

Full Length Research Paper

Induction of cinnamyl alcohol dehydrogenase in bacterial spot disease resistance of tomato

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Cinnamyl alcohol dehydrogenase (CAD) is known to be involved in the defense related responses in many host-pathogen systems. Induction of CAD enzyme in bacterial spot disease resistance in tomato (*Solanum lycopersicum* Mill.) was investigated in the present studies. Twenty different tomato cultivars were screened for resistance against bacterial spot disease incited by *Xanthomonas axonopodis* pv. *vesicatoria* under field conditions. Field screening was performed by artificially inoculating *X. axonopodis* pv. *vesicatoria* suspension to four-week-old tomato seedlings and observed for the typical symptoms of bacterial spot disease. They were categorized into highly resistant, resistant, susceptible and highly susceptible cultivars on the basis of disease incidence under field conditions. Tomato cultivars were subjected for estimating CAD - a defense-related enzyme. Temporal pattern of the enzyme was estimated using four cultivars representing each category of tomato cultivars by inoculating with six isolates of *X. a. vesicatoria*. Native PAGE analysis of CAD was carried out for the time course of enzyme activity and also by selecting three different tomato cultivars, after infecting with the pathogen. Based on the inducible amounts of the enzyme upon pathogen infection, the tomato cultivars were correlated with that of disease incidence under field conditions. A significant ($P \leq 0.05$) correlation was observed between the degree of host resistance and the enzyme level. In highly resistant tomato cultivar the enzymatic level was increased in comparison with highly susceptible tomato cultivars. Isoform analysis of CAD enzyme indicated a clear difference between the number of isoforms and also the intensity of each isoform after pathogen infection with the resistant and susceptible tomato cultivars. A possible regulation of CAD in imparting host resistance is discussed here.

Key words: Tomato, *Solanum lycopersicum*, bacterial spot, *Xanthomonas axonopodis* pv. *vesicatoria*, cinnamyl alcohol dehydrogenase, resistance, susceptibility.

INTRODUCTION

Seed-borne pathogens are the most serious disease causing agents in tomato (*Solanum lycopersicum* Mill.)

among which *Xanthomonas axonopodis* pv. *vesicatoria* (Doidge) Dye (*Xav*) causes bacterial spot of tomato. When the environmental conditions are favorable for the pathogen, the disease is established in the field and it affects stem, leaves and fruit which finally leads to yield loss of up to 52% (Jones et al., 1998; El-Hendawy et al., 2005). Tomato is an important popular vegetable,

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because of its high nutritive value, diversified use, nutritionally significant source of vitamin A and C. Red tomatoes have an important compound called lycopene, which has antioxidant properties and has the ability to cure human diseases like cancer and heart disease (Barone, 2003). The tomato production in India is 17.35 million tones with the productivity of 8.63 mth⁻¹ and the area covered under production is 0.497 million hectares (FAO, 2006).

Plants have to develop a broad range of complex defense systems to struggle against pathogenic infections, because they have being confined to the place where they grow. The defense mechanism includes inducible response of pathogenesis related enzymes, pre existing physical and chemical barriers that have become marked by the action upon infection. The pathogen spread is controlled by the deposition of lignin at the point of infection by the plants when they are infected by the pathogen (Vance et al., 1980; Ride, 1975). Lignin plays an elementary role in higher plants by providing mechanical support, solute conductance and disease resistance (Barber and Mitchell, 1997). The effectual barrier to pathogen ingress and spread are the lignified cell walls (Ride, 1983). The composition and quantity of lignin varies between cell types and between tissues with the same plant (Whetten et al., 1998). The coordinated regulation of the three biosynthetic pathways, the Shikimate pathway, the general phenylpropanoid pathway and the lignin branch pathway leads to the biosynthesis of lignin. Among the enzymes involved in lignin biosynthesis, Cinnamyl alcohol dehydrogenase (CAD; E.C. 1.1.1.195) of phenylpropanoid metabolism specific for lignin synthesis has become the focus of a number of molecular analyses of lignification. Cinnamyl alcohol dehydrogenase catalyses the last step of monilignol biosynthesis, the reduction of the hydroxycinnamyl aldehydes to hydroxycinnamyl alcohol, which is considered to be highly specific marker for lignification (Mitchell and Barber, 1994). Cinnamyl alcohol dehydrogenase has been purified from tobacco (Halpin et al., 1992), *Eucalyptus gunii* (Goffner et al., 1992; Hawkins and Boudet, 1994), Wheat (Pillonel et al., 1992) *Lobolly pine* (O'Malley et al., 1992) and *Aralia cordata* (Hibino et al., 1993). Cinnamyl alcohol dehydrogenase deficiency causes drastic changes in the accumulation and nature of soluble phenolics; it also alters the structure of the lignin polymer that is deposited in the cell wall. The enzyme has a high affinity for coniferaldehyde and much lower affinity for sinapaldehyde (Sederoff et al., 1999).

Taking into consideration the role of CAD and lignin in imparting disease resistance to the plants, our study was

aimed to investigate the possible role of CAD enzyme in imparting disease resistance to the plant, role of lignin deposition in disease resistance against bacterial spot in tomato and to correlate their activity with disease resistance of tomato to bacterial spot.

MATERIALS AND METHODS

Collection and screening of tomato seed samples

Twenty different tomato cultivars were procured from local seed agencies, Mysore, India. The collected seed samples were subjected to screening in the laboratory following direct plating method on semi-selective Tween B medium (Peptone 10 g/L, KBr 10 g/L, CaCl₂ 0.25 g/L, H₃BO₃ 0.30 g/L and Agar 15 g/L). After autoclaving; Tween 80 10 ml/L, Cyclohexamide 100 mg/L, Cephalexin 65 mg/L, 5-fluorouracil 12 mg/L and Tobramycin 0.4 mg/L was aseptically added (Mc Gurie et al., 1986). Tomato seed samples were plated directly on to the Tween B medium after surface sterilization with 70% ethyl alcohol, followed by repeated washing with sterile distilled water and blot dried. Plated seeds were incubated for 36-48 h at 28±2°C to observe typical yellow colonies with clear lipolytic zones around the seeds and sub-cultured onto the Tween B media. The number of seeds showing these typical *X. axonopodis vesicatoria* colonies was recorded. The experiments were conducted in four replicates of 100 seeds each and repeated thrice.

