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In vitro and *in vivo* production of polygalacturonase, polymethylgalacturonase and cellulase enzymes by *Alternaria solani* at different incubation periods

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The production of polygalacturonase (PG), polymethylgalacturonase (PMG) and cellulase (Cx) enzymes have been studied at different incubation period. The fruit rot pathogen of tomato *Alternaria solani* was cultured at 28°C on semi ripe tomato fruit broth medium for 2, 4, 6, 8, 10 and 12 days. Isolated pathogen *A. solani* has a capability of producing PG, PMG and Cx enzyme within short incubation period, that is, 2 days incubation. Production of these enzymes gradually increased by increasing the length of incubation period up to 6 days and further increase in length of incubation up to 12 days, did not show any effect on the production of PG, PMG and Cx. The 6 days incubation was found to be the best incubation period for the production of all these three enzymes. The production of PG and Cx was also more in comparison with PMG enzyme. The enzyme activity in healthy and diseased semi ripe tomato fruits was also assayed at different incubation periods and it was found to be maximum in diseased tomato fruits in comparison with healthy tomato fruits. It was also found that PG and PMG enzymes were produced in higher concentration than the Cx enzyme. Among six incubation periods, 6 days incubation period was found best for maximum production of all these three enzymes.

Key words: *Alternaria solani*, semi ripe tomato fruit medium, semi ripe tomato fruit, incubation period.

INTRODUCTION

Fungal diseases of fruit and vegetable plants are known to cause great damages all over the world. Tomato (*Lycopersicon esculentum* Mill.) is the most ancient among the vegetable fruits. Among the fungal diseases, fruit rot is the most severe disease of tomato, which is caused by *Alternaria solani* (Mehta, 1973; Chaurasia, 2001; Chaurasia and Chaurasia, 2010 and Chaurasia et.al., 2013a).

Pectin is present in the middle lamella of cell wall, in the form of magnesium and calcium pectate. They are complex and colloidal in nature and mostly comprises of anhydroglacturonic acid units, linked together by α-1,4

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glycosidic linkages to form a chain like structure of polygalacturonic acid units. The middle lamella is degraded by pectolytic enzymes secreted by the phytopathogens. However, the production and activity of pectolytic enzymes by fungal pathogens is an important process of pathogenesis (Gupta, 1956; Wood, 1960; Brown, 1965; Bateman and Millar, 1966; Mehrotra et al., 1988).

Next to pectin, the cellulose is the major component and basic unit of structural framework of plant cell walls, which act as barrier. The pathogens feed successfully only after breaking this barrier with the help of cellulolytic enzyme. Cellulolytic enzymes secreted by pathogen play on important role on living plant tissues in softening them and may further participate indirectly by hydrolysing cellulose into soluble saccharides which serve as food for pathogen. The production of cellulolytic enzymes and degradation of cellulose by several fungi has been studied by many workers like Reese (1956, 1963), Basu and Ghose (1960), Gascoigne and Gascoigne (1960), Bateman (1964), Norkrans (1967), Singh and Singh (1988), Kaur et al. (1989) and Sohail et al. (2011). Production of cellulolytic enzymes by several pathogenic fungi and its role in the development of diseases has also been reported (Lucas and Sherwood, 1966; Hasija and Batra, 1982; Sanghi and Rajak, 1987; Singh et al., 1991; Singh and Shukla, 1999; Sharma, 2000; Chaurasia, 2001; Shukla and Dwivedi, 2012; Jat et al., 2013, Chaurasia et al., 2013b, c).

Keeping the above facts in mind, in the present paper, the production and activity of pectolytic and cellulolytic enzymes by *A. solani* in culture and in the healthy and diseased tomato fruits at different incubation periods have been investigated.

MATERIALS AND METHODS

Organism

A. solani (Ellis & Marts) Jones & Grout was isolated from the diseased fruits of tomato (Chaurasia and Chaurasia, 2010). The organism was maintained on potato dextrose agar slants.

