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Confirmation of Proteins Encoding Genes Important in Bacterial Wilt Resistance

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Authors' contributions

This work was carried out in collaboration between all authors. Author GA performed the experiments. Authors NZ and SAK completed antioxidant assays. Author MZ wrote the protocol and designed primers. Author AA designed the study and wrote the final manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background and Aim: Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important soil borne plant pathogenic bacteria globally imparts decrease in crop yield per year. Situation can be improved by exploring defense mechanism against bacterial wilt. Aim of study was to confirm the expression of some defense genes differentially expressed in bacterial wilt resistant in comparison to medium resistant varieties.
Place and Duration of Study: Study was done in Department of Biochemistry and Molecular Biology, University of Gujrat Pakistan for one year.
Study Design: RNA extraction followed by cDNA synthesis was done following salicylic acid (SA) (1 mM) application on *Solanum lycopersicum* var. Roma (medium resistant) and Riogrande

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(resistant variety) for three consecutive days. Expression analysis studies of iron ATP-binding cassette (ABC) transporters, ATP synthase and chaperonin was done using RT-PCR along with measurement of antioxidants enzyme activities.

Results: In control Iron ABC transporter and ATP synthase were expressed in both varieties though chaperonin was expressed in Roma only. Iron ABC transporter was expressed in both varieties within 1st day; while chaperonin was expressed after two and three days of SA treatment in Roma and Riogrande respectively. ATP synthase was expressed after one and three days of SA treatment in Roma and Riogrande respectively. Peroxidase and catalase activity increased in both varieties after one and three days more prominently in Riogrande; with little decrease after two days of SA treatment.

Conclusion: Differential expression of ATP synthase and chaperonin in response to SA in resistant and medium resistant varieties of tomato signifies importance of these genes in response to bacterial wilt in tomato.

Keywords: Salicylic acid; tomato; ATP synthase; iron ABC transporter; chaperonin.

ABBREVIATIONS

SA: Salicylic Acid; SAR: Systemic Acquired Resistance; ABA: Abscisic Acid; GA: Gibberellic Acid; ET: Ethylene; JA: Jasmonic Acid; PR: Pathogenesis Related; CAT: Catalase; POD: Peroxidase; RTPCR: Reverse Transcription Polymerase Chain Reaction; ABC Transporters: ATP-binding Cassette Transporters.

1. INTRODUCTION

Tomato (*Solanum lycopersicum)* is second most significant vegetable crop; and for last 25 years total world production and consumption of this important crop had raised moderately. Small genome, short life cycle similar to many commercially vital plants (potato, eggplant, peppers and tobacco) made it important; as studies conducted on tomatoes are equally valid to these plants [1,2]. It is challenged by various biotic stresses and abiotic stresses; became leading reason for tomato loss worldwide [3]. Tomato respond to biotic/abiotic stresses by multiple defense mechanisms including cellular reactions generated by the pathogens [4]. Plant immune systems are dependent on resistance or susceptibility of variety toward stress that in turn led to various defense related pathways [5].

Plant pathogen interactions led to defense related pathways via signaling molecules e.g. auxins, cytokinins, salicylic acid (SA), abscisic acid (ABA), gibberellic acid (GA), ethylene (ET), jasmonic acid (JA). Defense reaction included production of protein related to defense, growth and development and production of pathogenesis related proteins (PR) proteins [6,7]. SA had an important role in plant defense along with regulation of physiological and biochemical processes (plant growth, photosynthesis, respiration, thermos tolerance, stomatal responses, nodulation, nitrate metabolism, flowering), and ET biosynthesis [7,8]. Increased SA in plant cells is coupled with augmentation of defense and metabolism related proteins with organization of the systemic acquired resistance (SAR) after pathogens infections [9,10,11,12]. SA had been reported to produce oxidative stress in plants at high concentration but at low concentrations enhanced antioxidative capability [13]. Antioxidant enzymes such as polyphenol oxidase, peroxidase (POD), catalase (CAT) were reported to be maximum after 1.5 mM SA treatment [14]. Higher antioxidant activity after SA treatment can be a measure of resistance or susceptibility of the varieties.

