Potentials of nutritional factors on production of cellulose enzyme by post-harvest fungal pathogens on sapodilla fruit

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ABSTRACT

Present paper describes the cellulase activity of post-harvest pathogenic fungi on Sapodilla (*Achras sapota* L.) fruit. Dominant three fungi viz. *Aspergillus niger, Geotrichum candidum* and *Rhizoctonia solani* were isolated and selected for this investigation. It was studied by using different nutritional sources like carbon, nitrogen, phosphorus, sulphur, antibiotic and vitamins. Carbon sources, starch and CMC (Carboxy methyl cellulose) inhibited the growth of fungi while Fructose, Sucrose, Maltose were induced the production of cellulase enzyme in all test fungi. In nitrogen sources, Sodium nitrate inhibited the production of cellulase enzyme activity whereas Sodium nitrite, Ammonium phosphate, Urea, Peptone stimulated the production of cellulase enzyme. Among Phosphorus, Amonium biphosphate and Sodium dihydrogen phosphate proved inhibitory whereas Disodium hydrogen phosphate and Potassium Hydrogen phosphate stimulated the enzyme production in all tested fungi. In case of sulphur, Ferrous sulphate is reduced the growth of cellulose production whereas Calcium sulphate is stimulatory. Among vitamins, Folic acid is inhibitory while thiamin and pyridoxine was stimulatory. In Amino acids, Argenine is reduced the enzyme production while aspartic acid is favorable. Among five tested antibiotics, all antibiotics are found favourable for the production of cellulose enzyme except ampicilin that reduced the production of enzyme.

KEY WORDS*:* *Achras sapota,* cellulase enzyme, nutritional factors, post- harvest pathogens

INTRODUCTION

Plant pathogens produce a range of enzymes capable of degrading plant cell wall components (Ryan, 1973; Baer and Gudmestad, 1995). Extracellular proteins secreted by fungus are able to macerate tissues and degrade cell wall components. They must thus contain the enzymes corresponding to the types of glycosidic linkages present in the cell wall polysaccharides. Extracellular enzymes are important to fungi not only for digestion but also in many instances for the pathogenic process the enzymes may function in overcoming the natural resistance of the host

as well as in providing soluble products that can be absorbed and used as food (Griffin, 1994).The production of extracellular proteases by plant pathogenic fungi is also well documented, and it has been proposed that in some fungus-plant interactions these enzymes may function as pathogenic factors (Raymond *et al.,*1959). Studies of enzyme production by a phytopathogenic fungus are complicated by the presence of plant, particularly by the presence of plant enzymes and microbial enzyme inhibitors that occur in the plants. The most practical way to study the production of enzyme by a fungus is therefore to study the production of its enzymes on artificial growth media that contain no plant or enzyme inhibitors produced by the plant. The purpose of this study was to determine the influence of growth conditions and medium composition on the cellulase enzyme production by post harvest pathogenic fungi.

MATERIALS AND METHODS

A) Isolation of fungi from sapodilla fruits

i) Isolation of fungi

Post harvested diseased fruit samples were collected at regular interval from field and market places of various localities of Thane District of Maharashtra State viz..Gholwad, Dahanu, Palghar, Saphale, Virar and Vasai. The study was carried out during 2010-2011. The collected samples were kept separately in pre-sterilized polythene bags and brought into laboratory. The fruit rot pathogens were isolated by using agar plate method. In this method pre-sterilized petriplates of 9cm diameter were poured with 20 ml autoclaved Potato Dextrose Agar (PDA). The diseased portions of fruit were cut aseptically into 1-2mm pieces using sterile blades. These pieces were surface sterilized with 0.1% Mercuric chloride, washed thrice with sterile distilled water repeatedly and inoculated. These plates were incubated at 27 ± 2 ^{0}C for 7 days. Growth and sporulation of the fungi were observed.

ii) Identification of fungi

The fungi occurring on each and every diseased tissue portion in plates were identified preliminary on the basis of sporulation characters like asexual or sexual spores and or fruiting structure with the help of stereoscopic binocular microscope. In some cases the infected tissues were stained by cotton blue and Lactophenol (McCleary and Glennie-Holmes, 1985) and observed under compound microscope. The identification and further confirmation of the fungi were made by preparing slides of fungal growth and observing them under compound microscope. The identification was made with the help of manuals recommended by (Ellis, 1971; Barnett, 1960) and symptoms were confirmed by Koch's postulates. Pure culture of these fungi were prepared and maintained on PDA agar slants.