Production of enzymes *in vitro*

The semi ripe tomato fruit medium tested by Chaurasia et al. (2013b) was used to study polygalacturonase (PG), polymethyl galacturonase (PMG) and cellulase (Cx) enzyme production *in vitro* at different incubation periods. The pathogen *A. solani* was grown in 150 ml Erlenmeyer flask containing 25 ml of semi ripe tomato fruit medium. The flasks were sterilized at 15 lb/sq in pressure for 15 min. After sterilization, each flask was inoculated by a 8.0 mm disc taken from the periphery of four day old colony of the pathogen growing on potato dextrose agar medium. The inoculated flasks were incubated for 2, 4, 6, 8, 10 and 12 days under stationary conditions at 28°C. Three replicates were taken in each case. After desired incubation, fungal mat was removed from the medium and the culture fluid was diluted with 35 ml of distilled water. The filtrates thus obtained was centrifuged for 15 min at 10,000 rpm and the supernatant was used as crude enzyme extract.

Production of enzymes *in vivo*

Semi ripe tomato fruits were used for this purpose. Healthy semi ripe tomato fruits were first surface sterilized with 0.01% mercuric chloride and then washed several times with sterilized distilled water. The surface sterilized fruits were inoculated with *A. solani* by cavity method (Granger and Hornes, 1924; Chaurasia et al., 2009; Chaurasia and Chaurasia, 2010). The inoculated fruits were kept in sterilized moist chambers having 98-100% relative humidity. These chambers were incubated at 28°C. Healthy surface sterilized fruits were kept as control. After 2, 4, 6, 8, 10 and 12 days of incubation, the infected tissue was taken out with the help of a spatula. Thirty grams of diseased tissue was mixed with 30 ml distilled water and the mixture was homogenized in Waring blender for 10 min. The homogenate thus obtained was centrifused for 20 min at 10,000 rpm. The supernatant was used as enzyme extract. Enzyme extract from uninoculated healthy tissue was also prepared in a similar manner.

Assay of enzyme activity

Enzyme activity was measured by using standard viscometric method (Hancock and Millar, 1965; Capellini, 1966; Chaurasia et.al., 2013c).

Oswald viscometers were clamped in stands which were fixed vertically in water bath, with temperature adjusted to 28°C. For the assaying of PG, PMG and C_X enzymes, the following freshly prepared substrate components were used:

1. Polygalacturonase (PG): 1.2% sodium polypectate - 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH 4.6) - 1.5 ml;
2. Polymethylgalacturonase (PMG): 1.2% citrus pectin - 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH4.6) - 1.5 ml.
3. Cellulase (C_X): 1.2% carboxymethyl cellulose- 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH 5.5) - 1.5 ml.

At the time of determination of PG, PMG and C_X enzyme activity, desired substrate component was taken into the stalk bulb of viscometer. Then, 1.5 ml of freshly prepared enzyme extract was poured into viscometer and then efflux time of the enzyme reaction mixture was determined at the intervals of 0, 20, 40, 60 and 80 min. Efflux time for 8.0 ml of distilled water was also noted in each viscometer.

Determination of percent loss in viscosity

Percent loss in viscosity was calculated with the help of the following formula (Capellini, 1966; Chaurasia et.al. 2013c): where, ET_0 = Efflux time in seconds at zero time/control. ET_t = efflux time in seconds at any specific interval of time. ET_w = efflux time

$$\text{Percent loss in viscosity} = \frac{ET_0 - ET_t}{ET_0 - ET_W} \times 100$$

Table 1. Production of PG, PMG and Cx enzymes by *A. solani* in semi ripe tomato fruit medium at different incubation periods.

Days after incubation	Polygalacturonase (PG)					Polymethylgalacturonase (PMG)					Cellulase (C_X)				
	Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)				
	Reaction time (min)					Reaction time (min)					Reaction time (min)				
	20	40	60	80	REA	20	40	60	80	REA	20	40	60	80	REA
2	32.5	43.4	50.3	53.2	65.01	7.2	15.3	19.4	21.2	0.00	42.5	52.3	58.2	62.2	85.03
4	53.4	66.3	76.0	78.3	106.83	9.3	19.3	25.4	30.0	16.93	50.4	62.3	68.2	71.4	100.80
6	56.3	68.2	77.4	80.2	112.61	10.4	22.4	30.2	40.3	20.13	66.6	78.4	84.4	88.3	133.33
8	37.5	47.3	52.1	55.1	75.01	8.2	13.4	16.3	18.3	0.00	31.2	41.3	47.5	50.2	62.42
10	32.3	42.4	48.3	51.4	64.64	8.0	11.3	14.6	16.2	0.00	20.3	29.4	35.4	38.2	29.40
12	28.0	32.2	35.3	37.3	56.02	7.4	9.8	11.2	13.4	0.00	18.0	26.2	31.3	33.6	26.20

in seconds for distilled water.

