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Differential Expression of Metabolites in Response to Infection of Bacterial Wilt Pathogen *R. Solanacerum* (E.F. Smith) Yabuuchi in Resistance and Susceptible Eggplant (*Solanum melongena* L.) Cultivar

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Abstract

Phytopathogen infection leads to changes in primary metabolism as well as secondary metabolism based on the modification in the metabolites levels leads to change in the growth and development of the plant. This will lead to death of the plant and loss. Qualitative changes in the phytohormones, polyamines of the Eggplant (*Solanum melongena*) during pathogenesis caused by *Ralstonia solanacerum* were investigated chromatographically. There was remarkable alteration in the level of various metabolites during interaction. Our data revealed a striking increase in brinjal polyamine putrescine and spermidine in susceptible Pusa Purple Long than resistant Arka Nidhi. In this addendum, we describe that changes in polyamine metabolism take place even in earlier stages of brinjal plant infection with *Ralstonia solanacerum*. However, after 24hpi the level of both hormones goes in opposite direction in susceptible plant. In our study, ABA, IAA and GA3 emerged as a central factor in the regulation and integration of plant immune responses, although little is known about the underlying mechanisms. As in our case, we have detected increase concentration of these phytohormones in susceptible plant than resistant plant during pathogenesis. Our results clearly indicate that, there was suppression of SA mediated defence pathway. There was reduction of JA in susceptible inoculated plant than resistant inoculated plant, indicated their role in imparting susceptibility towards disease at higher concentration.

Keywords: *Ralstonia solanacerum*; *Solanum melongena*; Metabolites; Bacterial Wilt Pathogen

Introduction

Bacterial wilt of eggplant (*Solanum melongena* L.) caused by *Ralstonia solanacerum* (E.F. Smith) Yabuuchi et al. (formerly known as *Pseudomonas Solanacerum*), is one of the most destructive and widespread diseases of the crop. This pathogen has one of the largest known host ranges for any plant pathogenic bacterium in tropical, subtropical and warm temperate regions of the world. Besides Solanaceae, several dicotyledonous and monocotyledonous families have members susceptible to *R. Solanacerum* [1,2]. This organism is the causal agent of brown rot of potato, bacterial wilt or southern wilt of tomato, tobacco, eggplant, groundnut, tobacco, Bird of paradise and Moko disease of banana [3,4].

R. Solanacerum is considered a 'species complex' due to significant destructiveness which is attributed by its widespread variability, the existence of different strains, its exceptional ability to survive in soil and its broad host range including nearly 54 families comprising more than 450 plant species [2]. New hosts of the pathogen are continually being added [1,5]. *R. Solanacerum* enters the plant through wounds in the roots from cultivating equipment, nematodes, insects, and through cracks where secondary roots emerge [3]. The bacteria reach the large xylem elements and are spread into the plant, where they multiply. Once established in the xylem vessels, the bacteria are able to enter the intercellular spaces of the parenchyma cells in the cortex and pith in various areas of the plant. *R. Solanacerum* is able to dissolve the cell walls and create slimy pockets of bacteria and cell debris. Production of highly polymerized polysaccharides increases the viscosity of the xylem, which results in plugging [6]. *R. Solanacerum* is difficult to manage because of its soil-borne nature and high host range. Development of resistance is only possible approach to manage the disease; however, development of durable resistant is very difficult because of high genetic diversity of the

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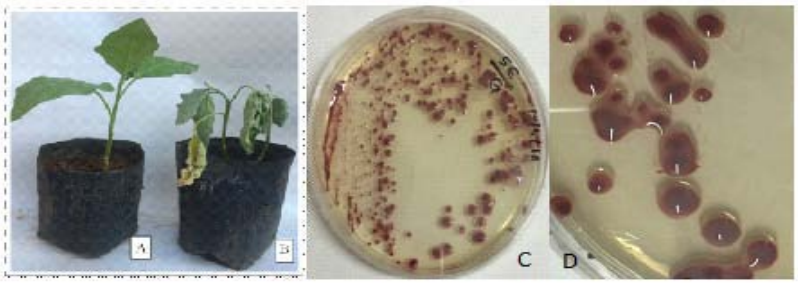

Figure 1: Eggplant Plants were grown under *in vitro* condition. (A) Healthy plant; (B) Wilted eggplant plant; (C) and (D) Bacterial growth on TZC medium and characteristic colonies of *Ralstonia solanacerum*.

bacteria. Other methods like intercropping and crop rotation are often hampered due to a wide range of pathogens [7].

