of attacked cells and their neighbouring cells and cell necrosis, varies with specific pathogen–plant interactions (McRoberts *et al.*, 1996). Pathogen activities focus on colonization of the host and utilization of its resources, while plants are adapted to detect the presence of pathogens and to respond

with antimicrobial defenses and other stress responses (Jinrong *et al.*, 2002). Early detection and correct identification of fungal pathogens is of primary importance in determining the most effective course of treatment to prevent the spread of fungi causing plant disease and post harvest storage losses. Thus in many aspects of plant pathology, there is a need for assay systems such as immunoassays that are specific, sensitive and quick to confirm visual systems, to detect early and latent infections before symptoms are visible (Dewey *et al*., 1989; Xia *et al*., 1992; Brill *et al*., 1994). Most of the antibodies in fungal antisera that are non specific are of the immunoglobulin class IgM, whereas many of the antibodies that are species specific are IgG antibodies (Cahil and Hardham 1994; Thronton *et al*., 1993, 1994). Use of such highly specific antibodies can help in identification of pathogen races and thus can help in pathotyping (Mohan, 1988). ELISA has been proven to be useful for monitoring the antigenic properties in the process of population shift during the sexual development of fungus together with the determination of genetic diversity among the pathogens and subsequently for immunopathotyping. Using *Stachybotrys chartarum* as a model fungus Schmechel *et al*. (2006) demonstrated that monoclonal antibodies (mAbs) can provide species specific diagnostic reagents and also be used to investigate immunological cross-reactivity patterns among fungi. They demonstrated that mAbs and immunoassays have the potential to detect *S. chartarum* species-specifically. Kamraj *et al*. (2012) studied on *Aspergillus oryzae*, extracted protein from the fungi and used it as an antigen to developed probe. This probe was used to develop a sensitive, rapid, and inexpensive screening test to detect *Aspergillus* in agricultural commodities. Dewey *et al*. (1990) studied on *Penicillium islandicum*, a fungus commonly isolated from stored rice grains in South-East Asia. They raised monoclonal antibodies (mAbs) against *Penicillium islandicum* and hybridoma supernatants were screened by ELISA. Most of the mAbs raised cross-reacted with other storage fungi or uninfected rice grains but three were species-specific. One of these, PIO1, was used to develop ELISA and DIP-STICK assays for the detection of *P. islandicum* in individual grains. Fox *et al*., (2004) also developed an indirect immunofluorescence spore assay (IFSA) to detect urediniospores of *Phakopsora pachyrhiz*. Shamala *et al.* (2006) studied that mAbs raised against *G. boninense* were not specific as the mAbs gave positive signals through the cross-reactivity test with all fungi tested in the cross-reactivity. Schmechel *et al*. (2008) studied on *A. alternata*. They developed pAbs (polyclonal antibodies) against *A. alternata* which showing cross-react broadly with related and nonrelated fungi.

In our study polyclonal anti-mycelial antibodies is developed against mycelia antigen(s) of *A. brassicae*. Concentration of mycelial antigen(s) and antimycelial antibodies were optimized for development of enzyme immunoassay. Cross reactivity studies was conducted with different isolates of *A. brassicae*.

## Materials and Methods

### Source of plant material

Seeds of two *B. juncea* cultivars one PAB 9511 (tolerant) and the other Varuna (susceptible) used in this study were obtained from CRC Pantnagar, India and grown in pots containing mixtures of soil: sand: vermicompost (2:1:1). Plants were kept in a glass house at 22ºC for 16 h photoperiod.

### Isolation of *Alternaria brassicae* isolates

Ten genotypes of *A. brassicae* were taken, viz Ashirwad (AS), Divya (DV), JD6 (JD), Kanti (KA), Kranti (KR), Local Mustard (LM), NDRE4 (ND), PRE2007 (P7), PRE2010 (P10), Varuna (VR) obtained from Crop Research Centre, Pantnagar, India. Isolates were grown on potato dextrose broth media and incubated at 22°C in the dark for the 15 days to allow growth of the pathogen.

### Artificial inoculation of pathogen

At the four leaf stage, plants of PAB 9511 and Varuna cultivars were inoculated with spore suspension ($10^5$ per ml) of *A. brassicae*. Fungal Spores were taken from infected leaves of *B. juncea* cultiver Varuna. *A. brassicae* inoculated plants were placed in moist chamber in a greenhouse at 22°C and 100% of relative humidity (RH). Control treatment was treated with sterilized distilled water.