Field experiments

Field experiments were conducted in the experimental plot of the Department of Applied Botany and Biotechnology, University of Mysore, Manasagangotri, Mysore to evaluate the tolerance ability of all the twenty cultivars against *X. axonopodis vesicatoria* infection under field conditions. Four week-old seedlings were transplanted to well prepared field from raised beds (1x 4 m). The seedlings were planted leaving 0.5 m distance between plant to plant and 1.3 m between row to row. Each replicate consisted of single row, which was 10 m long with 18–20 plants per row. Plants were irrigated through furrow irrigation as and when required. A randomized complete block design was employed with four replicated rows.

Inoculation procedure

X. axonopodis pv. *vesicatoria* isolates were preserved on Tween B medium in the absence of light at 4°C. The inoculum was prepared by growing the bacteria in nutrient broth incubated at 28±2°C for 36 h on rotary shaker at 100 rpm (Janked and Kunkel, IKA Labortechnik, Germany). 36 h-old culture was pelleted by centrifugation (thrice at 5000 rpm for 5 min) using bench top centrifuge (UniCen, 15 DR, Herolab GmbH, Germany). Inoculum was prepared by adjusting the bacterial concentration with sterile distilled water to 1 x 10⁸ cfu/ml at A₆₁₀ nm using UV-visible spectrophotometer. Field plots were inoculated with 36 h-old *X. axonopodis vesicatoria* suspension (1x10⁸ cfu/ml) at early morning

and late evening as spray inoculation to completely run-off the plants, 48 h after transplantation (EPPO, 1998). All the normal agronomical practices were maintained throughout the experiment viz., application of fertilizer once in every 20 days, weeding and irrigation as and when required etc.

Disease scoring

Disease assessment in field plots was done by counting infected plants at the intervals of 10 days upto 50 days starting from the appearance of first symptoms viz., spots surrounded by yellow hallow, necrotic lesions that occur on leaves, stems and flower parts. The disease was confirmed by observing the infected leaf section for the bacterial ooze under compound microscope, plating the pieces of plant parts showing symptoms on to Tween B media and subjecting the isolated bacteria to various biochemical, physiological, hypersensitive response and pathogenicity tests. Individual genotypes 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 becoming brown; susceptible (S), with 10.1 to 20.0% of plants showing sectorial spots and 20-40 % of leaves being brown; and highly susceptible (HS), with > 25% of the plants showing pronounced leaf collapse and more than 40% of leaves become brown. Experiments were conducted in two consecutive seasons and average disease incidence was calculated.

Determination of Cinnamyl alcohol dehydrogenase activity

Temporal pattern study of enzyme

To study the temporal pattern of CAD enzyme, the tomato seedlings of Safal, Rasi, PKM-1 and Golden seeds was selected from each category and were raised, by plating the seeds onto moist blotter discs inserted into the petridish (9 cm diameter), at the rate of 25 seeds per plate following standard procedures of International Seed Testing Association (ISTA, 2003). The plates were incubated at $28 \pm 2^\circ\text{C}$ for 8 days until cotyledonary leaf completely opens. The eight-day -old- tomato seedlings were covered with polythene sheet, 2 h before inoculation to increase the humidity. The tomato seedlings were root dip and spray inoculated with all the six isolates of *X. axonopodis vesicatoria* (1×10^8 cfu/ml) followed by polythene sheet covering (EPPO, 1998; AVRDC, 1999). The seedlings were harvested at different time intervals viz., 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 upto 72 h after pathogen inoculation and stored at -20°C until enzyme assay. Respective distilled water treated samples served as control.

Preparation of cell free extract

One gram tomato seedlings was macerated to a fine paste in a ice cold mortar with 1 g of acid purified sand in 100 mM Tris-HCl pH 7.5; 20 mM β -mercaptoethanol, 5% (w/v) Polyvinylpyrrolidone, 2% (w/v) polyethelene glycol 6000, 5 mM DTT. The crude extract was clarified by centrifugation twice (10,000 rpm/ 10 min) at 4°C and the supernatant was used directly for enzyme assay (Sarni et al., 1984)

Cinnamyl alcohol dehydrogenase assay

Cinnamyl alcohol dehydrogenase activity was determined by measuring the increase in absorbance at 400 nm when coniferyl alcohol was oxidized to coniferaldehyde (Wyrambik and Grisebach, 1975). The assay was performed for 10 min at 30°C in a total volume of 1 ml containing 100 mM Tris-HCl (pH 8.8), 100 μM coniferyl alcohol (Alfa Aesar, Johnson Matthey company, India), 200 μM NADP (Himedia, Laboratories limited Mumbai, India) and 200 μl enzyme extract. The values were expressed as a specific activity basis taking into account of proteins in the sample.

