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RESEARCH ARTICLE

Study on induced defense related enzymes in capsicum annuum Infected with *Xanthomonas* vesicatoria

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Abstract

Xanthomonas vesicatoria potential bacteria responsible for bacterial spot a major seed borne disease in many plants. This study focused on the time course of defense related enzyme like peroxidase (PO), polyphenol oxidase (PPO) in bacterial spot pathogenesis of Capsicum annuum. The results indicate, that increased peroxidase and polyphenol oxidase level in chilli, upon treatment with Xanthomonas vesicatoria than control. The increase peroxidase activity was about 14.1 nm/mg protein at 25h and 14.5nm/mg protein at 30h in case of polyphenol oxidase. The subsequent decrease in activity of both the enzymes was observed. The increased activity in chilli seeds may be due to the accumulation of phenols, were it acts as a cofactor of enzyme and hence one of the important biochemical parameters for disease resistance.

Introduction

16.5 million metric tons of Chilli (Capsicum annuum L) is grown for fresh consumption and use in condiments worldwide. India ranks first among all the spice with a share of 33.7% in the total production (Meena *et al.*, 2008).The crop is infected by several fungal, bacterial and viral diseases. Bacterial spot is one of the most devastating diseases of pepper and tomato, appears on foliage, stems, and fruit. *Xanthomonas* vesicatoria is responsible *for bacterial* spot a major seed borne disease. 10 to 15 % seeds carry the bacteria which initiate the disease through seedlings. The disease appears in water soaked areas as small, circular or irregular spot on leaves, stems, and fruits, which turn brown as they mature. It is impossible to control the disease and prevent major fruit loss when present in the crop according to Kucharek (1994).

Plants respond to bacterial pathogen attack by various defense responses that serve to prevent pathogen infection. The mechanisms include preexisting physical and chemical barriers as well as inducible response related enzymes that become activated upon infection. Resistance to pathogen(s) is associated with the accumulation of several factors like enzymes, antibiotics and inhibitors etc. The interaction between the pathogen and host plant induce some changes in cell metabolism, primarily in the enzyme activities, including that of Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Polyphenol oxidase (PPO), Lipoxygenase etc.(Chandrashekar and Umesha, 2012). The level of defense related enzymes are known to play a crucial role upon the degree of host resistance. Increasing activity and accumulation of these enzymes depends mainly on the inducing agent, the plant genotype, physiological condition and the pathogen (Tuzun, 2001).

The objectives of the present study include the isolation of pathogen from seeds and investigation on the changes in enzymes like peroxidase and polyphenol oxidase in seedlings against bacterial spot pathogen *Xanthomonas vesicatoria*

Materials and method

Seed sample collection

The seeds of chilli were obtained from local seed agencies Bangalore, Karnataka, India and seeds samples for pathogen isolation were collected from infected chilli fruit.

Isolation and identification of pathogen *Xanthomonas vesicatoria*

Xanthomonas vesicatoria was isolated from chilli seeds by plating on to the semi-selective medium (Tween B) after surface sterilization with 3% (v/v) sodium hypochlorite solution for 4 min was then washed with distilled water. Plated seeds were incubated for 24 to 48h. Yellow colonies with clear hydrolytic zone seen around the seeds, were observed and subcultured onto the Tween B media and the suspected colonies were subjected to different biochemical tests for confirmation.

Preparations of crude enzyme extracts

The seeds of the chilli were plated in petridish (9 cm diameter) onto moist blotter discs, at a density of 25 seeds per plate following standard procedures of International Seed Testing Association. The plates were incubated at 28±2ºC for 7 days until cotyledonary leaf completely opens. The 7^{th} day old seedlings were used for further experiments.

Bacterial inoculum was prepared by adjusting the bacterial concentration with sterile distilled water to 1 x 10^8 cfu/ml at A_{610} nm using UV-visible spectrophotometer. Noninoculated chilli seedlings were used as a control.

Chilli Seedlings (1g) raised from untreated and pathogen treated seeds were carefully uprooted and stored at -20ºC until enzyme assay. Seedlings were homogenized in 10mM phosphate buffer (pH 6.0) in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was used directly in the enzyme assay.