## Serological studies

### Preparation of antigen

The antigen was prepared from total protein extracted from mycelium of *A. brassicae* isolate NDRE4. The mycelial mat was filtered through muslin cloth, washed with phosphate buffer saline (pH 7.4) and lyophilized. Lyophilized mycelium (1 mg) was ground with liquid $N_2$. PBS was added to the ground mycelium (El-Nashaar *et al*., 1986) and the suspension was centrifuged at 12000 rpm for 30 min at 4°C. The supernatant containing total protein was obtained and protein concentration was determined by Bradford estimation (Bradford, 1976). Total protein concentration was adjust to 1 mg $mL^{-1}$ and stored at -20°C.

### Preparation of Antimycelial antibody

Antiserum against *A. brassicae* was prepared using New Zealand white rabbit by four weekly subcutaneously injections with mycelial antigen of 1 mg mL-1. First dose was given by antigen emulsified with an equal volume of Freund´s complete adjuvant (Difco lab, USA) and for subsequent boosting doses were used by Freund´s incomplete adjuvant. The doses 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 and 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 -20ÚC until use (Srivastava and Arora, 1997).

### ELISA for detection of changes in antigenic determinants

The ELISA was developed with minor modification as described initially by Engvall and Perlmann, (1971) and later by Clark and Adams (1977). Lyophilized mycelium was dissolved in PBS at the rate of 20 mg/ml. Polystyrene micro plates were coated with mycelia fragments and plant extract treated mycelium harvested at 14$^{th}$day and allow incubating overnight at 4ÚC. The plates were washed with PBS+Tween-20 (0.01%). The plates were filled with PBS containing 5% skimmed milk for 2hr. at RT. 100µl (1:250 dilution) of 1$^{st}$ antibody (Raised against respective antigens) were added and incubated for 2 hrs at RT. Washing was done thrice with PBS + Tween-20 (0.01%) + 0.25% BSA. 100µl of 1:1000 diluted alkaline phosphate conjugated secondary antibody was incubated for 2hr at room temperature. plates washed with PBS containing 0.25% BSA + Tween-20 (0.01%) thrice. Alkaline phosphate activity was assayed with p-nitrophenyl phosphate sodium salt. The plates were incubated for 30 min in dark and the reaction was stopped with 100µl of 1.5 M NaOH solution. Reading was taken at 405nm in ELISA reader.

## Results and Discussion

### Immuno pathotyping

Polyclonal antibodies raised against whole lyophilized mycelia of virulent pathogen of *A. brassicae*. Antimycelial antibody was used for development of immunoassay systems, viz indirect Enzyme linked immunosorbent assay (ELISA) procedure for pathogen identification and characterization. The immunoassays were developed performing antigen concentration kinetics and antibody dilution curve analysis. These assays were employed for immunological based characterization of diversity among these isolates of pathogen by studying antibodies reactivity pattern of pathogen isolates.

### Optimization of Antigen kinetics through ELISA

In order to determine the virulence level of *A. brassicae* isolate/strains, the causative agent of Alternaria blight disease of Brassica and differentiating it from similar, *A. brassicae* isolate, mycelial

Table S1: Tabular representation of antigen kinetics of fungal antigen

| Mycelial antigen in ng | Absorbance OD at 405 nm |
|---|---|
| 50 | 0.638 |
| 100 | 0.659 |
| 250 | 0.7545 |
| 500 | 0.608 |
| 1000 | 0.4515 |
| 2000 | 0.4595 |
| 4000 | 0.3835 |
| 8000 | 0.378 |

