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A STUDY OF ESTIMATION OF THE ACTIVITY OF OXIDIZING ENZYMES IN JUTE

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Abstract:
The pathogenesis affects biological oxidation in plant tissue. The changes in the activity of various oxidases by the pathogens are generally accepted as secondary effect due to cellular derangement. But these changes alter the concentrations of the various substrates and the products. These reactions are chosenly associated with the defense mechanism of plants.
Keyword: oxidation, pathogens, metabolism, Respiration, sterilization, cell.

Introduction:

The rise in the activity of the respiratory metabolism of the host irrespective of the type of pathogens, has been reported by number of authors (Yarwood, 1934: Pratt, 1938, Shaw and Samborski, 1957; Lakshamanan and Venkata Ram, 1951, Allen and Goddard, 1958; Uritani and Akazawa, 1959, Bushnell and Allen 1962; Hutchison and Buchanan, 1983, Prasad et al., 1983; Sinha et al., 1991)

A number of parameters such as (6) uptake of O. (b) release of CO, (iii) uncoupling of ADP and inorganic phosphate to form ATP (iv) increase in the activity of oxidative enzymes and (V) rise in the activity of dehydrogenase of respiratory acids of The Krebs cycle, has been adopted to observe the change in the level of respiratory metabolism. For ascertaining the metabolism, it is not necessary to work out all the parameters. The keen observation of a few of them is sufficient to infer any change in the respiration of the diseased plant.

following observations were made for evaluating the change in the resipiratory metabolism of C.capsularis due to C.copsid 1, capsularis.

(i) Dehydrogenase of pyruvic acid and a - ketoglutaric acid
(ii) Laccase
(III)Phenol oxidase
(IV)catalase and peroxidase

In contrast to the hydrolyzing enzymes, the oxidative enzymes will be worked out after inoculating C.capsici f. capsularis to C.capsularis.

MATERIALS AND METHODS

ESTIMATION OF THE ACTIVITY OF OXIDIZING ENIMES

procedure of inoculation

the fifth and sixth leaves of one and half month old plant raised in the garden were surface sterilized with 0.1% mercuric chloride for 1 min and washed first with tap water three times and finally two times with autoclaved distilled water. The surface sterilant and the water for washing was used with all glass atomizers. Adherant water on the surface of leaves was soaked with sterilized dry white blotting sheet.

For the purpose of inoculation, the cotton swal was used. Absorbant cotton was introduced in the cork borer of 5 mmn diameter and the fluffy surface of the cotton was cut with sharp scissiors. The whole cork borer was dipped in rectified spirit for 30 min and then dried at 70 ° C for 12 hr. This treatment made the cotton and the cork borer sterilized and also free from alcohol. This was tested by touching the surface on potato dextrose agar medium without any microbial growth for 4 days.

The surface of the cotton was touched to the culture of the fungus C.capsici f. capsularis so that the spores may adhere to the surface of cotton. This was touched to the surface of the sterilized leaves at four points. The leaves were covered with black polythene pocket internally sprinkled with water to maintain high humidity. These leaves were left in this position for 2 days. Then the inoculated area of the leaves were covered with moist sterilized blotting sheet. This sheet was covered with polythene sheet and the margin was shield. This was done for carrying high RH at the inoculated spot and to maintain natural condition of the plant. After six days the blotting was removed and symptoms of disease was observed. Mild symptom of the disease i.e. browning was observed at the spot of inoculation.

(1) Estimation of the activity of pyruvic acid and a ketoglutaric acid dehydrogenase. The procedure is based on the use of picric acid as H acceptor in buffer and the enzyme extract separately with the noted acids (sodium salt) in the reaction mixture and reading the OD of the resulting picramic acid (reduced picric acid) as suggeted by Snell. and Snell (1971).

Reagents

(I) Buffter: 0.67 M phosphate buffer, pH 7.5

(ii) 0.4% Picric acid solution

(iii) 0.1 M Sodium pyruvate

(iv) 0.1M Sodium a - ketoglutarate

500 mg of infected leaf was ground with 2 ml of the buffer and centrifuged at 10,000 rpm for 10 min. The supernatant served as the enzyme 1 ml

following were taken in test tube

Enzyme extract	-	1 ml
Buffer	-	1 ml
Picric acid	-	1 ml
Sodium pyruvate	-	1 ml
Sodium a - ketoglutarate	-	1 ml
Water separately	-	1 ml

These were mixed and incubated at 37 ° C for 12 hr transport the control of the boiled enzyme extract. After the expiry of incubation, 5 ml of acetone was mixed and picramic acid formed as a result of the reduction of picric acid, was read at 550 nm. The result was recorded as O.D. due to picramic acid formad in Table 6.

