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RESEARCH ARTICLE / *ARTIGO*

Synergistic effect of acetyl salicylic acid and DL-Beta-aminobutyric acid on biocontrol efficacy of *Bacillus* strains against tomato bacterial wilt

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ABSTRACT

This study was conducted to assess the biocontrol efficacy of *Bacillus subtilis* (strain 4812) and *Bacillus methylotrophicus* (strain H8) individually or in combination with two plant defense inducers including Acetyl salicylic acid (ASA) and DL-Beta-aminobutyric acid (BABA) against tomato wilt caused by *Ralstonia solanacearum*. The pathogen was significantly inhibited by all treatments in the greenhouse and *in vitro* tests. The treatments H8+ASA and 4812+H8 were found to be the most effective treatments in *in vitro* tests. Applied on tomato seeds and as soil drenching, the disease was most inhibited by H8 whereas 4812+H8+ASA was the least effective treatment. High activity of phenylalanine ammonia-lyase was observed in the plants treated with 4812+H8, H8+ASA and 4812+H8+ASA. The highest expression of peroxidase and polyphenoloxidase enzymes was found in the plants treated with H8, 4812+H8 and H8+ASA. The result of real time PCR showed that concentration of the pathogen in stem tissues was significantly reduced in all treated plants and H8+ASA was the most effective treatment. This study revealed that combined application of *Bacillus* strains can be considered as a more effective biocontrol agent against tomato bacterial wilt. Furthermore, the synergistic effect of plant defense inducers was variable to different *Bacillus* strains.

Key words: *Bacillus* spp., *Ralstonia solanacearum*, *Solanum lycopersicum*, chemical inducers, ISR, real-time PCR.

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is an important disease of tomato in tropical, subtropical and temperate regions of the world. This pathogen is able to systematically infect over 200 plant species including a wide range of crop plants, ornamentals and weeds. The early symptom of this disease is wilting of the youngest leaves during the hottest part of the day. As the disease develops, the entire plant may wilt quickly and desiccate although dried leaves remain green, leading to general wilting, yellowing of foliage and eventually plant death. Many methods such as soil solarization, field sanitation, crop rotation, and use of bactericides have been applied for control the disease, but limited success has been reported (Ciampi-Panno et al., 1989). Although resistant cultivars have used as an important component of integrated disease management, it is known that they lack stability or durability against this soil-borne pathogen. Furthermore, the high variability of *R. solanacearum* strains and the influence of environmental factors on host–pathogen interactions often restricts the resistance against the pathogen in specific regions (Hayward, 1991).

The conventional methods cannot effectively control this disease stimulating a search for more efficient strategies such as application of biological control agents and plant defense inducers (Adesemoye et al., 2009; Hassan et al., 2011).

It is well known that plant growth promoting rhizobacteria (PGPR) help plants to increase resistance against pathogens by secreting biologically active compounds and/or elicitation of induced systemic resistance (ISR) (Heil & Bostock, 2002). It is reported that the application of single biocontrol agent often results in inconsistent field performance due to the variability of soil conditions and agricultural ecosystems (Raupach & Kloepper, 1998). In fact the application of a mixture of several strains may result in more stable rhizosphere community and provide several mechanisms of biological control (Latha et al., 2009). Acetylsalicylic acid and β-aminobutyric acid have been reported as plant immune inducers against many bacterial, fungal and viral pathogens. Many researches have shown that these chemical inducers can result in high concentration of pathogen related (PR) proteins in plants (Cohen & Gisi, 1994; Heil & Bostock, 2002; Klessig & Malamy, 1994; Yun et al., 1999). Although there are some researches showing the efficacy of integration management using *Pseudomonas* spp. and acibenzolar-S-methyl against tomato bacterial wilt (Abo-Elyousr et al., 2012; Anith et al., 2004), no information was found about the probable synergistic or

antagonistic interaction between PGPRs and other plant defense inducers against this wilt disease. The objective of this study was to assess the interaction between two plant defense inducers mentioned above and two antagonistic *Bacillus* strains *in vitro* and under greenhouse conditions. In addition to some biochemical analyses, quantitative real time PCR was used to determine the inhibitory effect of different treatments against the actual concentration of *R. solanacearum* in the tissues of infected plants.

MATERIALS AND METHODS

Origin of bacterial strains, plant materials and chemical inducers

A virulent strain of *R. solanacearum* T-91 (race 1 biovar 3), *Bacillus subtilis* (strain 4812) and *Bacillus methylotrophicus* (strain H8) were provided by the Institute of Biotechnology, Zhejiang University, China. *Ralstonia solanacearum* and *Bacillus* spp. were routinely cultured on yeast extract peptone glucose agar (YPGA) (Xue et al., 2009) and Luria bertani agar (LBA), respectively. Tomato seeds (cv. "He zuo") known to be susceptible to *R. solanacearum* (Soad et al., 2004) were obtained from Horticulture Department, Zhejiang University, China. Two plant defense inducers including Acetyl salicylic acid (ASA) and DL-Beta-aminobutyric acid (BABA) were purchased from Sangon Company (Shanghai, China). ASA and BABA were dissolved in sterile deionized water and serially diluted to obtain solution with a final concentration 1mM (Yang et al., 2011).

Compatibility trials

In order to assess likely antagonistic activity among biocontrol agents used in this study, the compatibility/ incompatibility test was conducted on LBA according to the method explained by Fukui et al. (1994). The compatibility between the chemical inducers and biocontrol agents was tested based on the disk diffusion method (Duraipandiyan et al., 2006). This experiment was repeated three times in three consecutive weeks and there were three replications for each treatment.