Determination of relative enzyme activity (REA)

Values for percent loss in viscosity were determined for 0, 20, 40, 60 and 80 min reaction time. These values were then plotted against the reaction time, thus a curve was obtained and from this curve, the time to bring a 25% loss in viscosity was determined. REA was then calculated using the following formula:

$$REA = \frac{1000}{t}$$

Where, t = represent the time in min to reach 25% loss in viscosity. Thus:

$$\mathbf{REA = \frac{1000}{25}}$$

RESULTS AND DISCUSSION

Production of PG, PMGand Cx enzymes *in vitro*

The pathogen *Alternaria solani* was cultured in the semi ripe tomato fruits medium and incubated for 2, 4, 6, 8, 10 and 12 days at 28°C. In obtained culture filtrates, the activity of PG, PMG and Cx enzymes was assayed and results are presented in Table 1 and Figure 1. It is clear that pathogen *A. solani* was able to produce PG, PMG and Cx enzymes with a short period of incubation, that is, within 2 days. The gradual increase in incubation period up to 6 days, the activity of PG, PMG and Cx enzymes significantly increased in the culture filtrates. The 6 day of incubation period was found to be the best for the maximum production of PG, PMG and Cx enzymes as 80.2 (REA 112.61), 40.3 (REA 20.13) and 88.3 (REA 133.33) percent loss in viscosity has been recorded respectively at 80 min of reaction time. After 6 days, further increase in incubation period up to 12 days, has no effect and production of PG, PMG and Cx enzymes declined gradually. Mehta et al. (1974) has reported the maximum production of PG, PMG and Cx enzymes in between 4 to 12 days, in culture filtrates of *A. solani* and *Alternaria tenuis*. Mehta and Mehta (1985) also reported maximum PG, PMG and Cx enzyme activity in 6 days old culture of *Fusarium oxysporium*. The gradual fall in production of PG, PMG and Cx enzymes in long incubation period could be due to the slow inactivation of PG, PMG and Cx enzymes by the appearance of oxidized phenols in the semi ripe tomato fruit medium. The same explanation could hold for the observation of Harter and Weimer (1921) on the production of pectic enzyme by *Rhizopus nigricans* and *Rhizopus artocarpi*, in sweet potato broth. Similar observations were made by Balasubramanian and Srivastava (1973).

Production of PG, PMG and Cx enzymes *in vivo*

Data (Table 2 and Figure 2), indicates the activity of PG, PMG and Cx enzymes in the diseased and healthy tissue of semi ripe tomato fruits, in different incubation periods. From the results, it is evident that PG, PMG and Cx enzymes present in healthy tomato fruits and maximum activity of these enzymes were recorded in 6 days incubition period. After 6 day incubation, with the age, the activity of PG, PMG and Cx enzymes was decreased gradually in healthy tissues. It was also observed that the activity of PG and Cx enzymes has completely disappeared in healthy tomato fruits kept for 12 day incubation.

From the above result, it is concluded that the presence of PG, PMG as well as Cx enzyme in healthy tissues shows their constitutive nature. Comparatively, PG enzyme has been found to be

