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Biochemistry of post harvest spoilage of sweet potato (*Ipomoea batatas* L.) tubers. 3. Pectolytic enzymes production *in vitro* and in infected tubers by fungi

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The fungi, *Botryodiplodia theobromae* and *Rhizopus oryzae*, isolated from rotted sweet potato tubers produced several pectolytic enzymes i.e. polygalacturonase(PG), polymethylgalacturonase(PMG), pectin lyase(PNL) and pectate lyase(PL) in cultures when grown on pectin-polypectate mineral medium. PG and PMG were found to be produced in more quantity than the lyase enzymes. These two pectinases were also detected in substantial quantities from the extracts of sweet potato tubers rotted by each of the two test fungi. Enzyme containing culture filtrates and extracts from rotted tissues induced rot symptoms when applied onto sterilized healthy tubers whereas the extracts from healthy tissues did not. *R. oryzae* produced more PG and PMG than *B. theobromae.*

Key words : Polygalacturonase(PG), Polymethylgalacturonase(PMG), pectin lyase(PNL), pectate lyase (PL), sweet potato

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is susceptible to many post harvest diseases (Ray and Balagopalan, 1997 ; Snowdon, 1991). *Botryodiplodia theobromae* Pat. and *Rhizopus oryzae* Went & Prins. Geerl. are the two most frequently occurring associated fungi with spoilage of sweet potato tubers in tropics (Ray and Balagopalan, 1997 ; Ray and Misra, 1995).

Some of the pectolytic enzymes associated with cell separation and tissue maceration in soft rot diseases include polygalacturonase (PG) and polymethylgalacturonase (PMG) which are reported from culture filtrates of *Rhizopus stolonifer* and potato and sweet potato tissues rotted by the fungus (Srivastava *et al.*, 1959 ; Amadioha and Oladiran, 1993), pectin methylesterase (PME) reported from citrus fruit rot caused by *Aspergillus aculeatus* and *B.theobromae* (Adisa and Fajola, 1982), pectin lyase (PNL) reported from culture fluids of *Erwinia aroideae* (Dean and Wood, 1967) etc. While the production of many of these pectinases has been shown to occur in culture filtrates of the rot organisms (*in vitro*), only a few have been found tobe associated with the rotted tissues (*in vivo*) in concentrations sufficient to induce rot symptoms. This paper deals with the ability of *B. theobromae* and *R. oryzae* to produce pectolytic enzymes (PG, PMG, PNL and PL) *in vitro* and *in vivo,* and their role in the development of post harvest tuber rot of sweet potato.

MATERIAL AND METHODS

Fungal isolates

The isolates of *B. theobromae* (IMI 361228) and *R. oryzae* (IMI 361235) used in this study were earlier isolated from the post harvest rotted tissues of sweet potato tubers (Ray and Misra, 1995). Spore suspension of both the fungi were prepared separately from 6 days old cultures grown on potato dextrose agar (PDA). Spores were harvested in sterile distilled water and diluted to a concentration of 5.5×10^6 spores ml^{-1}. The same concentration was used in all the experiments.

Sweet potato tubers

Freshly harvested sweet potato tubers (var. Pusa Safed) were collected from the experimental farm of the Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar during rabi (January-February) season of 1998-1999. The tubers were used within 3-4 days after harvest.

Enzyme study

Pectolytic enzyme production *in vitro* was studied using a liquid pectin-polypectate mineral medium containing KH_2PO_4, 1.0 g ; $NaNO_3$. 2.0 g ; $MgSO_4$. $7H_2O$, 0.5 g ; KCl, 0.5 g ; $FeSO_4$. $7H_2O$; 0.01 g ; yeast extract, 10.0 g ; apple pectin, 5.0 g ; sodium polypectate, 0.5 g ; distilled H_2O, 1 L and pH was adjusted to 6.0. The 250 ml Erlenmeyer flasks containing 100 ml of the above medium were sterilized by autoclaving at 121°C for 15 min. Two ml spore suspension (5.5×10^6 spore ml^{-1}) of the fungi (*B. theobromae* or *R. oryzae*) were inoculated into the flasks and the flasks, in triplicates, were incubated for 12 days at room temperature (28 ± 2°C). It took 2-3 days for spore to grow into mycelium with pectin-sodium polypectate as carbon source, and after 4 days of inoculation and at regular intervals the contents of the growth flasks were pooled and filtered. The culture filtrates were used as the enzyme source.