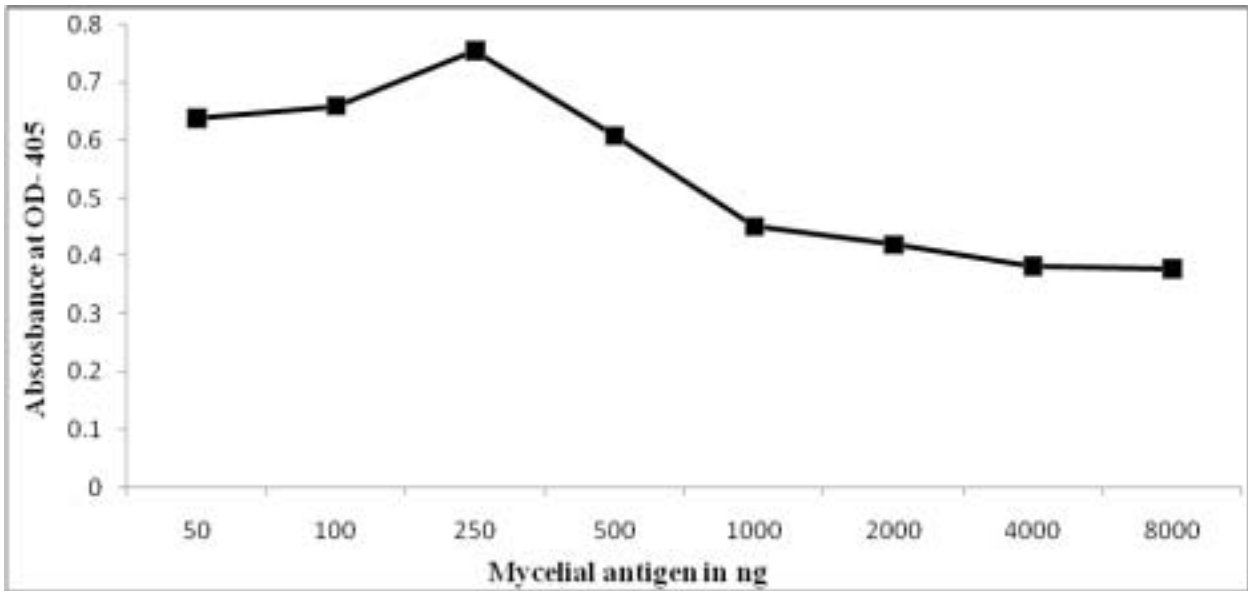


Figure 1: Antigen kinetics for determination of optimal mycelial antigen concentration of *A. brasiccae* for development of immuno-assay using anti-mycelial antibodies

fragments antigen from all isolates were subjected to indirect ELISA, standardization of antigen concentration for mycelial antigen was performed on ELISA plates. According to the result obtained, It was concluded that 250 ng/well of antigen concentration was appropriate for determining the virulence level of *A. brassicae* (fig. 1).