B) Cellulase production

Isolated fungi were grown on liquid medium containing 1% carboxy methyl cellulose (CMC), KNO_3 – 0.1 % KH_2PO_4 – 0.1 % and $MgSO_4.7H_2O$ – 0.5 % at pH 6.0 and distilled water 1000ml. Treatments of

different nutritional sources such as Carbon (0.5%), Nitrogen (0,25%), Phosphorus, Sulphur (0.5%), Vitamins (100 ppm), Amino acids (100 ppm) and antibiotics (100 ppm) were given to above basal medium. Twenty five ml of the medium was poured in 100 ml conical flasks. These conical flasks were autoclaved at 15 lbs for 1 hour and allowed to cool. After this the flasks were inoculated with 1 ml spore suspension obtained from 7 days old culture of isolated fungi on PDA slants. Three replications were made for each species. The inoculated flasks were incubated at 27 ± 2 ^{0} C for 7 days in BOD incubator. After the incubation period the flasks were harvested by filtering the content through Whatman filter paper No. 1. The filtrate obtained were collected in pre sterilized bottles and considered as crude enzyme preparation.

Assay for cellulase (Cup-plate method)

The cup-plate method followed by (Dingle *et al.,* 1953; Szecsi, 1969) was used. The assay medium contains 1% CMC and 2% difco agar, was poured in Petri plate (20ml/plate) and allowed to solidify in the centre; a 6 mm diameter cup/cavity was made with pre-sterilized cork borer (No.4). The cup was filled with 0.1m culture filtrate and incubated at room temperature for 48 hours. The activity zone was developed flooding the plates with 3% lead acetate solution (10-15ml/plate). Milky white coloured activity zones were clearly seen on removing lead acetate solution with distilled water after a period of 30 minutes. The diameter of zone was measured in mm. Cultures of *Aspergillus niger, Geotrichum candidum* and *Rizoctonia solani* were used in this study which was isolated from post-harvest infected sapodilla fruit. Statistical analyses of the experiments were performed as per Mungikar (1997).

RESULTS AND DISCUSSION

A) Isolation of fungi from sapodilla fruits

Sapodilla fruits of different varieties viz. Kalipati, Kutchh and Cricket ball were collected from various market places of Thane District of Maharashtra during 2010-2011 and studied for the association of different fungi,. Dominant three fungi were observed *viz., Geotrichum candidum* Link ex Fries, *Aspergillus niger* Van Tieghem, *Rhizoctonia solani* Kuhn. and *Rhizopus stolonifer* (Ehrenb) Vuill.

B) Cellulase production

Carbon Sources

In order to study the effect of nutrients on cellulase production, Carbon sources other than glucose were tested by supplementing them individually in the basal medium of which three sources belongs to monosaccharides, two belongs to disaccharides and two polysaccharides were selected and tested at 0.5% concentration and the results are summarized in table 1.It was found that carbohydrate sources like Starch and CMC (Carboxy methyl cellulose) inhibited the growth of fungi for cellulase production while Fructose, Sucrose, Maltose induced the production of cellulase enzyme in all the tested fungi. While in disaccharides, Sucrose was proved to be

stimulatory for cellulose production in all the three tested fungi *Aspergillus niger* (14mm), *Rhizoctonia solani* (14mm), *Geotrichum candidum* (12mm).

Nitrogen sources

Various sources of nitrate forms, nitrite forms, ammonium forms, amide forms and organic forms at 0.25% concentration were incorporated separately in the basal medium and their effect on cellulase production was recorded. Basal medium containing potassium nitrate served as control (Table 2). It was noticed that in nitrogen sources like Sodium nitrate inhibited production of cellulase in case of *Aspergillus niger* (9mm), *Geotrichum candidum* (10mm) and *Rhizoctonia solani* (8mm).While in Sodium nitrite *Aspergillus niger* (14mm) and *Rhizoctonia solani* (13mm) showed maximum production of cellulase. Ammonium phosphate proved stimulatory for cellulose production in *Aspergillus niger* (16mm) and *Geotrichum candidum* (18mm). In Amide forms, Urea proved stimulatory for cellulase production in *Aspergillus niger* (15mm). It was also found that Gelatin produced maximum cellulase in *Aspergillus niger* (14mm) and *Geotrichum candidum* (15mm).While Peptone favored maximum production of cellulase in *Aspergillus niger* (14mm) and *Geotrichum candidum* (15mm) and minimum production of cellulase in *Rhizoctonia solani*(10mm).

Phosphorus sources

Five different phosphorus sources were added at 0.1% concentration to the basal medium by replacing Dipotassium hydrogen phosphate and their effect on cellulase production was studied. Results are showed in table 3 that Sodium dihydrogen phosphate proved inhibitory for cellulase production in *Aspergillus niger* (10mm), *Geotrichum candidum* (8mm) and *Rhizoctonia solani* (9mm). Whereas Disodium hydrogen phosphate stimulated the cellulase production in *Aspergillus niger* (14mm), *Geotrichum candidum* (13mm) and *Rhizoctonia solani* (12mm). Potassium hydrogen phosphate inhibited cellulase production in *Rhizoctonia solani* (9mm) whereas it induced maximum production of cellulase in *Aspergillus niger* (13mm) and *Geotrichum candidum* (11mm). Ammonium phosphate was proved inhibitory for cellulase production in *Geotrichum candidum* (8mm) and *Aspergillus niger* (10mm) and *Rhizoctonia solani* (9mm). Ammonium biphosphate was proved inhibitory for cellulose production in all the three tested fungi.