Taking into account the mechanisms which plants employ to defend themselves against pathogens will enable the researchers to design novel strategies to improve disease resistance in plants. ATP synthase, iron ATP-binding cassette (ABC) transporters and chaperonin related to protein destination, storage synthesis and photosynthesis expression were some of the important proteins expressed in plants resistant to biotic stress in response to SA treatment [15,16]. Previously SA induced proteins were explored in susceptible and resistant varieties of tomato (Roma and Pant Bahr) [11]. In current study ATP synthase, iron ABC transporters and chaperonin expression was confirmed using reverse transcription

polymerase chain reaction (RTPCR) in *Solanum lycopersicum* cultivars Riogrande and Roma after one, two and three days of SA treatment. Along with these protein encoding genes, antioxidant enzymatic assays (POD, CAT) was carried out.

2. MATERIALS AND METHODS

2.1 Plant Material

Seeds of tomato (*S. lycopersicum*) cvs. Riogrande (resistant) and Roma (medium resistant) were obtained from National Agriculture Research Centre (NARC), Islamabad. Seeds were surface sterilized in 0.8 % (v/v) "Clorox" bleach (Sodium hypochlorite) [11]. Seedlings were planted in pots containing sand and soil, grown in growth chamber under white fluorescent light (600 mol m^{-2} s^{-1}; 16 h light/8 h dark) at 25°C and 70% relative humidity.

2.2 SA Treatment, RNA Isolation and cDNA Synthesis

SA (Wako) (1 mM) in water (50 mL) was used to treat three-week-old seedlings of Roma and Riogrande in growth chamber. Total RNA was isolated from leaf cells of controlled and treated tomato plants using the TRI Reagent (Cat. No. TRI. 118; Lot No. 4221) for three consecutive days following SA treatment. RNA was precipitated by mixing with isopropanol, centrifuged (12000 g) for 8 min at 4-25°C. RNA pellet was washed with 75% ethanol, centrifuged, dissolved in nano-pure water and stored. cDNA was prepared from total RNA samples using cDNA synthesis kit (Invitrogen, USA).

2.3 RT-PCR Analysis

Accession number of three proteins (Iron ABC transporter, ATP synthase and 60-KDa chaperonin) were analyzed using bioinformatics tools, and suitable length of aligned region of PCR primers were designed using Primer3 online software (Table 1). cDNA was amplified with gene-specific primers using PCR. Confirmation of the desirable sequences in extracted cDNA from all species was done by running PCR reaction with five set of primers under specific conditions (Table 1). 20 µL PCR reaction mixture contain 1.5 µL template cDNA, 2 µL Taq buffer, 1 µL $MgCl_2$, 0.5 µL dNTP mixture, 0.5 µL of each forward and reverse primer, 1 µL Taq polymerase and 13 µL nanopure water. Initial denaturation step for PCR was restricted to (95°C) for 4 minutes, followed by 40 cycles of denaturation at (95°C) for 30 seconds, annealing temperature (55°C for ATP synthase, 53°C 60-kDa chaperonin and iron ABC transporter) for 1.5 minutes and extension (72°C) for 30 sec, and a final extension of 7 min (72°C). The amplified PCR products were analyzed by 1% (w/v) agarose gel electrophoresis.

2.4 Enzymatic Assays

2.4.1 POD activity

POD activity was determined following the dehydrogenation of guaiacol as a substrate [17]. Plant material (2 g) was grinded in pre-chilled mortar with 0.1 M phosphate buffer (pH 7). Homogenate was centrifuged at 18000 g for 15 min at 5°C and supernatant used as enzyme source within 2-4 h. 3 ml of the buffer solution was pipetted along with 0.05 ml guaiacol solution (20 mM), 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide solution (12.3 mM) in cuvette. Absorbance was taken by spectrophotometer at 750 nm.

2.4.2 CAT assay

CAT was assayed by monitoring the decrease of absorbance as described by Aebi [18]. The reaction mixture consisted of enzyme (50 µL), potassium phosphate buffer (50 mM; 2.5 mL; pH 7.6), hydrogen peroxide (12.5 mM; 200 µL) and homogenate sample (50 µL), along with distilled water (250 µL) were taken in a cuvette. Absorbance was measured at 240 nm three times for each sample with an interval of 30 seconds. One unit activity of CAT was described as an absorbance change of 0.01 as unit per minute.

