EFFECT OF PECTIN ON PROTOPECTINASE PRODUCTION BY RHIZOCTONIA SPECIES ASSOCIATED WITH ROOT ROT OF COTTON

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Introduction

The root rot disease of cotton has been a subject of investigation by plant pathologists since a number of years and so far no conclusive data are available either on the mode of disease causation or its control. Vasudeva who has done most extensive work on the problem ascribed the disease to *Rhizoctonia solani* and *Rhizoctonia batatícola*, the two fungi which are most predominantly associated with diseased roots. Sattar and his co-workers found that it was not possible to reproduce the disease under artificial conditions by these two fungi. Khan who took up the work in 1950 reported that wilting is actually caused by a hitherto unidentified fungus designated 608, but it does not produce the symptoms of shredding of root bark which is typical of the diseases. Physiological studies on the problem were therefore taken up by the author under a scheme financed by the Pakistan Central Cotton Committee to determine the role of the fungi associated with the disease under laboratory conditions in the first instance.

Researches on the physiological aspect of the disease carried out in U.K. and U.S.A. have shown that root rot and wilt diseases are caused by enzymes or vivotoxins secreted by pathogens during their process of growth. This vivotoxin has been identified as protopectinase which converts protopectin, the structure representative of mid lamella of cell walls, into soluble products, thereby bringing about the loss of coherence of tissues. The interesting feature is that conditions of production of protopectinase vary with various pathogens and it may be produced either readily or under certain specific conditions only. An investigation of its mode of production and properties sometimes results in important findings as regards the occurrence of the disease in nature.

Techniques Used

(*a*) *Preparation of Culture Filtrates.*—Forty ml. of the medium to be used was taken in a 12-oz. medicinal bottle and sterilised media were inoculated with three discs of uniform size, removed by a cork borer from the margin of a five-day old culture colony on potato-dextrose-agar medium. After incubation at 30°C. for a fortnight, the cultures were passed through muslin before centrifuging at 5,000 r.p.m. for 10 minutes. The cell-free filtrate thus obtained was stored in the refrigerator and was used when required. The mycelial weights were taken according to the usual procedure on an Oertling's electrical balance.

(*b*) *Protopectinase Activity.*—This was determined by using potato tuber or turnip root tissues. Cylindrical plugs were cut out and injected with water under the vacuum pump for 30-45 minutes. From these plugs 0.5 mm. thick discs were cut out, using a rotary microtome. Protopectinase activity was given as the time taken for five such discs to lose coherence at the slightest tension. Protopectinase activity has been given in the table as $A = 1000/RT$ where RT is reaction time in minutes.

Toxic Activity.—The determination of toxicity was based on the principle that a cell was assumed to be dead when it no longer plasmolysed (Trible 1955). Ten discs of the tissues were immersed in the test solution. At regular intervals, the discs were removed and transferred to a plasmolysing solution, containing a vital stain (molar KNO_3, 8.5 ml.; 0.1% neutral red chloride, 1.0 ml.; phosphate buffer of pH 7.6, (0.5 ml.). In this solution the protoplasts which plasmolysed appeared as dark red spots after 15-20 minutes and were easily visible to the naked eye. Toxic activity was assessed according to the following scale:

0—Discs colourless, no cells plasmolysed.
1—Occasional cells alive and therefore only occasional red spots.
2,3 and 4—Proportional increase in the number of red spots or plasmolysed cells.
5—Discs uniformly red, all cells plasmolysed.

Experimental

A. Production of Protopectinase and Toxins

1. *Effect of Pectin.*— Experiments reported earlier had shown that protopectinase production is influenced by the source of carbon and nitrogen present in the nutrient medium and

that the active solutions were obtained when glucose was used as the source of carbon and ammonium nitrate was used as the source of nitrogen. An experiment was set up to study the effect of pectin on the protopectinase activity of the fungi. The following basal medium which had so far given best activity was used:—

Potassium chloride	0.05%
Magnesium sulphate	0.05%
Potassium dihydrogen phosphate	0.10%
Glucose	1.5%
Ammonium nitrate	0.1%
Calcium carbonate	0.75%

Calcium carbonate was used to keep the pH near about neutral. The cultures were incubated for a fortnight at 30°C. and the results obtained are given in Table 1.