Sulphur sources

Seven different sources of sulphur at 0.5% concentration were tested againstthe ten post-harvest fungi for cellulase production and the results are given in table 4. It is interesting to note that all seven sources of sulphur play more or less equal role in the enzymes production. Ferrous sulphate and Zinc sulphate inhibited the cellulase production whileremaining sulphur sources induced the production of cellulase enzyme. Sodium sulphate induced cellulase production in *Aspergillus niger* (14mm) and *Rhizoctonia solani* (12mm). Calcium

sulphate induced cellulase production in *Aspergillus niger* (13mm) and *Rhizoctonia solani* (14mm). Ammonium sulphate induced cellulase production in *Aspergillus niger* (13mm), *Rhizoctonia solani* (12mm) and Potassium sulphate for *Aspergillus niger* (13mm) and *Geotrichum candidum* (12mm).

Vitamins

Vitamins are very important in many activities of microorganisms. Therefore five commonly used vitamins at 100 ppm concentrations were tested for cellulose production in tested post-harvest fungi It was indicated that vitamins like Folic acid and Riboflavin has been retarded cellulase action while Ascorbic acid, Thiamine and Pyridoxine induced cellulose action (Table 5). Ascorbic acid stimulated cellulase production in *Geotrichum candidum* (13mm) and *Aspergillus niger* (11mm) whereas it inhibited cellulase production in *Rhizoctonia solani* (8mm). Thiamin was proved to be stimulatory for *Aspergillus niger* (12mm) and *Rhizoctonia solani* (11mm). Pyridoxine inhibited cellulose production in *Rhizoctonia solani* (9mm) whereas it proved stimulatory in *Geotrichum candidum* (14mm) and *Aspergillus niger* (12mm).

Amino acids

In order to study the effect of amino acids at 100 ppm concentration five different amino acids were tested with three post-harvest fungi (Table 6). Result indicated that Arginine monochloride and Threonine inhibited enzyme action in *Rhizoctonia solani* (7mm & 8mm) and *Geotrichum candidum* (8mm & 10mm). Aspartic acid induced production of cellulase in *Aspergillus niger* (10mm) as compared to control (9mm).

Antibiotics

Effect of antibiotics on cellulase production was studied for these five different sources of antibiotics at 100 ppm concentration were employed separately against three post-harvest fungi and results are given in table7. It was observed that in the presence of Mox cellulase production was stimulated in *Geotrichum candidum* (20mm) while in *Rhizoctonia soloni* (18mm) stimulation of cellulase production was due to Terramycin. Streptomycin showed stimulation of cellulase production in *Aspergillus* niger (20mm). In *Aspergillus niger* (18mm) and *Rhizoctonia soloni* (17mm), Doxycyclin proved stimulatory for cellulase production. It is interesting to note that Ampicillin inhibited cellulose.

A capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi. The characters are restricted to a few species among several major taxa (Gooday, 1979). It was studied the lipase enzyme activity of storage fungi under the influence of carbon and nitrogen sources (Chavan and Kakde,2009). They found that carbon sources as like fructose and sucrose induces lipase activity while starch, lactose and carboxyl methyl cellulose (CMC) inhibits lipase activity. Nitrogen sources as like nitrate, nitrite, amide, ammonium, and protein affect in different ways on lipase enzyme of fungi. It was observed that carbohydrates were good source of growth of *Rhizopus oligoporus* but

low lipase production was obtained (Ely, 1988). The impact of nutritional factors on lipase enzyme production was reported (Kakde *et al.,* 2009). It was observed that fructose and sucrose stimulates lipase activity while lactose, carboxyl methyl cellulose and starch inhibited lipase activity (Kakde, and Chavan, 2011) Carbohydrate sources affects protease enzyme activity of *Helminthosporium, Curvularia* and *Alternaria* sp (Sharma and Satyanarayana, 1980). Fructose and sucrose stimulated protease production in *Alternaria alternata* but glucose was found to no effect on protease activity (Patil and Shastri, 1982). The secretion of several enzymes provides this phytopathogenic fungus with the ability to attack hosts which differ in their polysaccharide cell wall compositions (Riou *et al.,* 1991). It has been suggested that the proteases may facilitate located penetration of the plant cell wall by breaking down the glycoproteins that contribute to cell wall stability (Carpita and Gibeaut, 1993). Some phytopathogenic fungi such as *Fusarium*, *Alternaria,* and *Rhizoctonia* produced serine alkaline proteases, which are indispensable for their growth (Pekkarinen,*et al.,*2000) Carboxy methyl cellulose (CMC), ferrous sulphate, calcium sulphate, sodium sulphate, copper sulphate and sodium dihydrogen orthophosphate retarded the enzyme action of some *Alternaria* species (Rathod and Chavan,2010). Enzymes such as cellulase, amylase and lipase were obtained in terms of measurement of activity zones formed by reaction between substrate and culture filtrate of *Trichoderma* species and found that maximum cellulase exhibited by *T.pseudokoningi* (32mm) than others (Bhale and Rajkonda,2012).

