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

Prabin K. Sahoo[1], Amrita Masanta[1], K.Gopinath Achary[2], Shikha Singh[3*]

[1]*Fish Nutrition & Physiology Division, ICAR-CIFA, Bhubaneswar, Odisha, India.*

[1]*Centre for Biotechnology, Siksha O Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India*

[2]*Imgenex India Pvt.Ltd, E-5 Infocity, Bhubaneswar, Odisha, India*

[3*]*Rama Devi Women's University, Bhubaneswar, Odisha, India.*

Abstract

Early detection and perfect diagnosis of neck rot in onions authorized for early treatment which can increase production and storage. In the current status, polyclonal antibody (pAb) raised against the protein extract from *Botrytis allii* was fixed for the detection of neck rot using serological assays. The pathogenic proteins were identified by ELISA with high sensitivity (60 ng). Correlation efficient between infected onions from different agroclimatical stages with antibody titers were taken as the primary endpoint for standard protocol. Highest positive correlation (r ¼ 1.502) was observed in stage I infected samples of south zone, whereas low negative correlation (r ¼ _0.087) was found in stage III infected samples of south zone among developed pAb. Linear positive correlations between antigenic proteins at all the stages of infection and the developed pAb titres. Hence, at an early stage the developed pAbs should be used to detect for the presence of the fungal pathogen. This point of study recommends the use for developed antibodies in rapid diagnosis of neck rot in onions.

Keywords: Neck rot, onions, *Botrytis allii*, Polyclonal antibody, ELISA, rapid diagnosis.

1. 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's very good taste to spicy dishes and also exhibits a number of therapeutic properties such as antibacterial, antifungal, anti hermitic, anti inflammatory, antiseptic and antispasmodic etc. [1]. In the world, India being the second largest onion growing country, mainly harvest in November to January and January to May. In the year between 2015-16 Indian has produced 12, 01,245.29 million tons of fresh onion to the world for worth of Rs. 2,747.41 corers in the international market because there is a huge demand of Indian onions all over the world [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 rots losses to the onion industry but 50% losses of some periodical or variable loss on individual crops is due to some capricious season. Onion produced neck rot species of *Botrytis* species is

disseminated in all the areas of the world where onions are produced but the temperate regions have been reported of great losses [3]. Although predominant species like *B. allii* and *B. aclada* have been recorded to have cause of neck rot of onion, it's very difficult to discriminate because of similar growth patterns on agar media, and overlapping spore sizes [4].

These distinct groups are confined to be associated with neck rot in the ribosomal internal transcribed spacer (ITS) region of the genome of *Botrytis* spp. is proved recent studies [5]. These include a smaller-spore group with 16 mitotic chromosomes, (*B. aclada* AI), a larger-spore 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 AII are referred to as *B. aclada* and *B. allii*, respectively [7]. *B. allii* usually having features which 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, sclerotic, 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 occurrence of neck rot disease in onion. Species of fungus *Botrytis aclada* and *B. allii* causing neck rot .*Botrytis allii* is a plant pathogenic fungus cause's neck rot in stored onions (*Allium cepa*) and related crops. The greatest commercial losses of *Botrytis* associated with onion in storage but the rot caused by *B.allii* and *B. aclada* [9]. *Botrytis aclada* and *B. allii* become a problem in storage in more than 30% yield loss results when onions are harvested immature. On first stage scales become water-soaked in the neck area and growing *Botrytis* fungus, at the neck turn brown scales. *Botrytis* grows through the bulb to the basal plate and the scales turn brown and dry up. Sclerotic 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. In the neck area infected bulbs may have a sunken appearance due to the dried up tissue and feel spongy. Despite of being a major contribution of onion production, very few steps have been taken to control this fungal pathogen. The reports available are limited with using fungicide or antifungal solutions. However plant faced the disease in spite of sparing 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 of 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 highly sensitive and cost effective method to detect 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?

2. 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 Orissa (Figure 1,2, Table 1). The fungal pathogens, *B. allii* were identified based on its morphological features.

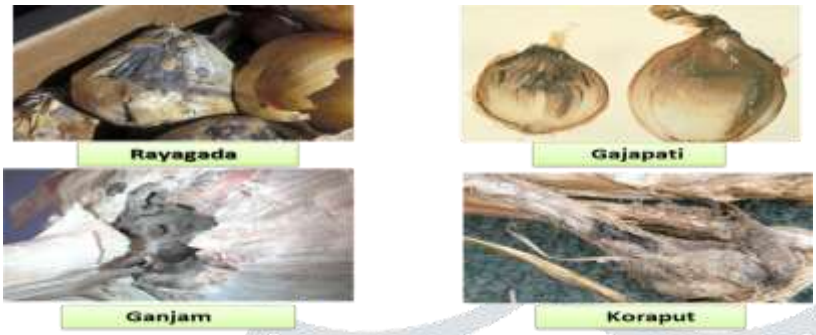

Figure 1: Infected onion samples showing neck rot symptoms collected from the different location of Odisha.

