The Pharma Innovation Journal 2022; 11(2): 1973-1977

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(2): 1973-1977

www.thepharmajournal.com
Received: 08-12-2021
Accepted: 16-01-2022

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Enzymatic activity in fruits of chilli infected by *Colletotrichum capsici*

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Abstract
Anthracnose of chilli is a serious disease affecting fruit yield and quality of fruit. India is the world leader in chilli production followed by China, Thailand and Pakistan. The experiment was conducted at department of plant pathology, college of agriculture, Bikaner and find out changes in host related enzymes and pathogen related enzymes. The activity of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase were increased in diseased fruits compared to healthy fruits in resistant variety Pusa Sadabahar, moderate resistant (Mathaniya) and susceptible (Pusa Jawala) varieties. The pathogen related enzymes polygalacturonase trans-eliminase (PGTE), pectin trans-eliminase (PTE), polygalacturonase (PG) and cellulolytic (Cx) enzymes activities were found lower at initial stages of observation.

Keywords: Peroxidase, Catalyse, Sadabahar, Jawala, Mathaniya, polygalacturonase

Introduction
Chilli commonly known as *Capsicum annuum* L. is a prominent vegetable crop belonging to the family *solanaceae*. It is an important spice, vegetable as well as cash crop in India grown in *Kharif* season which gives good returns to the cultivators. Chilli is good source of vitamins like A, B, C and minerals like Ca, P, Fe, Na and Cu in trace amounts. It is grown in tropical and subtropical regions of the world for its pungent fruits which are used both green and ripe. Chilli is also used in pickles, sauces, ketchup, essence, oleoresins and is an inevitable ingredient in Indian dishes. Alkaloid capsaicin is extracted from chilli, which has medicinal values. These properties increase the demand for chillies all over the world. India is the world leader in chilli production followed by China, Thailand and Pakistan. In our country, dry and green chilli are cultivated on an area of about 840 thousand hectares with an annual production of 2096 MT and about 316 thousand hectares with an annual production of 3634 MT, respectively (Anonymous, 2016-17) [1]. The important chilli growing states in our country are Andhra Pradesh, Odisha, Maharashtra, West Bengal, Karnataka, Rajasthan and Tamil Nadu (Subbiah and Jaykumar, 2009)[2]. In Rajasthan, the areas under dry and green chilli are about 9.95 thousand hectares with annual production of 20.15 MT and 8.58 thousand hectare with annual production of 27.73 MT (Anonymous, 2019-20) [3]. In Rajasthan, the major chilli growing districts are Jaipur, Jodhpur, Swai Madhopur, Ajmer, Bhilwara, Sikar, Tonk, Pali and Nagaur (Anonymous, 2019-20) [3]. The disease has been observed to occur in two phases, which are (i) leaf spot and dieback (ii) fruit rot. Typical anthracnose symptoms on chilli fruit include sunken necrotic tissues with concentric rings of acervuli. Host related enzymes like, peroxidise, polyphenol oxidase, L-phenylalanine ammonia lyase and catalase which are present in plant system, known to impart resistance against disease. These constituents are also sometime known to be produced under higher concentration in response to infection by pathogens. The biochemical's *viz.*, Total phenols, sugars, total soluble protein, oxidative enzymes etc. are known to play important role in the defense mechanisms / resistance against the biotrophic pathogens, hemi-biotrophic and necrotic plant pathogens (Hasabnis *et al*., 2004) [4].

Material and Methods
The experiment was conducted at Plant Pathology laboratory, College of Agriculture, SKRAU, Bikaner. To find out enzymatic activity changes in host as well as in pathogen.

1. Changes in host related enzymes
Estimation of peroxidase activity: Peroxidase activity was assayed by the method described by Thimmaiah (1999) [5].

One gram leaf sample was ground in previously chilled mortar and pestle with 10ml ice cold 0.1M phosphate buffer, pH 6.0. The homogenate was strained through two fold of muslin cloth and centrifuged at 16,000 rpm for 20 minutes of 4 ^{0}C. The supernatant was used as enzyme source. In order to assay the enzyme activity, 1 ml of 0-dianisidine, 0.5 ml of H_2O_2, 1 ml of phosphate buffer and 2.4 ml of distilled water were pipette in test tubes. The blank was prepared by excluding H_2O_2 and adding additional volume of water in place of H_2O_2. The reaction was initiated by adding 0.2 ml of enzyme extract (supernatant) and incubating at 30 ^{0}C for 5 minutes. The reaction was stopped by adding 1 ml of 2N H_2SO_4. The absorbance was measured at 430 nm against reagent blank. The activity of enzyme was expressed as min^{-1} g^{-1} sample.