It will be seen that all media supported good mycelial growth and that mycelial growth increased with the addition of pectin. Protopectinase activity was only shown by *Rhizoctonia spp.* and was completely absent in the case of culture 608 which is in conformity with previous results. There was a significant increase in protopectinase activity with the addition of pectin particularly in the case of *Rhizoctonia bataticola*, where maceration time was reduced from 3 hours to 2 hours. This shows that protopectinase production is of the adaptive type and is produced activity only under certain specific conditions. It will be interesting to see whether the fungus can be made to cause the disease readily under artificial conditions if the inoculum for infection is grown on medium giving best protopectinase activity or alternatively, by adding the fungus to soil supplemented with ingredients that promote this activity.

It was also found that toxicity is closely associated with protopectinase activity and that composition of medium has no effect on wilting activity.

2. *Effect of Type of Pectin.*—As protopectinase activity is also known to sometimes vary with the type of pectin an experiment was set up to note its effect and also to confirm previous findings. A low methoxyl pectin and a high methoxyl pectin at the concentration of 1% were incorporated in the medium and cultures set up according to the usual technique. The results obtained are given in Table 2.

It will be seen that both high methoxyl and low methoxyl pectin gave similar activity with the

TABLE 1.—EFFECT OF ADDITION OF PECTIN ON PROTOPECTINASE ACTIVITY, TOXICITY AND WILTING ACTIVITY OF CULTURE SOLUTIONS.

Treatment	Culture No.	Wt. of myc. (mg.)	Initial pH	Final pH	Proto-pectinase activity	Toxicity index			Wilting activity (hrs.)
						4 hrs.	6 hrs.	24 hrs.	
Ammonium nitrate — Glucose	608	52	7.2	7.2	0.0	5	5	4	7-8
	672	393	7.2	7.0	3.3	5	3	1	7-8
	RBC	252	7.2	6.6	6.0	3	2	0	7-8
Ammonium nitrate — Glucose 1% Pectin + M.M.	608	148	6.0	6.2	0.0	5	5	4	7-8
	672	398	6.0	6.2	4.0	5	3	1	7-8
	RBC	324	6.0	6.0	9.0	2	1	0	7-8
Controls	—	—	—	—	0.0.	5	5	5	None

TABLE 2.—EFFECT OF TYPE OF PECTIN ON PROTOPECTINASE ACTIVITY, TOXICITY AND WILTING ACTIVITY OF CULTURE SOLUTIONS.

Treatment	Culture No.	Wt. of myc. (mg.)	Protopecti-nase activity	Toxicity index			Wilting activity (hrs.)
				4 hrs.	6 hrs.	24 hrs.	
Ammonium nitrate-glucose +1% low methoxyl pectin	608	190	0.0	5	5	4	7-8
	672	382	4.0	5	2	0	7-8
	RBC	265	9.0	2	1	0	7-8
Ammonium nitrate-glucose +1% high methoxyl pectin	608	170	0.0	5	5	4	7-8
	672	376	4.0	5	2	0	7-8
	RBC	294	9.0	2	1	0	7-8
Controls	—	—	0.0	5	5	5	None

fungi and supported good growth of the mycelium. Again maximum protopectinase activity and toxicity was shown by *R. bataticola* and was completely absent in the case of 608. Wilting was however caused by all the three fungi.

3. *Effect of Growing R. bataticola in Combination with Other Isolates.*—The experiment was set up to see the effect of association of 672 and 608 with *R. bataticola* on protopectinase production and toxic activity. The cultures were made in the basal medium with 1% pectin. The results obtained are shown in Table 3.

It was observed that the fungi could grow well in association with each other. The pH always moved towards alkalinity during the process of growth. The protopectinase activity and toxicity slightly increased when *R. bataticola* and *R. solani* (672) were grown together, were the same when *R. bataticola* were either grown singly or with both 608 and 672, and was reduced when it was grown along with 608 only. There was no effect on the wilting activity of solutions when the fungi were grown in different combinations.

This showed that either the lethal properties of *R. bataticola* can be increased if it is associated with certain fungi or else the increase is due to the combined effect of both the fungi. This needs to be investigated further with some other important isolates associated with root rot disease.

4. *Effect of Different Concentrations of Pectin.*—Further investigations on protopectinase production were made with *R. bataticola* only as it was confirmed by previous results that it gives the best activity. In nature also this isolate is most prominently associated with the root rot disease.

In this experiment high methoxyl pectin was used at four different concentrations and cultures set up according to the usual technique. The results obtained are given in Table 4.