Sodium Pyruvate $\frac{Pyruvi\ c\ acid}{de hydrogenase}$ H + Picric acid.

Picramic acid

(OD of this acid was read)

Table 6: - Pyruvic acid and a - ketoglutaric acid dehydrogenase activity of inoculated and control leaves (expressed as 0.D. due to picramic acid formed)

Enzymes	Inoculated leaf	control leaf
Pyruvic acid dehydrogenase	0.048	0.027
a - ketoglutaric acid dehydrogenase	0.031	0.018

Result (Table 6, Fig. 2) (ii) Estimation of the activity of laccase Laccase oxidizes a wide range of substrates such as monophenols, ortho- and para-diphenols, triphenols and ascorbic

acid (vitamin C). Most of the fungi possess the laccase activity. Some fungi possess both extracellular and intracellular laccase activity

Procedure: The three pathogens namely C.capsici f. capsularis, M.phaseolina and S.rolfs were grown in Richard solution having the following composition. (Fahmy, 1923).

Composition of Richard solution

Potassium nitrate	10 g
Potassium monobasic phostate	5 g
Magnesium sulphate	2.5 g
Ferric chloride	0.02 g
Sucrose	50 g
Distilled water	1000 ml

The fungi were cultured on CDA medium for 7 days at 30 ° C 100 ml of the medium was taken in the conical flasks of 200 ml capacity and autoclaved. Two conical flasks were maintained for each pathogen and the control. 4 mm bit of each culture was dropped in the flasks and allowed to grow for 10 days as a procedure adopted by Mahadevan and Sridhar (1996). The metabolite of the fungi were filtered through Whatman No.1 filter paper setting in Buchner funnel.

Reagents

Buffer: 0.1 M phosphate buffer, pH 6

Guaiacol

In a test tube containing 5 ml of sodium phosphate buffer containing 10 mM of guaiacol was taken and left at 30 ° C for 30 min. 0.1 ml of the enzyme source (culture filtrate) was taken. After 5 min of reaction the absorption at 470 nm was taken. The control was maintained with the enzyme extract boiled for 5 min. The result was recorded as OD at an interval of 1 min and continued for 10 min in Table 7.

Table 7: Activity of laccase of the pathogens (expressed as change in OD at interval of 1 min)

Pathogens	Time in min				
	1st	3rd	5th	7th	9th
C.capsici f. capsularis	0.020	0.060	0.080	0.110	0.130
M.phaseoli	0.010	0.030	0.050	0.070	0.090
S.rolfsi	0.005	0.015	0.025	0.035	0.045

Results (Table 7, Fig. 3)

It appears that the activity of laccase was maximum in C.capsici f. capsularis followed in succession by M.phaseoli and S.rolfsi.

Estimation of the activity of phenol oxidase

Phonel oxidase catalyses the oxidation of monophenols and Ortho-diphenols. Monophenols particularly tyrosine and para cresol and ortho- phenols such as caffeic acid, pyrogallol and substituted catechols are important substrate of the enzyme. Phenol oxidase is also known as polyphenol oxidase, tyrosinase, DOPA oxidase, catechol oxidase etc. This enzyme is present is all plant species (Mahadevan and Sridhar, 1996). Immediately after inoculation to the host, the activity of phenol oxidase has been found increased. The pathologists have found alteration in oxidation-reduction potential as the first reaction which the pathogens bring in the host. Buffer: - 0.1 M, Phosphate buffer pH 6.0 Reagent: - Catechol 0.01 M dissolved in the noted phosphate buffer. 0.5 g of leaf of C.capsularis showing the symptom of anthracnose due to C.capsici f. capsularis was extracted with 5 ml of the buffer. This was centrifuged at 10,000 rpm for 15 min.The supernatant served as the enzyme extract.

2 ml of the enzyme extract and 3 ml of buffer was taken in a cuvette and mixed and set in the spectrophotometer at 495 mm. The absorbance was set at zero. 1 ml of catechol solution was mixed

in the cuvette and it was again put in the instrument and change in absorbance was recorded at every 30 sec for 3 min. The control was maintained using the enzyme extract boiled for 5 min. The result was noted in Table 8.