Lignin analysis

One gram of tomato seedlings was extracted with methanol (2 ml) with two changes for 24 h. The supernatant was discarded, the solids centrifuged at 10,000 rpm for 10 min were dried at 37°C for 48 h and their dry weights were determined. The wall-bound phenolic acids from the dried samples were hydrolyzed by extracting in 2 ml of 0.2 M NaOH for 24 h. The incubated mixture was neutralized with 0.5 ml of 2 M HCL and the residue collected by centrifugation, washed twice with distilled water and resuspended in methanol (5 ml) and allowed to air dry. The air-dried pellet was resuspended in 5 ml of 2 M HCl to which 0.5 ml of thioglycolic acid was added. The tubes were sealed and placed in a 95°C water bath for 4 h, then cooled on ice and the solids collected by centrifugation. The solids were washed twice with 2 ml of distilled water each time, followed by centrifugation. The solids were then suspended in 0.5 M NaOH (5 ml) overnight at 4°C and again collected by centrifugation and washed twice with 2 ml water. The water washes were added to the NaOH supernatant, acidified with 1 ml of concentrated HCl, and the solution placed at 4°C for 4 h to precipitate the lignothioglycolic acid (LTGA). The precipitate was collected by centrifugation and washed twice with 0.1 M HCl. The final pellet was dissolved in 0.5 M NaOH (3 ml) and insoluble material was removed by centrifugation. The absorbance of a 1 ml solution of LTGA was determined at 280 nm and the lignin content was expressed as absorbance at $280 \text{ nm ml}^{-1} \text{ g}^{-1}$ dry weight (Cahill and McComb, 1992).

Protein estimation

Protein contents of the extracts for the estimated enzyme was determined using the standard procedure of Bradford (1976) using BSA (Sigma, USA) as standard.

Nondenaturing PAGE and activity staining

Native PAGE (10% Polyacrylamide gel) was performed for the prepared enzyme extract (150 μg) and electrophoresis was performed as described by Laemmli (1970) but without SDS in buffers. Activity staining was performed as described by Mansell et al. (1974). Samples (150 μg) were loaded onto 10% (w/v) polyacrylamide gels with a vertical mini-gel electrophoresis unit (Biometra, Gottingen, Germany). The electrode buffer was Tris-base (3.0 g Tris-base, 7.2 g glycine and 1lt distilled water). Electrophoresis was performed at a constant voltage of 50 V initially

Table 1. Reaction of tomato cultivars for bacterial spot disease under field conditions.

Tomato cultivars	Bacterial spot incidence %	Categorization
Safal	0	HiR
Indam	8± 0.2 ^b	R
Vignesh	6±0.3 ^a	R
Rasi	5±0.3 ^a	R
Pradhan	9±0.3 ^a	R
Naveen	8±0.3 ^a	R
Pioneer seeds	4 ±0.4 ^a	R
Rukshita	11±0.6 ^c	S
Marglobe	18±0.7 ^c	S
PKM-1	13±0.6 ^c	S
Rohini	15±0.5 ^c	S
SCL-4	14±0.5 ^c	S
Utsav	17±0.6 ^d	S
Leadbeter	13±0.6 ^c	S
Arka vikas	19±0.7 ^d	S
Madanapalli	30±0.9 ^e	HS
Heemsona	32±0.9 ^e	HS
Vajra	38±0.8	HS
Amar	35±0.7 ^e	HS
Golden	32±0.9 ^e	HS

Seeds of all the tomato cultivars were sown using Random Block Design and pathogen was sprayed 48 h after transplantation. Plants were observed for typical symptoms of bacterial spot at 10 days intervals upto 50 days. Tomato cultivars were categorized into highly resistant (HiR), resistant (R), susceptible (S) and highly susceptible (HS) based on the disease incidence. Values are the means ± S.E. of four replicates of 18-20 plants each. Values followed by same letters do not significantly differ at 5% level according to Fisher's least significant difference ($p= 0.05$).

for 1 h and 100 V to complete electrophoresis. The gels were incubated for 2 h at room temperature in darkness in 10 ml of a reaction mixture that contained 100 mM Tris-HCl (pH 8.8), 1.5 mg of nitrobluetetrazolium, 0.1 mg of phenazinemetho-sulphate, 2.5 mg of NADP and 2.5 mg of coniferyl alcohol.

Statistical analysis

All experiments were performed three times with similar results. The data from field experiments were analysed separately for each experiment and were subjected to two – way 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

The reactions of the twenty tomato cultivars for bacterial spot disease recorded varied degree of disease

incidence under field conditions. Cultivar Safal was exceptional which was completely free from the disease in both the seasons (Table 1). The bacterial spot incidence in the remaining cultivars ranged from 4 to 38%, accordingly tomato cultivars were categorized as Highly resistant (HiR), Resistant (R), Susceptible (S) and Highly susceptible (HS), based on the degree of host-resistance (Table 1).

Temporal pattern study of enzyme

The temporal activity of CAD enzyme in all the categorized tomato cultivars treated with *X. axonopodis* pv. *Vesicatoria*1 showed that in the HiR cultivar the CAD activity began to increase and reached the peak activity at 24 h with 126.6 Δ OD at 400 nm / min /mg protein where its control showed 24 Δ OD at 400 nm / min /mg protein. The R cultivar showed 113.7 Δ OD at 400 nm /

