JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2008. Vol. **2**(2), p. 447-450

Production and Characterization of Proteases by Seed Borne Fungi in Pulses

S.M. More, A.V. Girde and M.M.V. Baig[1]*

Department of Microbiology, [1]Department of Botany and Department of Biotechnology, Yeshwant Mahavidyalaya, Nanded - 431 602, India.

(Received: 11 May 2008; accepted: 24 August 2008)

The changes in the seed contents due to seed infestation with the common and dominant seed-borne fungi lead to deterioration of seed quality. The protein content and dry weight of the seeds of soybean were found to be greatly affected. The deterioration of soybean seeds rich in protein was correlated with extracellular production of protease by seed borne fungi. Five dominant fungi viz. *Alternaria alternata, Aspergillus flavus, A. niger, F. oxysporum, Penicillium digitatum* were studied for protease synthesis. These fungi synthesized proteases in Czapek medium supplemented with soybean powder and casein hydrolysate separately. The fungi synthesized more enzymes in soybean powder supplemented medium over casein hydrolysate supplemented medium. The enzyme production was affected by the pH and temperature. The optimum pH was found to be in the range of 5.0 to 6.5 and 45°C to 50 °C was optimum temperature.

Key words: Proteases, Fungi, Soybean.

The loss of grain in our country is approximately 10% of total production. Beside fungi, these losses can be attributed the unsanitary storage state and elevated dampness level of seeds or absorption of moisture during storage. These condition poses suitable conditions for deterioration of seed especially in pulses and the losses can be attributed to the enzyme synthetic ability of fungi associated. Seed-borne fungi cause losses in terms of seed quality and quantity in most of the crops. The associated fungi also reduce the germination and storability of the seed. They are responsible for seed rot, seedling blight, root/stem rot, foliar infection as well as pod blight diseases[1,2,3].

Seeds of many pulses crop are known to harbour large amount of mycoflora. Seed borne microorganisms affect farm produce in the field as well as in storage. In several cases such mycoflora is found to affect adversely the seed germination, vigour and quality and quantity[4,5]. Seed deterioration due to mycoflora is common feature leading to loss of viability and numerous fungi develop on stored seed[6]. To investigate this aspect fungi associated with soybean was isolated and the dominant fungi were screened for the production of proteases.

MATERIAL AND METHODS

Isolation of fungi

Untreated seeds were obtained from various sources – breeders, retailer, farmers etc. these seeds were assessed for presence of fungi using standard blotter method as recommended by International Seed Testing Association[7,8,9].

Culture and Protease production medium

The associated fungi were maintained on Czapek medium-broth supplemented with casein hydrolysate instead of sucrose as carbon sources. Czapek medium-broth supplemented with 1% casein hydrolysate or 1% soybean powder was used as enzyme production medium. The pH was adjusted to 6.0 by adding dilute 0.1N HCl in all cases[10].

* To whom all correspondence should be addressed.
Tel.: +91-9422170641
E-mail: mmvb@indiatimes.com

Preparation of enzymes

Fifty ml of medium was poured in 250 ml flasks and was inoculated with 0.5 ml of spore suspension of the fungi. The flasks were incubated for 27±2°C for 10 days. Flask were drawn after regular time interval and the contents were filtered through Whatman No. 1. The culture filtrate was collected, centrifuged at 5000 rpm for 15 minutes to remove spore and suspended matter. The filtrate was dialysed against running tap water for 24 hours. The partially purified solution was used as crude enzyme source and was stored at 4 °C for further use.

Measurement of Protease activity

1% Casein dissolved in 0.1M phosphate buffer at pH 7 (as casein is sparing soluble in water. it is dissolved in a minimal quantity of 0.1 NaOH and the volume was raised to 100 mL with the buffer. the pH was adjust)

Ten mL of casein solution was taken into a test tube of 0.1 M phosphate buffer, pH 7 and 5 mL of enzyme preparation was added. The contents were mixed thoroughly and the mixture was incubated at 30 C in a water bath. Boiled enzyme at zero time served as control. Aliquots of 1 ml was withdrawn at various intervals and the reaction was stopped by the addition of ninhydrin reagent (0.1%). The amino acid content of the solution was estimated from a standard curve prepared from aspartic acid or glutamic acid standards. The enzyme activity was determined as the amount of amino acids released/unit time/ g of protein. One unit of enzyme was defined as the amino acid released / unit time / gm of protein.

