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Research Article

INDUCTION OF ANTIOXIDANT ENZYMES ASSOCIATED WITH BACTERIAL SPOT PATHOGENESIS IN TOMATO

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ABSTRACT
Plant–pathogen interactions are mediated by a complex network of molecular and cytological events that determine a range between susceptibility and resistance. The production of reactive oxygen species, via consumption of oxygen so-called oxidative burst, is one of the earliest cellular responses following successful pathogen recognition. The present investigation was focused on the role of antioxidant enzymes in imparting resistance in tomato against bacterial spot pathogen *Xanthomonas axonopodis* pv.*vesicatoria*. Ten different tomato cultivars were collected from private seed agencies and screened for resistance to bacterial spot disease, using artificial inoculation technique under greenhouse conditions. Involvement of antioxidant enzymes in bacterial spot pathogenesis was studied in resistant, susceptible and highly susceptible tomato cultivars. Eight-day-old seedlings were root-dip and spray inoculated with the inoculum (1 x 10^8 cfu/ml) and harvested at different time intervals (0, 3, 6, 9, 12, 15, etc., up to 72 h) and assayed for two antioxidant enzymes. Temporal accumulation of ascorbate peroxidase and catalase enzymes showed maximum activity at 12 and 21h after pathogen inoculation (hpi) in resistant cultivar, whereas in susceptible and highly susceptible cultivars it increased at 18 and 36h for ascorbate peroxidase and 42 and 54 h for catalase respectively. Furthermore, Isoforms analysis of APX and CAT enzymes indicated the clear difference between resistant and susceptible cultivars. Resistant cultivar showed higher enzyme activity after pathogen inoculation when compared to uninoculated control and also the susceptible cultivars. The role of these two antioxidant enzymes in imparting resistance to tomato against bacterial spot pathogenesis is discussed.

Key Words: *Bacterial spot, Xanthomonas axonopodis pv.vesicatoria, Antioxidant Enzymes, Tomato, Ascorbate peroxidase, Catalase*

INTRODUCTION
Tomato (*Solanum lycopersicum* Mill.) which belongs to the family Solanaceae is one of the most important vegetable grown and consumed worldwide. Tomatoes constitute as an excellent food and as a natural diet. It is considered as a protective food because of its nutritive value. Plant diseases become limiting factor in tomato production in many parts of the world.
Plants are constantly exposed to a wide array of environmental stresses that cause major losses in productivity. Resistance and susceptibility to these biotic and abiotic stresses are complex phenomena, in part because stress may occur at multiple stages of plant development and often more than one stress simultaneously affects the plant. To cope with various environmental challenges, plants execute a number of physiological and metabolic responses (Bohnert *et al*., 1995).
Tomato is the target of many infectious diseases that cause severe yield losses. Among them, bacterial spot of tomato, incited by *Xanthomonas axonopodis pv.vesicatoria*, is one of the most important diseases, especially when weather conditions are suitable for its development (Pohronezny and Volin, 1983). This particular pathogen also affects peppers (*Capsicum* spp.) and has been reported worldwide wherever tomatoes and peppers are grown (Stall, 1995a).
Seed-borne bacteria constitute one of the major risk factors for the production of the healthy crop. Infact, bacteria ranks second next to fungi when damage to crops is considered. Bacterial spot is primarily