CONCLUSION

It was concluded that starch, ammonium sulphate, ammonium biphosphate, ferrous sulphate, folic acid, arginine and ampicillin hold back the production of cellulose activity due to post harvest pathogens such nature of reticence of these may be useful to control the spoilage of fruits by fungi. If the cellulose is supplied in the nutritive media, fungi prefer to grow on it and the cellulase enzyme is also produced by the fungi.

Table 1: Effect of carbohydrates sources on cellulase production

Carbohydrates sources (0.5%)	**Post-harvest fungi (Activity zone in mm)**		
Monosaccharide	***A. niger***	***G. candidum***	***R. solani***
Glucose	12	14	10
Fructose	13	12	12
Xylose	12	13	11
Sucrose	14	12	14
Maltose	13	14	12
Starch	10	08	09
CMC (Control)	11	10	12
SEm ±	4.27	4.79	4.24
CD @5%	12.16	12.30	10.89

Table 2: Effect of nitrogen sources on cellulase production

Nitrogen Sources (0.25%)	Post-harvest fungi (Activity zone in mm)		
	A. niger	*G. candidum*	*R. solani*
Sodium nitrate	09	10	08
Sodium nitrite	14	11	13
Ammonium phosphate	16	18	10
Ammonium sulphate	10	13	09
Urea	15	12	08
Peptone	14	15	10
Gelatin	14	15	10
Potassium nitrate (Control)	10	13	11
SEm ±	0.95	0.91	0.59
CD @5%	2.23	2.15	1.38

Table 3: Effect of phosphorus sources on cellulase production

Phosphorus Sources (0.1%)	Post-harvest pathogens(Activity zone in mm)		
	A. niger	*G. candidum*	*R. solani*
Sodium dihydrogen phosphate	10	08	09
Disodium hydrogen phosphate	14	13	12
Potassium hydrogen phosphate	13	11	09
Ammonium phosphate	10	08	09
Ammonium biphosphate	09	09	08
$KH_2(PO_4)_2$ (Control)	12	10	11
SEm ±	4.26	3.60	3.49
CD @5%	10.93	9.24	8.96

Table 4: Effect of sulphur sources on cellulase production

Sulphur sources (0.5%)	Post-harvest pathogens(Activity zone in mm)		
	A. niger	***G. candidum***	***R. solani***
Zinc sulphate	10	09	10
Sodium sulphate	14	09	12
Calcium sulphate	13	11	14
Ferrous sulphate	09	08	10
Ammonium sulphate	13	09	12
Potassium sulphate	13	12	08
$MgSO_4$ (Control)	12	11	10
SEm ±	0.70	0.56	0.74
CD @5%	1.64	1.32	1.75

Table 5: Effect of vitamin sources on cellulase production

Vitamins sources (100 ppm)	Post-harvest pathogens(Activity zone in mm)		
	A. niger	***G. candidum***	***R. solani***
Ascorbic acid	11	13	08
Folic acid	09	09	10
Riboflavin	11	09	09
Thiamin	12	11	11
Pyridoxine	12	14	09
Control (C)	10	12	10
SEm ±	3.98	4.26	3.37
CD @5%	10.23	10.95	8.69

Table 6: Effect of amino acids on cellulase production

Amino acids sources (100 ppm)	Post-harvest fungi (Activity zone in mm)		
	A. niger	***G. candidum***	***R .solani***
Alanine	08	12	07
Arginine	07	08	07
Threonine	09	10	08
Aspartic acid	10	12	08
Methionine	08	12	09
Control (C)	09	12	10
SEm ±	2.93	4.08	2.80
CD @5%	7.56	10.50	7.20

Table 7: Effect of antibiotics on cellulase production

Antibiotics (100 ppm)	Post-harvest fungi(Activity zone in mm)		
	A. niger	*G. candidum*	*R. solani*
Ampicillin	12	13	10
Streptomycin	20	15	19
Terramycin	19	16	18
Mox	14	20	16
Doxycyclin	18	20	17
Control (C)	17	18	16
SEm ±	6.68	6.81	6.40
CD @5%	17.17	17.50	16.43

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[MS received 12 February 2014;
MS accepted 21 March 2014]

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