**Determination of titre of Anti-mycelial antibody**

To determine the optimal antibody dilution, the

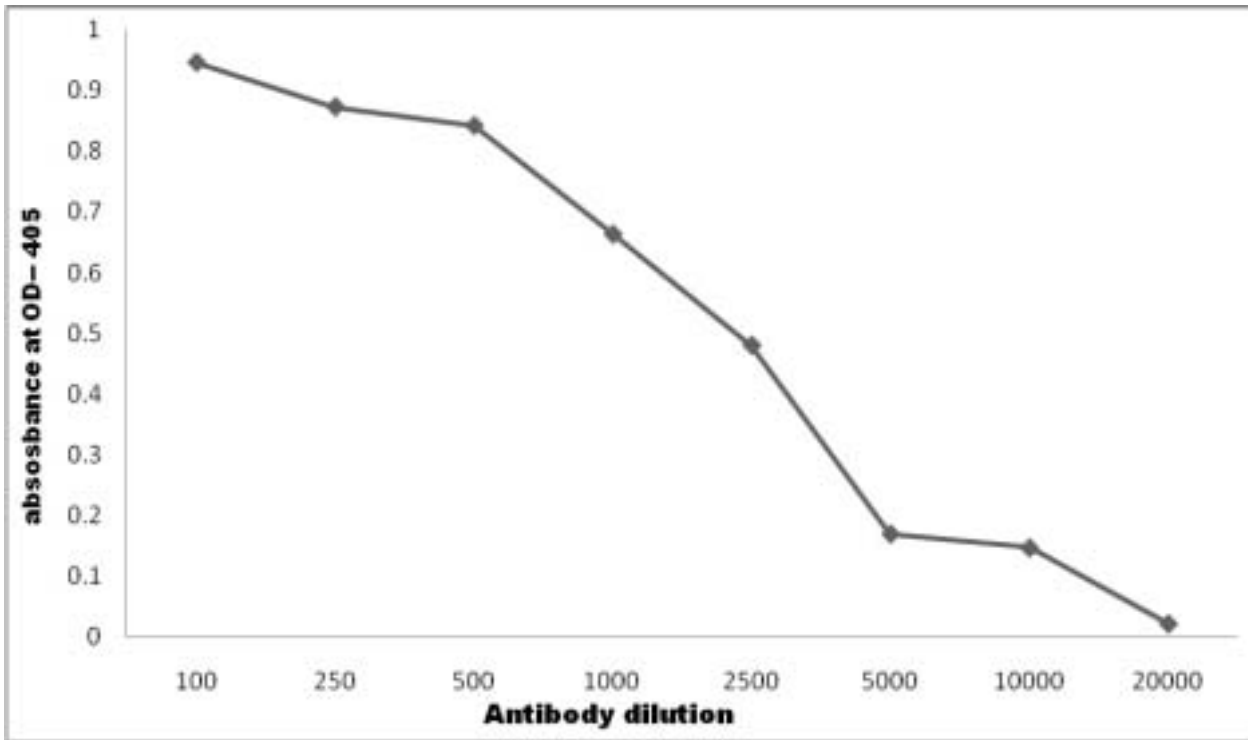


Figure 2: Determination of titre of Anti-mycelial antibody using indirect ELISA

Table S2: Tabular representation of anti-mycelial antibodies dilution as indirect ELISA absorbance values (405 nm)

| Anti-mycelial antibodies dilution | Absorbance OD at 405 |
|---|---|
| 1:100 | 0.946 |
| 1:250 | 0.873 |
| 1:500 | 0.842 |
| 1:1000 | 0.663 |
| 1:2500 | 0.480 |
| 1:5000 | 0.169 |
| 1:10000 | 0.146 |
| 1:20000 | 0.021 |

micro-titre plates were coated with optimal amount of mycelial antigen that falls within the detection range of indirect ELISA i.e. 250 ng per well. Antibody dilutions for anti-mycelial antibody raised was used ranging from 1:100 to 1:20,000. At dilution, 1:5,000, reactivity corresponding to $O.D.^{405}$ was observed 0.169 for mycelial antigen of *A. brassicae*. It was observed that the straight line was obtained after the antibody dilutions of 1:5000 and almost stagnant immuno reactivity in the antibody dilution range of 1:5000 to 1:20,000 indicating that dilutions higher than this will not improve the binding activity. Thus 1:5000 was determined as titre of anti-mycelial antibody raised against mycelial antigen. The titre of antibodies is defined as maximal dilution of antibodies at which reactivity was visible. It was concluded that 1:250 antibody dilution is appropriate for determine the virulence level of *A. brassicae* (fig 2).

## Cross reactivity patterns of different isolates of *Alternaria brassicae* using indirect ELISA

In order to increase the sensitivity of the analysis, compares the ELISA reactivity of anti-mycelial antibody against 10 isolates of *A. brassicae* pathogen and *A. solani.* These intra-species and intra-generic pathogen were showed cross-reactivity with anti-mycelial antibody raised against whole mycelial antigen of *A. brassicae*. Antimycelial antibodies strongly crossed react with mycelium of *A. brassicae* isolate and *A. solani*. Results indicated that different fungi express different amount per unit biomass of the antigen-antibody

