Isolation and characterization of *Botrytis* antigen from *Allium cepa* L. and its role in rapid diagnosis of neck rot

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Abstract: **Early and accurate diagnosis of neckrot in onions permits early treatment which can enhance yield and its storage. In the present study, polyclonal antibody (pAb) raised against the protein extract from *Botrytis allii* was established for the detection of neck rot using serological assays. The pathogenic proteins were recognized by ELISA with high sensitivity (50 ng). Correlation coefficient between infected onions from different stages and from different agroclimatic zones with antibody titres was taken as the primary endpoint for standardization of the protocol. Highest positive correlation (r ¼ 0.999) was observed in stage I and II infected samples of North-western zone, whereas low negative correlation (r ¼ _0.184) was found in stage III infected samples of Western zone with developed pAb. Linear positive correlations (R2) exist between antigenic proteins at all the stages of infection and the developed pAb titres. Hence, the developed pAbs could be used to detect the presence of the fungal pathogen at an early stage. This study recommends the use of these developed antibodies in rapid diagnosis of neckrot in onions.**

Keywords: **Neckrot, onions, *Botrytis allii* , Polyclonal antibody, ELISA, rapid diagnosis**

I. INTRODUCTION

Onions (*Allium cepa.L*) regarded as "queen of kitchen" are an important food crop worldwide. It is commonly used for cooking purposes by almost all the population. It ensures excellent taste to dishes and also exhibits a number of therapeutic properties such as antibacterial, antifungal, anti - helmintic, anti inflammatory, antiseptic, antispasmodic, etc. [1]. India being the second largest onion growing country in the world, mainly harvest it in November to January and the second harvesting from January to May. There is a lot of demand of Indian Onion in the world, the country has exported 12,01,245.29 million tons of fresh onion to the world for the worth of Rs. 2,747.41 crores during the year 2015-16 [2]. However this value decreased as compared with the early and expected value due to neck rot infection in onions during storage period. Worldwide neck rot losses to the onion industry but variable and sporadic with some seasons bringing greater than 50% losses to individual crops. Neck rot species of *Botrytis* are distributed in all areas of the world where onions are produced, but greatest losses have been reported from temperate regions [3]. Although *B. allii* and *B. aclada* are the predominant species reported to cause neck rot of onion, these species are difficult to distinguish morphologically because of similar growth patterns on agar media, and overlapping spore sizes [4].

Recent studies of the ribosomal internal transcribed spacer (ITS) region of the genome of *Botrytis* spp. associated with neck rot of onion have confirmed the existence of three distinct groups [5]. These include a smaller-spored group with 16 mitotic chromosomes, (*B. aclada* AI), a larger-spored group with 16 mitotic chromosomes (*B. byssoidea*) and a group with intermediate-sized spores with 32 mitotic chromosomes (*B. aclada* AII) [6]. *B. aclada* AI and *B aclada* AII be referred to as *B. aclada* and *B. allii*, respectively [7]. *B. allii* usually having features infectious are symptomless, grows from the leaves into the bulb during curing, leading to rots of bulbs in storage with necroses developing in the neck region of the bulb during storage and transport, reducing the quality of export crops [8]. However, conidiophores, sclerotia, neck and basal rots on bulbs are occasionally noted in the field.

In few decades the production has been repeated to decline because of frequently the occurance of neckrot disease in onion. Neck rot is caused by *Botrytis acclada* and *B. allii* species of fungus .*Botrytis allii* is a plant pathogen a fungus that causes neck rot in stored onions (*Allium cepa*) and related crops. *Brotrytis* associated with onion in storage but the rot induced by *B.allii* and *B. aclada* causes the greatest commercial lossess [9]. *Botrytis aclada* and *B. allii* become a problem in storage when onions are harvested immature and can result in more than 30% yield loss. Initially, scales become water-soaked in the neck area and then as the fungus grows, the scales turn brown at the neck. *Botrytis* continues to move through the bulb to the basal plate and the scales turn brown and dry up. Sclerotia can develop on the outside of an infected bulb or between the scales. Frequently, *Botrytis* will produce spores on the outside of the bulb and gray mycelium can sometimes be seen growing between the scales. Infected bulbs may have a sunken appearance due to the dried up tissue and feel spongy in the neck area. In spite of being a major contribution for onion production. very few steps has been taken to control these fungal pathogen. The reports available