characterized by the occurrence of greasy-appearing, water-soaked, circular lesions on leaves, stems, and fruits. These lesions vary in size and shape, and normally develop into necrotic spots. As a final consequence, leaf abscission in pepper plants or necrosis of tomato leaflets may also occur (Stall, 1995b).
Bacterial diseases can be devastating since bacteria have a high multiplication rate. Seed borne disease is of great significance as seeds provide numerous location of infection. An infected seed not only provides disease initiation but also creates a number of additional inoculum sources for the future generation of crops by introducing the pathogen to soil and other host plants. Seed-borne bacteria thus acquire considerable economic significance (Gitaitis and Walcott, 2007).
Bacterial spot disease, which is very difficult to manage, causes reduced plant growth, fruit yield, and quality. Despite many efforts to control this disease, not a single method has been completely effective. The efficacy of chemical to control with copper compounds and streptomycin has been marginal. The rise of resistant strains of *X.axonopodis* pv.*vesicatoria* to both of these chemicals is also responsible for reduced control. Thus, management of bacterial spot relies essentially on exclusion of the disease by using pathogen-free seeds and seedlings, sanitation, and resistant varieties (Sahin and Miller, 1996). The inability to control the disease with cultural practices and/or antibacterial agents leaves resistance as one of the most important alternatives for controlling this disease.
Evolution has provided plant pathogens with a significant number of mechanisms to enhance their pathogenic potential and to ensure their survival. Likewise, plants have developed an equally diverse set of counter measures to avoid their own demise. Throughout time, this co-evolution between host and pathogen has given form to what is defined today as plant-pathogen interactions.
Plant–pathogen interactions are mediated by a complex network of molecular and cytological events that determine a range between susceptibility and resistance (Lamb *et al.*, 1989). When plants are attacked by pathogens they respond by activating a variety of defense mechanisms, including the rapid production and accumulation of reactive oxygen species (ROS). Generation of ROS is thought to be an early event that can fundamentally influence the balance of the interaction between the plant and the pathogen (Gayoso *et al*., 2004)
One of the first defense responses to the bacterial infection is rapid generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical (Mittler, 2004). ROS have direct antimicrobial activities which reduce pathogen viability. On the otherhand enhanced ROS production can also lead to oxidative damage of pigments, proteins, nucleic acids and lipids (Mandal *et al*., 2008). However, ROS are inevitable by products from the essential aerobic metabolisms, and they need to be maintained under sub lethal levels for normal plant growth. Hence, plants have developed efficient protective mechanisms against oxidative stress which utilizes enzymatic and non-enzymatic compounds in order to scavenge excess ROS**.** Multiple antioxidant enzymes systems are involved in the enzymatic scavenging of ROS. Superoxide dismutases (SOD, EC 1.15.1.1) react with the superoxide radical to produce H_2O_2. Hydrogen peroxide is scavenged by catalases (CAT, EC 1.11.1.6) and peroxidases (POX, EC 1.11.1.7). Among peroxidases, ascorbate peroxidases (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9) which uses ascorbate and glutathione as electron donors, respectively, are well known for their role in H_2O_2 detoxification in plant (Apel and Hirt, 2004).
APX utilizes ascorbic acid (AsA) as its specific electron donor to reduce H_2O_2 to water. To date, there are only a few reports on the role of APX in protecting plants from the damage resulting from drought stress and also in host-pathogen interaction. APX genes have been isolated from some plants, such as strawberry fruit, spinach leaves, potato tubers, and rice, and their expression has been determined under fruit ripening, oxidative stress, and low temperature, respectively (Lu *et al.*, 2005).
APX protects cells against H_2O_2 under normal as well as stressful conditions. Increased activity of APX in response to environmental stresses such as NaCl salinity, chilling, metal toxicity, drought, heat, etc. has been reported in different plant species which suggests its possible role in eliminating H_2O_2 from cells (Davis and Swanson, 2001) whereas fewer reports are available during plant-pathogen interaction and APX enzyme relationship. Isoforms of APX have been purified from spinach chloroplasts (Nakano and

Asada, 1987) tea leaves (Chen and Asada, 1989), plastids of tobacco, and their enzymatic and molecular properties have been studied. Distinct differences occur in physico-chemical and kinetic properties of APX isoforms (Sano *et al.*, 2001).

Catalase is a tetrameric heme containing enzyme that is found in all aerobic organisms and serves to rapidly degrade H_2O_2 into water and oxygen. The enzyme is abundant in the glyoxisomes of lipid-storing tissues in germinating barley, where it decomposes H_2O_2 formed during the β-oxidation of fatty acids (Fazeli *et al.*, 2007) and in the peroxisomes of the leaves of C_3 plants, where it removes H_2O_2 produced during photorespiration by the conversion of glycolate into glyoxylate (Jayakumar *et al.*, 2008). This is also due to the fact that there is proliferation of peroxisomes during stress, which might help in scavenging of H_2O_2, which can diffuse from the cytosol (Lopez-Huertas *et al.*, 2000). A third class of CAT is located in vascular tissues and may be involved in protection against environmental stress (Fu and Huang, 2001). The H_2O_2 scavenging system represented by APX and CAT are more important in partitioning tolerance than SOD as reported in oxidative stressed wheat varieties (Lafitte *et al.*, 2007).

Peroxidase and catalases are involved in the defense mechanisms of plants in response to pathogens either by their direct participation in cell wall reinforcement, or by their antioxidant role in the oxidative stress generated during plant-pathogen interaction (Mehdy, 1994). Catalase and ascorbate peroxidase are induced by oxidative stress, since they convert H_2O_2 to H_2O. Since the early stages of plant pathology, natural resistance to plant pathogens has been considered a desirable trait for selection of crop plants. The first attempts to study resistance in plants focused on finding new sources of resistance throughout the world. Those findings were then used to establish breeding programs in order to introduce resistance into commercial varieties. Recently, the focus of this type of research has been partially shifted towards the exploration of the biochemical and molecular basis of resistance, and ultimately to the improvement of the ability to genetically engineer durable resistance into commercial crops. However, no reports have been published on the possible involvement of APX and CAT in the tomato seedlings upon infection of *X.axonopodis pv.vesicatoria* and screening of different tomato cultivars for their resistant and susceptible nature.

In this study, the aim is to investigate two antioxidant enzymes APX and CAT in different tomato cultivars upon *X.axonopodis* pv.*vesicatoria* inoculation to differentiate between highly resistant (HiR), resistant (R), susceptible (S) and highly susceptible (HS) cultivars which can be used as biochemical markers in host resistance screening.

MATERIALS AND METHODS

Seed sample

Ten different tomato cultivars were procured from different seed traders, Mysore, India. All seed samples used in the experiment were surface sterilized with 3% (v/v) sodium hypochlorite solution for 4 min and washed with distilled water three times.