3. RESULTS AND DISCUSSION

Bacterial wilt is one of the important bacterial stress faced by tomato, potato, pepper, banana, ginger, cowpea, peanut, papaya, cashew, and olive restraining its production [19]. Different genes had been identified which are expressed in tomato varieties resistant and susceptible to bacterial wilt after JA and SA application [11]. SA mediated defense pathway play a key role in response to biotic stresses [12,14,15,16,20].

RTPCR is widely used for confirmation of gene or protein expression; so was used for confirmation of Iron ABC transporter, ATP synthase and chaperonin 60 in Riogrande and Roma. RNA extraction was done from 3-W-Old seedlings leaves, followed by cDNA synthesis and RTPCR of Iron ABC transporter, ATP synthase and chaperonin 60 in resistant and medium resistant varieties of tomato (Fig. 3; Fig. S1A and S1B)

3.1 Morphological Variation

Leaves became light green after 1, 2 and 3 days of SA treatment in comparison to control in Roma (Fig. 1A; B; C; D). Riogrande leaves remained same as control (Fig. 1E; F; G; H). Reduction in chlorophyll in semi-resistant variety (Roma) show its more sensitiveness to water stress, as the leaves were treated with aqueous solutions of SA in comparison to control. It is in line with observation for Roma [11].

3.2 Antioxidant Enzymes Assay

POD and CAT activity was increased one day after SA treatment, decrease after 2 days and again increased after 3 days of SA treatment in Riogrande and Roma (Fig. 2A; 2B). CAT and POD help to scavenge the stress damage were upregulated in these two varieties after SA treatment. Antioxidant activity was more prominent in resistant variety suspects its role to reduce the ROS damage. Higher induction of antioxidant enzymes (POD, PPO) along with generation of H_2O_2 were reported in response to SA (up to 1.5 mM) in *Cicer arietinum L.* [14]. Antioxidative enzymes POD, thioredoxin, superoxide dismutase, glutathione S-transferase were reported in tomato after SA treatment to protect plant from damage [21,22]. Immune response is suppressed by inhibition of host cell's POD activity, thereby inhibiting ROS production [23]. So, increased enzymes activity after SA treatment more prominent in Riogrande; display its role in tomato resistant cultivar immunity.

3.3 Iron ABC Transporter

Iron ABC transporter was found to be expressed within one day of SA treatment in both Roma and Riogrande (Fig. 3A). Iron ABC transporter, was also upregulated in the proteomics analysis of tomato cvs and soybean in response to application of either JA or SA [11,16]. Iron ABC transporter had a role in stress resistance in plants, and induced in response to bacteria, fungus, heavy metal stress, ET, SA, JA, ABA, resistance to HR, iron homeostasis, cell viability and iron acquisition in plants [22,24]. Iron ABC transporter, photosynthesis related gens, defense genes, antioxidant enzymes were induced in response to SA treatment can be used as genetic tools to investigate disease resistance [25]. Iron ABC transporter is also induced in JA dependent pathway in response to infection by bacterial and fungal infections in tobacco [26]. Its expression in response to JA, SA, bacteria and fungus imparts it importance in biotic stress related pathway for tomato.

3.4 ATP Synthase

ATP synthase was found to be expressed within one day of SA treatment in Roma along with control, while in Riogrande it is expressed in control and 3 days after SA treatment (Fig. 3B). It is in line with report that characterize upregulation of ATP synthase in susceptible cultivars after 1st day and after 2 days of SA treatment in resistant cultivar [15]. Proteomic approach show reduced expression of ATP synthase after SA treatment in medium resistant cv. Roma [11]. Wu et al. [7] via gel free techniques (LC MS/MS) reported phosphoproteins such as ATP synthase and iron ABC transporters were upregulated by SA in maize. ATP synthase was expressed in response to biotic/abiotic stresses for production of resistance in plants by suppressing HR response, along with increased metabolism of glucose [27]. Transgenic tobacco with ATPase exhibited resistance in response to *P. syringae*, suggesting its role in defense via JA or SA [28]. Upregulation of ATP synthase after 1st day of SA treatment in susceptible (Pant Bahr) and medium resistant (Roma) and after 2 days in resistant variety (Riogrande) of tomato implied its importance as defense gene in response to bacterial stress. This is supported by its role in insect herbivory, suppression of HR, and resistance to bacterial stress made it a good candidate for transgenic tomato with enhanced resistance to bacterial wilt.