are limited with using fungicide or antifungal solutions. However plant faced the disease in spite of sparging these solutions because of the vivacious spread of this fungus over all plants. Early diagnosis can only be utilize the protect this plant at initial stages of infection and prevent the specialty of pathogen. The methods for early diagnosis of *Botrytis* infection in onions are reported very few.

Immunological methods has genus-specific monoclonal antibodies, particularly quantitative laboratory-based plate-trapped antigen ELISAs, allow large numbers of samples to be processed .ELISA tests can sometimes be quantitative when used in conjunction with an optical plate reader [10].Similarly infectected onion samples could be assayed for the presence of *B. allii* DNA by using the polymerase chain reaction (PCR) which again will be specific and sensitive, but more expensive and time taken. So rapid sensitive and inexpensive method to diagnose the botrytis infection in onion is urgently required. Keeping this problem in view, our study demonstrate the isolation of *Botrytis* antigen from infected onion samples, its characterctisation and development of polyclonal antibodies against those isolated antigens. Further in future, this study recommended the use of these antibodies in rapid and protection, onsite detection of botrytis disease in onions. The usefulness of qualitative tests for bulbs going into storage is not good because *B. allii* is ubiquitous in commercial onion fields. Just testing for the presence of *B. allii* on a large scale would likely yield near 100% positive results.

II. MATERIALS AND METHODS

2.1 Collection of infected onions samples:

Botrytis infected onion bulbs were collected from rural and urban area of vegetable farms of Odisha (Table 1). The fungal pathogens, *B. allii* was identified based on its morphological features.

2.2 Identification of pathogen sample:

The identification of *Botrytis allii* to the species level by traditional methods has been complicated by morphological variation within the genus [11] distinguished at least three *Botrytis* species associated with neck rot, *B. aclada* (syn. *B. allii*), *B. byssoidea* (teleomorph, *Botryotinia allii*), and *B. squamosa. Botrytis allii* belonging to Sclerotiniaceae family were morphologically described by [12]. *Botrytis allii* has main agronomic host is the onion control this disesase, most agricultural utilize the fungicide benomyl.

2.3 Pathogenecity test of fungal sample:

To determine pathogenecity, the isolates were cultured on Potato dextrose broth (PDA) plates for 10days at 20^0C. The cultured isolates were then suspended in sterile distilled water at a concentration of $1x10^5$ conidia/mL. After incubation period, fungi were collected for pathogenic test. Fungi were homogenized using mini pestle and 500 microliter of urea extraction buffer and incubate 4hours at 4^0C with 15min vortex. The mixture was centrifuged at 10000 rpm for 30min at 4^0C and supernatant was collected [13,14,15].

2.4 Isolation antigen from infected onion leaves:

The healthy and infected leaves were cut into smaller sizes and washed with tap water. The pieces were homogenized using liquid nitrogen (-196^0C) in a motar and pestle using urea extraction buffer. The mixture were transferred to centrifuge tubes and centrifuged at 10000 rpm for 30min at 4^0C. The supernatant were collected and stored for further analysis.

2.5. Extraction of fungal proteins from bulbs:

The infected onion bulbs were washed carefully with tap water to remove adhering soil and then cut into smaller sizes. The pieces were then dried on sterile filter paper for 10 min. All preparations were carried out in biosafty cabinet. The sterilized pieces were homogenized using liquid nitrogen (-196^0c) in a motar and pestle using urea extraction buffer. The mixture was centrifuged at 10000 rpm for 30 min at 4^0C and supernatant was collected. The protein content was estimated by dye binding method [16] using bovine serum albumin as the standard (Table 2).