Estimation of polyphenol oxidase activity: The polyphenol oxidase activity was determined the method of Mayer *et al.* (1965) [6]. One gram fruit sample was homogenized in 2 ml 0.1M sodium phosphate buffer pH 6.5 and centrifuged at 16,000 g for 15 minutes at 4 ^{0}C. The supernatant was used as enzyme source. The reaction mixture was consisted of 0.2 ml of enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 0.2 ml of 0.1 M catechol was added and the change in absorbance was recorded at 30 second interval up to 3 minutes at 495 nm. The activity of enzyme was expressed as change in optical density at 495 nm in min^{-1} g^{-1} sample.

Estimation of L-phenylalanine ammonia lyase activity: L-phenylalanine ammonia-lyase activity was assayed by the method described by Thimmaiah (1999) [5]. Three gram fruit sample was macerated in a mortar in presence of 2.6 ml of 0.2M sodium borate buffer (pH 8.7), containing 2-mercaptoethanol (0.8 ml/liter). The homogenate was filtered through cheese cloth. Acetic acid (0.1 M) was added drop by drop to bring the pH of filtrate 5.5. Protamine sulphate solution (0.002 g + 0.008 ml of 1M of sodium acetate buffer pH 5.5 diluted 0.1 ml) was added to filtrate and stirred for 10 minutes followed by centrifuged at 7,000 rpm for 10 minutes. The supernatant was used as the enzyme source. In order to determine the enzyme activity and assay mixture consisting of 1 ml of 0.05 M Tris-HCL buffer, (pH 8.8) 0.5 ml of 0.01M L-phenylalanine and 0.4 ml of water was incubated at 30 ºC for 60 minutes. The blank without L-phenylalanine was run. The reaction was stopped by adding 0.5 ml of 1N HCL. The mixture was extracted twice with 3.5 ml of ether each time. The ether phase was removed, pooled and dried. The residue so obtained, was dissolved in 3 ml of 0.05N NaOH. The absorbance was recorded at 290 nm. A standard curve was prepared using different concentrations of cinnamic acid. The specific activity of the enzymes was express as µ moles cinnamic acid produced min^{-1} g^{-1} sample.

Estimation of Catalase: Catalase activity was measured by adopting the procedure of Sinha (1972) [7]. The reaction mixture containing 0.4 ml of 0.2 M H_2O_2, 0.1 ml of enzyme extract and 0.5 ml of 0.01 M phosphate buffer (pH 7.0) was incubated at 37 ^{0}C for 1 min along with continuous shaking and then added 3 ml of dichromate reagent (5 per cent potassium dichromate; glacial acetic acid, 1:3). The mixture was heated for 10 min. in a boiling water bath. After cooling, the intensity of colour was measured at 570 nm. The enzyme activity has been expressed as µ moles of H_2O_2 utilized min^{-1} g^{-1} sample.

2. Changes in pathogen related enzymes

Enzyme preparation: Pathogenic fungi varying in virulence were studied for pectinolytic and cellulolytic enzymatic activity after growing them on medium. The medium was inoculated by using 5 mm pieces (cut by cork borer) from petri dishes containing culture of test pathogen. These were incubated for 10 days at 27 ± 2 ^{0}C. After incubation period mycelial mat were harvested by filtering through filter paper (whatman-42), filtrate thus obtained were centrifuged at 5000 rpm for 30 minutes to separate spores. The filtrate was then dialysed by using double distilled water at 4 ^{0}C for 24 hours (by changing the water at every 8 hours). After 24 hours the dialysed culture filtrates was immediately used for assaying pectinolytic and cellulolytic enzymes, the preparation was kept at 4 ^{0}C, when not in use. In Richard's medium for estimation of pectinolytic enzymes sucrose was replaced by 1% citrus pectin, while for estimation of cellulolytic enzymes it was replaced by 1% carboxyl methyl cellulose (CMC). A control was also maintained by using sucrose in medium.