Screening of Tomato Cultivars against Bacterial Spot Disease

Tomato cultivars were screened against bacterial spot under green house conditions following the standard procedure as previously explained Soylu *et al.*, (2003). Tomato seedlings were raised in plastic pots (9 cm diameter) filled with mixture of sterilized soil, sand and farmyard manure (2:1:1). For each cultivar, 15 plants in four replicates each were maintained.

Bacterial inoculum was prepared by growing bacteria on Tween B medium (Tween B medium; Peptone 10 g/l, KBr 10g/l, $CaCl_2$ 0.25 g/l, H_3BO_3 0.30 g/l and Agar 15 g/l. After autoclaving; aseptically add Tween 80 10 ml/l, Cyclohexamide 100 mg/l, Cephalexin 65 mg/l, 5-fluorouracil 12 mg/l and Tobramycin 0.4 mg/l) (McGuire *et al.*, 1986) for 36 h at 30^0 C. 36h-old-culture was pelleted by centrifugation (thrice at 5000 rpm for 5 min) using bench top refrigerated centrifuge (UniCen, 15 DR, Herolab GmbH, Germany). Inoculum was prepared by adjusting the optical density (OD) of the bacterial suspension to 0.45 (A_{610}nm) to obtain approximately $1x10^8$ cfu/ml with the help of UV- visible spectrophotometer (Hitachi U-2000, Tokyo, Japan). Fifteen milliliters of bacterial suspension

($1x10^8$ cfu/ml) was sprayed onto the four week old tomato seedlings using spray inoculation method during early hours of the day.

Plants were inspected for bacterial spot symptoms daily up to 50 days following inoculation. Based on disease incidence the tomato cultivars were categorized into highly resistant (HiR), with no plants (0 %) showing any symptoms of bacterial spot disease; resistant (R), with 0.1 % to 10.0 % of plants showing slight marginal spots and 1- 20 % of leaves become brown; susceptible (S), with 10.1 % to 20.0 % of plants showing sectorial spots and 20-40 % of leaves become brown; and highly susceptible (HS), with > 25 % of the plants showing pronounced leaf collapse and more than 40 % of leaves become brown (Kavitha and Umesha, 2008).

Temporal Pattern Study of Enzymes

Three tomato cultivars were plated in Petri dishes onto moist blotter discs, at a density of 25 seeds per plate following standard procedures of the International Seed Testing Association (ISTA, 2005). The plates were incubated at 28 ± 2^0 C for eight days until cotyledonary leaves completely opened. The 8-day-old seedlings were used for further experiments. *X. axonopodis* pv. *vesicatoria* inoculum was prepared as explained above and 8-day-old tomato seedlings were covered with polythene sheeting 2h before inoculation to increase the humidity. The tomato seedlings were root-dip and spray inoculated with the inoculum ($1x10^8$ cfu/ml) and kept covered with polythene sheeting.

To study the temporal pattern of APX and CAT enzymes, three different cultivars of tomato, that were highly resistant (Solar cultivar), susceptible (Amulya cultivar) and highly susceptible (Quality cultivar) category based on the disease incidence under greenhouse conditions were selected. The tomato seedlings were raised as explained previously and seedlings were harvested at different time intervals: 0, 3,6,9,12,15, 18, 21, and 24 up to 72h after pathogen inoculation (hpi). Distilled water inoculated samples served as control.

Ascorbate Peroxidase Assay

One gram of tomato seedlings was homogenized in 1 ml of 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA, 1 mM PMSF, 5 mM Ascorbic acid and 5 % (w/v) PVP in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 13,000 rpm, for 10 min at 4^0 C and the supernatant served as enzyme source. All the experiments were carried out 4^0 C.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano & Asada by monitoring the decrease in the absorbance at 290 nm over 2 min. The enzymatic reaction was started by adding 10 µL of 12 mM H_2O_2 in 990 µL of the reaction mixture. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7.0) with 0.1 mM EDTA, 50 mM ascorbic acid and 100 µL of enzyme extract.. One unit of APX activity is defined as the amount of enzyme that oxidizes 1 µmol min^{-1} ascorbate under the above assay conditions. All the experiments were conducted three replicates and were repeated thrice.

Catalase assay

One gram of tomato seedlings was homogenized in 1ml of sodium acetate buffer (pH 7.0) in a pre- chilled mortar and pestle on ice. The homogenate was centrifuged at 12, 000 rpm for 10 min at 4^0C and the supernatant served as enzyme source. All the experiments were carried out at 4^0C.

Catalase was estimated by following the procedure of Beers and Sizer (1951). The 2.9 ml reaction mixture contained 50 mM potassium phosphate buffer and 100mM H_2O_2 and the reaction was initiated by adding 100 µl of enzyme extract and the decrease in A_{240} was measured for 3 min. The specific activity of catalase was expressed as ΔOD at 240 nm min^{-1} mg^{-1} protein. All the experiments were conducted three replicates and were repeated thrice.

Protein Estimation

Protein contents of the extracts were determined according to standard procedure of Bradford, (1976) using BSA (Sigma, USA) as standard.