2.6. SDS-PAGE Analysis :

Fungal proteins ranging from 40 microliter were loaded into a single well of a multi-welled 10% acrylamide gel. Samples were separated by SDS-PAGE using a BIO-RAD Mini-Protean vertical electrophoresis system [17]. A standard protein marker was run parallel along with the samples for molecular weight determination. Protein separation was carried out under a constant voltage of 120V. The gels were stained with Coomassie Brilliant Blue R-250 (Sigma) to visualize the fungal proteins.

2.7. Development of Antibody

The animal was immunized with 500 mg of fungal proteins mixed with mineral oil. The initial dosage was given containing 1 mg of the protein antigen. Three subsequent booster doses were given at an interval of 15 days. Collection of antisera were done 7 days after the 2^{nd} and 4th immunization. The reactivity of antiserum was checked using ELISA .

2.8. Indirect ELISA

The cross-reactivity against the fungal proteins isolated from infected bulbs of samples antiserum was tested by indirect ELISA. Healthy onion bulb samples were used as negative control and proteins containing *Botrytis* as positive control. The antiserum was diluted using 1X PBS. 2 mg of fungal proteins were coated per well by re-suspending in 100 ml of coating buffer and incubated for overnight at 4^0C. The plates were washed twice with Phosphate Buffer Saline-Tween 20 (PBS-T) and blocked by 5% skimmed milk for 1 h at room temperature. 100 ml of antiserum diluted in blocking buffer at different dilutions was added to each well and incubated at

room temperature for 2 h. After washing, again the plates were incubated with 100 ml of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1:10000 for 1 h at room temperature. Finally, the plates were washed with PBS-T and incubated with 100 ml of TMB (3,30, 5,50-Tetramethylbenzidine) substrate per well for 5 min in dark for development of color The reaction was stopped by adding 50 ml of 2 N sulfuric acid to each well. The optimum density of the developed color was measured at 450 nm in a micro plate reader (Mindray).

2.9. Statistical analysis

The experimental results were analyzed and Botrytis proteins isolated from infected onions samples collected at different stages of infection with antibody titres were calculated at a significant level of $P < 0.01$. All experiments were done in triplicates.

2.10. Validation of ELISA technique

Specificity and sensitivity of the technique were determined by testing the samples by the developed assay and tabulating the test results in a two way (2 _2) table. Results of the tests on standard sera were categorized into true positive (TP) or true negative (TN) if they were in agreement with those of the "gold standard". Alternatively, they were classified as a false positive (FP) or false negative (FN) if they disagreed with the standard. Diagnostic sensitivity was calculated as TP/(TP þ FN) whereas diagnostic specificity was TN/(TN þ FP); the results of both calculations were usually expressed as percentages are shown in Table 3. Table 3 is a hypothetical set of results from which diagnostic sensitivity and diagnostic specificity estimates were obtained.

III. RESULTS AND DISCUSSION

3.1. Total antigenic protein concentration estimation from infected onion samples:

The protein content of both infected (collected from various stages of disease development) and healthy onions were estimated and compared. The protein concentration of the infected bulbs was found to be considerably higher than the healthy bulbs (Table 2). The increase in protein content of the infected samples may be due to accumulation of chemical constituents and soil borne microbes in the bulbs of onions in infected plants [18].

3.2 SDS-PAGE analysis:

The separation of the isolated proteins from *Botrytis allii* having concentration (2-7 mg/ml)was done by SDS-PAGE followed by Coomassie bule staining. It resulted in identification of multiple proteins with molecular masses ranging from 10-110 kDa [19, 20].. The number and intensity of bands in the antigenic protein isolated from stage III infected samples were seen to higher in comparison to stage I and II samples.

3.3 ELISA analysis:

In ELISA test the anti sera was tested at 1:1000, 1:2000 and 1:5000 dilutions against Botrytis antigenic protein concentrations. As a result antiserum was very specific to detect up to 10 ng of fungal protein at 1:5000 dilution. From these results, standardized experimental conditions were selected for the further experiments: 1:2000 antiserum dilutions and 60 ng of fungal antigen per well, with 0.7 absorbance unit was kept as reference value.