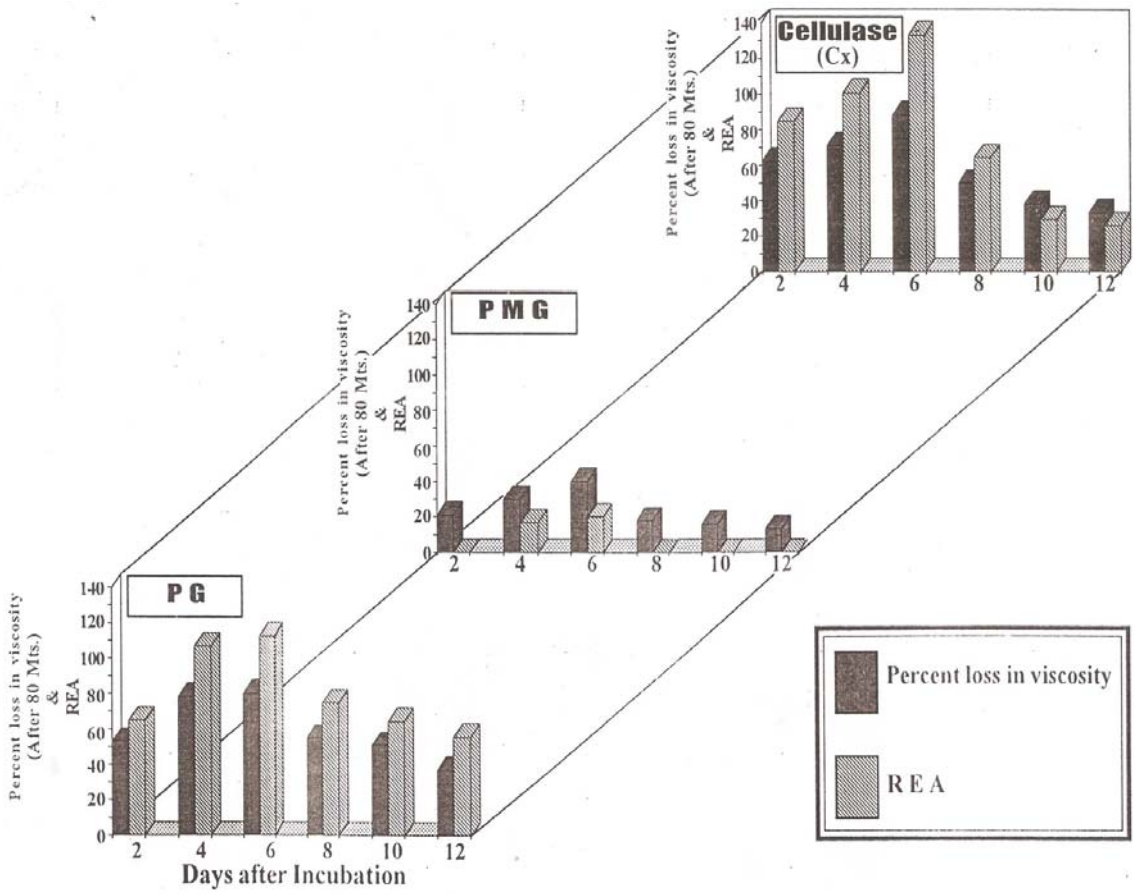

Figure 1. Production of PG, PMG and Cx enzymes by *A. solani* in semi ripe tomato fruit medium at different incubation periods.