Plants, like all other organisms, are constantly exposed to and challenged by numerous potential pathogenic micro-organisms. However, only a relatively small number of them can successfully establish an infection and cause disease. This is due to variously preformed defense systems and a suite of elaborate induced defense responses that plants possess.

The first line of induced defense is the basal immune system that is activated by molecules that are conserved among many pathogens [8]. *R. Solanacerum* possesses a very large repertoire of genes contributing to virulence, including genes involved in aero- and chimio-tactism [9,10], reactive oxygen species (ROS) detoxification [11], multidrug efflux pumps [12], and Tat secretome [13]. Secreted proteins (effectors) distributed by the T3SS constitute, the key virulence factors [14].

Arka Nidhi is one of the resistant varieties, whereas, Pusa Purple long is highly susceptible variety of the eggplant to the *R. Solanacerum* wilt. During the present investigation, the differential expression of metabolites in response to infection of bacterial wilt pathogen *R. Solanacerum* was studies in resistance (Arka Nidhi) and susceptible (Pusa Purple Long) eggplant cultivars.

There were two bacterial wilt resistant (Arka Nidhi) and susceptible (Pusa Purple Long) varieties used in these experiments in order to see the metabolites changes during progression of disease.

Materials and Methods

Isolation of Bacterial strain of *Ralstonia solanacerum*

The plant showing typical wilting symptoms was collected from the field Navsari Agricultural University farm. The crop was planted in 0.5ha land and was showing 20 per cent infection. Field diagnosis of diseased samples was done by critically observing the bacterial wilt symptoms by checking the bacterial ooze [15]. The diseased plant parts were cut into several small pieces and placed on the Tetrazolium chloride (TZC) agar selective medium for *Ralstonia solanacerum* colonies [16].

Pathogenicity of isolated bacteria was confirmed by inoculation of bacteria by root dip method and re-isolation of the pathogen with the same characteristics.

Metabolic profiling

Metabolic profiling of control as well as pathogen infected roots was done to analyze differential expression of metabolites in response to pathogen by the eggplant. A time-course analysis was performed with the help of HPLC equipped with PDA detector. Root tissues were collected at 24hpi, 48hpi, 72hpi and 96hpi from pathogen inoculated resistant and susceptible plants. Heat map was generated to figure out the possible metabolite production as a result of infection in both the varieties (Table 1). Alterations in the metabolites level were analyzed at each time point under study by fold change with control.

Table 1: Analysis of different metabolites by its fold change with control.

	Fold change								
	24	48	72	96		24	48	72	96
	SI/SC	SI/SC	SI/SC	SI/SC	RC/SC	RI/RC	RI/RC	RI/RC	RI/RC
SA	0.69	0.15	0.08	0.016	0.78	0.69	0.53	0.39	0.12
JA	2.21	1.32	1.26	1.25	1.23	1.03	2.34	1.33	0.59
GA3	1.83	3.82	3.96	5.54	0.45	2.98	3.22	4.32	5.15
ABA	11.25	57	73.75	87.5	1.5	2.17	2.83	3.17	3.33
IAA	5.44	5.36	5.53	6.01	0.73	2	2.57	2.79	3.08
Zeatin	0.2	0.04	0.004	0.026	0	0	0	0	0
Spd	0.59	1.4	1.36	1.69	1.51	0.54	0.45	0.26	0.28
Put	2.31	2.34	4.1	2.72	0.93	1.52	1.56	1.48	1.48

Results and Discussion

Isolation of pathogen

The bacterial wilt pathogen *R. Solanacerum* was isolated from the infected sample of eggplant (Local cultivar) collected from the field of NAU, Navsari (Gujarat). Representative plant was collected and pathogen was isolated by streak plate method on Nutrient Agar (NA) and maintained on NA slant for the study (Figure 1C and 1D). Pathogenicity of isolated bacteria was confirmed by inoculation of bacteria by root dip method and re-isolation of the pathogen with the same characteristics. During the progression of the host–pathogen interaction, there was little browning occurs at the root-stem interphase in susceptible plants (Figure 1A and 1B) and no visible symptoms were observed on any of the mock inoculated variety. The mock-inoculated plant did not show any physiological and morphological changes (Figure 2). All the plants were kept under same environmental conditions and experiment performed under uniform reaction conditions.