Native-Page Analysis of APX and CAT

The isoforms profiles of APX were determined by discontinuous Native polyacrylamide gel electrophoresis (Native-PAGE) following the procedure of Mittler and Zilinskas, (1993). Enzyme extracts (100 µg protein) of HiR, S and HS tomato cultivars at 12h of both inoculated and control were loaded onto 8% (w/v) polyacrylamide gels with a vertical mini-gel electorphoresis unit (Biometra, Gottingen, Germany). The electrode buffer was Tris-base (6.0 g Tris-base, 14.4 g glysine and 1 L distilled water). Electrophoresis was performed at a constant voltage of 50V initially for 1 h and 100 V to complete electrophoresis.

The isoforms profiles of CAT were examined by discontinuous Native polyacrylamide gel electrophoresis (Native-PAGE) following the standard procedure (Yang *et al.*, 1999). Enzyme extracts (100µg protein) of HiR, S and HS tomato cultivars at 21h were loaded onto 8% (w/v) polyacrylamide gels with a vertical mini-gel electorphoresis unit (Biometra, Gottingen, Germany). The electrode buffer was Tris-base (6.0 g Tris-base, 14.4 g glysine and 1 L distilled water). Electrophoresis was performed at a constant voltage of 50V initially for 1h and 100V to complete electrophoresis.

Activity Staining For APX and CAT

APX was stained according to the method described in Mittler and Zilinskas, (1993), which is based on the inhibition of NBT (Nitro blue tetrazolium chloride) reduction by ascorbate. Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM sodium ascorbate for 30 min (the buffer was changed with each 10 min). Thereafter, gels were incubated in the above buffer amended with 4 mM sodium ascorbate and 2 mM H_2O_2 for 20 min.

Gels were then washed in the phosphate buffer alone for 1 min, stained in 50 mM potassium phosphate buffer, pH 7.8, amended with 28 mM TEMED and 2 mM NBT, and agitated gently for 2-3 min up to appearance of clear bands on an intense blue background due to NBT reduction by ascorbate.

CAT activity staining was performed following the procedure of Yang *et al.* (1999). The gel was first rinsed three times with distilled water and then incubated in 0.003 % H_2O_2 for 10 min. The gel was then stained with 2 % ferric chloride and 2 % potassium ferricyanide; when a chromatic bands begin to form, the stain was poured off and the gel was rinsed extensively with tap water to stop the reaction and then washed with distilled water. Achromatic bands demonstrated the presence of CAT activity.

Validation of APX and CAT Activity in different Tomato Cultivars

Tomato seedlings of 10 different cultivars were raised as explained previously on wet blotters. The seedlings were harvested at 12 hpi and 21 hpi for both APX and CAT analysis. The spectrophotometric enzyme assay for both APX and CAT were done as previously explained.

Statistical Analysis

All experiments were performed three times with similar results. The data obtained from greenhouse experiments were anaylsed separately for each experiment and were subjected to two-way (Treated and Control) analysis of variance (ANOVA) using the statistical software SAS (version 9.0) for Microsoft windows. The means were compared for significance using Fisher's LSD. Significant effects of pathogen inoculation on enzyme activities were determined by the magnitude of the F-value ($p \leq 0.05$).

RESULTS

Screening of tomato cultivars against bacterial spot disease: Among the 10 different tomato cultivars screened, a varied level of disease incidence was found which ranged between (0-40%). The tomato cultivar Solar (HiR) is completely free from bacterial spot disease. Two cultivars (Sun hybrid and Indam) were found to be resistant (7-13%), four cultivars (Ashwini TA-4, Amulya, PKM-1 and Rohini-TR) were susceptible (20-26%) and three cultivars (Golden, Ujwala and Quality seeds) were highly susceptible (33-40%) to bacterial spot disease (Table 1).

Temporal Changes in APX Activity

The temporal changes in APX activity of highly resistant (HiR), susceptible (S) and highly susceptible (HS) seedlings with or without pathogen shown in figure (Fig. 1). Varying patterns of APX activity were

observed in inoculated and control seedlings HiR, S and HS cultivars. A gradual increase in APX activity was observed in all type of cultivars. In highly resistant seedlings, after pathogen inoculation a drastic increase in APX activity was noticed initially and reached its peak at 12h (0.35 units) whereas, in susceptible and highly susceptible seedlings, APX activity was found to be 0.28 units at 15h and 0.23 units at 38h, respectively after pathogen inoculation (Fig 1).

Native Page Analysis of APX

The protein samples of HiR, S and HS seedlings of tomato were analyzed for expression of APX isoforms. A total of three isoforms of APX were expressed and its intensity varied between cultivars and even between control and inoculated seedlings. The APX isoforms were less intense in S and HS seedlings when compared to HiR seedlings (Fig. 5). There was no difference in number of isoforms in all the three categories of seedlings used

Temporal Changes in CAT Activity

The temporal changes in CAT activity of highly resistant (HiR), susceptible (S) and highly susceptible (HS) seedlings with or without pathogen shown in figure (Fig. 2). Varying patterns of CAT activity were observed in inoculated and control seedlings HiR, S and HS cultivars. A gradual increase in CAT activity was observed in all type of cultivars. In highly resistant seedlings, after pathogen inoculation a drastic increase in CAT activity was noticed initially and reached its peak at 21h (3.5 units) whereas, in S and HS seedlings. CAT activity was found to be 2.7 units at 42h and 2.0 units at 54h respectively after pathogen inoculation.