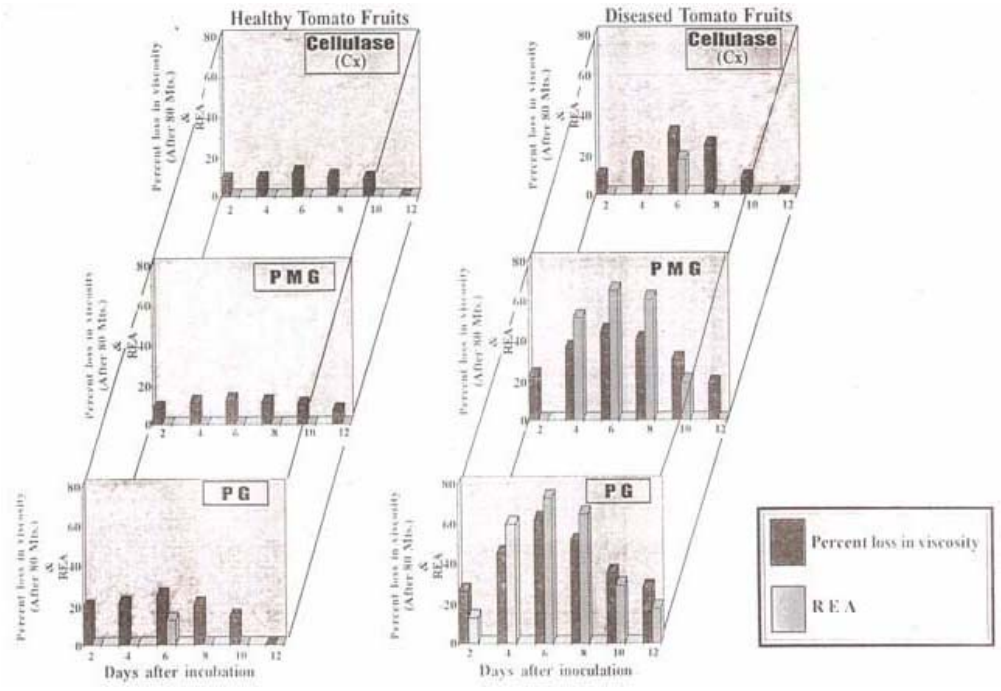

Figure 2. Production of PG, PMG and Cx enzymes in healthy and diseased semi ripe tomato fruits at different incubation periods.

Table 2. Production of PG, PMG and Cx enzymes by *A. solani* in healthy and diseased semi ripe tomato fruits at different incubation periods.

Days after incubation	Polygalacturonase (PG)					Polymethylgalacturonase (PMG)					Cellulase (Cx)				
	Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)				
	Reaction time (min)					Reaction time (min)					Reaction time (min)				
	20	40	60	80	REA	20	40	60	80	REA	20	40	60	80	REA
	Healthy tomato fruits														
2	8.00	14.30	18.00	19.00	0.00	6.00	7.00	8.00	8.00	0.00	4.60	6.00	7.50	8.50	0.00
4	8.00	16.00	19.20	21.10	0.00	6.20	8.00	10.00	11.00	0.00	5.00	7.5.	8.60	9.00	0.00
6	8.20	17.40	22.80	25.20	12.60	6.40	8.60	10.20	12.10	0.00	7.20	9.30	11.40	12.00	0.00
8	8.00	15.10	18.30	20.30	0.00	6.10	8.00	10.00	11.20	0.00	7.00	8.00	9.50	10.00	0.00
10	6.20	10.20	13.40	14.00	0.00	5.40	7.20	9.50	10.00	0.00	6.50	7.00	8.00	9.00	0.00
12	0.00	0.00	0.00	0.00	0.00	4.50	6.00	7.00	7.00	0.00	0.00	0.00	0.00	0.00	0.00
Days after inoculation	**Diseased tomato fruits**														
2	16.40	21.40	24.10	26.30	13.15	12.20	17.50	20.80	22.00	0.00	5.40	7.00	8.00	9.00	0.00
4	30.00	39.60	43.30	45.20	7.02	25.60	31.40	34.60	36.10	51.20	6.00	11.30	15.30	17.50	0.00
6	36.50	48.20	57.70	62.20	73.04	32.60	38.30	42.50	44.20	65.23	13.30	21.60	26.50	30.30	17.66
8	32.60	43.30	48.30	51.30	65.23	30.50	35.00	38.30	40.00	61.01	11.70	18.40	22.30	24.60	0.00
10	19.00	29.40	33.40	35.40	29.40	18.60	24.60	28.40	30.30	18.93	4.50	6.50	7.80	8.00	0.00
12	12.40	22.10	26.50	28.10	17.66	9.40	14.00	17.00	18.00	0.00	0.00	0.00	0.00	0.00	0.00

more active in healthy tissues than the PMG and Cx enzyme. Ulrich (1958), Mc Cready et al. (1955) has also reported the higher activity of PG enzyme in healthy tissues of various fruits.

In inoculated diseased semi ripe tomato fruit, the activity of PG, PMG and Cx enzymes were found to be higher in comparison with healthy tissue at various given incubation period. Within a short incubation period, that is, within 2 day, activity of PG, PMG and Cx enzymes has been noted in diseased tissues, which increased with increased in incubation period up to 6 days. The 6 days incubation period was found to be the best for the production of PG, PMG and Cx enzymes in diseased tissues, in which 73.04, 65.23 and 0.0 REA were observed respectively. After 6 day incubation, further increase in incubation period had no effect on the production of PG, PMG and Cx enzymes. In 12 day old infected tissues, the Cx activity has completely disappeared which indicates the inactivation of Cx enzyme in a very long incubation period.