In vitro evaluation of antagonists and plant defense inducers against *R. solanacearum*

The inhibitory effect of chemical inducers (ASA and BABA) and *Bacillus* spp. were tested alone or in combinations against the pathogen by the dual culture technique (Latha et al., 2009). There were 11 treatments in this experiment including *B. subtilis* (4812), *B. methylotrophicus* (H8), ASA, BABA, 4812+H8, 4812+ASA, 4812+BABA, H8+ASA, H8+BABA, 4812+H8+ASA and 4812+H8+BABA. Briefly, *R. solanacearum* (R.s.) was grown on YPG for 18 h. Then bacterial culture was centrifuged at 1000 rpm for 10 min and cell pellets were suspended with sterile distilled water and finally adjusted to OD_{600} = 0.9, CFU mL^{-1}~1×10^{8} . Subsequently, 100 µL of bacterial suspension were swabbed uniformly on agar surface and plates were left in a laminar-flow hood at 22-25°C for 2 h to dry. Sterilized filter paper discs were placed in equidistance to each other on the seeded medium and spotted by 6 µL of each antagonistic strains suspension (OD_{600}=1 and CFU~10^{7}) obtained from overnight bacterial culture and chemical inducers (1 mM) individually. In order to prepare the suspensions of *Bacillus* spp., overnight bacterial culture was spun for 8 min at 9000 rpm then resuspended in sterile distilled water. For combinations, 3 µL of each component in double component treatments and 2 µL of each component in triple component treatments were loaded on sterilized filter paper discs. In control, filter paper discs were treated with 6 µL of sterile distilled water. Plates were incubated at 30°C. The incubated plates were checked for appearance of clear haloes around the spotted discs, and inhibition zone diameter was calculated after 72 h for each treatment. This experiment was repeated two times with three replicates for each treatment in a completely randomized design.

Pot experiments

This experiment was conducted from 14th Oct, 2011 to 15th Jan, 2012 in a completely randomized block design and each treatment was repeated five times with 20 plants for each replicate. Pots (12 cm diameter × 15 cm height) filled by autoclaved potting mixture (1 field soil: 1 peat moss) were used in this experiment. Tomato seeds were surface disinfected by immersing in 2% sodium hypochlorite for 2 min and washed three times with autoclaved tap water. They were transferred to dry sterilized filter paper and allowed to dry in laminar flow for 8 h and pre-germinated on 0.6% water agar for 5 days at 24-26ºC with photoperiod of 12 h of light and 12 h of dark. Four pre-germinated seeds were sown in each pot and assessed for the entire test. In addition, micro-nutrition fertilizer was applied for three times. The pots were randomly placed in a greenhouse (70-80% humidity and 22 ± 2°C) equipped with Osram daylight to provide supplementary light for a 12 h photoperiod. The pathogen was grown on YPG medium at 30ºC in a rotary shaker with 160 rpm for 18 h, and was centrifuged at 10000 rpm for 10 min. Afterwards the cell pellets were harvested and suspended in sterilized saline (0.85% NaCl) water to obtain the final concentration of 1×10^{9} CFU mL^{-1}. The tomato plants in all treatments were inoculated with the pathogen at the third to fourth leaf stage by punching each plant with sterilized needle at the base of stem at 1-2 cm from the soil. Subsequently 80 mL of the bacterial suspension were poured in each pot over wounded areas. After inoculation, all pots were covered by polyethylene bags for 24 h to maintain high humidity (Algam et al., 2010). For each treatment, five pots were allocated for biochemical tests and were not considered in statistical analysis.

Six treatments including H8, 4812+H8, H8+ASA, H8+BABA, 4812+H8+ASA and 4812+H8+BABA were tested against tomato wilt under greenhouse conditions.

These six treatments had shown high efficacy against the pathogen in *in vitro* tests. In other words, they were able to show more inhibitory effect against the pathogen growth on YPGA. There were two sets of controls: C1 (treated only with pathogen) and C2 (treated with saline water).

The treatments were applied two times as bacterial strains suspended in saline water (OD_{600}=1, ~10^7 CFU mL^{-1}) and/or chemical inducers at concentration 1 mM. Before planting, tomato seeds were overnight immersed in the suspension of different treatments. Then treated seeds were transferred to dry sterilized filter paper and allowed to dry in laminar flow for 8 h. Before planting the treated seeds into the pots, they were pre-germinated on 0.6% water agar for 5 days at 24-26ºC with photoperiod of 12 h of light and 12 h of dark. The second application of treatments was soil drenching with 60 mL per pot of bacterial suspension (1×10^7 CFU mL^{-1}) or aqueous solution of ASA or BABA (1 mM) or their combinations were poured in each pot one week before inoculation with pathogen (Almoneafy et al., 2012). The plants were weekly observed and disease index was recorded for each treatment according to the method described by Park et al. (2007). Disease incidence was then calculated for each treatment after 15, 30, 45 and 60 days post inoculation using the formula (Xue et al., 2009):

Disease incidence = [(Disease index × number of diseased plants in this index)/ (Total number of plants investigated × the highest disease index)] × 100%. Based on disease incidence, the area under disease progress curve (AUDPC) was calculated for each treatment according to the midpoint rule (Garrett & Mundt, 2000):

$$\text{AUDPC}=\sum_{i-1}^{n-1} \left[0.5(x_i + x_{i+1})\right]\left[t_{i+1} - t_i\right],$$

where x_i is the percentage of disease incidence at i^{th} assessment, t_i is the time of the i^{th} assessment on days from the first assessment date and n is the total number of days that the disease was assessed. Because incidence (x) was expressed in percent and time (t) in days, AUDPC was expressed in %-days.

Biological control efficacy (BCE) was assessed based on AUDPC data by following this formula: BCE= $[(AUDPC_C - AUDPC_T)/AUDPC_C] \times 100\%$, where $AUDPC_C$ is the AUDPC value of control treatment and $AUDPC_T$ is the AUDPC value of treatment group (Aliye et al., 2008).

In addition to AUDPC and BCE, the effect of each treatment on fresh and dry weight of above-ground tissues was measured 60 days after inoculation. Plant tissues were dried at 60°C for three days. Compared with controls, the growth promotion efficacy was calculated to clarify the relative effect of different treatments using the formula: GPE (%) = $[(G_T-G_C)/G_C] \times 100$**,** where, GPE refers to growth promotion efficacy, G_T refers to growth parameter in treatment and G_C refers to growth parameter in control.

Biochemical analysis

For each treatment, tomato leaves were collected on the 1st, 5th and 9th days after inoculation with the pathogen, and stored at -80°C until used. All enzyme extraction procedures were conducted at 4°C. To assess the activity of Phenylalanine ammonia-lyase (PAL), 1 g of tissue was grounded and mixed with 2 mL extracting buffer [0.2 M boric acid buffer containing 10% (w/v) polyvinylpolypyrolidone (PVPP), 1 mM EDTA, and 50 mM β-mercaptoethanol, pH 8.8]. For polyphenoloxidase (PPO) and peroxidase (POD), 2 g of the tissue were grounded with 10 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of PVPP, homogenized and centrifuged at 12,000 rpm at 4°C for 30 min, and the supernatant was collected and used for enzyme assay.