Figure 2: Infected onion samples showing neck rot symptoms collected from the different location of Odisha.

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

Agro climatic zone	**Districts**	**No. of samples collected**	**Conc. Of Botrytis(mg ml^{-1})**		
			Stage I	**Stage II**	**Stage III**
Eastern Zone	Puri	01	0.948±0.961	1.300±1.399	1.359±1.458
	Kendrpada	01	0.866±0.867	1.230±1.276	1.248±1.298
	Jagatsinghpur	01	0.630±0.851	1.213±1.211	1.245±1.271
North Zone	Keonjhar	01	0.688±0.721	1.124±1.115	1.161±1.199
	Baripada	01	0.505±0.567	1.013±1.043	1.050±1.077
	Balasore	01	0.266±0.301	0.723±0.724	0.791±0.824

South Zone	Ganjam	01	0.067±0.117	0.489±0.482	0.631±0.638
	Koraput	01	0.322±0.298	1.113±1.234	**1.431±1.502**
	Rayagada	01	0.254±0.309	1.234±1.567	1.336±1.188
	Gajapati	01	**0.087±0.123**	0.823±0.998	0.912±0.945

Mean ± SD; n= 3.
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

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, *Botrytis aclada, Botrytis allii, Botrytis byssoidea* (*Botryotinia allii*), and *Botrytis squamosa, Botrytis cineria, Botrytis tulipae , Botrytis Porri* (Figure 3). *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.

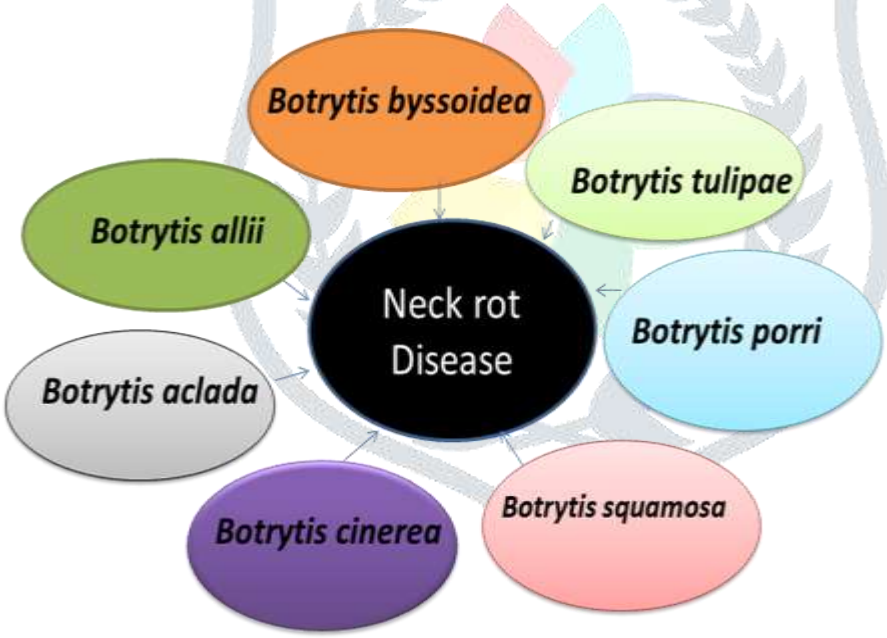

Figure 3: Group of *Botrytis* pathogen causing Neck rot Disease

2.3 Pathogen city test of fungal sample

To determine pathogen city, the isolates were cultured on Potato dextrose broth (PDA) plates for 10days at 20^0C (Figure 4). 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 µl 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].

Figure 4: Botrytis colony / hyphae on PDA plate and broth at 10^{-4} dilution.

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^{0}C) in a mortar and pestle using urea extraction buffer. The mixture were transferred to centrifuged 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 pathogenic proteins from bulbs

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

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%	

Table 2: Detection sensitivity calculated from a hypothetical set of results for samples tested from known infected and non-infected onion populations.

2.6. SDS-PAGE Analysis

Botrytis fungal proteins ranging from 40µl were loaded into multi-welled 10% acryl-amide gel through separated by SDS-PAGE using a vertical electrophoresis system under a constant voltage of 120V [17]. A standard protein marker and the samples were run parallel along molecular weight determination. The gels were stained over night in Coomassie Brilliant Blue R-250 Dye. According use this method after that visualize the fungal proteins (Figure 5).