Extraction of enzymes from infected sweet potato tubers

Healthy and freshly harvested tubers were taken and surface sterilized with 1 % sodium hypochlorite solution. These tubers were inoculated separately with 4 mm mycelial discs of either *B. theobromae* or *R. oryzae* as per the method described elsewhere (Ray and Punithalingam, 1996). The tubers were kept for 4-10 days at room temperature (18 ± 2°C) for rot development. Lesion areas and surrounding tissues were removed with a sterile scalpel, weighed into 10 g samples and extracted by grinding with distilled water in a morter and pestle in the ratio 1. : 3. The homogenate was strained through three layers of cheese cloth to remove the pulp. The liquid fraction (rot extract, RE) was cleared by centrifugation at 10,000 g in a refrigerated centrifuge and was adjusted to the volume. The RE served as the enzyme source.

Enzyme assay

PG

Relative polygalacturonase (PG) activity was assayed viscometrically (Mahadevan and Sridhar, 1988) using 2 ml enzyme preparation and 4 ml of buffered substrate (1.2 % Na-polypectate solution, pH 5.0) and 1 ml sodium acetate-acetic acid buffer solution (pH 5.0). Reduction in flow time of enzyme-substrate mixture was recorded using a viscometer after 30 min interval upto 120 min at room temperature (28 ± 2°C). Percentage loss in viscosity was calculated using the following formula :

$$V = \frac{T_0 - T}{T_0 - T_{H_2O}} \times 100$$

Where, V = Per cent loss in viscosity
T_0 = Flow time in seconds at zero time,
T = Flow time of the rection maxture at time T1 and
T_{H_2O} = Flow time of distilled water.

PMG

The substrate used for PMG was 1 % pectin in sodium acetate-acetic acid buffer at pH 5.2. The rest of the procedure was similar to that of PG.

PNL and PL

The substrates used for relative PNL and PL were 1.2 % pectin and 1.2 % sodium polypectate, respectively in boric acid – borax buffer at pH 8.7. The rest of the procedure was similar to that for PG.

Pectolytic enzymes and rot development

The effect of pectolytic enzymes on rot development was investigated using the pectinases from culture filtrates and extracts of rotted tissues as inocula on surface-sterilized healthy sweet potato tubers. Controls were inoculated with 5-8 drops of distilled water, heated culture filtrates or rotted tissue extracts. Incubation was done at 28°C in a Biological Oxygen Demand incubator while

observations were made regularly at 2 days interval for a period of 10 days.

RESULTS

The relative pectolytic enzymes i.e. PG, PMG, PL and PNL activities of the fungi *B. theobromae* and *R. oryzae* in the pectin-polypectate defined liquid medium is shown in Figure 1. Preliminary results have shown that yields of pectolytic enzymes was much higher in the pectin-polypectate medium than in potato-dextrose broth. The enzyme activity, moreover, increased with days of incubation from 4 to 12 days in most cases and *R. oryzae* produced more pectolytic enzymes than *B. theobromae* as was evident by the percentage loss in viscosity.

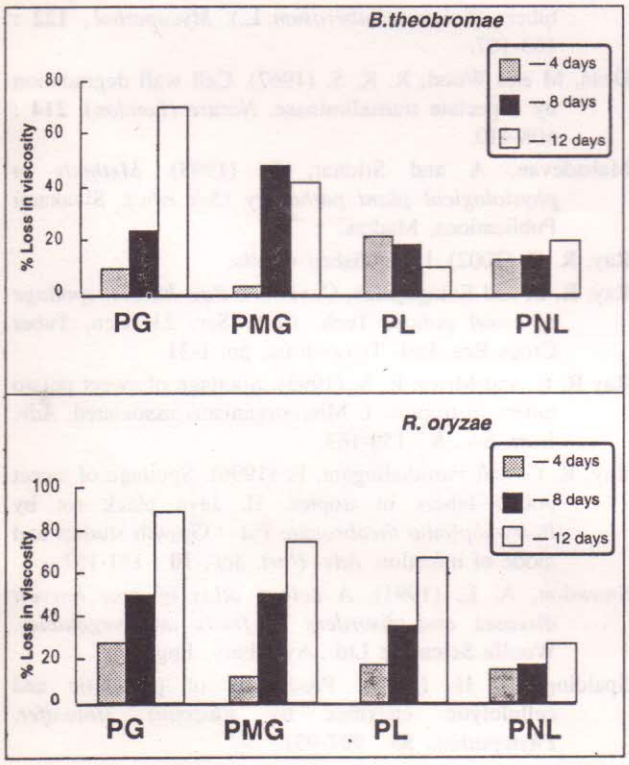

Fig. 1 : Pectolytic enzyme (PG, PMG, PL and PNL) activities of the culture filtrate of the fungi grown on pectin-polypectate liquid medium in terms of percentage loss in viscosity after 4, 8 and 12 days of incubation.