Phenylalanine ammonia-lyase (PAL) assay was investigated as described by Assis et al. (2001). The enzyme extract 300 µL was incubated with 1 mL 0.02 M L-phenylalanine (suspended in double distilled water) and 2 mL of the PAL extracting buffer at 24°C for 2 min, and absorbance at 290 nm was measured in an ultraviolet spectrophotometer. The PAL activity was expressed using the U290 = 0.01ΔOD290, where U290 is the unit of enzyme at 290 wave length, and ΔOD is the change of optical density per mg of protein per min.

Polyphenoloxidase PPO activity was determined according to the method described previously by Galeazzi et al. (1981) In this method, 100 µL of enzyme extract was incubated with 2 mL of 0.05 M phosphate buffer (pH 7.0) and 0.5 mL of 0.5 M catechol at 24°C for 2 min, and the absorbance at 398 nm was measured with an ultraviolet spectrophotometer. The PPO activity was expressed as U398, where U398 = 0.01ΔOD398, where U398 is the unit of enzyme at 398 wave length, and ΔOD is the change of optical density per mg of protein per min.

Peroxidase POD activity was determined using guaiacol as substrate. The reaction mixture consisted of 0.1 mL of crude extract and 2 mL of guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4) and was incubated for 30 min at 30°C. The increase in absorbance at 460 nm was measured after 1 mL H_2O_2 (24 mM) was added. The activities of POD were expressed as U460, where U460 = 0.01ΔOD460, where U460 is the unit of enzyme at 460 wave length, and ΔOD is the change of optical density per mg of protein per min (Ippolito et al., 2000).

The maximum POD and PPO enzymes activities were observed on 5th and 9th days after inoculation respectively, in H8, 4812+H8 and H8+ASA during spectrophotometer analysis. Therefore leaf samples in these treatments were collected on 5th and 9th day for POD and PPO respectively, and subjected to native-PAGE analysis to find out the expression of their isoforms. Briefly, each sample was homogenized with 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 10000 rpm for 20 min at 4°C. The protein contents of selected samples were determined by the Nanodrop ND-1000 Spectrophotometer adjusted

to Bradford procedure. Samples (50 µg protein) were loaded on 8% polyacrylamide gel, the gel was stained in 0.2 M sodium acetate buffer (pH 4.2) containing 0.05% benzidine for 30 min in the dark. Drops of H_2O_2 (0.03%) were added slowly with constant shaking to visualize the POD isoforms. After staining, the gel was washed with distilled water (Nadolny & Sequeira, 1980). For PPO, the gel was immersed in P-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer pH 7.0 for 30 min. Later on, 10 mM catechol was added and then kept in a shaker with gentle shaking. The appearance of dark brown protein bands was noticed after about 10-15 min (Shanmugam & Kanoujia, 2011). These assays were repeated two times and there were three replicates in each treatment.

Specific detection and quantification of *R. solanacearum* by real-time PCR

This experiment was conducted to estimate the inhibitory effect of the treatments said above for pot experiments on the concentration of pathogen inside the plant tissues. Plant DNA was extracted from treated plants according to the procedure described by Maciá-Vicente et al. (2009). According to Chen et al. (2010), *R. solanacearum* specific primers RSF/RSR produced based on the upstream region of the UDP-3-O-acyl-GlcNAc deacetylase gene were used. The standard curve of real-time PCR for detection and quantification of *R. solanacearum* from the plants tissues was generated. The overnight culture broth of the pathogen was centrifuged at 10000 rpm for 10 min then suspended with sterile distilled water and adjusted to 1×10^8 CFU mL^{-1}, and a 10-fold dilution series was made from each suspension (10^0-10^7). The number of the cells in the serial dilutions was confirmed by incubation on TZC agar plates at 30°C for 3 days. The DNA of *R. solanacearum* was extracted from each dilution using Tianamp Bacteria DNA kit (Tiangen Co., China) following the manufacturer's instructions, and DNA quantification was performed at real-time PCR (Applied Biosystems 7500 Real-Time PCR System, Foster City, CA, USA) to create standard curve. Moreover, acquired DNA from infected tomato stem tissue was also subjected to real-time PCR quantification. The 20 µL reaction mixture was used which was containing 10 µL SYBR Premix Ex Taq mix (TaKaRa Biotech.Co., Japan), 0.4 µL ROX Reference Dye II (50×), 0.4 µL of both forward and reverse primers (10 µM each) and 2 µL DNA template with following PCR conditions 95°C for 2 min, followed by 40 cycles at 95°C for 20 s, 62°C for 25 s, 72°C for 35 s, and 85°C for 3 s. Once the amplifications were completed, the melting curves were obtained based on a standard protocol and used to identify the characteristic peak of PCR product. This experiment was repeated twice and there were two replicates for each treatment.

Statistical analysis

SAS software Version 9.1 (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analysis. The data from all experiments were subjected to the analysis of variance (ANOVA) test to determine the significant differences among treatments means. Least significant difference test (LSD) was used to carry out individual mean comparisons. A significant level of $\alpha = 0.05$ was used in all analyses.

RESULTS

Compatibility tests

No inhibition zone was formed between two *Bacillus* strains in dual culture experiments. Two strains overgrew each other indicating that these strains were compatible. Moreover results showed that there was no inhibitory effect against *Bacillus* strains due to exposing them to ASA or BABA.

***In vitro* tests**

The results showed that most of treatments were significantly different from untreated control. H8+ASA was found to be the most effective treatment against growth of the pathogen, followed by 4812+H8, H8 and 4812+H8+BABA, respectively. Showing statically at par results, 4812+H8+ASA and H8+BABA were the next treatments in order of efficacy against the pathogen growth. The next treatments were 4812 and 4812+BABA which inhibited zone in diameter by 0.8 and 0.5 cm, respectively. Different from other treatments, the least inhibitory effect was observed in ASA, BABA and 4812+ASA with statically similar performance (Figure 1).

Greenhouse trials

Effect of treatments on AUDPC and disease incidence

Based on the results, all treatments significantly reduced the disease 15 days after inoculation. No significant difference was observed between treatments and control before 15 days. Compared with the controls, H8 was the most effective treatment against AUDPC and disease incidence. The next treatments were 4812+H8, H8+ASA, H8+BABA and 4812+H8+BABA with statically at par performance. The least efficacy against the disease was found in 4812+H8+ASA (Table 1, Figure 2).