Comparatively, the activity of PG and PMG enzymes were found to be always higher than the Cx enzyme in diseased tissues of various incubition periods, which indicated that PG and PMG enzymes played a significant role in pathogenesis of *A. solani*. To some extent, these results are similar and in agreement with the work of Mehta et al. (1974), Agarwal and Gupta (1978), Hasija and Chawdhury (1979), Sanghi and Rajak (1987) and Sharma (2000).

In general, production of PG, PMG and Cx was higher *in vitro* than *in vivo*. This could be due to the negligible inactivation of these enzymes by phenols present in the semi ripe tomato fruit medium or perhaps the fungus is forced to secrete the enzymes in large quantities to spread in the semi ripe tomato fruit medium. This situation does not prevail when the fungus is grown in host tissue. The ability of the fungus to secrete PG, PMG and Cx *in vitro* and *in vivo* indicates the importance of these enzymes in the pathogenesis of the fungus.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Agarwal GP, Gupta S (1978). Production of pectic enzymes by *Corynespora cassicola* causing fruit rot of papaya. Curr. Sci. 45(5):161-163.

Balasubramanian KA, Srivastava DN (1973). In vitro and in vivo production of pectic enzymes by *Rhizopus stolonifer*. Indian J. Exp. Biol. 11:127-128

Basu SN, Ghosh SN (1960). The production of cellulase by fungi on mixed cellulosic substances. Can. J. Microbiol 6:265-288.

Bateman DF (1964). Cellulase and *Rhizoctonia* disease of bean. Phytopathology 54(11):1372-1377.

Bateman DF, Millar RL (1966). Pectic enzymes in tissue disintegration. Ann. Rev. Phytopath. 4:119-146.

Brown W (1965). Toxin and cell wall dissolving enzymes in relation of plant disease. Ann. Rev. Phytopath. 3:1-18.

Capellini RA (1966). Growth and polygalacturonase producting by *Rhizopus stolonifer*. Phytopathol. 56:734-737.

Chaurasia Amit Kumar (2001). Studies on the fruit rot disease of tomato (*Lycopersicon esculentum* Mill.) caused by *Alternaria solani* (Ellis & Mart.) Jones & Grout. M.Sc. thesis, Dr. H.S. Gaur Univ. Sagar (M.P.) India pp. 68.

Chaurasia AK, Chaurasia Shirdha (2010). Effect of different inoculation methods and temperatures on tomato fruit rot pathogen *Alternaria solani* and its host range. Biozone Int. J. Life Sci. 2(1&2):236-243.

Chaurasia AK, Chaurasia Shridha, Chaurasia SC, Yadav VK (2009). Studies on the host range of apple isolate of *Botryodiplodia theobromae* Pat. Biozone Int. J. Life Sci. 1(1):34-38

Chaurasia Amit Kumar, Chaurasia Shridha, Chaurasia Sushmita, Chaurasia Shubha (2013a). Studies on the development of fruit rot of tomato caused by *Alternaria solani* (Ellis & Mart.) Jones & Grout. Int. J. Pharm. Life Sci. 4(6):2713-2716.

Chaurasia Amit Kumar, Chaurasia Shridha, Chaurasia Shubha, Chaurasia Sushmita (2013b). Production of polygalacturonase, polymethylgalacturonase and cellulase enzymes by *Alternaria solani*. Nat. J. Life Sci. in press.

Chaurasia Amit Kumar, Chaurasia Sushmita, Chaurasia Shubha, Chaurasia Shridha, Chaurasia SC (2013c). Influence of temperature on production of polygalacturonase, polymethylgalacturonase and cellulase enzymes by *Alternaria solani in vitro*. Int. J. Curr. Res. 5(7):1648-1650.

Gascoigne JA, Gascoigne MM (1960). Biological degradation of cellulase. Butler worths & Co. London.

Granger K, Horne AS (1924). A method of inoculating the apples. Ann. Bot. 38:212.

Gupta SC (1956). Studies in the physiology of parasitism XXII. The production of pectolytic enzymes by *pythium debaryanum* Hasse. Ann. Bot. 20:179-190.

Hancock JB, Millar RL (1965). Association of cellulotytic, Proteolytic and xylolytic enzymes with southern anthracnose, spraying block stem and *Stemphylium* leaf spot of alfalfa. Phytopathology 55:356-360.

Hasija SK, Batra S (1982). Production of cellulolytic enzymes by *Phoma destructiva*. Indian Phytopath. 35(3):384-387.