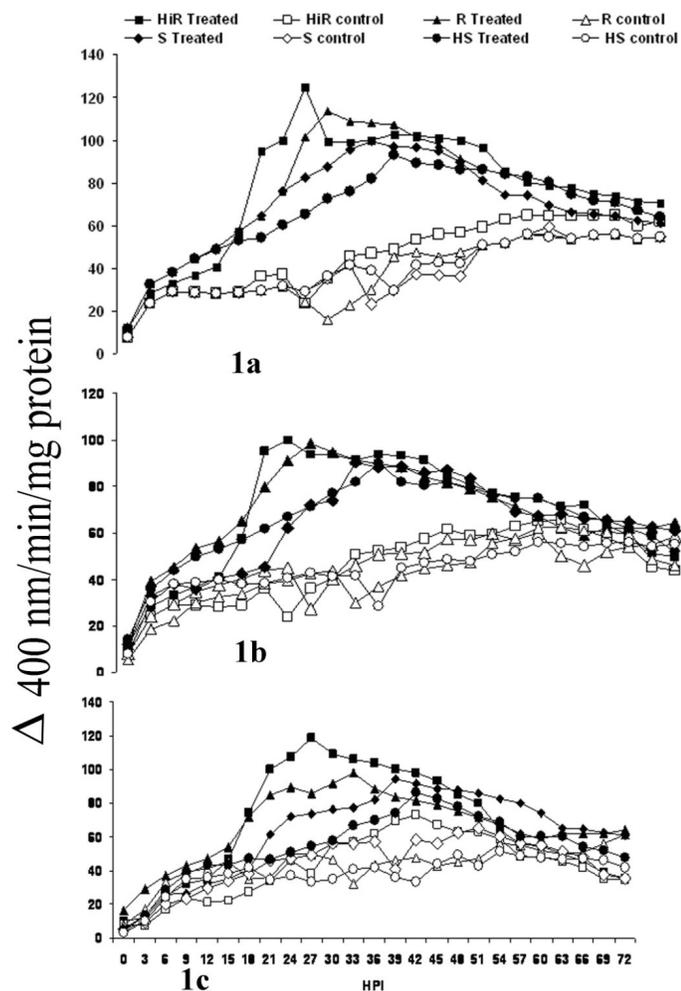


Figure 1a,b,c. The temporal pattern study of CAD activity in HiR, R, S, and HS tomato cultivars treated with Xav isolates 1, 2, and 3. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$).

min /mg protein and its control showed 16 Δ OD at 400 nm / min /mg protein upon pathogen inoculation at 27 h. In the S cultivar the activity was 99.4 Δ OD at 400 nm / min /mg protein and its control showed 23.6 Δ OD at 400 nm / min /mg protein at 33 h after pathogen inoculation. The HS cultivars showed the maximum activity of CAD at 36 h with 93.2 Δ OD at 400 nm / min /mg protein and its control showed 30 Δ OD at 400 nm / min /mg protein (Figure 1a)

The HiR cultivar showed the maximum activity of CAD

at 21 h when treated with Xav 2, with 100 Δ OD at 400 nm / min /mg protein and its control showed 23.6 Δ OD at 400 nm / min /mg protein. Similarly the R cultivar showed 98.3 Δ OD at 400 nm / min /mg protein CAD activity which was maximum when compared to its control with 27.4 Δ OD at 400 nm / min /mg protein. The S cultivars also showed a maximum activity of CAD at 30 h with 90.5 Δ OD at 400 nm / min /mg protein and its control showed 30 Δ OD at 400 nm / min /mg protein. The HS cultivar showed the increase in CAD activity at 33 h with 89.9 Δ OD at 400 nm / min /mg protein and its control showed 28.6 Δ OD at 400 nm / min /mg protein (Figure 1b).

When the HiR was treated with the Xav 3 the CAD activity was found to be increased at 27 h with 118.7 Δ OD at 400 nm / min /mg protein and its respective control showed 38.1 Δ OD at 400 nm / min /mg protein. The R cultivar showed the CAD activity to be maximum at 33 h with 97.9 Δ OD at 400 nm / min /mg protein and its control showed 32.6 Δ OD at 400 nm / min /mg protein. The S cultivar also responded for the inoculation with Xav 3 by mounting CAD activity at 39 h with 94.5 Δ OD at 400 nm / min /mg protein and its control showed 40.6 Δ OD at 400 nm / min /mg protein. The HS cultivar showed the maximum CAD activity at 42 h with 86.3 Δ OD at 400 nm / min /mg protein in treated and its control showed 33.5 Δ OD at 400 nm / min /mg protein (Figure 1c).

The tomato cultivars showed varied responses to the infection of Xav 4 where HiR showed 120.6 Δ OD at 400 nm / min /mg protein when compared to its control where it showed 54.4 Δ OD at 400 nm / min /mg protein CAD activity at 24 h. The R cultivar showed CAD activity to be maximum at 30 h with 100.9 Δ OD at 400 nm / min /mg protein and its control showed 47.7 Δ OD at 400 nm / min /mg protein. The S cultivar showed the CAD activity to be maximum at 36 h with 95.5 Δ OD at 400 nm / min /mg protein and its control showed 32 Δ OD at 400 nm / min /mg protein. The HS cultivar showed CAD activity at 39 h to be maximum with 90 Δ OD at 400 nm / min /mg protein and its control showed 24.6 Δ OD at 400 nm / min /mg protein (Figure 2a). The HiR cultivar showed the CAD activity to be maximum min /mg at 21 h with 115.3 Δ OD at 400 nm / min /mg protein and its control showed 27.5 Δ OD at 400 nm / min /mg protein when artificially inoculated with Xav 5. Similarly R cultivar showed maximum activity at 24 h with 103.6 Δ OD at 400 nm / min /mg protein and its control showed 35.4 Δ OD at 400 nm / min /mg protein. The S cultivar showed the maximum CAD activity at 33 h with 98.3 Δ OD at 400 nm / min /mg protein and its control showed 20 Δ OD at 400 nm / min /mg protein. The HS showed the maximum CAD activity at 42 h with 85.3 Δ OD at 400 nm / min /mg protein and its control showed 32.6 Δ