Native Page Analysis of CAT

The protein samples of HiR, S and HS seedlings of tomato were analyzed for expression of CAT isoforms. A total of three isoforms of CAT were expressed and its intensity varied between cultivars and even between control and inoculated seedlings. The CAT isoforms were faint in S and HS seedlings when compared to HiR seedlings (Fig. 6). There was no difference in number of isoforms in all the three categories of seedlings used.

Validation of APX and CAT Enzymes for different Tomato Cultivars

Seedlings of 10 different tomato cultivars were analyzed for APX and CAT with or without pathogen inoculation. All the cultivars showed an increased level of enzyme activity after pathogen inoculation. Highest activity APX of 0.38 units was observed in Solar cultivar followed by Sun hybrid and Indam, whereas least activity of APX was found in cultivar Quality seeds (0.15 units) after pathogen inoculation showing significant ($p=0.05$) difference between their respective controls. A moderate level of increased APX activity was found in the cultivar Amulya (0.21 units) after pathogen inoculation (Fig. 3). Similarly varied level of CAT activity was found between cultivar, which was ranged from 3.5 to 1.5 units. Significantly ($p=0.05$) highest CAT activity was found in cultivar Solar (3.5 units) seedlings after pathogen inoculation when compared to control and other cultivars. The least activity was noticed in cultivar quality (1.30 units) (Fig 4).

DISCUSSION

Vegetables are horticulturally important crops and hundreds of vegetables and fruits are grown in India. The diverse agro-climatic zones of India make it possible to grow almost all varieties of vegetables. Vegetables provide many essential vitamins and minerals. Additionally higher intakes of vegetables are associated with healthier lives including lower risks of cancer and coronary diseases. They contain valuable food ingredients which can be successfully utilized to build up and repair the body. They are valued mainly for their high vitamin and mineral contents.

Rapid and transient generation of AOS (active oxygen species), namely the oxidative burst, is a characteristic event in the early phase of many plant-pathogen interactions (Baker and Orlandi, 1995). In the present study, an attempt has been made to study the resistance and susceptible nature of different tomato cultivars against *X.axonopodis* pv.*vesicatoria* by considering APX and CAT as biochemical markers of host resistance. The 10 different tomato cultivars showed varying degrees of resistance to

bacterial spot pathogen. The cv. Solar was highly resistant as it is completely free from bacterial spot disease. Resistant cv. Sun hybrid and cv. Iandam and had minimum disease incidence, whereas cv. Quality was HS and had recorded maximum disease incidence. All cultivars reacted to pathogen inoculation by inducing antioxidant enzymes. Various studies conducted on host resistance in tomato against *X.axonopodis* pv.*vesicatoria* have revealed the resistance of commercial cultivars to various degrees. A highly resistant cultivar (Solar) showed maximum enzyme activity when compared with susceptible (Amulya) and highly susceptible (Quality) cultivars. These results are well correlated with the findings of Kavitha and Umesha, (2008).

Early and elevated levels of expression of various anti-oxidant enzymes are in important feature of plant resistance to pathogens. The production of reactive oxygen species (ROS) is one of the cellular responses following successful pathogen recognition. As part of the host defense responses, plants produce an oxidative burst. The burst occurs in two phases depending on the ability of the microbe to evade or suppress its host defense (Grant *et al.,* 2000). In response to invasion by microorganisms, plants employ various defense mechanisms to combat pathogen population growth. General defense reactions, such as production of reactive oxygen species (ROS), cell wall reinforcement accumulation of antimicrobial proteins etc are the most decisive factors governing the outcome of host-pathogen interactions.

Development of an antioxidant defence system in plants protect them against oxidative stress damage, by either the partial suppression of ROS production, or the scavenging of ROS which has already been produced (Ye *et al.,* 2006). Thus, various antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD) catalase (CAT) and ascorbate peroxidase (APX) are participate in ROS metabolism during the pathogen attack Results of the spectrophotometric assays indicate that infection by *X.axonopodis* pv.*vesicatoria* led to the substantial changes in the antioxidant status of tomato seedlings.

In the present study, we report the direct involvement of APX and CAT during host-pathogen interaction in tomato when inoculated with *X.axonopodis* pv.*vesicatoria*. Spectrophotometric assays of APX and CAT enzyme in resistant, susceptible and highly susceptible seedlings without pathogen inoculation recorded lesser activities compared with inoculated seedlings, indicating a possible role of these enzymes during pathogen infection and host-resistance. There are several examples that indicate the differences in the final outcome of a plant-pathogen interaction; either susceptibility or resistance might be due to the timing and intensity of the plant's defense responses (Tao *et al.,* 2003).