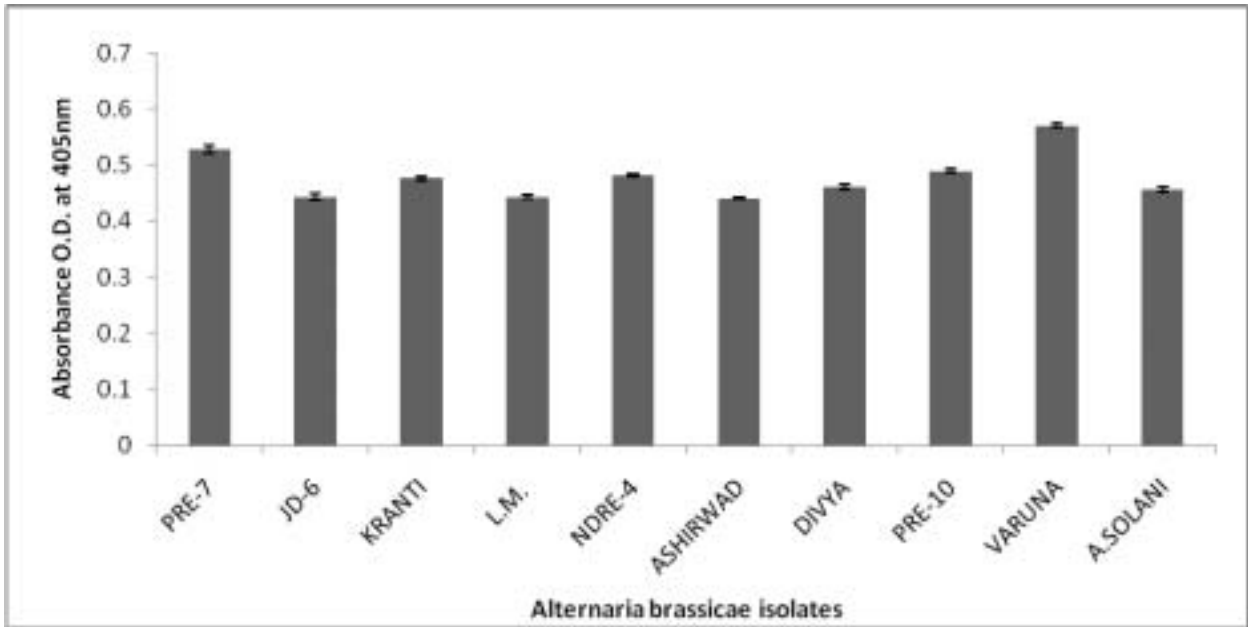


Figure 3: Cross reactivity studies between different strain of *Alternaria brassicae* by indirect ELISA assay using anti-mycelial antibody and Dilution of primary antibody is 1:250, Dilution of secondary antibody is 1:1000, Antigen concentration is 250ng in 100 µl.

Table S3: Tabular representation of cross reactivity of *A. brassicae* isolates.

| *Alternaria brassicae* isolates | Average Absorbance OD at405 nm |
|---|---|
| PRE-07 | 0.528 |
| JD-6 | 0.443 |
| KRANTI | 0.476 |
| LOCAL MUSTARD | 0.443 |
| MNRE-4 | 0.482 |
| ASHIRWAD | 0.441 |
| DIVYA | 0.462 |
| PRE-10 | 0.489 |
| VARUNA | 0.571 |
| *A.solani* | 0.456 |

binding sites for anti-mycelial antibody (fig. 3).

## Serological Detection of Alternaria blight during different time intervals/stages of infection of *Alternaria brassicae* in host species of *Brassica juncea*

Total protein was extracted from artificially inoculated *Brassica* leaves (varuna and PAB 9511) at 3 hpi, 1dpi, 2dpi and 3dpi). Total protein extracted from healthy Brassica leaves was used as control.

Table S4 : Serological test for *A. brassicae* antiserum as indirect ELISA absorbance values (405 nm) of total protein extracted from inoculated brassica leaves after different periods of inoculation with *A. brassicae*.

| Brassica cultivar Time post-inoculation | Absorbance values (405 nm) |
|---|---|
| Varuna (Susceptible) | |
| CV | 0.539333 |
| 6 h | 0.646333 |
| 1 D | 0.845333 |
| 2 D | 1.07 |
| 3 D | 0.889333 |
| PAB 9511 (tolerance) | |
| 6 h | 0.676667 |
| 1 D | 0.583333 |
| 2 D | 0.501667 |
| 3 D | 0.5 |

Indirect ELISA was used to detect Alternaria blight pathogen. *Alternaria brassicae* was used at a dilution of 1:250 and 1 µg protein per well in serological tests. The amount of mycelium of *A. brassicae* was estimated by indirect ELISA after different periods of inoculation with *A. brassicae* using polyclonal antiserum specific to *A. brassicae*, in leaves of tolerant (PAB9511) and