Time Course Studies on Defense Responses

Collection of plant samples: Chilli seedlings (1g) raised from untreated and pathogen treated seeds were carefully uprooted from 0 to 60 h at a difference of 5h time intervals after pathogen inoculation and stored at -20ºC until enzyme assay. Seedlings were homogenized in 10mM phosphate buffer (pH.6.0) in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was used directly in the enzyme assay.

Peroxidase assay (PO, E.C.1.11.1.7): PO activities were measured at room temperature, described previously (He et al., 2001) with minor modifications. The PO assay reaction contained 7.5 µL of 10 mM guaiacol in 50 mM sodium phosphate buffer (pH 6.0), 100 µL crude extract, 792.5 µL of 10 mM sodium phosphate buffer (pH 6.0), and 100 µL of 600 mM H_2O_2. The change in optical density at 470 nm was measured for 1 min. PO activity was calculated as change in absorbance units min^{-1} mg^{-1} protein.

Polyphenol oxidase assay (PPO, E.C. 1.14): Polyphenol oxidase activity was determined as per the procedure given by Gauillard et al (1993). The reaction mixture consists of 1mM catechol in 0.05M sodium phosphate buffer (pH 6.5) and 100µl of the enzyme extract. The activity was determined using catechol as substrate and the rate of increase in absorbance was measured at 405 nm. The activity was expressed as change in absorbance/min/mg protein.

Protein estimation

Protein contents of the extracts for all estimated enzymes were determined by using the standard procedure of Bradford (1976) using BSA as standard.

Results Discussion

Isolation and Characterization of *X. vesicatoria*

Colonies showing characteristic hydrolytic zone, with fried egg appearance around the seeds, on the semi selective media (Tween B) were isolated, pure cultures were obtained by streaking onto NA media. The biochemical characterizations of the isolate were tabulated in table 1.

Peroxidase Activity on Time Course Studies

Higher peroxidase activity was observed in pathogen treated seeds as compared to untreated and it was observed that activity increased sharply and peak was maxium at 25h with 14.1 units/min/mg protein. The activity of enzyme decreased gradually after 25h and sharp decline at 45h onwards almost reaching the activity of control (Fig 1).

Polyphenol oxidase Activity on Time Course Studies

PPO activity was determined by measuring the conversion of catechol to orhto quinone in enzyme assay. It was observed that there was a transient increase in activity till 40h after pathogen inoculation and showed a sharp decreasing trend after 45h. The maximum activity was showed at 30h (14.5units/min/mg protein), while at the same time; control showed lesser activity when compared to treated seedlings (Fig 2).

Discussion

Plant responds to bacterial pathogen attacks by transcriptionally activating a number of genes coding for proteins, which are thought to help the plant toward off the invader (Vidal *et al.,* 1997). It is generally assumed that wide variety of induced proteins play different roles in the defense of plants against bacterial pathogen. In literatures there are several reports in which, the level of defense related enzymes were well correlated with the host resistance. The level of defense related enzymes are known to play a crucial role upon the degree of host resistance (Shivakumar *et al*., 2000).

Peroxidase activity is associated with disease resistance in plants and an enhanced peroxidase activity was also demonstrated during pathogenesis and it leads to reinforcement of cell walls with phenol compounds (Scott-Craig *et al*., 1995) including Xanthomonad-induced resistance (Do *et al*., 2003). PPO is copper-containing enzyme, which oxidize phonemics to highly toxic quinies and involved in the terminal oxidation of disease plant tissue, which was attributed for its role in disease resistance (Kosuge, 1969). Along with other defense enzymes PPO catalyze the last steps in the biosynthesis of lignin and other oxidative phenols, which are major chemical and physical barrier of pathogen attack (Ramamoorthy *et al*., 2002).

Enhanced activity of peroxidase in banana infected by Banana virus was reported by Devanathan *et al.,* (2005). Increased peroxidase activity and polyphenol oxidase activities found to occur in diseased leaves of capsicum Infected with Geminivirus compared to healthy leaves as been reported by Meena *et al.,* (2008). The study by Ramanujam *et al.,* (2012) reported *B. subtilis* as induce defense related compounds in chilli plants against C. capsici.