CAT activity in tomato cultivars showed significant induction after pathogen inoculation. CAT activity was maximum at 24h after pathogen inoculation when compared to the control. Similarly Baker and Orlando, (1995) reported that there was an increase in antioxidant enzymes such as APX, CAT SOD etc in tomato plants when the tomato plants were inoculated with *Meloidogyne javanica* and induction of the antioxidant enzymes and oxidative stress are quite general defense responses. Catalase protects cells from the toxic effects of hydrogen peroxide. Pathogen induced CAT activity in tomato infected with *Botrytris cinerea* was shown to be maximum upon infection (Kuzniak and Sklodowska, 2005).

Our findings were similar with the earlier studies of Carmen *et al.,* (2002), where they have studied the role of antioxidant enzymes in resistant and susceptible cultivars of chickpea with *Fusarium oxysporum* f.sp. *ciceris.* The results presented here indicate that enzyme such as catalase and ascorbate peroxidase, which are normally induced by oxidative stress, are induced at the commencement of bacterial infection, but are subsequently suppressed. The induction was coincident with time which shows the early response and also defence role of antioxidant enzymes.

Elevated levels of SOD, CAT and APX in cucumber seedling appear to be correlated with the development of heat-shocked-induced chilling tolerance (Kang and Saltveit, 2002). The heat shock treatment could induce oxidative stress, which then induces an increase in the antioxidant capacity of the tissue (Li *et al*., 1999). Samia, (2007) reported that infection by *Fusarium oxysporum* significantly increased SOD, APX and CAT activities in leaves of tomato plants at different stages of growth as compared with non-infected control. Treatments with arbuscular mychorrhiza fungi and jasmonic acid markedly raised these activities and the highest activity was recorded when applied together.

Mutlu *et al.*, (2009) studied the effects of salicylic acid (SA) and salinity on the activity of apoplastic antioxidant enzymes were studied in the leaves of two wheat (*Triticum aestivam* L.) cultivars. Increased level of antioxidant enzymes; CAT and SOD activities were observed in both cultivars, compared to those of untreated control plants.

The fact that APX and CAT activity was higher in highly resistant seedlings than in susceptible and highly susceptible seedlings indicates that APX and CAT might have played a specific role in triggering the development of host resistance. The results from spectrophotometric estimation were further confirmed by native PAGE analysis to observe the change in isozymes pattern and intensity of the isoforms in susceptible and highly resistant seedlings with and without *X.axonopodis* pv.*vesicatoria* inoculation. The native PAGE analysis profile results correlate with results of Sang *et al.*, (2005) in which they observe significant increase in the activities of SOD, CAT, APX, POX, and Glutahione reductase in the NaCl-stressed barley root was highly correlated with the temporal regulation of the constitutive isoforms as well as the induction of new isoforms. The native PAGE analysis of the APX and CAT enzymes showed that there were three isoforms in resistant, susceptible and highly susceptible cultivar upon pathogen inoculation. But, the intensity of isoforms of APX and CAT increased in resistant cultivars when compared to susceptible and highly susceptible (Fig 5 and 6).

Our studies indicate that the antioxidant enzymes APX and CAT are actively involved in imparting resistance to bacterial spot of tomato. It was observed that upon inoculation of resistant seedlings, APX and CAT expression were increased. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to bacterial spot of tomato. Increased APX and CAT enzyme activities during host-pathogen interaction were well correlated with imparting resistance to bacterial spot of tomato. Further, APX and CAT can be used as a biochemical marker to indicate the resistance/susceptibility nature of tomato cultivars against bacterial spot disease of tomato. The differential expression of APX and CAT genes in different tomato cultivars upon infection with *X.axonopodis* pv.*vesicatoria* was worth pursuing.

Table 1: Screening of different tomato cultivars to bacterial spot disease under green house conditions*

Cultivars	Bacterial spot incidence (%)	Categorization
Solar	0	HiR
Sun hybrid	7±0.2	R
Indam	7±0.3	R
Ashwini TA-4	13± 0.5	S
Amulya	20± 0.9	S
PKM-1	20±0.7	S
Rohini-TR	26±0.8	S
Golden	33± 0.6	HS
Ujwala	33±0.7	HS
Quality seeds	40±0.9	HS

** Results of screening of different tomato cultivars for bacterial spot disease incidence under green house condition. Tomato plants were raised in plastic pots for 15 days and inoculated with Xanthomonas axonpodis pv.vesicatoria. Bacterial spot incidence was screened up to 50 days after pathogen inoculation. Values are the replicates means ±SE of 4 replicates of 15 plants each and repeated thrice.*