As will be seen from Table 4, it was confirmed that protopectinase activity of *R. bataticola* increases when pectin is added to the medium at 1%. It remained the same at 1.5% concentration and showed a slight increase when 2% pectin was used. The toxic activity closely followed the trend of protopectinase activity and wilting was caused in 4-5 hours at all concentrations. Mycelial growth showed a gradual increase with the increase in pectin concentration thereby indicating that it is a good source of carbohydrate for the fungus. As only a small quantity of synthetic pectin had been obtained from California it was used at 1% concentration only in further experiments.

5. *Effect of Two Different Strains of R. bataticola.*—Recent studies have shown that different strains of the same fungus may show difference in their infection capacity. During the author's tour to Lyallpur the Assistant Plant Pathologist gave him a culture of *R. bataticola* (419) (RBC) which was reportedly giving better pathogenicity as compared with other strains. This strain was similar to the one being used in these studies previously

TABLE 3.—EFFECT OF GROWING R. BATATICOLA IN COMBINATION WITH OTHER ISOLATES ASSOCIATED WITH ROOT ROT.

Cultures	Wt. of myc. (mg.)	Initial pH	Final pH	Protopectinase activity	Toxicity index				Wilting activity (hrs.)
					2 hrs.	4 hrs.	6 hrs.	24 hrs.	
RBC	294	5.8	6.2	9.0	4	2	1	0	7-8
RBC+672	354	5.8	7.8	11.0	3	2	1	0	7-8
RBC+608	300	5.8	6.2	3.0	5	5	4	0	7-8
RBC+608+672	370	5.8	7.6	9.0	4	2	1	0	7-8
Control	—	—	—	0.0	5	5	5	5	None

TABLE 4.—EFFECT OF DIFFERENT CONCENTRATIONS OF PECTIN ON PROTOPECTINASE ACTIVITY, TOXICITY AND WILTING ACTIVITY OF RBC.

Pectin conc.	Wt. of myc. (mg.)	Protopectinase activity	Toxicity index			Wilting activity (hrs.)
			2 hrs.	4 hrs.	24 hrs.	
0.0%	257	4.0	5	5	1	4-5
0.5%	284	4.0	5	5	1	4-5
1.0%	337	9.0	4	2	0	4-5
1.5%	398	9.0	4	2	0	4-5
2.0%	411	11.0	3	1	0	4-5
Controls	—	0.0	5	5	5	None

TABLE 5.—COMPARISON OF TWO DIFFERENT STRAINS OF RBC AS REGARDS THEIR ACTIVITY IN CULTURE SOLUTIONS.

Treatment	Culture No.	Wt. of myc. (mg.)	Protopectinase activity	Toxicity				Wilting activity hrs.
				2 hrs.	4 hrs.	6 hrs.	24 hrs.	
Basal medium +1% pectin	RBC (X)	337	9.0	4	2	1	0	4-5
	RBC (Y)	210	11.0	3	1	1	0	4-5
Basal medium +2% pectin	RBC (X)	411	11.0	3	1	1	0	4-5
	RBC (Y)	366	16.5	2	0	0	0	4-5
Control	—	—	0.0	5	5	5	4	None

except that it had comparatively more profuse hyphal growth, and the sclerotia were larger and rather round in shape. Protopectinase production was compared at 2 different concentrations, viz., 1.0% and 2.0% and the results obtained are given in Table 5.

As will be seen from Table 5, it was found that greater protopectinase activity and toxicity was shown by the strain RBCY. At both the pectin concentrations the increase was significant. This result is quite important as in nature although several strains may be occurring together it might be a particular strain which is mainly responsible for causing the infection. This needs to be investigated further with various isolates of *Rhizoctonia spp.*

B. PROPERTIES OF ACTIVE VIVO-TOXIN

These studies were made with the culture solutions of *Rhizoctonia bataticola* giving protopectinase activity of 9.0, i.e., which brings about the loss of coherence of living tissues in 2 hours and death of 50% of the cells in four hours.

1. *Effect of Heating.*—The solutions were heat-treated for 5 minutes at the various temperatures, chilled in ice-cold water and frozen till such time as used for the assessment of properties. The results obtained are given in Table 6.

It was found that like many other enzyme solutions the protopectinase solution was very sensitive to heat-treatment. The protopectinase activity was reduced from 9 to 5 at 60°C. and was deactivated at 70°C. Toxicity followed a similar trend, and it was confirmed that death of the cells is brought about as a result of enzyme action. Again, heating or autoclaving had no effect on wilting activity of the solutions.

2. *Effect of Dilution.*—This experiment was made to see as to how the concentration of the protopectinase present in the solution affects the activity. The various concentrations were made up with autoclaved enzyme solution. The results obtained are given in Table 7.