Table 1. Production of proteolytic enzyme by dominant fungi on seed powder medium

Age of culture filtrate (Days)	Proteolytic activity (U/ml)				
	Alternaria alternata	*Aspergillus flavus*	*Aspergillus niger*	*Fusarium oxysporum*	*Penicillium digitatum*
1	0.00	0.00	0.00	0.00	0.00
2	0.00	0.02	0.00	0.00	0.00
3	0.00	0.04	0.02	0.03	0.00
4	0.02	0.06	0.05	0.05	0.02
5	0.03	0.08	0.06	0.06	0.04
6	0.05	0.11	0.11	0.10	0.07
7	0.9	0.14	0.12	0.11	0.10
8	0.11	0.17	0.13	0.12	0.12
9	0.11	0.17	0.13	0.12	0.12
10	0.11	0.17	0.13	0.12	0.12

Table 2. Production of proteolytic enzyme by dominant fungi on Czapek agar with casein hydrolysate

Age of culture filtrate (Days)	Proteolytic activity (U/ml)				
	Alternaria alternata	*Aspergillus flavus*	*Aspergillus niger*	*Fusarium oxysporum*	*Penicillium digitatum*
1	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00
3	0.01	0.03	0.02	0.02	0.01
4	0.02	0.05	0.03	0.05	0.02
5	0.03	0.06	0.05	0.06	0.04
6	0.05	0.08	0.08	0.11	0.08
7	0.07	0.10	0.10	0.12	0.11
8	0.07	0.10	0.10	0.12	0.13
9	0.07	0.10	0.10	0.12	0.13
10	0.07	0.10	0.10	0.12	0.13

RESULTS AND DISCUSSION

Fungi are known for their capacities to synthesize a variety of enzyme depending upon availability of substrate. The isolated fungi were maintained on seed meal agar and the most dominant were used for assessment of protease production by these fungi. A series of experiments were undertaken to assess the ability of fungi to degrade protein present in the soybean seed by secretion of protease.

All the fungal sps used in the present study and few non sporutation forms were isolated from the seeds. Among them the most dominant in terms of their raidal growth on the plate, they were selected for further studies. *Alternaria alternata, Aspergillus flavus, A. niger, F. oxysporum, Penicillium digitatum* were selected. These selected fungi were grown on soybean powder containing medium. The 8 days old culture filtrate was used as crude enzyme source.

Alternaria alternata, Aspergillus flavus, A. niger, F. oxysporum, Penicillium digitatum synthesizes proteases in both the media.

Table 3. Effect of pH on production of proteolytic enzyme by dominant fungi soybean powder medium

pH	Proteolytic activity (U/ml)				
	Alternaria alternata	*Aspergillus flavus*	*Aspergillus. niger*	*Fusarium oxysporum*	*Penicillium digitatum*
3.5	0.09	0.08	0.10	0.09	0.08
4.0	0.10	0.09	0.11	0.10	0.09
4.5	0.11	0.11	0.12	0.11	0.11
5.0	0.12	0.12	0.13	0.12	0.12
5.5	0.12	0.17	0.14	0.12	0.13
6.0	0.12	0.18	0.14	0.12	0.13
6.5	0.10	0.18	0.14	0.10	0.11
7.0	0.08	0.08	0.12	0.08	0.08
7.5	0.05	0.05	0.10	0.05	0.05
8.0	0.02	0.03	0.05	0.02	0.03
8.5	0.03	0.00	0.02	0.03	0.00
9.0	0.00	0.00	0.00	0.00	0.00
9.5	0.00	0.00	0.00	0.00	0.00
10.0	0.00	0.00	0.00	0.00	0.00
10.5	0.00	0.00	0.00	0.00	0.00

Table 4. Effect of Temperature on production of proteolytic enzyme by dominant fungi soybean powder medium

Temp (°C)	Proteolytic activity (U/ml)				
	Alternaria alternata	*Aspergillus flavus*	*Aspergillus. niger*	*Fusarium oxysporum*	*Penicillium digitatum*
20	0.08	0.07	0.08	0.07	0.07
25	0.10	0.09	0.11	0.09	0.09
30	0.11	0.11	0.12	0.10	0.11
35	0.12	0.12	0.13	0.11	0.12
40	0.12	0.17	0.14	0.12	0.13
45	0.12	0.18	0.14	0.12	0.13
50	0.10	0.18	0.14	0.10	0.11
55	0.09	0.12	0.12	0.08	0.08
60	0.04	0.05	0.08	0.05	0.05
65	0.03	0.03	0.05	0.02	0.03