Table 1. Primers for genes encoding protein sequences, melting temperature ™ and their functional category

Protein name	Protein database/ Tm	Forward primer	Functional category
ATP synthase epsilon chain (F)	gi|114610/ 55°C	5-3' TGACCTTAAATCTTAGTGTACTGACC	Glycolysis pathway
ATP synthase epsilon chain (R)		5-3' TTATGAAATCGGATTGATAGCC	
60 kDa chaperonin (F)	gi|603912/ 53°C	5-3' GAGATTGTGTTCGACCAGGAG	Protein destination and storage
60 kDa chaperonin (R)		5-3' ACACGCCATCGTTGATAACC	
Iron ABC transporter (F)	gi|57504871/53°C	5-3' ATGAAAAAAATTTTAATCATTATGAGTTTA	Electron transport and photosynthesis
Iron ABC transporter (R)		5-3' TTAAAAATTTTGATTTTCTAATCTTGTTTT	

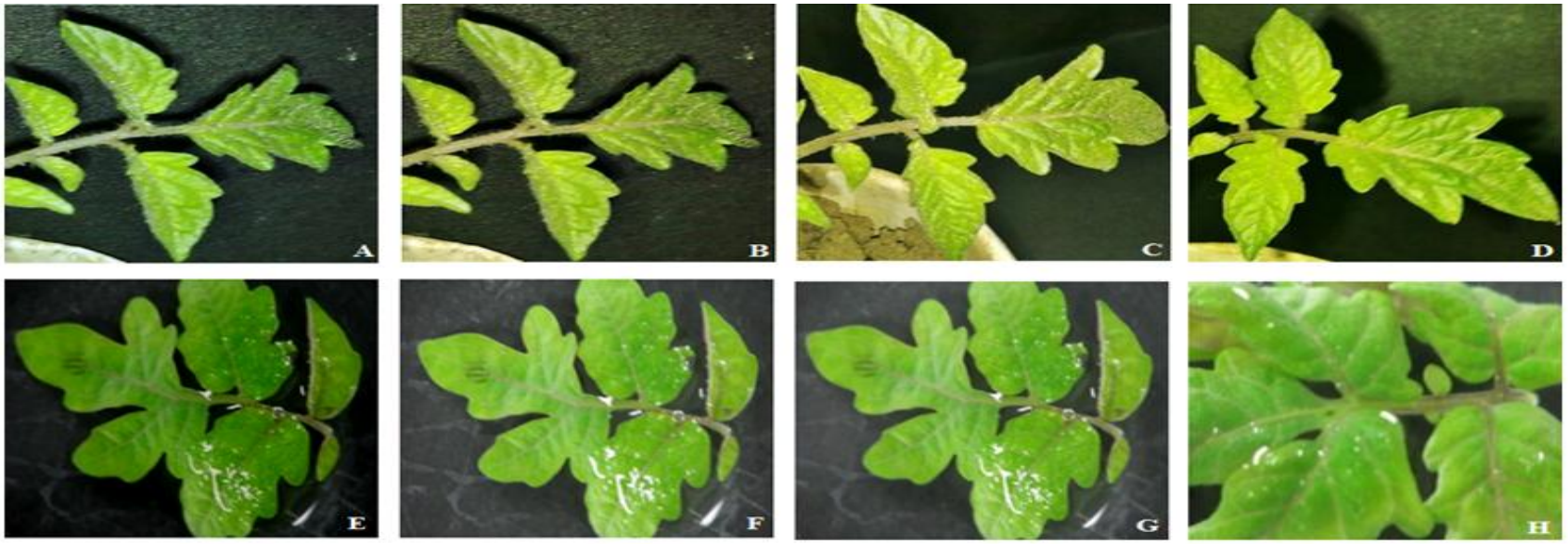

Fig. 1. Morphological variation of *Solanum lycopersicum* cvs Roma (a, b, c and d) and Riogrande (e, f, g & h) in control and 1, 2 and 3 days after SA treatment