The PG and PMG activities of the rot extracts are shown in Figure 2. Comparative studies on culture filtrate and extracts of rotted tissues showed that PG and PMG activity was more in culture filtrate than in the RE as was evident from the loss in viscosity. However, the rot extracts either from *B.*

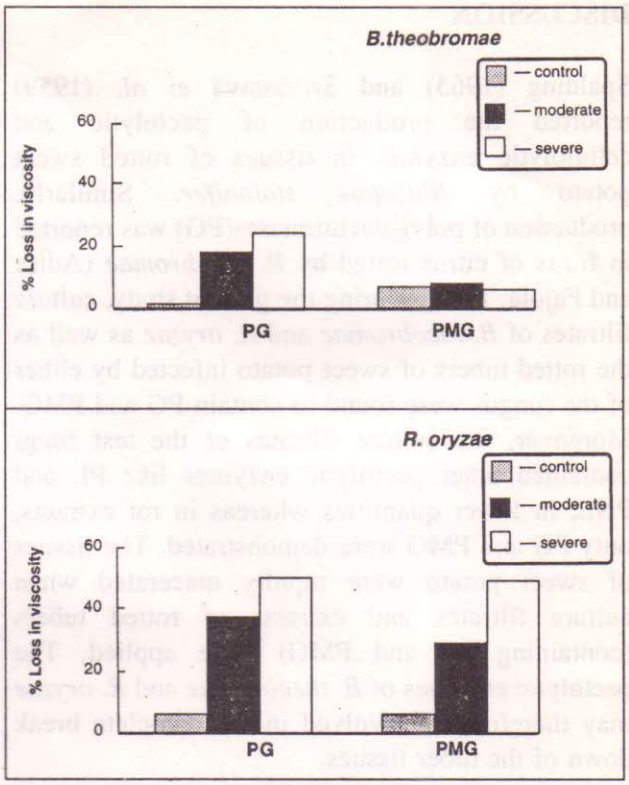

Fig. 2 : PG and PMG activities of the rot extract of sweet potato tubers infected with the fungi. Control (healthy, non-infected), moderate (<50 % rotten) and severe (<90 % rotten).

theobromae or *R. oryzae* had very little PNL and PL activity (> 10 %) although these enzymes were produced substantially in culture medium. The results of the effect of pectolytic on rot development showed that the culture filtrates and extracts of rotted tissues were able to induce rot symptoms whereas the extracts from healthy tissues did not show any symptom of rot when applied to sweet potato tubers (Table 1). The results show the pectolytic enzymes (PG and PMG) from the two preparations caused tissue maceration of sweet potato tubers.

Table 1 : Effect of pectolytic enzymes obtained from different sources on rot development in sweet potato tubers after 10 days of incubation

Treatments	*B. theobromae*	*R. oryzae*
Extracts from healthy tubers	– *	–
Enzyme extract from rotted tubers	++	++++
Culture filtrates of the test fungi	+++	++++

* – not rotten ; ++, < 40% rot ; +++, < 60% rot ; ++++, < 95%

DISCUSSION

Spalding (1963) and Srivastava *et al.* (1959) reported the production of pectolytic and cellulolytic enzymes in tissues of rotted sweet potato by *Rhizopus stolonifer.* Similarly, production of polygalacturonases(PG) was reported in fruits of citrus rotted by *B. theobromae* (Adisa and Fajola, 1982). During the present study, culture filtrates of *B. theobromae* and *R. oryzae* as well as the rotted tubers of sweet potato infected by either of the fungus were found to contain PG and PMG. Moreover, the culture filtrates of the test fungi contained other pectolytic enzymes like PL and PNL, in lower quantities whereas in rot extracts, only PG and PMG were demonstrated. The tissues of sweet potato were rapidly macerated when culture filtrates and extracts of rotted tubers (containing PG and PMG) were applied. The pectolytic enzymes of *B. theobromae* and *R. oryzae* may therefore be involved in the complete break down of the tuber tissues.

In our study, both rot organisms produced PG. However, *R. oryzae* produced more PG and PMG than *B. theobromae.* The differences in the amount of the two enzymes produced by *R. oryzae* and *B. theobromae* might explain the differences in the rate of rot development in sweet potato. In our study on cellulolytic enzymes, *R. oryzae* was similarly found to produce more cellulases than *B. theobromae* (Ray, 2002). It was of interest that after 10 days of inoculation *R. oryzae* caused severe rot whereas *B. theobromae* induced only a moderate rot.

The role of individual enzymes like PG, PMG and others in maceration of the cells needs to be further investigated.

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