Table 8: Activity of catechol oxidase in C.capsularis showing the symptom of anthracnose (expressed as OD)

	Time in sec					
Inoculated / Control	**30**	**60**	**90**	**120**	**150**	**180**
Inoculated		0.030	0.052	0.075	0.070	0.138	0.162
Control		0.000	0.001	0.001	0.002	0.003	0.004

Result (Table 8, Fig. 4)

(1) It appears that the rate of oxidation of catechol is considerably faster in the inoculated host in comparison to the control ie uninoculated host. In the latter the rate of oxidation of catechol is neghligible.

(iv) Estimation of the activity of catalase and peroxidase.

The two enzymes are authentically consideved as oxidizing enzymes. Catalase catalyses the breakdown of hydrogen peroxide H: 02) to water and molecular oxygen. This is known as catalytic activity of catalase. H2O2 is peroxidatively converted to water in presence of Hydrogen donor.

Catalytic activity of catalase

$H_2O_2 \longrightarrow H_2O + 1/2\ 0_2$

Peroxidative activity of catalase

$H_2O_2 + AH_2 \longrightarrow A + 2H_2O$

Though the importance of peroxidative removal of H2O2 is still uncertain but the removal of H2O2 is important as it is toxic to cells of the host. The increase in the activity of catalase has been recorded in seveval host-parasite interactions (Sarkar and Joshi, 1977, Sasikumaran et al., 1979; Agarwal, 1979; Kar and Mishra, 1976; Vidhyasekaran, 1988). The activity of catalase has been found to be augmented in lablab bean seed due to Aspergillus niger and A.niveus (Prasad, 1984) and in mustard due to Aspergillus flavsus (Kumar, 1988).

peroxidase has been reported to catalyse the oxidation ofVarious Hydrogen donors such as para- cresol, benzidine, guaiacol, ascorbic acid, nitrite and cytochromec in presence of H2O2. The representative reaction is given below.

$H_2O_2 + 2AH \longrightarrow 2A + 2H_2O$

peroxidases are widely distributed in the plant kingdom (Addy and Goodman, 1972; Mali et al., 1989; Mahadevan and Sridhar, 1996).

Estimation of catalase activity

The catalase activity is measured by estimating the residual H2O2 in the reaction mixture either by spectrophotometric method or by permanganate titration method.

In spectrophometric method, the UV light absorption of H2O2 solution is easily measured at 240nm. On decomposition of H2O2 by catalase, the absorption decreases with time. The rate of decrease denotes the amount of H2O2 tending to decrease. The method is based on Luck (1974).

Enzyme extract

0.5 of the inoculated tissue was extrated with 5 ml of the buffer in porcetain mortar with pestle. The extract was centrifuged at 10.000 rpm for 10 min. The supernatant served as the enzyme extract.

Buffer:

0.067 M phosphate buffer, pH 7.0
Substrate: 0.16 ml of H2O2 was dibuted to 100 ml with the noted
buffer.
2.5 ml of the buffer was taken in the cuvette and 0.1 ml of H2O2 (substrate) and 0.2 ml of the enzyme extract was mixed. The change in absorbance at 240 nm for 75 sec. at 15 sec interval was noted. The first reading was recorded after 15 sec. of adding H2O2. For zero absorbance the enzyme extract and buffer were mixed. The control was maintained with the enzyme extract boiled for 5 min. The reading was noted in Table 9 as absorbance at every 15 sec for 75 sec.

Table 9: Catalase activity in the diseased and control tissue of the host (expressed as change in OD)

Inocualated / control	time in sec				
	15	30	45	60	75
Inoculated	0.38	0.35	0.32	0.28	0.24
Control	0.42	0.41	0. 40	0.39	0.38

Result (Table 9, Fig . 5) It appears that the rate of decrease of H2O2 due to the enzyme extract of the inoculated leaf is clearly faster as compared to the control indicating highly augmented activity of catalase in inoculated plant as compared to the control.
(v) Estimation of the activity of peroxidase
as peroxidase catalyses the dehydrogenation of large number of organic compounds, phenol, aromatic amins, hydroquinones etc., Guaiacol was used as substrate for the assay of peroxidase,

$$\text{Peroxidase Guaiacol} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Oxidized guaiacol} + 2H_2O$$