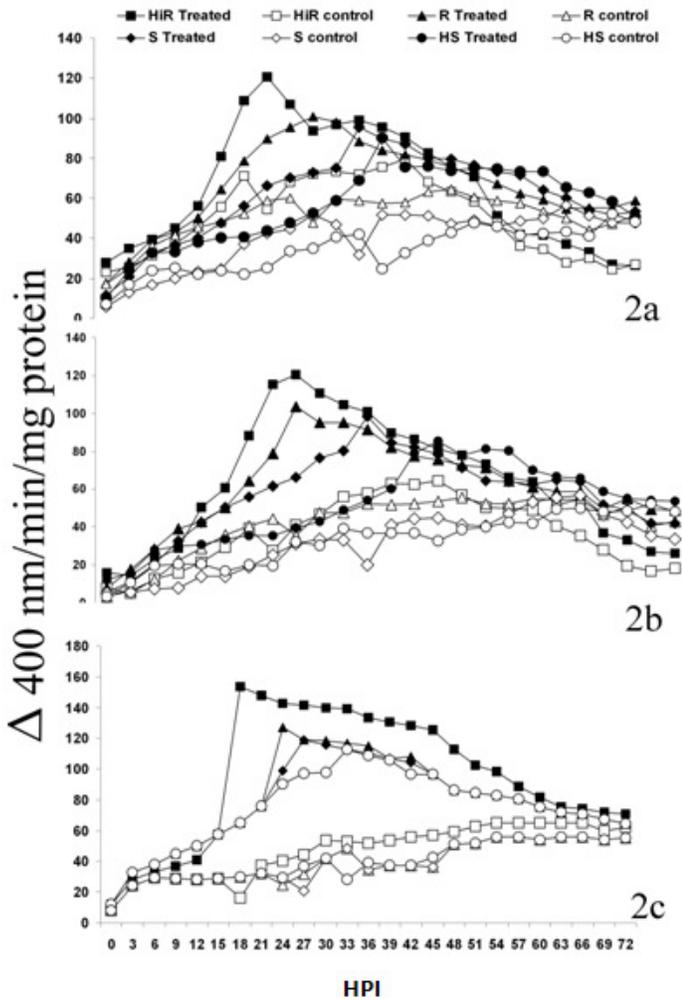


Figure 2a,b,c. The temporal pattern study of CAD activity in all the four category of tomato cultivars treated with *Xav* isolates 4, 5, and 6. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$).

OD at 400 nm / min /mg protein (Figure 2b).

The first increase in CAD activity in HiR began at 3 h after inoculation with the 6th isolate of *Xav*, and peaked at 18 h with 153.8 Δ OD at 400 nm / min /mg protein where its respective control showed 16 Δ OD at 400 nm / min /mg protein. The R cultivar showed 127.1 Δ OD at 400 nm / min /mg protein and its control showed 24.7 Δ OD at 400 nm / min /mg protein at 24 h. The S cultivars showed maximum CAD activity at 27 h with 118.8 Δ OD at 400 nm / min /mg protein and its control was 20.6 Δ OD at

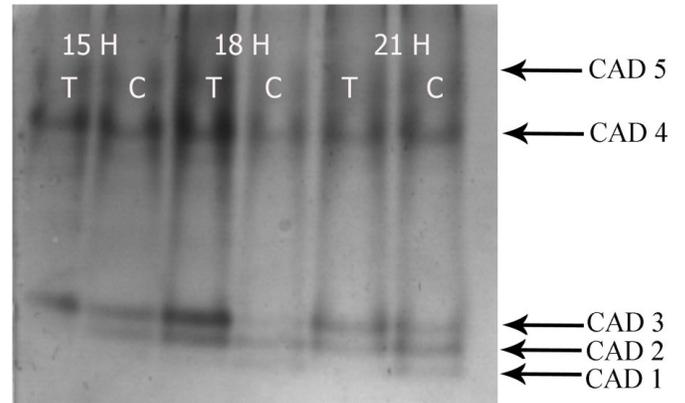


Figure 3. Native PAGE profile of CAD isoforms in tomato seedlings at different time intervals with and without pathogen inoculation. Total protein (150 μ g) was loaded in each lane of 10% polyacrylamide gel. T= Treated; C = Control.

400 nm / min /mg protein. The HS cultivar showed 112.8 Δ OD at 400 nm / min /mg protein and its control showed 28 Δ OD at 400 nm / min /mg protein at 33 h after pathogen inoculation (Figure 2c). Our temporal pattern results show that the *Xav* 6 had maximum CAD activity in all the cultivars when compared to all the other isolates. So we selected the sixth isolate for confirming the results using Native PAGE analysis where the 18 h treated seedlings showed more intensified bands with maximum isoforms (5), when compared to its control where it showed 3 isoforms and also with less intensified bands. The 15 and 21 hpi treated and control lane showed 3, 4, 4 and 5 isoforms, respectively but not significant as that of 18 hpi treated lane (Figure 3).

CAD assay and host resistance of tomato cultivars to bacterial spot disease

Among the twenty tomato cultivars subjected for CAD assay, Safal being the HiR cultivar showed the maximum activity with 222.3 Δ OD at 400 nm / min /mg protein where its control showed 50.8 Δ OD at 400 nm / min /mg protein (Figure 4a). Among the resistant cv. Rasi showed CAD activity of 36.8 Δ OD at 400 nm / min /mg protein in control which significantly ($P\leq 0.05$) increased to 92.5 Δ OD at 400 nm / min /mg protein at 24 hpi (Figure 5a). Similarly in the susceptible cv. group PKM-1 showed 53.5 Δ OD at 400 nm / min /mg protein in treated seedlings where its control showed 24.7 Δ OD at 400 nm / min /mg