Identification of pathogen

PCR based detection of *Ralstonia solanacerum* using Rs specific primers:

759 5'- GTCGCCGTCAACTCACTTTCC-3'

760 5'- GTCGCCGTCAGCAATGCGGAATCG-3'

Table 2: Analysis of different metabolites at different time point after infection using HPLC.

Sr No	Metabolite	Control PPL	24 hpi PPL	48 hpi PPL	72 hpi PPL	96 hpi PPL	Control AN	24 hpi AN	48 hpi AN	72 hpi AN	96 hpi AN
1	SA	9.30 ± 0.70	6.50 ± 0.50	1.48 ± 0.26	0.75 ± 0.22	0.15 ± 0.15	7.20 ± 0.20	4.98 ± 0.02	3.83 ± 0.08	2.82 ±0.01	0.90 ± 0.10
2	JA	0.64 ± 0.14	1.42 ± 0.01	0.85 ± 0.05	0.81 ± 0.01	0.80 ± 0.00	0.79 ± 0.25	0.81 ± 0.06	1.85 ± 0.50	1.05 ±0.04	0.47 ± 0.18
3	GA3	25.71 ±1.00	47.20 ± 0.10	98.32 ± 0.18	102.00 ± 0.42	142.45 ± 12.20	11.51 ± 2.29	34.28 ± 0.08	37.11 ± 1.11	49.67 ±3.18	59.29 ± 2.29
4	ABA	0.04 ± 0.02	0.45 ± 0.04	2.28 ± 0.12	2.95 ± 0.09	3.50 ± 0.02	0.06 ± 0.03	0.13 ± 0.02	0.17 ± 0.05	0.19 ± 0.02	0.20 ± 0.02
5	IAA	0.83 ± 0.02	4.52 ± 0.60	4.45 ± 0.02	4.59 ± 0.21	4.99 ± 0.24	0.61 ± 0.00	1.22 ± 0.12	1.57 ± 0.17	1.70 ± 0.07	1.88 ± 0.11
6	Zeatin	2.28 ± 0.02	0.47 ± 0.07	0.10 ± 0.02	0.01 ± 0.00	0.06 ± 0.02	0.00 ± 0.00	0.00± 0.00	0.05 ± 0.00	0.50 ± 0.05	0.70 ± 0.08
7	Put	0.29 ± 0.02	0.67 ± 0.01	0.68 ± 0.05	1.19 ± 0.06	0.79 ± 0.06	0.27 ± 0.05	0.41 ± 0.08	0.42 ± 0.07	0.40 ± 0.05	0.40 ± 0.04
8	Spd	0.49 ± 0.06	0.29 ± 0.06	0.69 ± 0.03	0.67 ± 0.02	0.83 ± 0.05	0.74 ± 0.06	0.40 ± 0.01	0.33 ± 0.03	0.19 ± 0.03	0.21 ± 0.05

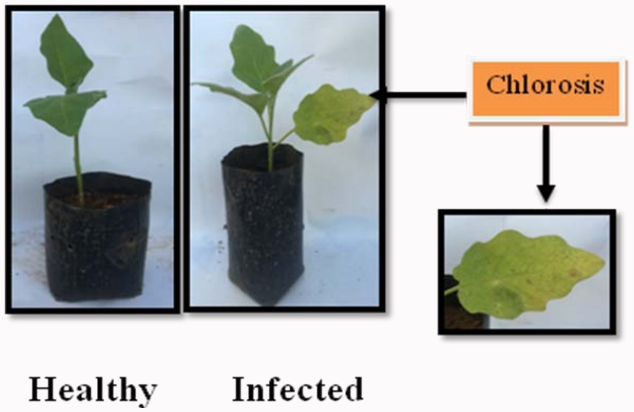

Figure 2: Confirmation of Hypersensitive Reaction. Healthy plant and infected plant of Brinjal.