The synthesis increased with increase in time of incubation in both the media; however the amount of enzymes varied. Maximum enzymes were secreted in soybean powder medium (Table 1) followed by Czapek agar with casein hydrolysate containing medium (Table 2). The quantity of enzyme secreted in soybean powder medium increased during the study period, the maximum amount was detected on 8th day. It was 0.11 µ/ml by *Alternaria alternata*, 0.17 µ/ml *Aspergillus flavus,* 0.13µ/ml by *A. niger,* 0.12µ/ml by *F. oxysporum* and *Penicillium digitatum.* In Czapek agar with casein hydrolysate containing medium the synthesis of enzymes increased up to 7 days and thereafter it remained constant. The synthesis was 0.07 µ/ml by *Alternaria alternata*, 0.10 µ/ml *A. flavus* and *A. niger* 0.12µ/ml by *F. oxysporum* and 0.11µ/ml by *Penicillium digitatum.*

In the study, effect of temperature and pH on the synthesis of proteolytic enzymes, it was found that 5.0 to 6.5 (Table 3) was optimum pH and 45°C-50°C (Table 4) was optimum temp.

The assessment of soybean in storage for the quantity of total protein content present revealed that, there was reduction in total protein content in soybean seeds. The dominant fungi associated were mostly internal fungi. This indicates the role of fungi in deteriorating total protein content and quality by secreting proteases. In earlier studies, it was shown that the qualitative estimation indicates the decrease in some amino acids with increase in time and accumulation of some amino acids with storage period[10].

This indicates the proteolytic abilities of the dominant fungi associated with the soybean. The study of the dominant fungi for synthesis of proteases revealed that they were ardent producers of proteases. So the deterioration of seed can be attributed to the proteolytic ability of fungi. Similarly the decrease in total protein content indicates their utilization as substrate by these fungi.

The results indicates that *A. alternata, Aspergillus flavus, A. niger, F. oxysporum, Penicillium digitatum* were found to play decisive role in altering nutritive value. The growth of these fungi caused significant deterioration in the quality of the seeds. This clearly suggests well equipped enzyme make up of these fungi to degrade and utilize any kind of storage chemical present in the seeds. However, the degree of enzyme production was found to be variable among the mycoflora. This may be related with their adaptation potential which might be different in these fungi. Similar type of reports regarding production of enzymes in seed borne pathogens has been reported in literature[11-13].

REFERENCES

1. Lambat, A.K. and Ram A. Seed borne infection of *Curvularia* causing a new blight disease of Jowar. *Indian phytopath*. 1969; **20**: 382-383.
2. Agrawal, V.K., Joshi, A.B. A preliminary note on the purple stain disease of of extracted crude oil. *J. Amer. Oil Chem. Soc*. 1972; **57**(10): 339-342.
3. Agrawal, V.K., Singh, O.V. Fungi associated with sunflower seed. *Indian phytopath*. 1974; **27**(2): 240-241.
4. Ward, H.S. Jr. and Diener, U.L. Biochemical changes in shelled peanuts caused by storage fungi. I. Effect of *Aspergillus tamari*. Four species of *A. glaucus* group and *Penicillium citrinum*. Phytopath, 1961; **57**: 244-250.
5. Kadian, O.P., D.Suryanarayana. Studies on seed microflora of oil seed crops (i) Linseed. *Indian phytopath.* 1972; **24:** 457-490.
6. Lalithakumari, D., Govindaswami, C.V., Vidhyasekaran, P. Isolation of seed-borne fungi from stored groundnut seed and their role in seed spoilage. *Madras agric. J.* 1970; **57**(9): 27-28.
7. ISTA. International rules for seed testing. *Proc. Int. Seed Asso.* 1966; **32:** 565-589.
8. De Tempe, J. The blotter method of seed health testing. *Proc. Int. Seed Test. Asso.* 1953; **28**: 133-151.
9. Neergard, Paul, Seed pathology. Revised edition. The Mcmillan press Ltd., London 1979.
10. Pople P. U., Studies on Seed Borne Fungi of Soybean (*Glycine Max* (L.) Merril) Ph.D. thesis, Swami Ramanand Teerth Marathwada University, Nanded, 2004.
11. Shukla, D.N. and Bhargawa, S.N. Some studies on *Fusarium solani* isolated from seeds of pulses and oil crops. *Proc. Nat. Acad. Sci. Ind.* 1977; **47**(4): 199-203.
12. Cherry, J.P. Protein Degradation during Seed Deterioration. *Phytopath.* 1982; **73**: 317-321.
13. Bilgrami, K.S., Sinha, R.K., Prasad, T., Jamaluddin, Roy, A.K. Studies on deterioration of some pulses by fungi. Indian Phyopath. 1976; 29: 374-377.