The rate of formation of oxidized guaiacol is the measure of peroxidase activity. The procedure of estimation of peroxidase activity was adopted as outlined by Putter (1974).
Buffer: 0.1 M phosphate buffer, pH 7.0
Enzyme extract
0.5 g of inoculated leaf was extracted with 3 ml of the buffer by grinding in pre - chilled mortar and pestle. It was centrifuged at 15,000 rpm for 10 min in refrigerated centrifuge. The supernatant was used as enzyme extract.
Substrate
240 mg of Guaiacol was dissolved in 100 ml of water.
H_2O_2
H_2O_2 solution: 0.14 ml of 30% H_2O_2 was dissolved in 100 ml of water.
Procedure In a cuvette 3 ml of buffer, 0.5 ml of guaiacol solution, 0.1 ml of the enzyme extract and 0.03 ml of H_2O_2 were taken. The buffer was equiliberated at 25 ° C before use. After mixing, the reading was taken at 436 nm. The reading was noted at an interval of 20 sec. and total for 180 sec.
The reading of the uninoculated leaf (control) was recorded in Table 10 side by side the value of peroxidase activity in the inoculated leaf.

Total 10: Activity of peroxidase in inoculated and the control leaf (expressed as OD / 20 sec)

Inoculated / Control	Time in sec								
	20	40	60	80	100	120	140	160	180
Inoculated	0.018	0.020	0.025	0.030	0.035	0.040	0.045	0.050	0.058
Control	0.008	0.009	0.010	0.011	0.012	0.013	0.014	0. 015	0.016

Results (Table 10, Fig. 6) It appears that the rate of dehydrogenation (or oxidation) of guaiacol in the inoculated leaf was distinctly faster as compared to observed in the control leaf. This indicates high rate of peroxidation in inoculated leaf than non - inoculated one.

Result and Discussion:

The pathogenesis affects biological oxidation in plant tissue. The changes in the activity of various oxidases by the pathogens are generally accepted as secondary effect due to cellular derangement. But these changes alter the concentrations of the various substrates and the products. These reactions are chosenly associated with the defense mechanism of plants. The significance of various oxidative enzymes in discase resistance has been reviewed (Cruickshank and Perrien, 1964: Kue, 1966; Agrios, 1980; Singh, 1984)

The increase in respiration starts soon after inoculation and rises to a maximum rate coincident with the speculation of the pathogen and then declines to normal or subnormal (Yarwood, 1934, Shaw and Samborski, 1957; Allen and Goddard, 1958; Bushnell and Allen, 1962; Hutchison and Buchanan, 1983; Singh, 1988, Kumar, 2013). Uncoupling of oxidative phosphorylation causes enhanced uptake of 02. Certain substances such as 2, 4 dinitrophenol prevents formation of ATP from ADP, resulting in accumulation of ADP in the tissue and causes increased respiration. In diseased plants various metabolic processes are accelerated for which energy is required and therefore, there is breakdown of ATP. This results in rise in the level of ADP and inorganic phosphate. The respiratory metabolism hos also been recorded stimulated in seeds due to storage fungi such as Aspergillus and Fusarium ..

Increase in O2 uptake and CO2 release, uncoupling of ADP and inorganic phosphate to form ATP, increase in the activity of oxidative enzymes and dehydrogenase of respiratory acids of Krebs cycle are some parameter to access the respiratory metabolism. The dehydrogenase activity of pyruvic acid, after that its entrance in the Krebs cycle, and dehydrogenation of a-ketoglutaric acid caupled with decarboxylation to form succinic acid, are the steps in the chain reactions of respiration metabolism which have been reported to be due to the pathogenesis. . This agrees with the previous finding of Rohringer (1964). Daly (1976) has reported stimulated activity of Glucose 6- phosphate dehydrogenase due to biotrophic organism. Lucas (1998) has reported stimulation of the activity of pentose phosphate pathway.

The augmented activity of laccase, catechol oxidase, catalase and peroxidase all come under oxidative metabolism, are the sign of spurt in respiratory metabolism in the host due to the pathogens.

The increase in the respiratory metabolism in response to the fungal invasion has been reported due to the involvement of toxins secreted by the pathogens (Yabuta et al., 1934; Scheffer, 1976). In seeds, too, the high respiratory activity has been correlated with the activity of toxins secreted by storage fungi (Harman and Drury 1973; Prasad and Prasad, 1986; Sao et al., 1989).

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