Polygalacturonase Trans-eliminase (PGTE): To find out the PGTE activity, viscometer methods described by Ayers *et al.* (1966) [8] was used. The reaction mixture consists of 5 ml of 1 per cent sodium polypectate at pH 8.7 in boric acid borax buffer, 2 ml of borate buffer at pH 8.7 and 2 ml of enzymes preparation. Reduction in the water bath at 30 ^{0}C for 5, 10, 30, 60, 120 and 240 minutes and expressed in per cent loss in viscosity which was calculated by using the formula:

$$\text{Per cent loss in viscosity} = \frac{T_0 - T_1}{T_0 - T_w} \times 100$$

Where

T_O =Flow time of reaction mixture with heated enzymes

T_1 = Flow time of reaction mixture at time

T_W =Flow time of distilled water

Flow time indicated time taken by the liquid to move from the upper end to lower end of the bulb of the viscometer.

Pectin trans-eliminase (PTE): It was assayed by measuring the loss in viscosity of 1 per cent pectin in boric acid borax buffer at pH 8.7 in viscometer (Albersheim *et al.*, 1960) [9]. The same method used for PGTE was employed except 1 per cent sodium polypectate. Each observation was replicated thrice and percent viscosity loss was recorded after 5, 10, 30, 60, 120 and 240 minutes as specified and viscosity was calculated by the formula given earlier.

Polygalacturonase (PG): To find out the PG activity viscometeric method described by Bateman (1966) [10] was used change in viscosity of sodium polypectate were employed to estimate the PG activity. To 5 ml of 0.75 per cent sodium polypectate at pH 4.6 in acetate buffer, 2 ml of sodium acetate acetic acid buffer at pH 4.6 and 2 ml of enzyme preparation were added. The PG activities were determined in a viscometer kept in water bath at 30 ^{0}C at interval of 5, 10, 15, 30, 60, 120 and 240 minutes and expressed in per cent loss in viscosity calculated by the formula given earlier.

Cellulolytic enzymes: To evaluate production of cellulolytic enzymes, the same procedure was described earlier to assay the pectinolytic enzymes. However, for the cellulolytic enzymes, the carbon source of each medium was replaced with the carboxyl methyl cellulose (CMC). The enzyme filtrate was centrifuged at 2000 rpm for 30 minutes for separating the spores and then estimated for enzymes activity as method reported by Hussain and Rich (1956) [11], Koti and Mahadeven (1967) [12]. The loss in viscosity of carboxyl methyl cellulose was determined in a viscometer. To 5 ml of 0.5 per cent CMC in acetate buffer at pH 5.6, 2 ml of acetic acid acetate buffer at pH 5.6 and 2 ml of enzymes preparation were added and immediately transferred to the viscometer kept in a water bath at 5, 10, 15, 30, 60, 120 and 240 minutes and expressed in per cent loss of viscosity was calculated by formula earlier.

Results and Discussion

Changes in host related enzymes

Peroxidase: Peroxidase enzyme (Table 1) in healthy and diseased fruits of chilli varieties were estimated at 75 DAT. The peroxidase activity were recorded higher 0.55, 0.43 and 0.21 min^{-1} g^{-1} sample in diseased fruits as compared to healthy (0.41, 0.35 and 0.19 min^{-1} g^{-1} sample) one of Pusa Sadabahar, Mathaniya and Pusa Jawala, respectively. The peroxidase was increased from 14.29 to 32.26 per cent in all the infected fruits as compared to healthy ones. The peroxidase was increased maximum (+32.26%) in infected fruits of Pusa Sadabahar variety. It was followed by Mathaniya (+23.08%) and minimum in Pusa Jwala (+14.29%) as compared to healthy ones. From the results, peroxidase activity was recorded higher in resistant variety Pusa Sadabahar followed by moderate resistant variety Mathaniya and lower in susceptible variety Pusa Jawala. Similar changes in peroxidase activity as a consequence of fungal infection were reported by other investigators. Prasath and Ponnuswami (2008) [13] recorded the activity of peroxidase enzyme was highest in the resistant genotype followed by moderate resistant hybrids and least enzyme activity was recorded in the susceptible genotype. However, following infection, several workers reported a rapid rise in peroxidase activity, which appears to be more connected with susceptibility than resistance. (Gupta *et al.*, 1990) [14].

