# NOTE: **Phenylalanine Ammonia Lyase Activity in Tomato Seedlings and Its Relationship to Bacterial Canker Disease Resistance**

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Phenylalanine ammonia lyase (PAL) activity was studied in different genotypes of tomato with varying degrees of resistance and susceptibility to bacterial canker disease after inoculation with *Clavibacter michiganensis* ssp. *michiganensis*. In resistant genotypes the enzyme activity increased significantly 21 h after bacterial inoculation, whereas in the susceptible genotypes the activity decreased. The increase or decrease in PAL activity correlated well with the degree of host resistance along with total phenol contents. The role of PAL in imparting resistance to tomato against bacterial canker disease is discussed.
KEY WORDS: Phenylalanine ammonia lyase; *Clavibacter michiganensis* ssp. *michiganensis*; tomato; bacterial canker.

Plants possess a variety of active defense responses, which contribute to resistance against a range of pathogens. Strengthening the endogenous defense capabilities of plants, *viz.*, synthesis of pathogenesis-related (PR) proteins, phytoalexins, accumulation of active oxygen species, rapid alterations in cell walls and enhanced activity of various defense-related enzymes, has been advanced as a promising strategy for crop protection (3). Plants respond to bacterial pathogen attacks by transcriptionally activating a number of genes coding for proteins, which are thought to help the plant to ward off the invader. The development of disease resistance has been correlated with the accumulation of the inducible plant proteins in many plant–bacteria interactions (12). The Gram-positive bacterial pathogen *Clavibacter michiganensis* ssp. *michiganensis (Cmm)* is responsible for bacterial canker of tomato, one of the world's major diseases of tomato (2). Disease control is difficult because of a lack of commercially acceptable resistant tomato cultivars. Phenylalanine ammonia-lyase (PAL, E.C. 4.1.3.5) catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid, which

is the first step in the phenylpropanoid pathway which supplies the precursors for phenolics, lignin and furanocoumarin, phytoalexins (11). A literature survey indicates that there are very few reports on the involvement of PAL in plant bacterial pathogenicity. In the present study we report that the compatible and incompatible reactions between the tomato genotypes and *Cmm* involve rapid induction and reduction of PAL activity along with the phenol content.

The seeds of 30 tomato genotypes (Table 1) were procured locally from seed traders in Mysore, Karnataka, India and were surface sterilized using sodium hypochlorite followed by a thorough washing with sterile distilled water (SDW). Seeds of all the genotypes were sown in earthen pots filled with soil, sand and manure and maintained in a greenhouse. Surface-sterilized seeds were germinated on moist blotter discs inserted into Perspex plates and incubated in a seed germinator at 25±2°C (4).

*Clavibacter michiganensis* ssp. *michiganensis* isolate *SERC 10* was preserved on nutrient broth yeast extract (NBY) agar at 4°C. Broth cultures of *Cmm* were grown in liquid NBY

medium for 24–48 h in an orbital shaker at 200 rpm at 27 ± 2°C. Bacteria were subsequently pelted by centrifugation (thrice at 5000 rpm for 5 min at 4°C) using a bench top refrigerated centrifuge (UniCen, 15 DR, Herolab GmbH, Wiesloch, Germany). Bacterial concentration was adjusted with SDW until a relative optical density of 0.225 at $A_{610}$ nm was obtained with a UV-visible spectrophotometer (Hitachi, U-2000, Tokyo, Japan), corresponding to $2.5 \times 10^8$ cfu $ml^{-1}$, as verified by plate counting. The pair of first true leaves of tomato seedlings were cut at the tip and inoculated by dipping into such suspension (10) with SDW-inoculated controls. Pots were maintained under greenhouse conditions and typical symptoms were monitored over several weeks. Plants were rated for bacterial canker incidence on an arbitrary scale of 0 to 4, as follows: 0, no plants/leaves showing any of the typical symptoms of bacterial canker disease; 1, slight marginal wilting, 1–20% of leaves wilting; 2, sectorial wilting, 20–40% of leaves showing wilting associated with chlorosis; and 4, pronounced leaf collapse, 40% of leaves showing wilting. Individual genotypes were categorized into highly resistant (HiR), with no plants (0%) showing any of the symptoms of bacterial canker disease; resistant (R), with 0.1% to 10.0% of plants showing slight marginal wilting and 1–20% of leaves wilt; susceptible (S), with 10.1% to 20.0% of plants showing sectorial wilting and 20–40% of leaves wilt; and highly susceptible (HS), with >25% of the plants showing pronounced leaf collapse and more than 40% of leaves wilt. Experiments were conducted in five replicates of 25 plants each and repeated thrice. Average disease incidence was calculated.