Table 1: Biochemical characterization of *Xanthomonas vesicatoria*

Characterization of isolate	**Observation**	**Inferences**
Colony morphology	yellow color, hydrolytic zone, appears like fried egg	*Xanthomonas vesicatoria*
Biochemical tests		
Gram staining	Pink red	gram negative
Strach	No clear zone of hydrolysis	negative
KOH solubility	thin viscoid mucoid strand	positive
Kovac's oxidase	No violet color	negative
Lipase activity	white precipitate around the colonies	positive
Arginine dihydrolase	orange to red after 3 days of incubation	positive
Gelatin hydrolysis	liquefaction	positive

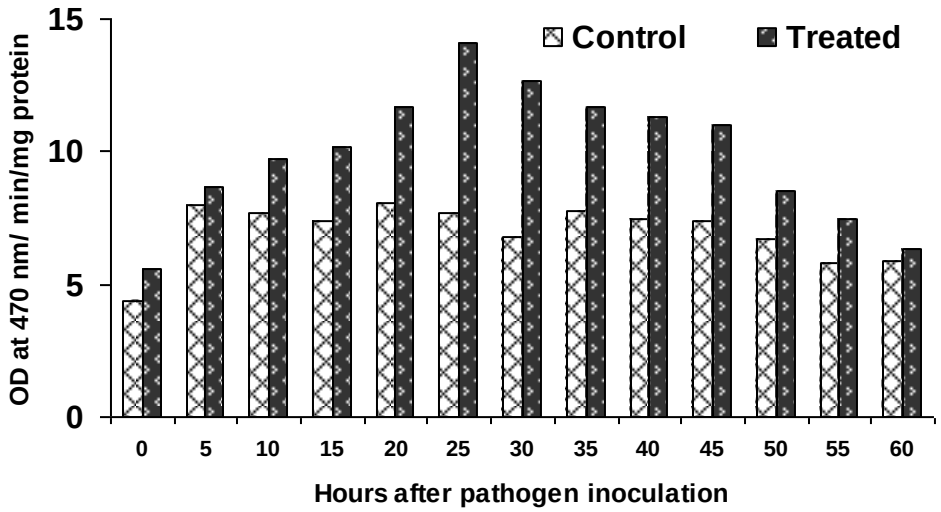

Figure 1: Peroxidase activity (nm/min/mg protein) on a time course after pathogen inoculation

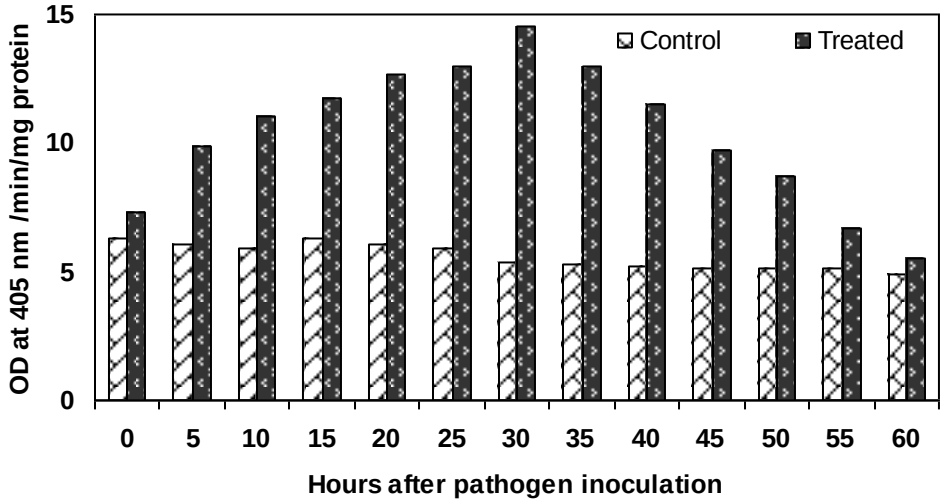

Figure 2: Polyphenol oxidase Activity (nm/min/mg protein) on a time course after pathogen inoculation

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