Effect of treatments on plant growth parameters

Compared with infected control, application of tomato plants with the treatment 4812+H8 was able to significantly increase the plant height. The treatments 4812+H8+BABA, H8+BABA, H8+ASA and H8 were the next treatments in order of superiority. In addition, all treatments significantly increased the fresh and dry weight of above-ground tissues compared with infected control (Table 2). The results showed that H8 treated plant show fresh and dry weight even superior to uninfected saline control. The highest value for fresh weight of tissues was observed in the plants treated with H8 whereas 4812+H8+BABA, H8+ASA, H8+BABA,

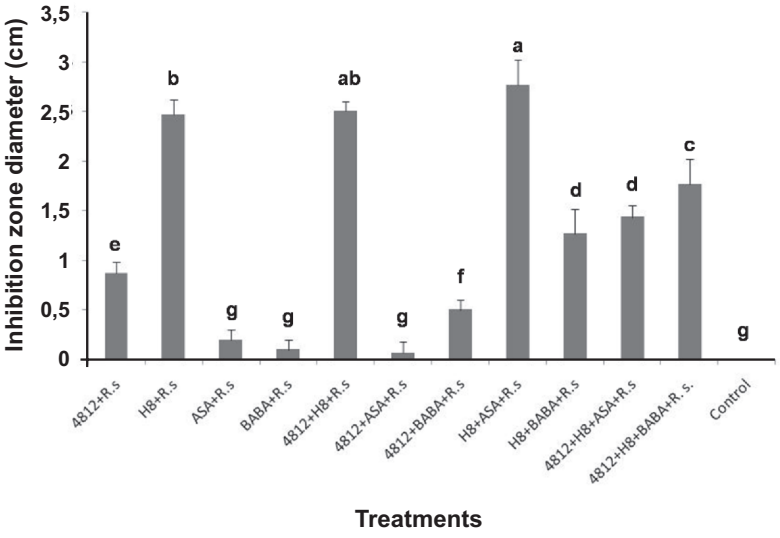

FIGURE 1 - *In vitro* inhibitory effect (cm) of two *Bacillus* strains (4812 and H8) and two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) against *Ralstonia solanacearum* growth on yeast extract peptone glucose agar (YPGA) medium after 72 h incubation at 30ºC in laboratory. Columns with the same letters are not significantly different based on LSD test (P< 0.05), error bars upper each column represent the standard error within same treatment.

TABLE 1 - Tomato bacterial wilt progress expressed as area under disease progress curve (AUDPC) in the tomato plants inoculated by *Ralstonia solanacearum* (R.s.) after treatment with two *Bacillus* strains (4812 and H8) and two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) individually or in combinations

Treatment	AUDPC (%-days)	Biocontrol efficacy (%)
H8+R.s.	306.48 ± 54.79 c	75.72 ± 4.34 a
H8+ASA+R.s.	420.92 ± 154.68 bc	66.65 ± 13.69 ab
H8+BABA+R.s.	544.28 ± 360.77 bc	56.88 ± 19.90 ab
4812+H8+R.s.	404.08 ± 172.79 bc	67.99 ± 12.26 ab
4812+H8+BABA+R.s.	568.52 ± 251.16 bc	54.96 ± 28.58 ab
4812+H8+ASA+R.s.	669.43 ± 280.75 b	46.96 ± 22.24 b
R. s.	1262.20 ± 21.51 a	0 c

Data represented as a mean of five replicates and each replicate contains four plants. Values with same letters within each column means no significant difference according LSD test (p<0.05).

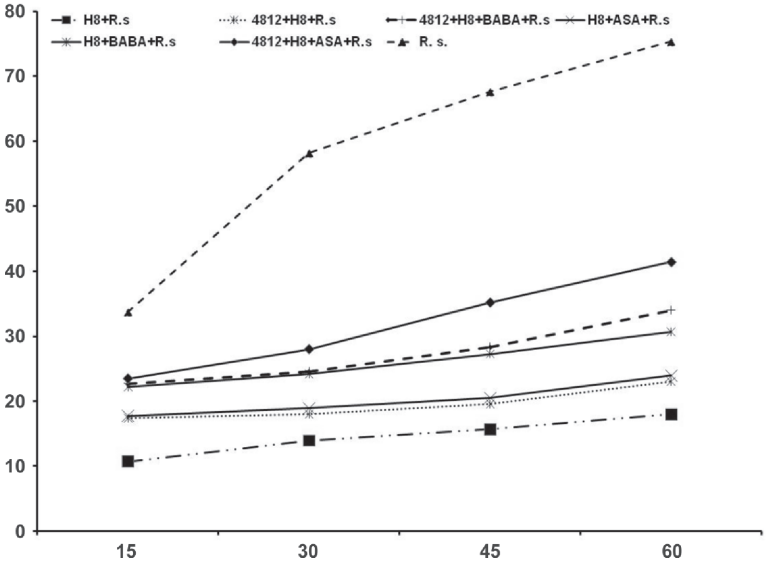

FIGURE 2 - Progression of bacterial wilt symptoms termed as disease incidence on tomato plants treated by two *Bacillus* strains (4812 and H8) and two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) individually or in combinations after being inoculated by *Ralstonia solanacearum* T-91. (ns) and (*) indicate absence and presence of significant differences (Least Significant Difference Test, α = 0.05) among treatments at a particular day after treatment, respectively.

4812+H8 and 4812+H8+ASA were the next treatment in order of superiority. As the most effective treatment, dry weight of tissues was recorded as 1.77 g in H8 and other treatments showed statically similar results (Table 2).

Biochemical analysis

PAL activity was increased significantly in the plants treated with 4812+H8, H8+ASA and 4812+H8+ASA after 5 and 9 days of inoculation compared with other treatments and controls (Figure 3). For PPO activity, all treatments showed significant increase of PPO activity over inoculated and non-inoculated controls especially 5 and 9 days after inoculation. However, the highest induction of this enzyme in H8, 4812+H8 and H8+ASA was observed 9 days after inoculation (Figure 4). Meanwhile, POD enzyme activity was considerably increased in all treatments which followed the same trend of PPO activity. The highest POD activity was observed in the plants treated with H8, 4812+H8 and H8+ASA at 5th day after inoculation (Figure 5).