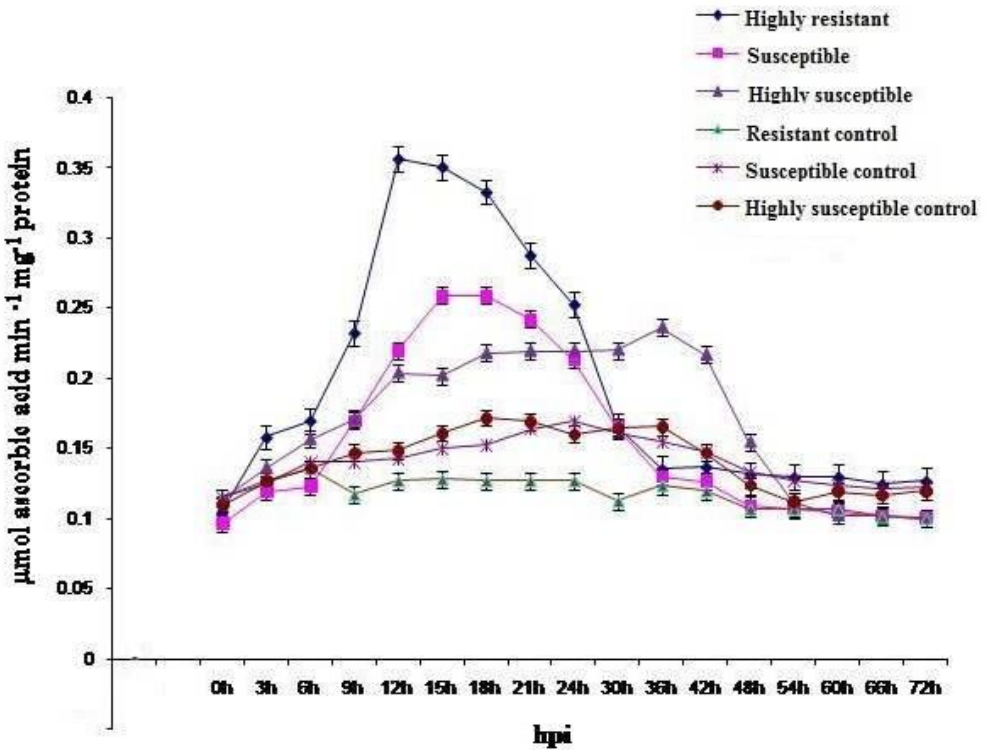

Figure 1: Temporal pattern study of APX activity in highly resistant (HiR), susceptible (S) and highly susceptible (HS) cultivars with and without pathogen inoculation. The data expressed as the average of three independent experiments with three replicates each. Bars indicate standard error

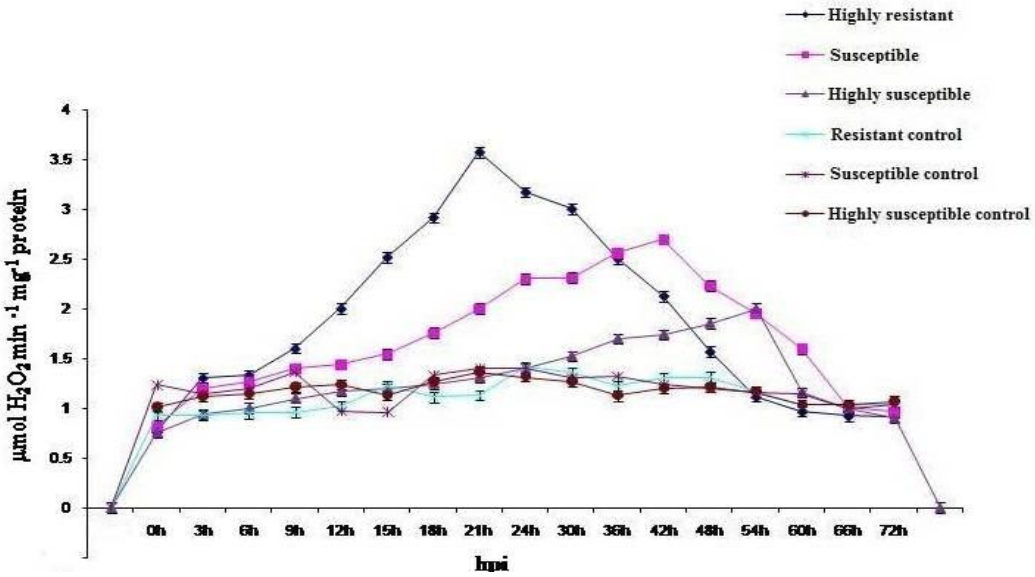

Figure 2: Temporal pattern study of CAT activity in highly resistant (HiR), susceptible (S) and highly susceptible (HS) cultivars with and without pathogen inoculation. The data expressed as the average of three independent experiments with three replicates each. Bars indicate standard error.