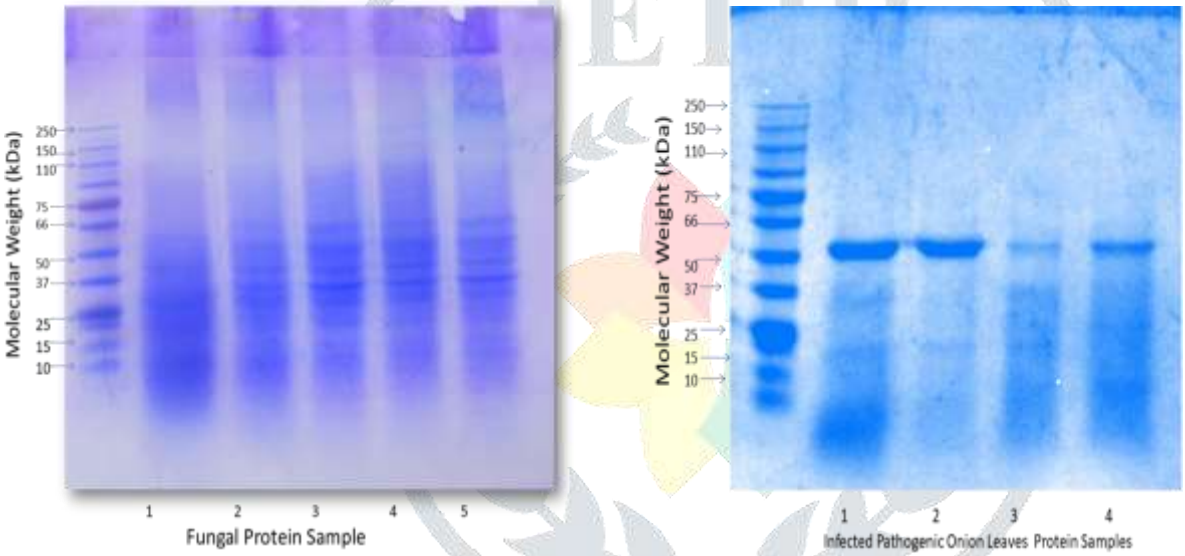

Figure 5: Protein banding pattern of fungal isolates after SDS-polyacrylamide gel electrophoresis.

2.7 Development of Antibody

The animal was immunized 600 mg of *Botrytis* proteins mixed with mineral oil. The initial dosages were given containing 2 mg of the protein antigen. Within interval of 15 days used three subsequent booster doses. Collections of antisera were within 7 days after the 2nd and 4th immunization. The reactivity of antiserum was checked by ELISA.

2.8. Indirect ELISA

Indirect ELISA Tested were infected bulbs of samples antiserum isolated the cross-reactivity against the fungal proteins. *Botrytis* marked as a positive control of proteins and healthy onion bulb samples marked as a negative control proteins. The antiserums were diluted using 2mg of 1X PBS. Fungal proteins were coated per well by re-suspending in 100 ml of coating buffer and incubated for overnight at 4^{0}C. 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, 3', 5, 5'-Tetramethylbenzidine) substrate per well for 5 min in dark for color development. 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.

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 titers 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 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 2. Table 2 is a hypothetical set of results from which diagnostic sensitivity and diagnostic specificity estimates were obtained.

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. 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 (Figure 6) [18].

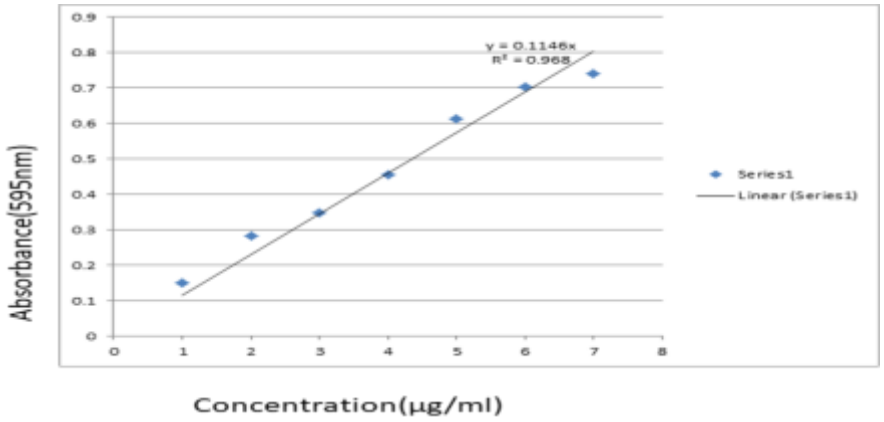

Figure 6: Standard Curve for fungal protein

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 96%. Hence it was concluded that the obtained antibody raised in rabbit against *Botrytis allii* was highly sensitive and allow the detection of signals in neck rot infected onions.