Table 1: Change in peroxidase, phenylalanine ammonia lyase, polyphenol oxidase and catalase activity in fruits of chilli infected by *C. capsici*

Variety	Disease reaction	Peroxidase (min^{-1} g^{-1} sample)		Phenylalanine ammonia lyase (min^{-1} g^{-1} sample)		Polyphenol oxidase (min^{-1} g^{-1} sample)		Catalase (min^{-1} g^{-1} sample)	
		Healthy	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
Pusa Sadabahar	Resistant	0.41*	0.55* (+32.26) #	5.70*	7.32* (+28.48) #	0.27*	0.37* (+36.92) #	0.28*	0.41* (+46.58) #
Mathaniya	Moderate resistant	0.35	0.43 (+23.08)	4.97	5.84 (+17.44)	0.19	0.24 (+23.91)	0.23	0.32 (+36.07)
Pusa Jawala	Susceptible	0.19	0.21 (+14.29)	3.11	3.54 (+13.70)	0.14	0.16 (+11.76)	0.16	0.19 (+21.95)
		S.Em.±	C.D. (P=0.05)	S.Em.±	C.D. (P=0.05)	S.Em.±	C.D. (P=0.05)	S.Em.±	C.D. (P=0.05)
Variety		0.010	0.032	0.049	0.154	0.004	0.014	0.005	0.016
Healthy/Diseased		0.008	0.026	0.040	0.126	0.004	0.011	0.004	0.013
Variety×Healthy/Diseased		0.014	0.045	0.069	0.218	0.006	0.019	0.007	0.023

*Mean of three replication, (+) = Per cent increase in enzyme activity, #Values in parentheses indicate per cent deviation in diseased fruits over healthy fruits of corresponding variety.

L-Phenylalanine ammonia lyase (PAL): The Phenylalanine ammonia lyase activity was increased in diseased fruits compared to healthy ones in all the chilli varieties used (Table 1). The PAL activity in diseased fruits of resistant variety Pusa Sadabahar showed more (7.32 min^{-1} g^{-1} sample) as compared to (5.70 min^{-1} g^{-1} sample) in healthy fruits. Similar trend observed in moderate resistant and susceptible varieties, where diseased fruits recorded 5.84 and 3.54 min^{-1} g^{-1} sample against healthy fruits of 4.97 and 3.11 min^{-1} g^{-1} sample of PAL activity, respectively. Increase in PAL activity in diseased fruits of resistant variety (+28.48%) followed by moderately resistant (+17.44%) and in susceptible variety (+13.70%). Also, the maximum increase in PAL activity was observed in diseased fruits of resistant Pusa Sadabahar variety in comparison to moderately resistant Mathaniya and susceptible Pusa Jawala. Anand *et al*. (2008) [15] found similar trend for PAL activity in fruit rot of chilli. Higher increase in PAL activity in inoculated incompatible host than in susceptible one.

Polyphenol oxidase: The table 1 indicated that the polyphenol oxidase acivity in resistant, moderate, susceptible varieties of healthy fruits ranged from 0.14 (Pusa Jawala) to 0.27 min^{-1} g^{-1} sample (Pusa sadabahar). It was increased in diseased fruits of *C. capsici* which ranged from 0.16 to 0.37 min^{-1} g^{-1} sample. The per cent increased in diseased fruits was higher in Pusa Sadabahar variety (+36.92%) which followed by Mathaniya (+23.91%) and Pusa Jawala (+11.76%) as compared to healthy fruits. The increase in polyphenol oxidase activity in response to disease in resistant to susceptible variety is in agreement with the observations of Jabeen *et al.* (2009) [16] recorded activity of polyphenol oxidase enzyme was highest in the resistant genotype followed by moderate resistant and least enzyme activity was recorded in the susceptible genotypes. Also reported sharp increase in PPO activity due to infection by anthracnose pathogen in chilli plants. Several other investigators have also been reported that the polyphenol oxidase activity was relatively more in resistant variety as compared to susceptible ones (Bharti *et al*., 2004) [17].

Catalase: The catalase activities in all three varieties (resistant, moderately resistant and susceptible) are presented in table 1. The maximum catalase activity was recorded in diseased fruits of Pusa Sadabahar (0.41 min^{-1} g^{-1} sample) followed by Mathaniya (0.32 min^{-1} g^{-1} sample) and it was