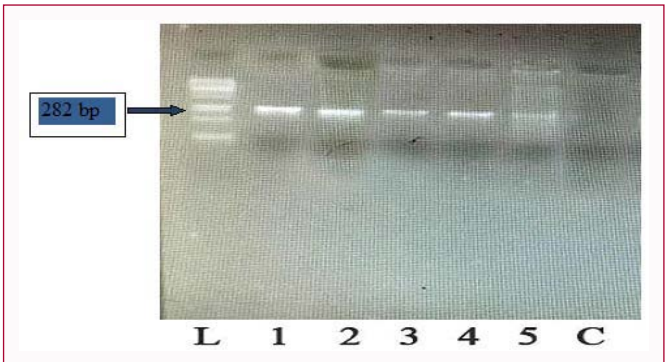

Figure 3: Amplification of fragments of approximately 282 bp generated by universal RS specific primers of 759/760.
where, L: Invitrogen 100 bp Ladder (Cat No 15628-019); Lane 1 to 5: Test DNA from wilted brinjal roots; C: Negative Control.

Rs specific primers for the amplification of target genomic DNA of *Ralstonia solanacerum* isolated from infected brinjal plants. The intensity of amplified product using Rs specific primer 759/760 [17] amplified 282bp in bacterial genome of all isolated bacterial samples of infected brinjal plants (Figure 3).

Metabolic profiling

Significant variations in the expression of different metabolites as presented hereunder were observed in resistance (Arka Nidhi) and susceptible (Pusa Purple Long) varieties of eggplant due to the infection of *R. Solanacerum*.

Expression of salicylic acid and jasmonic acid

The expression of salicylic acid in the Pusa Purple Long and Arka Nidhi in root tissue was decreased immediately after the inoculation of *R. Solanacerum*. Before inoculation salicylic acid concentration in root tissue was 9.3µmol/ml and 7.21µmol/ml in the in Pusa Purple Long and Arka Nidhi respectively. Within 48 hours its concentration was reduced up to 1.48µmol/ml and 3.84µmol/ml in Pusa Purple Long and Arka Nidhi respectively.

In contrary, the expression of Jasmonic Acid in Arka Nidhi remained almost constant till 24hrs (0.81µmole/ml), however, then it increase more than double and reached upto 1.85µmole/ml at 48 hours. In susceptible variety Pusa Purple Long, the level of Jasmonic Acid initially increased within 24 hours, and then decreased substantially.

Plant hormones

Gibberellin (GA_3): The alteration and response of GA_3 showed a similar pattern as Salicylic Acid in the two-eggplant varieties before and after infection of the pathogen. The level of GA_3 almost remains constant in resistant varieties Arka Nidhi which was initially 11.51µmole/ml at the time of inoculation and reached maximum upto 59.29µmole/ml at the 96 days after inoculation. In contrary the level of GA_3 was constantly increased in the susceptible variety Pusa Purple Long which was initially 25.71µmole/ml at the time of inoculation which reached to 142.45µmole/ml at 96 hour after the inoculation.

The level of ABA almost remained constant in resistant varieties Arka Nidhi which was initially 0.06µmole/ml at the time of inoculation and reached maximum upto 0.2µmole/ml at the 96 days after inoculation. In contrary the level of ABA was constantly increased in the susceptible variety Pusa Purple Long. It was initially 0.04µmole/ml at the time of inoculation which became 3.5µmole/ml at 96 hour after the inoculation.

The level of IAA almost remained constant in resistant varieties Arka Nidhi which was initially 0.61µmole/ml at the time of inoculation and reached maximum upto 1.88µmole/ml at the 96 days after inoculation. In contrary the level of ABA was constantly increased in the susceptible variety Pusa Purple Long which was initially 0.83µmole/ml at the time of inoculation which became 4.99µmole/ml at 96 hour after the inoculation.