Native gel electrophoresis of POD and PPO

The results of this experiment determined the number of isoforms as the expression level of PPO and POD. The role of these two enzymes has been reported in many ISR-related studies. The treatment H8+ASA expressed two high intensity isoforms of PPO which were named as PPO1 and PPO2. In addition, 4812+H8 expressed PPO1 and PPO2 with less intensity as compared with H8+ASA. The treatment H8 expressed only one PPO2 (Figure 6).

All three tested treatments expressed two isoforms of POD, which were designated as POD1 and POD2. However H8+ASA and H8 expressed higher intensity POD2 compared with low intensity for 4812+H8 (Figure 6).

Quantification of *R. solanacearum* in infected tissues by real-time PCR

The real-time PCR showed that all treatments were significantly able to decrease the concentration of *R. solanacearum* cells in the plant tissues 60 days after inoculation ($P<0.0001$). Compared with control, the highest reduction of pathogen concentration was observed in the treatment H8+ASA (2.72×10^1 CFU g^{-1} plant tissue). As the next treatments in order of superiority, pathogen concentration was recorded by 6.03×10^1, 8.66×10^1 and 8.97×10^1 CFU g^{-1} plant tissue in 4812+H8, H8+BABA and H8, respectively (Figure 7). In this experiment, the pathogen concentration was least inhibited by 4812+H8+ASA. All obtained PCR products (132 bp) showed an equal Tm (melting temperature) value of 90.23 ± 0.20°C.

DISCUSSION

Enhancement of activity of PGPR strains to improve their beneficial effect for plants whether by applying them in compatible mixtures or by involving them within combined treatments beside other resistant inducer agents is a new approach in crop protection to reduce the disease damage level in economically important crops (Latha et al., 2009; Senthilraja et al., 2010). In this study, two *Bacillus* strains and two chemical inducers were applied individually or in combinations to test their synergistic/antagonistic biocontrol effects against tomato bacterial wilt. This is the first study of synergistic effects of ASA and BABA on PGPRs against tomato bacterial wilt. In *in vitro* tests, *B. methylotrophicus* H8 showed more biocontrol efficacy than *B. subtilis* against *R. solanacearum*. Except for interaction between H8 and ASA, combination with chemical inducers was not able to increase biocontrol efficiency of *Bacillus* strains against *R. solanacearum* in *in vitro* tests. Contrary to previous studies demonstrating antimicrobial effects of chemical inducers against plant pathogens under *in vitro* conditions (Lopez & Lucas, 2002; Nair et al., 2007), no inhibitory effect against *R. solanacearum* was observed by individual application of ASA or BABA. Combined with Acetyl salicylic acid, *B. methylotrophicus* was found to be one of the most effective treatments in *in vitro* tests. Moreover dual mixture of *Bacillus* PGPR strains was found to probably have more efficacy against the pathogen. This finding is consistent with previous studies reporting greater antibacterial activity of dual or triple mixture of PGPR compared with individual application (Jetiyanon, 2007; Latha et al., 2009; Ryu et al., 2007; Shanmugam & Kanoujia, 2011). Under greenhouse

TABLE 2 - Effect of application by two *Bacillus* strains and two chemical inducers individually or in combinations on plant growth parameters in the tomato plants inoculated by *Ralstonia solanacearum* (R.s.)

Treatment	Plant height (cm)	GPE (%)	Plant fresh wt.(g)	GPE (%)	Plant dry wt.(g)	GPE (%)
H8+R.s	21.42 ± 3.77 abc	7.4	14.76 ± 2.91 a	129.48	1.77 ± 0.34 a	105.75
4812+H8+R.s	24.13 ± 3.57 a	20.99	10.76 ± 0.62 b	67.40	1.36 ± 0.08 ab	58.27
4812+H8+BABA+R.s	23.52 ± 2.49 ab	17.96	11.94 ± 1.28 ab	85.76	1.58 ± 0.15 ab	83.79
H8+ASA+R.s	22.83 ± 1.76 abc	14.51	11.75 ± 1.23 ab	82.78	1.51 ± 0.12 ab	75.71
H8+BABA+R.s	23.5 ± 3.13 ab	17.85	10.77 ± 1.67 b	67.44	1.46 ± 0.18 ab	69.25
4812+H8+ASA+R.s	19.96 ± 1.68 c	-	10.73 ± 1.76 b	66.84	1.43 ± 0.37 ab	66.67
R.s.	19.94 ± 1.85 bc	-	6.43 ± 2.91 c	-	0.86 ± 0.4 c	-
Saline solution	24 ± 2.48 a	-	9.1 ± 1.38 bc	-	1.27 ± 0.2 bc	-

Data represented as a mean ± standard error of five replicates and each replicate contains four plants. Values with same letters within each column mean no significant difference according LSD test ($p<0.05$).