**Determination of PAL activity in the tomato seedlings** Eight-day-old seedlings of the selected genotypes were root-dip inoculated with $2.5 \times 10^8$ cfu $ml^{-1}$ concentration of bacterial suspension. Seedlings were removed after 21 h of inoculation and used to determine the enzyme activity. One gram of inoculated/uninoculated seedlings was macerated to a fine paste in an ice-cold mortar with 1 g of acid-purified sand in 25 mM Tris-HCl buffer (pH 8.8) and 32 mM $\beta$-mercaptoethanol. The weights of the seedlings and of the buffer were selected

to be at a ratio of 1:2 (w/v). The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was used directly for enzyme assay.

PAL activity was assayed by a slight modification of the procedure developed by Lisker *et al.* (5). The enzyme activity was determined by measuring spectrophotometrically the production of *trans*-cinnamic acid from L-phenylalanine. The reaction mixture of 1 $cm^3$ contained 0.1 $cm^3$ enzyme extract, 0.5 $cm^3$ substrate – 50 mM L-phenylalanine and 0.4 $cm^3$ 25 mM Tris-HCl buffer (pH 8.8). After incubation for 2 h at 40°C, the activity was stopped by addition of 0.06 $cm^3$ 5N HCl and absorbance at 290 nm read against the same volume of reaction mixture without L-phenylalanine. Protein content of the extracts was determined by using the standard procedure of Bradford (1) using BSA (Sigma, St. Louis, MO, USA) as standard. The enzyme activity was expressed as $\mu$mol of trans-cinnamic acid $mg^{-1}$ protein $h^{-1}$. Phenol content of the extract was estimated according to Malick and Singh (6) and expressed as mg phenol $g^{-1}$ material. Experiments were conducted in four replicates and repeated thrice. Data were subjected to analysis of variance and means were compared for significance (Tukey's B test, DMRT; $P=0.005$).

Results presented in Table 1 depict the bacterial canker disease reaction of different tomato genotypes and the amount of *trans*-cinnamic acid released in healthy and *Cmm*-inoculated tomato seedlings. A correlation was observed between disease reaction and PAL enzyme activity. Data reported herein indicate that PAL activity is pathogen-induced in tomato seedlings, in incompatible combination with *Cmm*. Such an induction in enzyme activity increases with the degree of host resistance to bacterial canker disease, the maximum increase being recorded in highly resistant genotypes. In several host-pathosystems, PAL activity is known to increase in incompatible interactions (7,8). Increase in PAL activity is often associated with progressive incorporation of phenolic compounds with the cell wall during incompatible plant–microbe interaction. The reinforcement of the plant cell wall by phenolics and lignin increases plant resistance to cell wall-degrading enzymes and toxins

TABLE 1. Comparison of bacterial canker incidence and PAL activity of tomato genotypes