It was observed that the activity increased with the increase in concentration of protopectinase. Toxic activity also followed a similar trend.

TABLE 6.—EFFECT OF HEATING ON PROTOPECTINASE ACTIVITY AND TOXICITY OF ACTIVE RBC CULTURE SOLUTION.

Heating at °C.	Protopectinase activity	Toxicity index				Wilting activity hrs.
		2 hrs.	4 hrs.	6 hrs.	24 hrs.	
60	5	5	4	2	0	4-5
70	0	5	5	5	4	4-5
80	0	5	5	5	4	4-5
90	0	5	5	5	4	4-5
100	0	5	5	5	4	4-5
Autoclaved	0	5	5	5	4	4-5
No heating	9	5	3	2	0	None

TABLE 7.—EFFECT OF DILUTION ON PROTOPECTINASE ACTIVITY AND TOXICITY OF ACTIVE RBC CULTURE SOLUTION.

% Culture solution	Protopectinase activity	Toxicity index				
		1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.
0.0%	0	5	5	5	5	5
25.0%	3	5	5	5	4	3
50.0%	4	5	5	5	4	2
75.0%	6	5	5	3	2	1
100.0%	9	5	4	2	1	0

TABLE 8.—EFFECT OF TEMPERATURE ON PROTOPECTINASE ACTIVITY AND TOXICITY OF ACTIVE CULTURE SOLUTION.

Temperature °C.	Protopectinase activity	Toxicity Index			
		1 hr.	2 hrs.	3 hrs.	4 hrs.
10	0	5	5	5	5
30	7	5	5	3	1
32.5	9	5	4	2	1
35	10	5	3	1	0
50	0	5	3	2	2

3. *Effect of Temperature of Reaction.*—In this experiment the effect of solutions on living tissues was studied at various temperatures within the range when solutions remain active. The results obtained are given in Table 8.

The optimum temperature for activity was found to be 35 °C. where maceration of cells was brought about in 100 minutes. This is interesting as the optimum temperature for infection has also been reported to be between 35 °-38 °C.

Summary and Discussion

Several experiments were set up to investigate the effect of various factors on the protopectinase activity, toxicity and wilting activity of culture solutions derived from the three main fungi, viz., *R. bataticola*, *R. solani* and 608 associated with root rot. It was found that the addition of pectin in small quantities increases activity in the case of *Rhizoctonia spp.* particularly *Rhizoctonia bataticola* but has no effect in case of culture 608. Best protopectinase and toxin production was obtained when ammonium nitrate was used as the source of nitrogen and glucose and pectin were used as sources of carbon along with calcium carbonate. With this treatment culture solutions derived from *R. bataticola* could cause the living tissues to lose their coherence in 2 hours and caused death of 50% of the cells after 4 hours. Another interesting finding was that different strains of *Rhizoctonia bataticola* show variable activity and this provides a good means of assay for more active strains to be used in infection experiments.

These experiments show that the nature of protopectinase production in this case is adaptive, i.e., it is activated in the presence of certain specific conditions, and this might be the reason why it has been found difficult to reproduce the disease readily. It will be interesting to see if the disease can be reproduced by growing cultures on media giving good protopectinase activity and using them for artificial inoculations or alternatively inoculating plants in soil substrates after supplementing them by carbon and nitrogen sources which promote protopectinase activity.

A few experiments made on the properties of the active protopectinase solutions itself showed that it is deactivated after being heated at temperatures above 60°C., is reduced in activity on dilution, and reacts best on living tissues at a temperature of 35 °C. This is interesting as in nature also optimum temperature for disease causation is reportedly 35-38 °C.

References

1. W. Brown, *Studies in the physiology of parasitism*, Ann. Botany (London), **29**, 313-348 (1915).
2. A.E. Dimond and P.E. Wagonner, *On the nature and role of vivo-toxins in plant diseases*, Phytopathology, **43**, 229-235 (1953).
3. M. Kamal and R.K.S. Wood, *Enzymes secreted by Verticillium dahliae and their role in the development of wilt of cotton*, Ann. Appl. Biol., **44**, 322-340 (1956).
4. M. Kamal, *Studies in the physiology of root rot of cotton in Pakistan*, Pakistan Cottons, **4** (1), 1-5 (1960).
5. A. Sattar and A. Hafeez, Scientific Monograph No. 1, Pakistan Association for the Advancement of Science (1952).
6. R.S. Vasudeva, *Studies on the root rot disease of cotton in the Panjab*, Indian J. Agr. Sci., **5**, 946 (1935).