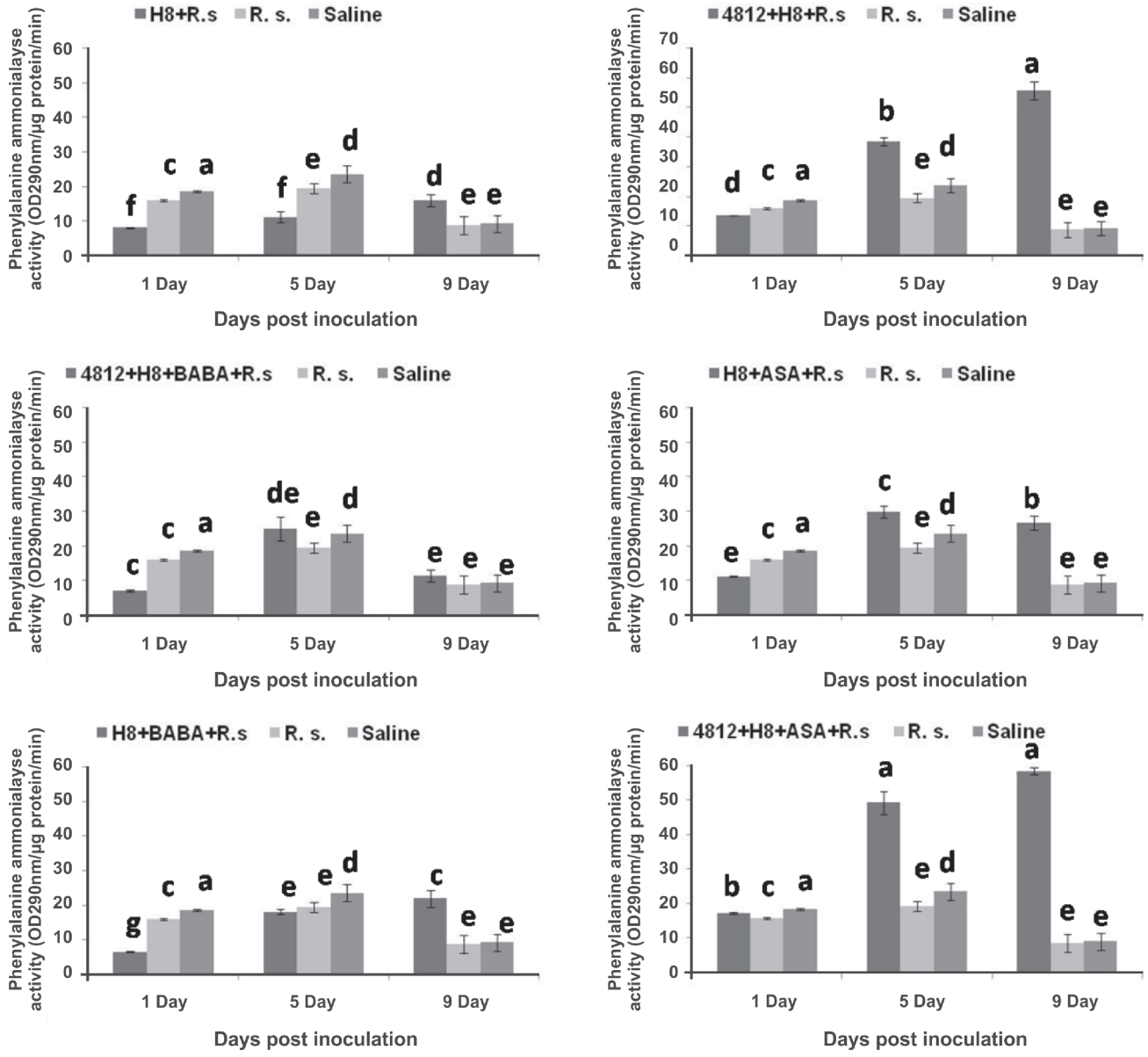

FIGURE 3 - Changes of Phenylalanine ammonia-layse (PAL) activities in the tomato plants treated with two *Bacillus* strains (4812 and H8) individually or combined with two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid against *Ralstonia solanacearum* (R. s.). Bars having the same letters are not significantly different at P = 0.05.

conditions, all treatments significantly reduced disease incidence compared with control. The treatment 4812+H8 significantly increased the height of plants compared with infected control. In addition, all treatments tested in greenhouse were remarkably able to enhance fresh and dry weights. These results confirms the researches carried out by Gupta et al. (2008) and Vogt & Buchenauer (1997) indicating that disease was significantly (p=0.05) suppressed due to integrated application of PGPR strains and chemical inducers compared with their individual applications.

Although defense responses of plants against pathogen have been well documented, little information is found related to the effect of PGPR mixtures on defense-related enzymes and it is possible that each rhizobacterium activates different defense mechanisms. In fact PGPR related researches which assess ISR as a mechanism of disease suppression have been mainly focused on fluorescent *Pseudomonas* spp. and there has been little emphasis on *Bacillus* strains (Kloepper et al., 2004). According to the results, maximum PAL activity was detected in the plants treated with 4812+H8, H8+ASA and 4812+H8+ASA at 5th and 9th day after inoculation. These results suggest that combination of some *Bacillus* strains with each other or with ASA increase the level of plant resistance