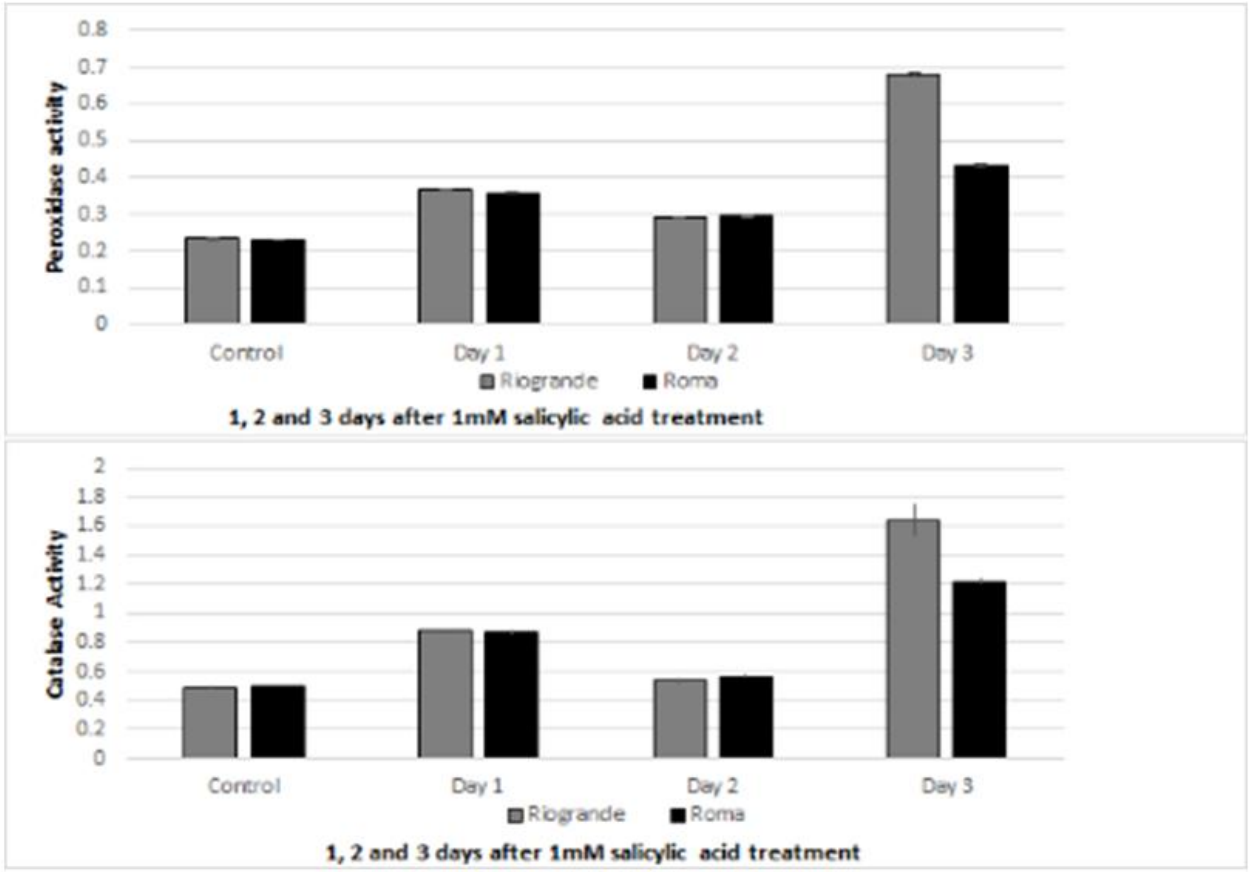

Fig. 2A. Peroxidase activity in *S. lycopersicum* cvs Roma and Riogrande 1, 2 and 3 days after 1 mM SA treatment.
2B. Catalase activity in tomato cvs Roma and Riogrande 1, 2 and 3 days after 1 mM SA treatment

3.5 Chaperonin

Chaperonin was expressed in control and 2 days after SA treatment in Roma (Fig. 3C) and after 3 days of SA treatment in Riogrande (Fig. 3D). Wu et al. [29] reported that after inoculation with *P. solanacearum*; ATP synthase was expressed in susceptible and chaperonin was expressed in resistant maize cultivar in SA pathway. Moshe et al. [30] reported up regulation of chaperonin in resistant tomato cultivar in response to tomato leaf curl virus infection in comparison to susceptible cultivar. Proteomics analysis of *Arabidopsis* associate chaperonin-60 with photosynthesis for increasing duration of stress [31]. Cueto-Ginzo et al. [32] reported increase in molecular chaperone in resistant cultivars in comparison to accumulation of ROS species in susceptible cultivars of tomato in response to virus attack and SA application. Chaperone was expressed in medium resistant early in comparison to resistant cultivars; more prominent in Riogrande (11; Fig. 3D). Proteomic approach show enhanced expression of chaperonin in susceptible variety (Pant Bahr) and unchanged expression in medium resistant variety (Roma) after SA treatment. Contrastingly RTPCR show enhanced expression of Chaperonin after 2 days of SA treatment in Riogrande and after one day in Roma. So chaperonin had possible role in tomato to cope up with bacterial stress.