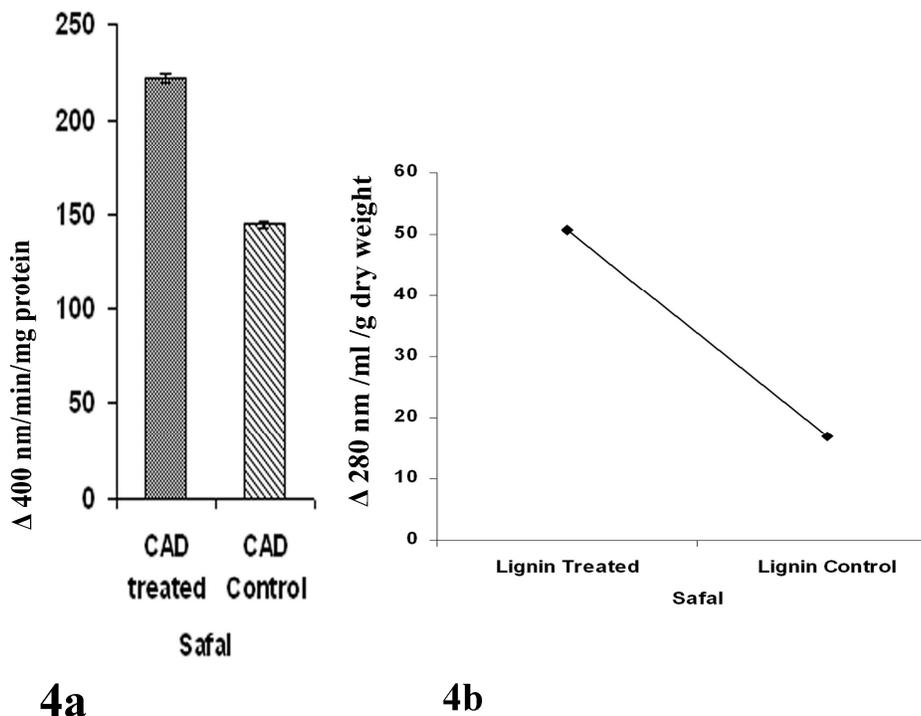


Figure 4a, b. CAD activity AND lignin content of HiR cultivar estimated spectrophotometrically in 8 day-old seedlings of HiR cultivar Safal in response to *X. axonopodis vesicatoria*. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$). Bars indicate standard errors.

protein which was greater than the treated seedlings (Figure 6a). Among the highly susceptible category cultivar Golden showed 23.1 Δ OD at 400 nm / min /mgprotein where its control seedlings showed 20.2 Δ OD at 400 nm / min /mg protein (Figure 7a).

Native PAGE analysis

The spectrophotometric results were supported by the native PAGE analysis. The HiR treated seedlings showed 6 isoforms with thick and high intensified bands where its respective control showed only 3 isoforms. In resistant cultivars the treated seedlings showed 5 isoforms and its respective control showed 3 isoforms. Four isoforms were visualized in HS treated seedlings where its control expressed 3 isoforms (Figure 8). When compared to the HiR treated lane with all the other lanes it showed maximum isoforms with thickest bands, thus representing

the maximum enzyme activity.

Lignin analysis

Lignin content of the tomato cv. Safal known to be highly resistant showed 17 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weights in the untreated control, which was significantly ($P \leq 0.05$) increased to 85.1 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight upon pathogen inoculation (Figure 4b). Cultivar Rasi showed 27.4 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight and 14.3 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight with and without pathogen inoculation respectively (Figure 5b). Susceptible cultivar PKM-1 showed 18.4 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight and its control showed 11 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight (Figure 6b). The cultivar Golden showed 10 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight in control whereas it remained unchanged upon pathogen inoculation with 10 Δ OD at 280 nm $\text{ml}^{-1} \text{g}^{-1}$ dry weight

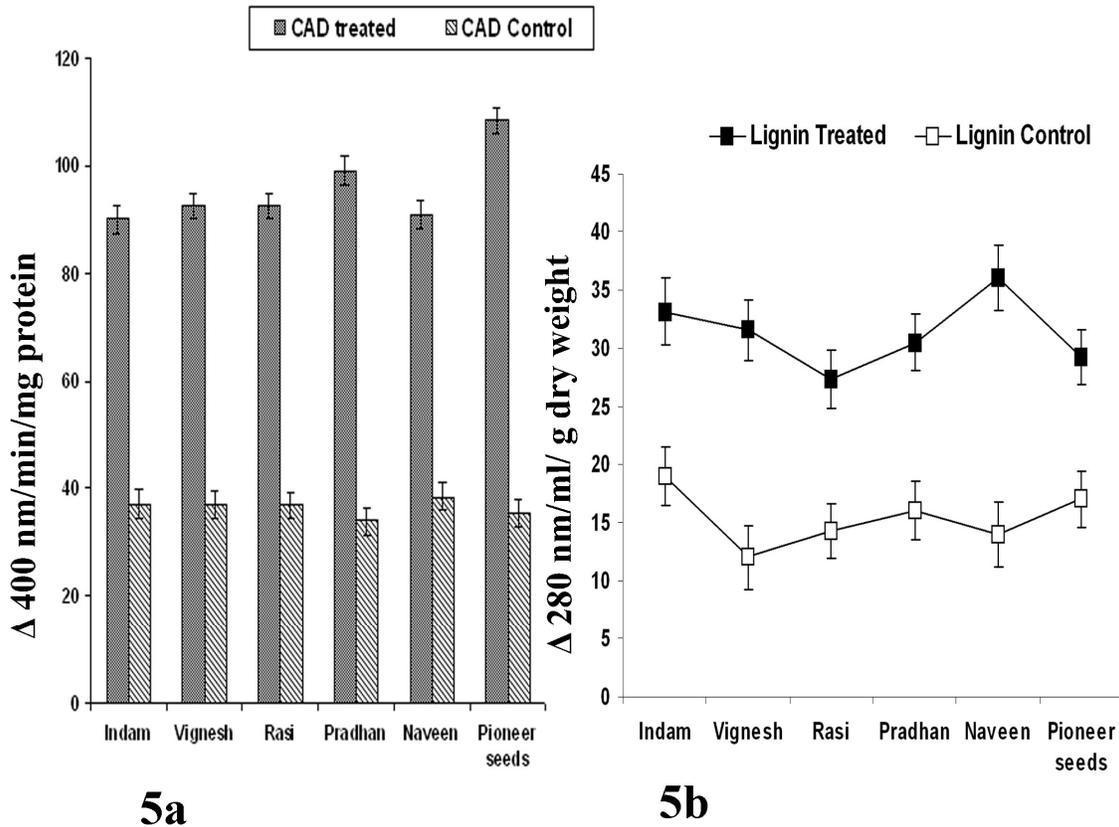


Figure 5a, b. CAD activity and lignin content of R cultivar in 8 day-old seedlings in response to *X. axonopodis vesicatoria*. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$). Bars indicate standard errors.