3.4. ELISA validation for diagnosis

The sensitivity of this detection test was calculated using a hypothetical set of results from known infected and non-infected onion samples. The test was conducted on a sample size of 50 consisting of both infected and non-infected onions where the diagnostic sensitivity was found to be 94.83%. Hence it was concluded that the obtained antibody raised in rabbit against *Botrytis allii* was highly sensitive and permitted detection of signals in neck rot infected onions.

IV. CONCLUSION

This study showed the effect of specific IgG polyclonal antibody upon the *Botrytis allii* infections in onion. Here we optimized the reaction conditions for the developed antibody in the control sera. 1:2000 dilutions with incubations for 30 min at 37°252 C were chosen as the parameters for the control sera. For the enzymatic transformation, TMB (3,3', 5,5'-Tetramethybenzidine) substrate was selected for color development. The optimum density of the developed color was measured at 450 nm. All these outcomes permitted us to develop a simple, rapid, sensitive and specific ELISA kit which can detect neck rot in onion at the mild stage of infection hence one can immediately prevent major crop loss.

ACKNOWLEDGMENTS

The authors are grateful to Prof. (Dr.) S.C. Si, Dean, Centre of Biotechnology and Prof. (Dr.) M.R. Nayak, President, Siksha 'O' Anusandhan University for the generous financial support.

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Table-1: Sample wise collection from different agroclimatic location of Odissa and content of protein in *Botrytis allii* infected onions at various stages of disease development.

Agro climatic zone	Districts	No. of samples collected	Sample codes	Conc. Of Botrytis(mg ml^{-1})		
				Stage I	Stage II	Stage III
Estern Ghat Highland	Koraput	01	PA1	0.948±0.961	1.300±1.399	1.359±1.458
	Nabarang pur	01	PA2	0.866±0.867	1.230±1.276	1.248±1.298
	Malkangiri	01	PA3	0.630±0.851	1.213±1.211	1.245±1.271
North Estern Ghat	Phulbani	01	PA4	0.688±0.721	1.124±1.115	1.161±1.199
	Rayagada	01	PA5	0.505±0.567	1.013±1.043	1.050±1.077
	Gajapati	01	PA6	0.266±0.301	0.723±0.724	0.791±0.824
	Ganjam	01	PA7	0.067±0.117	0.489±0.482	0.631±0.638
South Zone	Keonjhar	01	PA8	0.322±0.298	1.113±1.234	1.431±1.502
	Baripada	01	PA9	0.254±0.309	1.234±1.567	1.336±1.188
	Balesore	01	PA10	0.087±0.123	0.823±0.998	0.912±0.945

Mean ± SD; n= 3.

Values carrying same alphabet (aee) did not vary significantly from each other ($P < 0.01$).

Stages I, II and III represent stages of infection

Stage I (Mild): The early stage of infection, which was characterized by the appearance of water soaked bulbs.

Stage II (Acute): The intermediate stage of infection, which was characterized by water soaked and black color. Dryness in the leaves was observed during this stage.

Stage III (Chronic): The very late stage of infection, which was characterized by an increase in intensity of rotting leading to the destruction of entire bulb system, total yellowing & dryness of all leaves, wilting and death of the plants.

Table-2: Changes in the content of total protein (mg/g fr. Wt.) in healthy and *Botrytis allii* infected onion bulbs at various stages of disease development.

Samples	Total proteins
Healthy bulbs	0.283±(0.008)
Stage I infected bulbs	1.325±(0.050)
Stage II infected bulbs	4.935±(0.067)
Stage III infected bulbs	5.408±(0.079)

Table-3: Detection sensitivity calculated from a hypothetical set of results for samples tested from known infected and non-infected onion populations.

Test Results			
Negative		Positive	
Infected	Non-infected	Infected	Non-infected
45 (TP)	5 (FP)	3 (FN)	95 (TN)
Detection sensitivity TP/(TP+FN) : 90% Detection sensitivity TN/(TN+FP) : 96%			