Hasija SK, Chawdhury SR (1979). Pectic and cellulolytic enzymes by *Phomopsis vexans*. Acta biol. Acad. Sci. Hung. 30(1-2):161-163.

Jat BL, Sharma P, Gaur HN (2013). Production of enzymes and culture filtrates by *Colletotrichum gloeosparioides* penz. causing banana fruit rot. Proc. Nat. Acad. Sci., India 83B(2):177-180.

Kaur G, Gupta AK, Mehrotra RS (1989). Effect of different carbon sources on the activity of pectolytic and cellulolytic enzymes *Phtophthora drechsleri* var. *cajani*. Plant Dis. Res. 4(2):198.

Lucas GB, Sherwood RT (1966). Role of pectic enzymes of *Alternaria tenuis* in brown spot disease of tobacco. *Proc. IVth Inter.,* Tobacco scientific cong. Athens. pp. 410-416

Mc Cready RM, Mac Comb EA, Jansen EF (1955). The action of tomato and avocado polygalacturonase. Food. Res. 20:186-191.

Mehrotra RS, Agarwal A, Kaur G (1988). Enzymic degradation of host cell walls by different *Phytophthora* sp. with special reference to the work done in india. In current trends in physiological plant pathology. Association of plant pathologists of India, Lucknow. pp. 89-111.

Mehta A, Mehta P (1985). Production of pectolytic and cellulolytic enzymes by *Fusarium oxysporum* and *F. Moniliformae* under different cultivation conditions. Folia Microbiol. 30:42-50.

Mehta P (1973). Biochemical investigations of fruit rot diseases of tomatose (with special reference to the pathogenesis caused by *Alternaria solani* (Ellis & Mart.), Jones & Grout and *Alternaria tenuis* Auct.) Ph.D. Thesis, Saugar Univ. Sagar (M.P.) India pp. 413.

Mehta P, Vyas KM, Saksena SB (1974). Production of pectolytic and cellulolytic enzymes by *Alternaria* sp. during pathogenesis of tomato fruits. Hindustan Antibiot. Bull. 16(4):210-214.

Norkrans B (1967). Cellulase and cellulolysis. Adv. Appl. Microbiol. 9:91-130.

Reese ET (1956). A microbiological progress report enzymatic hydrolysis of cellulose. Appl. Microbiol. 4:39-45.

Reese ET (1963). Advances in enzyme hydrolysis of cellulose and related materials. Mcmillan & Co. New York.

Sanghi N, Rajak RC (1987). Production of pectic and cellulolytic enzymes by two strains of *Myrothecium roridum* causing fruit rot of tomato. Prof. G.P. Agarwal Festschrift Volume. Today and Tomorrow's printers and publishers, New Delhi -5 pp. 111-116.

Sharma RL (2000). "*In vivo*" and "*in vitro*" production of cellulolytic enzymes by *Monilinia* sp. causing brown rot of apple. Indian J. Mycol. Plant Pathol. 30(1):31-33.

Singh PL, Gupta MN, Singh AL (1991). Cell wall degrading enzymes production by seed brone fungi isolated from chilgoza. Indian J. Mycol. Plant Pathol. 21(3):263-267.

Singh RS, Singh OS (1988). Quantification of cellulase produced by *Macrophomina phaseolina* and Pythium butleri. Plant Dis. Res. 3(2):229-231.

Singh SN, Shukla P (1999). Variation in the production of enzymes by different isolates of *Myrothecium roridum* caused leaf spot of mungbean. Indian Phytopath. 52(1):63-65.

Sohail M, Ahmad A, Khan SA (2011). Production of cellulases from *Alternaria* sp. MS28 and their partial characterization. Pak. J. Bot. 43(6):3001-3006.

Shukla A, Dwivedi SK (2012). Pathogenic action of Cx, PG and PMG enzymes of *Fusarium udum* and *Fusrium oxysporum f. sp. ciceri*. Int. J. Curr. Res. 4(6):111-113

Ulrich R (1958). Post harvest physiology of fruits. Ann. Rev. Plant Pathology. 9:384-416.

Wood RKS (1960). Pectic and cellulolytic enzymes in plant disease. Ann. Rev. Plant Physiol. 11:299-322.