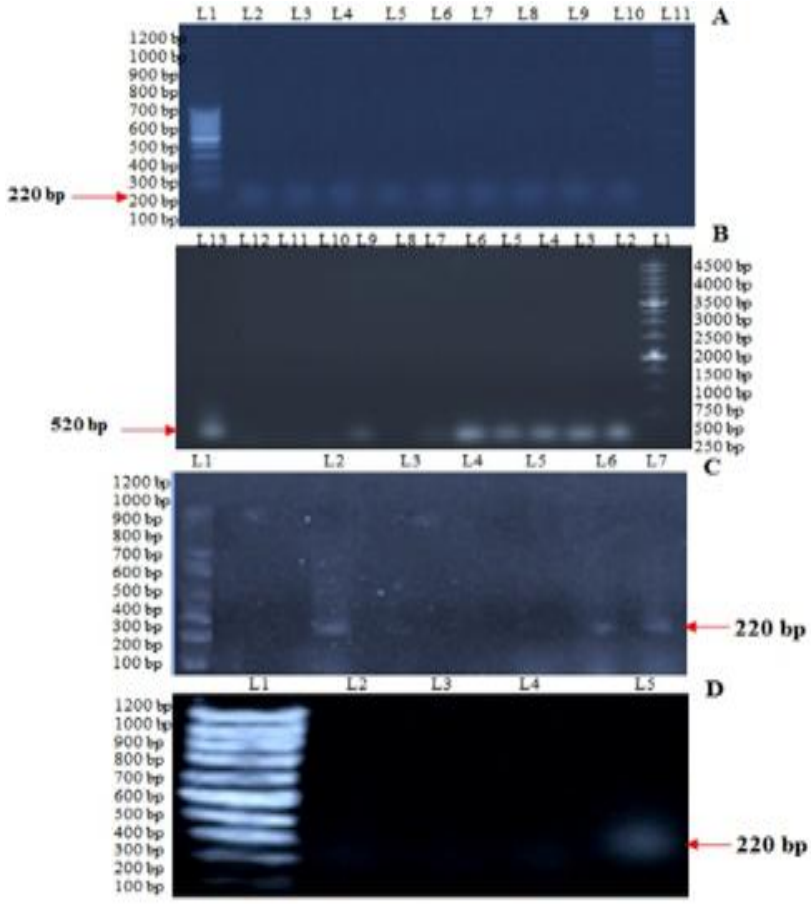

Fig. 3. RTPCR of 3-W-Old *S. lycopersicum* leaves after 1, 2 and 3 day of SA treatment with 3A. ABC transporter primer (220 bp). L1 100 bp marker, L 2-5: Roma control, 1, 2, 3 days after SA treatment. L 6-9: Riogrande control, 1, 2 & 3 days after SA treatment. 3B. ATP Synthase primer (520 bp). L 1: 1 Kb ladder, L2-L5: Roma control, 1, 2 & 3 days after SA treatment. L6-L9: Riogrande control, 1, 2 & 3 days after SA treatment; L11- 13: Riogrande 1, 2 & 3 days after SA treatment as repeat. 3C.Chaperonin primer (220 bp). L 1: 100 bp ladder, L2, L5-7: Roma control, 1, 2 & 3 days after SA treatment 3D. Chaperonin primer (220 bp). L 1: 100 bp ladder, L2-5: Riogrande control, 1, 2 & 3 days after SA treatment

4. CONCLUSION

ATP synthase and chaperonin early expression in Roma in comparison to Riogrande after SA application presents medium resistant variety sensitization to cope up with stress. Late expression of the said proteins related to energy and protein destination & storage could be more important in defense reactions. Antioxidants activity prominence in Riogrande revealed their importance in resistant variety immunity to bacterial stress in comparison to medium resistant one.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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