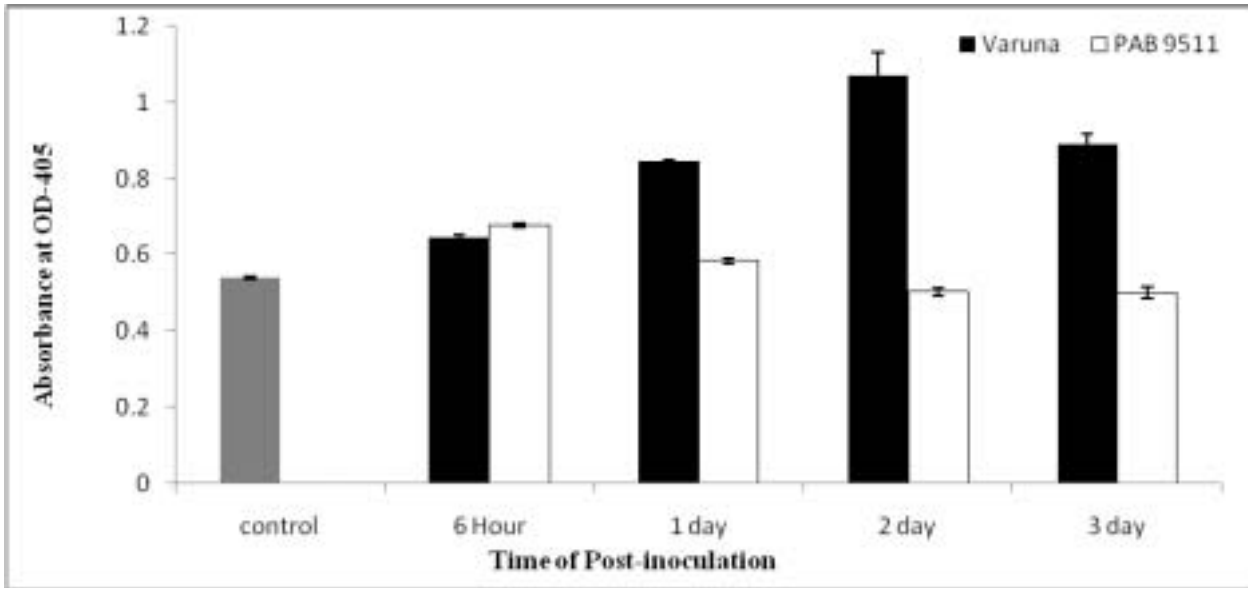


Figure 4: Serological Detection of *A. brassicae* in inoculated Brassica genotype of Varuna and PAB 9511 of different time interval

susceptible (varuna) *Brassica* cultivars, by comparing absorbance values obtained with those given by control samples for each isolate. 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 *Brassica* (varuna) until after 6 h post-inoculation in inoculated leaves (0.654). After that, a sharp increase in ELISA values was recorded with the time (fig-4). Low levels of ELISA value were obtained in case of tolerant *Brassica* cultivar (PAB 9511). The amount of antigen in leaves was sufficient to cause a positive ELISA reaction (0.853 to 1.46, respectively) only after 6 h post-inoculation. Results indicated that among the inoculated *Brassica* (6h to 3days post-inoculation), there was large amount of mycelium in leaves of the susceptible *Brassica* cultivar (Varuna), while the tolerant *Brassica* cultivar (PAB 9511) contained less. The experiment was repeated twice and ELISA absorbance values at 405 nm was average of two replicates each and 100ìl from the diluted *A. brassicae* antiserum (1:250 diluted in PBS) were used for each ELISA reaction. Serological testing can be useful for early detection of the presence of *A. brassicae* before appearance of the symptoms. Keeping in view the present study was carried out to generate polyclonal antibody against total mycelia of *A. brassicae* to develop ELISA based method for early detection and quantitation of mycelia biomass. It could be possible that polyclonal antibodies raised against specific strain of *A. brassicae* were showing cross reactivity with other strain of *A. brassicae* and other species of *Alternaria*.

From the above study it was concluded that polyclonal antibodies against mycelial antigen of *A. brassicae* was showing the interaction with *A. brassicae*. Optimal concentration of mycelial antigen of *A. brassicae* was found to be 250 ng/ well for development of indirect ELISA method for detection of Alternaria blight disease. It was concluded that 1:250 anti-mycelial antibody dilution was appropriate for anti-mycelial antibody to determine the virulence level of *A. brassicae*. The titer of anti-mycelial antibody generated against mycelial antigen was 1:5000. Cross reactivity studies was done by indirect ELISA. Due to polyclonal nature of anti-mycelial antibody cross-reactivity pattern was observed among different isolates of mycelial antigen of *A. brassicae*. Serological detection of *A. brassicae* was done in inoculated Brassica genotype of varuna and PAB 9511 with different time interval. It was observed that among the inoculated Brassica (6h to 3days post-inoculation), the mycelial biomass was increased with disease progression. Gemmrich and Seidel (1996) found out the detection of *Oidium* mycelium by using polyclonal antiserum. In the present study, it was also observed that relatively more amount of mycelium biomass was observed in inoculated leaves of susceptible Brassica cultivar (varuna), as compared to tolerance Brassica cultivar (PAB 9511).

In this study it was observed that the polyclonal antibody was helpful to determine pathogen biomass in infected leaves. By using ELISA we can determine the level of infection. For future prospective it could be used for quantitation of mycelial biomass in plant patho-system. Kennedy *et al.* (1999) developed detection method of *Mycosphaerella brassicicola* in infected crop of vegetable brassicas. We need to work at the production of additional mAbs against the purified antigen of *A. brassicae*. This will allow the development of a mAb-based sandwich assay for environmental sample analysis. In conclusion, the widespread cross-reactivity among fungi and the observed biological variability are considered to be major challenges for the successful implementation of accurate and quantitative immunoassays.

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