4. 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°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.

Acknowledgements

The author was thankful to all co-authors involved in the study and special acknowledge to K.Gopinath Achary and his team (Imgenex India Pvt.Ltd, E-5 Infocity, Bhubaneswar, Odisha, India) for immense platform for my research work.

References

1. Augusti, K.T., Therapeutic values of onion (*Allium cepa L.)* and garlic (*Allium sativum L.*). Indian journal of experimental biology, 1996. *34*(7), pp.634-640.
2. (http:// apeda.gov.in/aped website/ Subhead Products/ Onions.htm
3. Maude, R. B.; Presley, A. H. (1977). "Neck rot (*Botrytis allii*) of bulb onions". Annals of Applied Biology. **86** (2): 163–180. doi:10.1111/j.1744-7348.1977.tb01829.x

4. Chilvers, M. I., and du Toit, Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion. L. J. 2006, Online. Plant Health Progress doi: 10.1094/PHP-2006-1127-01-DG.
5. M. Chilvers ,S. J. Pethybridgen ,F. S. Hay Characterisation of *Botrytis* species associated with neck rot of onion in Australia, March 2004, Volume 33, Issue 1, pp 29–32
6. Nielsen, K. and D.S. Yohalem. Origin of a polyploidy Botrytis pathogen through interspecific hybridization between *Botrytis aclada* and *Botrytis byssoidea*. 2001, Mycological 33:1064–1071.
7. Yohalem, D.S., K. Nielsen, and M. Nicolaisen. Taxonomic and nomenclatural clarification of the onion neck rotting *Botrytis* species. Mycotaxon 2003, 85:175–182.
8. M. Chilvers ,S. J. Pethybridgen ,F. S. Hay Characterization of *Botrytis* species associated with neck rot of onion in Australia, March 2004,33:1- 29–32
9. Toit, Lindsey Du. *Botrytis* Species Associated with Neck Rot, Scape Blight, and Umbel Blight of Onion. Detection and Identification of Botrytis Species Associated with Neck Rot, Scape Blight, and Umbel Blight of Onion. Plant Health Progress, 27 Aug. 2006. Web. 17 Nov.2016
10. Lopez P. A novel germ line-specific gene of the phosducin-like protein (PhLP) family. A meiotic function conserved from yeast to mice. J Biol Chem 2003;278(3):1751-7
11. Walker, J. C. Two undescribed species of *Botrytis* associated with the neck rot disease of onion bulbs. Phytopathology 1925,15:708
12. *Botrytis allii Munn:* MycoBank. International Mycological Association.1917 Retrieved 2013-03-31.
13. Elena Perez-Nadales , Maria Filomena Almeida Nogueira , Clara Baldin ,Fungal model systems and the elucidation of pathogen city determinants Fungal Genetics and Biology 70 (2014) 42–67.
14. Draginja Pavlic-Zupanc Diversity, phylogeny and pathogen city of *Botryosphaeriaceae* on non-native *Eucalyptus* grown in an urban environment: A case 2017 studyhttp://dx.doi.org/doi:10.1016/j.ufug..04.009.
15. Jarvis, W. R. *Botryotinia* and *Botrytis* species: Taxonomy, physiology and pathogen city. 1977, Canada Department of Agriculture, Ottawa.
16. Bradford, M. M. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding.AnalyticalBiochemistry 1976, 72, 248e254.
17. Laemmle, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4 1970. Nature, 227(5259), 680e685.
18. Reddy, M. N., Sridevi, N. V., & Charitha Devi, M. Changes in the nitrogen fractions and amino acid metabolism of turmeric (*Curcuma longa* L.) roots infected with *Fusarium solani*. 2005 Plant Pathology Bulletin, 14, 221-226.
19. M. Amrita, A. Gopinath. K & S. Singh. Monitoring the advancement and evaluation of a novel diagnostic method for rapid detection of tuberculosis. 2020. Journal of Emerging Technology and Innovative Research, 130, 126-135, doi: http//10.6084/m9.figshare.
20. M. Amrita, A. Gopinath. K & S. Singh. Rapid detection of tuberculosis using urine sample: Development & validation of ELISA technique. 2020. International Journal of Research & Scientific Innovation, 9, 6-15.