(Figure 7b).

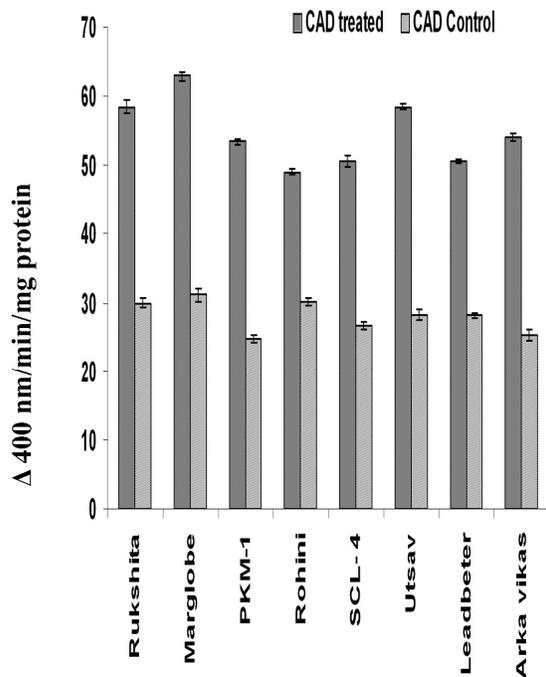
DISCUSSION

The present work is an attempt to correlate the CAD enzyme activity in tomato to bacterial spot disease resistance. The twenty cultivars used in this study clearly varied in the degree of host resistance to the pathogen. All the resistant cultivars sampled in this study reacted to pathogen inoculation by mounting CAD enzyme activity along with lignin. In this study, we report the direct involvement of CAD during host-pathogen interaction and our results also suggest that CAD is involved in lignin synthesis.

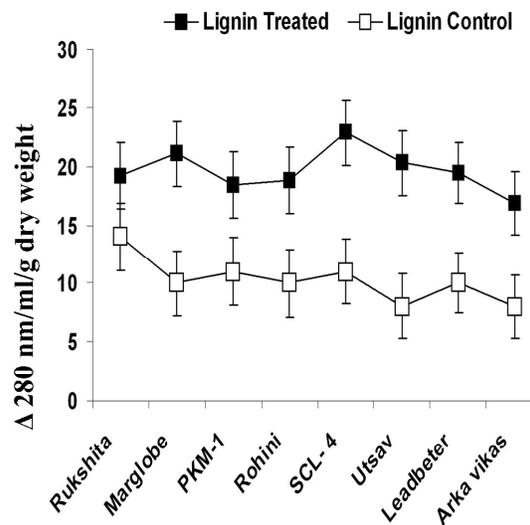
Cinnamyl alcohol dehydrogenase induction has been studied in relatively few plant defense responses (De Sa

et al., 1992; Grand et al., 1987; Moerschbacher et al., 1988; Walter et al., 1988). Mitchell et al. (1994) first reported the CAD activity expression during the plant defense response involving lignin deposition.

In HiR, the CAD activity was maximum than R, S and HS cultivars. Similarly the lignin content of HiR was maximum than the other three grouped cultivars. The above findings supports that as the CAD activity increases upon pathogen attack the lignin accumulation is also increased. Lignin plays an important role in protection and defense and is synthesized in response to pathogen attack (Bostock and Stermer, 1989; Vance et al., 1980) and mechanical wounding (Hawkins and Boudet, 1996). Our findings also support this hypothesis, where we found that where the lignin content was maximum the disease expression was minimum and where lignin content was moderately deposited the



6a



6b

Figure 6a, b. CAD activity and lignin content of S cultivar estimated in 8 day-old seedlings by artificially inoculating *X. axonopodis vesicatoria*. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$). Bars indicate standard errors.

disease expression was moderate and in highly susceptible cultivar where its lignin content was minimum or very less the disease expression was maximum.

Tomato cultivars with maximum CAD activity and maximum lignin content showed high resistance against the *X. a. vesicatoria*, similarly cultivars which showed less CAD activity and less lignin content was found to be susceptible to the bacterial spot disease. This is supported by the findings of Hawkins et al. (1997) who revealed that plants down regulated in CAD activity could be more susceptible to pathogen attack, even though their morphological development is apparently unaffected. Cinnamyl alcohol dehydrogenase is directly involved in lignification (Goffner et al., 1992; O'Malley et al., 1992). Our findings also support the above statement as we observed that CAD activity increased the lignin deposition increased in tomato seedlings. Our findings revealed that the HiR cultivar showed maximum lignin content upon pathogen infection when compared with its respective control and the R, S and HS cultivars. The

resistant cultivars showed moderate lignin content. The susceptible and highly susceptible cultivars showed minimal lignin content upon infection. We noticed that HiR cultivar showed maximum CAD activity and where there was maximum enzyme activity; the lignin deposition was also maximum, thus resisting the pathogen entry into the host. This support the findings of Mitchell et al. (1994) who found that the lignin deposition at wound margins in wheat leaves was consistent with the sequential induction of CAD.

Cinnamyl alcohol dehydrogenase isoforms have been demonstrated in a number of species including wheat, eucalyptus, soybean and bean (Mackay et al., 1995). Native PAGE analysis of CAD enzyme was performed to visualize the isoforms expressed in HiR, R, S and HS cultivars. The results showed that HiR showed maximum isoforms upon infection when compared to R, S and HS cultivars. Differential expression of isoforms with different specificities has been put forth as a potential mechanism account for lignin heterogeneity (Mansell et al., 1976).