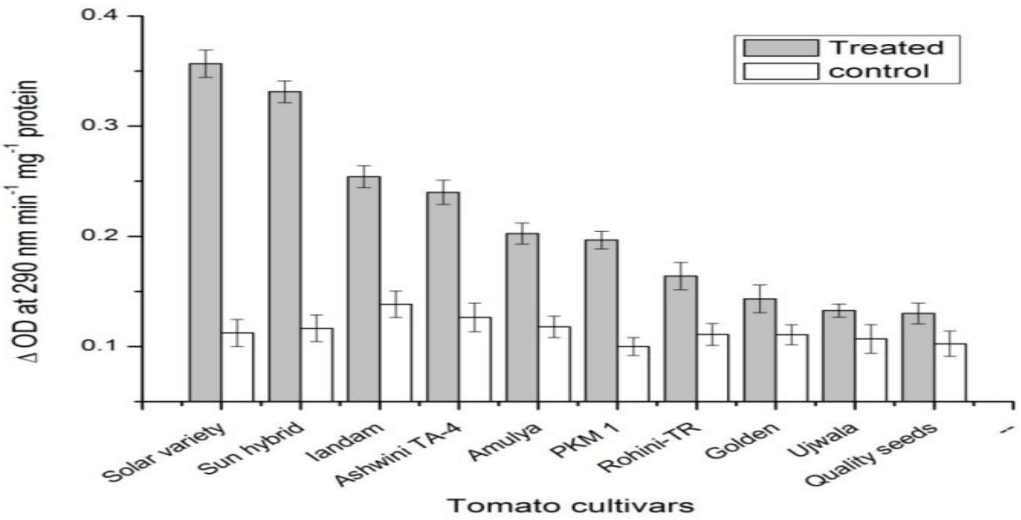

Figure 3: APX activity in different tomato cultivars at 12 h with pathogen inoculation. The data are expressed as the average of three independent experiments with three replicates each. Bar indicate standard error

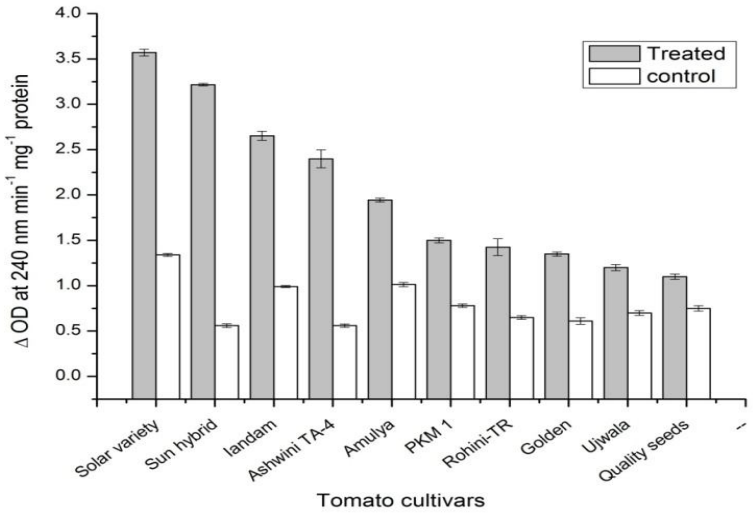

Figure 4: CAT activity in different tomato cultivars at 21 h with pathogen inoculation. The data are expressed as the average of three independent experiments with three replicates each. Bar indicate standard error

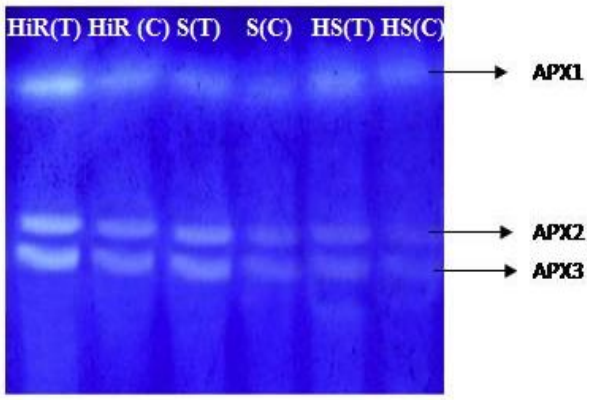

Figure 5: Differential expression of isoforms of APX in highly resistant (HiR), susceptible (S) and highly susceptible (HS) tomato culivars with and without pathogen *X.axonopodis* pv.*vesicatoria.* Each lane was loaded with 100 µg protein. HiR, highly resistant; S susceptible; HS, highly susceptible; C, control; T, treated

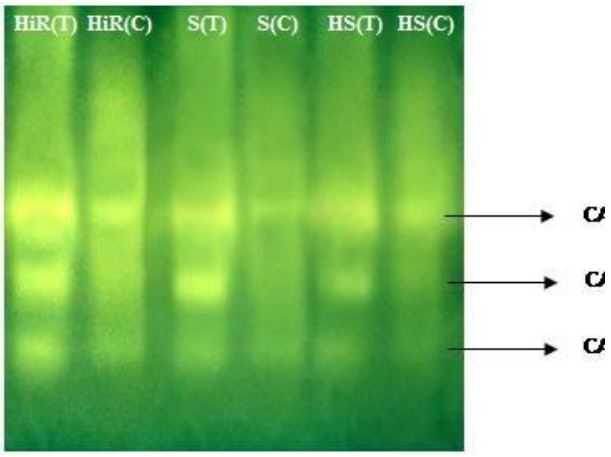

Figure 6: Differential expression of isoforms of CAT in highly resistant (HiR), susceptible (S) and highly susceptible (HS) tomato culivars with and without pathogen *X.axonopodis* pv.*vesicatoria.* Each lane was loaded with 100 µg protein. HiR, highly resistant; S susceptible; HS highly susceptible; C, control; T, treated

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