Polyamines

The level of polyamines viz., Putrescine and Spermidine were tested in susceptible and resistant varieties. Putrescine level was remained almost constant in resistant variety Arka Nidhi, which was initially 0.27µmole/ml which slightly elevated to 0.41µmole/ml at 24 hours after the inoculation and remained almost constant till 96 hours after inoculation. In susceptible variety PPL it was 0.29µmole/ml which increased upto 1.19µmole/ml after 72 hours

of post infection and then decreased to the level 0.79µmole/ml at 96 hours after infection. The level of Spermidine was initially higher in resistant variety (0.74µmole/ml) which was reduced to 0.21µmole/ml after 96 hours of post infection. In susceptible the level was initially 0.49µmole/ml and increased upto 0.83µmole/ml at 96 post inoculation.

Discussion

Decline in expression of salicylic acid was rapid in Pusa Purple Long as compared to Arka Nidhi and same trend remain continued till 96 hours after inoculation. Since there were decreasing trend in the Salicylic Acid, the resistance provided in the Arka Nidhi during the present investigation was not mediated by it. Salicylic acid indeed initiates pathogenesis-related gene expression and synthesis of defensive compounds involved in local resistance and systemic acquired resistance. Therefore, increase in the expression of Salicilic Acid provides resistant to the plants by induction of pathogenesis-related (*PR*) genes [18]. Further, there was no induction of pathogenesis-related (*PR*) genes in the present investigation. SA-dependent resistance response lead to hyper sensitive reaction and subsequently cell death is extremely effective in combating biotrophic pathogens. Since, *R. Solanacerum* is not a biotrophic reaction, even if there would be the increase in production of Salicylic Acid; it would have aggravated the disease development. Jasmonic Acid is a key signal in the transcription activation of defense-related genes (e.g., for the synthesis of proteinase inhibitor). Increased level of Jasmonic Acid after the inoculation in resistance reveals the resistance is mediated by the Jasmonic Acid mediate pathway. Immediately, after the infection, the bacteria population in the plant was not in the proportion to trigger the synthesization of Jasmonic Acid and induced resistance, therefore, the level of Jasmonic Acid within 24 hours of inoculation was not increased initially; however, subsequently it increased significantly. It has been observed that bacterial toxins, such as coronatine of *P. syringae*, toxin, disturbs salicylic acid, mimics Jasmonic Acid, and induces defense genes and defense compounds. Since it is mediated by the Jasmonic Acid-dependent pathways, it may be considered as induced resistance. This seams Salicylic Acid -independent signaling pathway(s), which activate other defense responses controlled by mechanisms dependent on Jasmonic Acid [19,20]. It has been demonstrated that there is an extensive cross-talk between SA and JA/ET signaling. Usually, this cross-talk is thought to be antagonistic [20]. *R. Solanacerum* is a necrotrophic pathogens and can grow better in HR-induced plants [21]. Therefore, to combat necrotrophic pathogens, plants usually activate JA/ET-dependent resistance responses [22].

Decreasing trend of Gibberellin in the resistant plant Arka Nidhi and increasing trend in the susceptible variety Pusa Purple Long indicated that it increases susceptibility of the plant. Gibberellin enhance the activities of cytoplasmic enzymes phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and catalase (CAT), stimulated plant innate defense responses and increase plant resistance against the nematode, *Meloidogyne javanica*. The plant parasitic nematodes are biotopes and do not survive on the dead tissue. PPO plays a novel and fundamental role in secondary metabolism and acts as an indirect regulator of cell death [23]. *R. Solanacerum* being necrotrophic pathogens and can grow better in HR-induced plants increase the susceptibility to the pathogen.

The alteration and response of ABA showed a similar pattern as GA3. ABA decreases the activities of activity of cytoplasmic enzymes phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and catalase (CAT), and does not provide resistance against the biotrophic fungi; however, *R. Solanacerum* can secrete the toxins and can kill the host for the better nutrition. Compared to the GA_3, ABA and IAA, the level Zeatin was reverse. The level of Zeatin was undetectable in resistant variety which was increased upto 0.7µmole/ml after the infection. In susceptible variety it was initially 2.28µmole/ml which was reduced to 0.067µmole/ml at 96 hours of post infection.

Spermidine is a polyamine derived from Putrescine that is involved in many biological processes, including the regulation of membrane potential, the inhibition of nitric oxide synthase (NOS) and the induction of autophagy. Increasing Polyamine biosynthesis enzymes shows the tolerance in tobacco against *Fusarium oxysporium* [24-28].

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