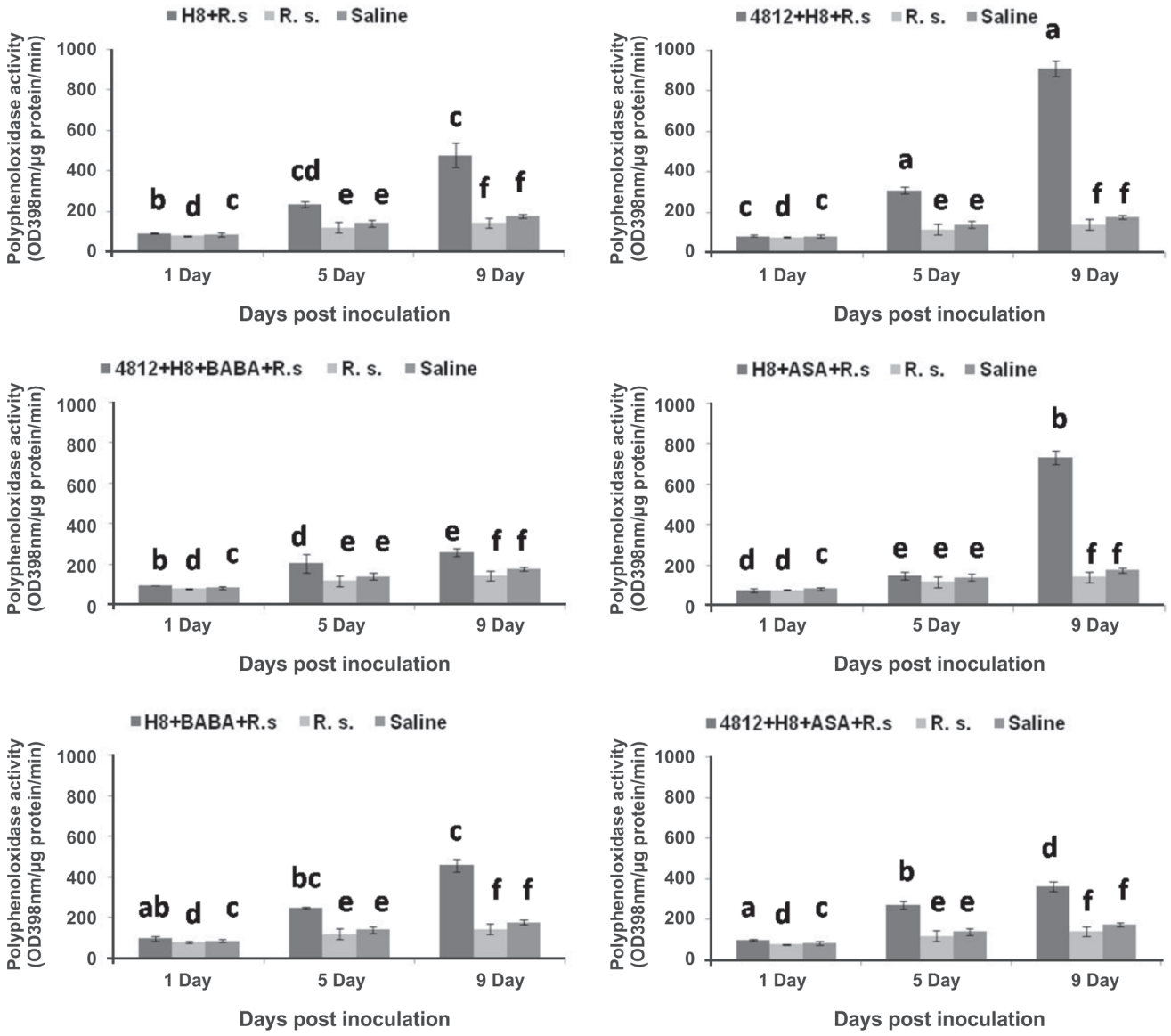

FIGURE 4 - Changes of Polyphenol oxidase (PPO) activities in the tomato plants treated with two *Bacillus* strains (4812 and H8) individually or in combination with two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) against *Ralstonia solanacearum* (R. s.). Bars having the same letters are not significantly different at P = 0.05.

to *R. solanacearum* and it is probably due to phenolic phytoalexins compounds produced through a PAL pathway (O'Neill & Saunders, 1994). It is well known that PAL is an essential enzyme in phenyl propanoid biosynthesis pathway leading to the synthesis of phytoalexins or phenols, which have defense functions in plants, such as fortification of plant cell wall (Nicholson & Hammerschmidt, 1992), antimicrobial activity and synthesis of signaling compounds such as salicylic acid (Wen et al., 2005). In this study, the highest induction of PPO enzyme was observed with the treatments H8, 4812+H8 and H8+ASA. Correspondingly, the maximum POD activity was detected in the plants treated with three treatments H8, 4812+H8 and H8+ASA. Contrary to PPO, the level of PAL was found to be less than controls 1 day after inoculation in all treatments. However, a considerable increase was occurred in PAL after 5 days of inoculation in most treatments. These results are in agreement with Govindappa et al. (2010) showing that PPO and POD activities increased in safflower plants treated by *Pseudomonas fluorescens* after inoculation with *Macrophomina phaseolina*. In addition, an increased level of PPO has been reported in pigeon pea plants when inoculated with *Rhizobia*, *Bacillus cereus* BS03 and *Pseudomonas aeruginosa* against *Fusarium* sp. (Dutta et al., 2008). Accordingly, we can conclude that the reduction in bacterial disease incidence is probably because of enhanced

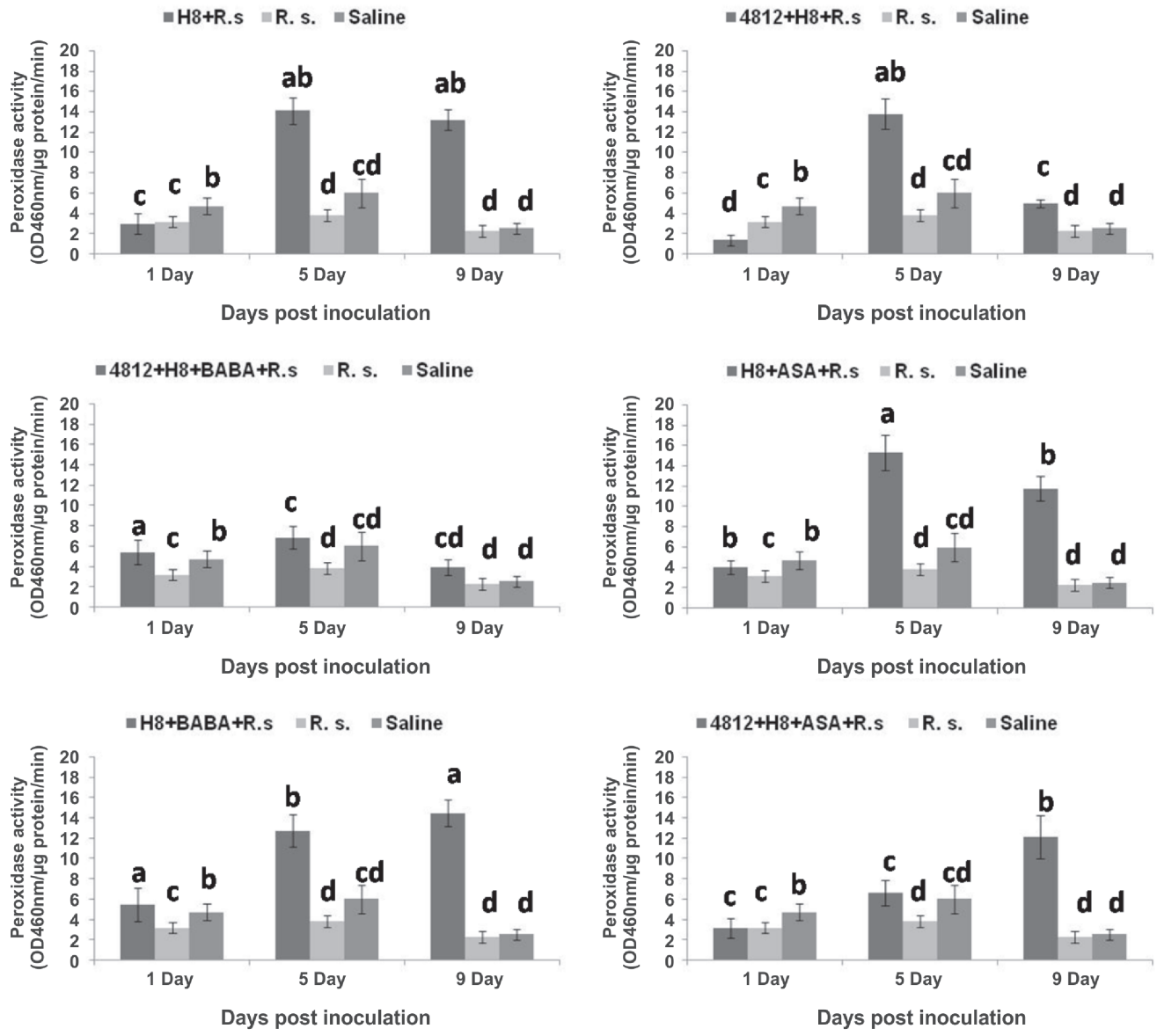

FIGURE 5 - Changes of Peroxidase (POD) activities in the tomato plants treated with two *Bacillus* strains (4812 and H8) individually or in combination with two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) against *Ralstonia solanacearum* (R. s.). Bars having the same letters are not significantly different at P = 0.05.