| Genotypes | Bacterial canker incidence (%) and categorization[z,y] | | PAL activity ($\mu$mol of *trans*-cinnamic acid mg$^{-1}$P h$^{-1}$)[y,x] | |
|---|---|---|---|---|
| | | | Healthy | Inoculated |
| Rukshita | 9±0.91f | R | 6898±0.19d | 6936±0.55d |
| Nam 25-35 | 18±0.91d | S | 5690±0.09d | 2096±0.14d |
| Higher 35-26 | 35±0.57b | HS | 4019±0.88ab | 1918±0.14d |
| PKM–1 | 8±0.40f | R | 7198±0.55d | 8936±0.84d |
| S-22 | 8±0.40f | R | 7549±0.55d | 8154±0.74d |
| S-72 | 9±0.91f | R | 6536±0.19d | 6694±0.19d |
| 619 | 6± 0.40f | R | 8398±0.55d | 9018±0.44d |
| NS 246 | 16±0.91f | S | 5492±0.09d | 4936±0.88ab |
| Utsav | 29±0.57c | HS | 3948±0.88ab | 1496±0.15cd |
| Vajra 1 | 28±0.57c | HS | 3636±0.76a | 1295±0.81cd |
| Ananya | 30±0.91c | HS | 3996±0.51a | 1190±0.17d |
| Ankush | 5±1.0g | R | 8129±0.94d | 9391±0.99d |
| TO–39 C | 2±0.40g | R | 9210±0.15b | 12030±1.44d |
| Jyothi | 0 | HiR | 9517±1.80a | 18024±2.03c |
| CKVT–F | 5±0.57g | R | 8324±0.04c | 9549±0.15b |
| Nandini | 26±0.57c | HS | 4108±0.88ab | 2001±0.44d |
| Ruchi | 43±0.57a | HS | 2835±0.48d | 1005±0.22d |
| Pusa Ruby | 28±0.91c | HS | 3433±0.49d | 1432±0.81cd |
| Vijay S-22 | 6±0.91f | R | 8592±0.15b | 9602±0.15b |
| Jyot-149 | 14±0.91f | S | 5528±0.09d | 5436±0.09d |
| Nandi | 3±0.91g | R | 9019±0.04c | 10958±0.93bc |
| JK Asha | 3±0.91g | R | 8998±0.80ab | 9632±0.19b |
| Nimbusona | 14±0.91f | S | 5328±0.09b | 5319±0.28a |
| Avinash | 12±1.4f | S | 5496±0.85b | 5378±0.09d |
| Allwounder | 16±0.91e | S | 5590±0.98b | 5236±0.19c |
| Chiranjeevi | 25±0.91c | HS | 3906±0.49d | 1801±0.33d |
| Rohini | 18±0.57d | S | 5409±0.09d | 1969±0.94d |
| Ashwini | 21±0.57d | HS | 3504±0.49d | 1104±0.81cd |
| Nunhems 1005 | 8±0.91f | R | 7324±0.46d | 8335±0.86d |
| Local | 12±0.91f | S | 5459±0.09d | 5148±0.09d |

[z] Values are the means of three independent experiments ± S.E. of five replicates of 25 plants each. Seeds were surface disinfected and sown in earthen pots. The pair of first true leaves were cut at the tip and inoculated by dipping in the bacterial suspension. Pots were maintained in greenhouse conditions and typical symptoms of bacterial canker were monitored over several weeks. Tomato genotypes were categorized as HiR (highly resistant), R (resistant), S (susceptible) and HS (highly susceptible) based on the bacterial canker incidence.

[y] Within columns, values followed by a common letter do not differ significantly according to analysis of variance and Tukey's B test at 5% level (DMRT; *P*=0.005).

[x] Activity assayed at 21 h post-inoculation.

produced by pathogens, and acts as a mechanical barrier to physical penetration of the cell (10). Constitutive hydrolases, peroxidases and PAL have been associated with resistance reaction in various plant-pathosystems (9). The results of the present investigation lead us to propose the probable involvement of PAL enzyme in the bacterial canker disease resistance in tomato and also provide a simple and reliable assay for the evaluation of tomato genotypes for bacterial disease resistance/ susceptibility.

It is intriguing to see a gradual increase and decrease in the PAL enzyme activity upon bacterial inoculation based on the disease reaction. A good correlation between the degree of resistance under greenhouse conditions by artificial inoculation technique was obtained with that of PAL activity. These results show the

S. Umesha

validity of the PAL enzyme assay in screening for resistance and susceptibility in tomato against bacterial canker disease.

Phenol estimation studies carried out in our work showed the presence of more phenol upon the pathogen inoculation in resistant tomato genotypes at 21 h after inoculation. PAL is the key enzyme in the phenylpropanoid pathway, and lignin biosynthesis might result in increased phenol accumulation and increased lignin synthesis, leading to a hypersensitive response at the site of infection in the incompatible interactions, thereby restricting the bacterial multiplication.

In conclusion, these studies of the PAL enzyme assay in tomato–bacterial canker host–pathogen interactions suggest the involvement of PAL in resistance of tomato genotypes to bacterial canker pathogen. Differential expression, *viz.*, constitutive and inducible activities of the enzyme in resistant and susceptible tomato genotypes, indicates the possible use of the PAL enzyme activity as a biochemical marker for screening tomato genotypes against bacterial canker disease.

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