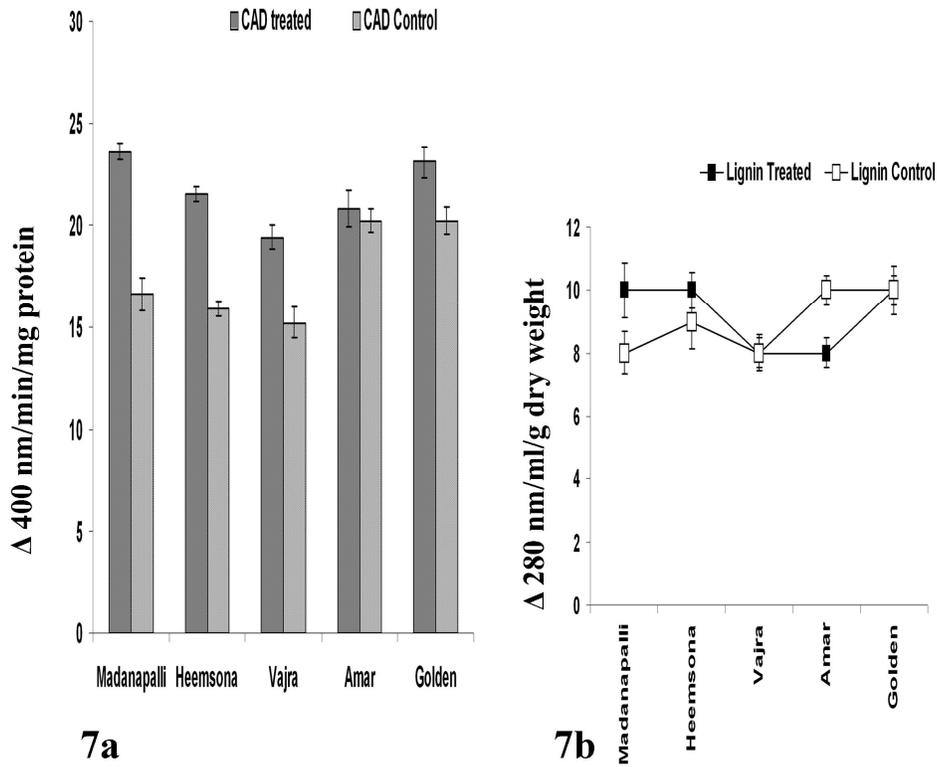


Figure 7a, b. CAD activity and lignin content of HS cultivar in 8 day-old seedlings in response to *X. axonopodis vesicatoria*. The data are expressed as the averaged of three independent experiments with three replicates each and subjected to Fisher's least significant difference ($p=0.05$). Bars indicate standard errors.

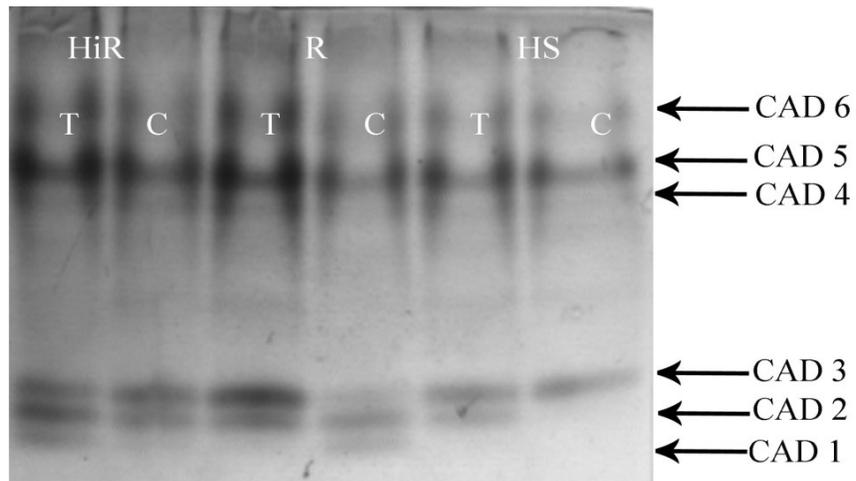


Figure 8. CAD isoforms visualized using Native PAGE of 10% in three tomato cultivars WITH and without *X. vesicatoria* inoculation. Total protein (150 μg) was loaded in each lane. HiR = Highly resistant, R= Resistant and HS = Highly susceptible

Some of these isoforms seems to be associated with the synthesis of defense lignin (Mitchell et al., 1994). Several studies have shown that CAD may be polymorphic with isoforms that differ not only on substrate affinity but on molecular mass also (Boudet et al., 1995; Goffner et al., 1992; Halpin et al., 1992; Luderitz and Griesebach, 1981; Mansell et al., 1974, 1976; Pillonel et al., 1992; Sarni et al., 1984). Dixon et al. (2001) and Higuchi et al. (1997) have found that in various species CAD isoforms are involved in monolignol biosynthesis. Two isoforms has been observed in Soybean (Wyrambik and Grisebach, 1975), *Eucalyptus gunii* (Goffner et al., 1992) and *Phaseolus vulgaris* (Pettenati et al., 1994).

It is clear from the present studies that the defense-related enzyme CAD is up-regulated in the highly resistant tomato cultivars along with lignin content, upon pathogen infection, whereas in susceptible tomato cultivars the level of defense enzyme and lignin content remains unchanged or down regulated. Hence we conclude that CAD enzyme is an enzyme, which prevents the plant from pathogen attack by inducing lignin deposition, where lignin is known to be a physical barrier, which prevents the pathogen entry into the host, and we have noticed that as CAD activity increases, lignin deposition also substantially increased. Thus we conclude that CAD is directly involved in lignification and helps the plant by imparting resistance to the plant against bacterial spot disease.

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