lowest in Pusa Jawala (0.19 min^{-1} g^{-1} sample) as compared to healthy (0.28, 0.23 and 0.16 min^{-1} g^{-1} sample) fruits of all three chilli varieties. The catalase activity in resistant variety Pusa Sadabahar was increased by +46.58 per cent followed by moderate resistant, Mathaniya (+36.07%) and susceptible variety, Pusa Jawala (+21.95%) as compared to healthy fruits. Results indicated that, catalase activity was recorded higher in resistant variety Pusa Sadabahar followed by moderate resistant variety Mathaniya and lower in susceptible variety Pusa Jawala. Similar results also found by Anand, *et al*., 2008 [15] and they demonstrated an increase in CAT activity in infected fruit indicated an increase in H_2O_2 concentration in host tissues. The detrimental effect of H_2O_2 buildup was eliminated due to increased CAT activity (Gangopadhyay *et al*., 1996) [18]. At 65 and 80 days after exhibiting, Saharan and Saharan (2003) [19] found that the healthy leaves of resistant clusterbean types had higher catalase activity than susceptible varieties, indicating that catalase plays a role in resistance.

Change in pathogen related enzymes

The pathogen *C. capsici* produced more cellulolytic and pectinolytic enzymes in this study, showing that cell wall destroying enzymes are important in pathogenesis (Table 2). Enzymes are significance in plant disease especially for blight types they are play an important role in pathogenesis. The activity of polygalacturonase transeliminase (PGTE), pectin transeliminase (PTE), polygalacturonase (PG) and cellulolytic enzymes were determined. Reduction in viscosity was determined after 5, 10, 15, 30, 60, 120 and 240 minutes. The present investigation was carried out to study the production of pectinolytic and cellulolytic enzymes by anthracnose of chilli causing by *C. capsici* under *in vitro* conditions. Per cent loss in viscosity was observed maximum in PGTE. The highest enzymatic activity of PGTE expressed as per cent loss in viscosity was 81.86 per cent after 240 minutes, while it was lowest 21.85 per cent after 5 minutes. It was observed that loss in viscosity of PTE activity was maximum 67.95 per cent after 240 minutes. The loss in viscosity of PG was maximum 67.25 per cent after 120 minutes and it was lowest 11.20 per cent after 5 minutes. Lowest activity of Cellulolytic was 19.99 per cent after 5 minutes; it was maximum 75.63 per cent after 240 minutes. The decrease in enzymatic activities were with the time appears to be due to the feedback inhibition of enzyme by the accumulation of the products from during the reaction and/or the decrease in the concentration of the substrate the reaction proceeds. The decrease in viscosity in response to pathogenesis is in agreement with the observations of Verma and Rawal (2014) [20]. Similar trend was also observed in case of soft rot of ginger caused by *P. aphanidermatum.* Because *C. capsici* is an intercellular fungus in the host, the production of these enzymes appears to facilitate the dissolution of a portion of the host cell wall and middle lamella, as well as the entry and establishment of a reaction with the pathogen in the host, and is possibly responsible for playing a key role in pathogenesis via cell wall degradation and tissue disintegration (Kushwaha and Narain, 2005) [21].

Table 2: Production of pectinolytic and cellulolytic enzymes by *C. capsici* under *in vitro* conditions

Time (minutes)	Loss of viscosity (%)			
	PGTE	PTE	PG	Cx
5	21.85*	15.90*	11.20*	19.99*
10	33.29	23.50	21.46	22.80
15	55.28	32.12	35.35	36.31
30	63.26	43.34	49.49	42.50
60	75.28	55.51	54.84	55.25
120	79.01	64.03	67.25	67.18
240	81.86	67.95	67.25	75.63
Control	0.00	0.00	0.00	0.00
S.Em.±	0.44	0.17	0.31	0.25
C.D. (P=0.05)	1.32	0.53	0.95	0.77

*Mean of three replications, PGTE= Polygalacturonase transeliminase, PTE= Pectin transeliminase, PG= Polygalacturonase, Cx= Cellulolytic enzyme

Conclusion

The activity of peroxidase, polyphenol, phenylalanine ammonia lyase and catalase were increased in diseased fruits compared to healthy ones in all the tested chilli varieties used. The maximum increase in peroxidase, polyphenol, phenylalanine ammonia lyase and catalase activity were observed in diseased fruits of resistant Pusa Sadabahar in comparison to moderately resistant Mathaniya and susceptible Pusa Jawala. The pathogen related enzymes polygalacturonase trans-eliminase (PGTE), pectin trans-eliminase (PTE), polygalacturonase (PG) and cellulolytic (Cx) enzymes activities were found lower at initial stages. In all cases, the rate of decreasing in viscosity was maximum in first 30-60 minutes and then showed down after 120-240 minutes, no further losses in viscosity was observed.

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