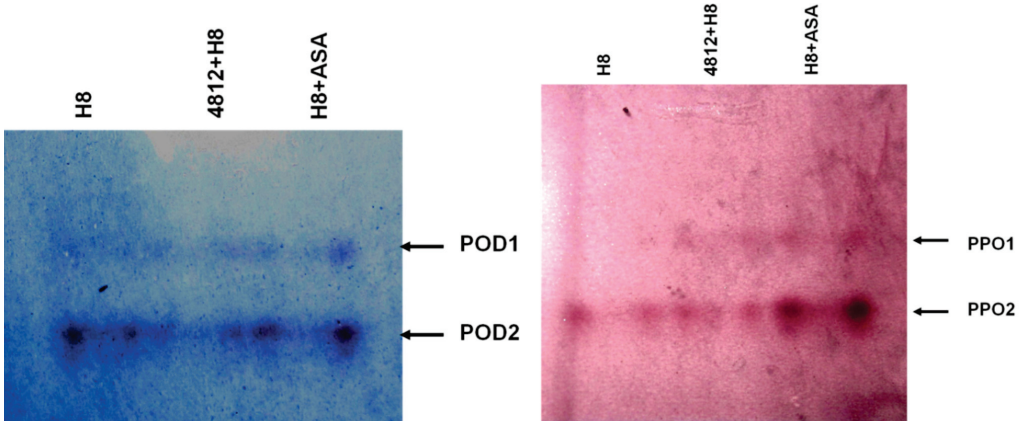

FIGURE 6 - Native PAGE profile of Peroxidase (left) and Polyphenoloxidase (right) isoforms induced in tomato plants in response to treatment with two *Bacillus* strains (4812 and H8) individually or in combination with two chemical inducers (Acetyl salicylic acid and DL-Beta-aminobutyric acid) against *Ralstonia solanacearum*.

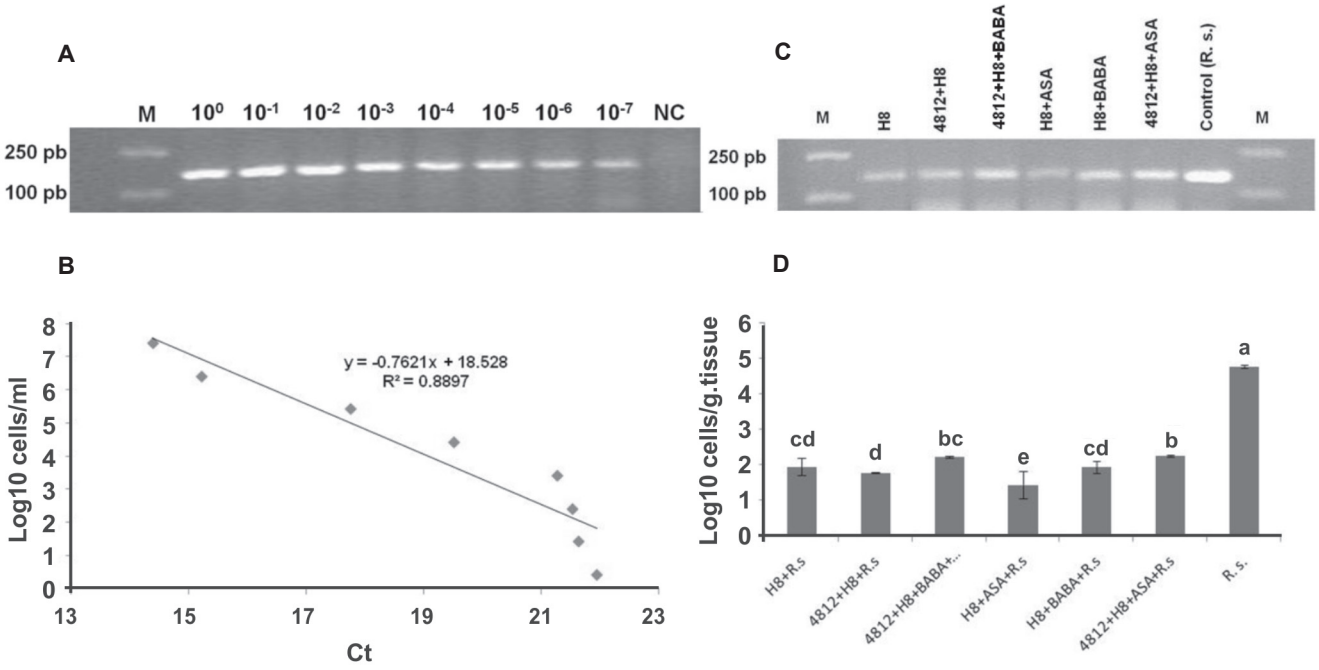

FIGURE 7 - Detection and quantification of *Ralstonia solanacearum* by real-time PCR. (a) Amplification of DNA extracted from different dilutions of culture suspension of strain T-91 by conventional PCR with primer pair RSF/RSR. (b) Standard curve created based on data obtained by plotting the log amount of *R. solanacearum* cells against the threshold cycle of each reaction detected by real-time PCR with primer pair RSF/RSR. (c) Example of amplification by conventional PCR with primer pair RSF/RSR of DNA extracted from tomato plants treated with two *Bacillus* strains (4812, H8) individually or combined with two chemical inducers (ASA and BABA). (d) Quantification results expressed by Log amount of *R. solanacearum* cells in above mentioned treatments, Columns with the same letters are not significantly different based on LSD test (P< 0.05), error bars upper each column represent the standard error within same treatment.

enzyme production elicited by the treatments 4812+H8 and H8+ASA. Induced systemic resistance is associated with the changes of activity of the three enzymes assessed in this study. It is well known that Polyphenol oxidases (PPO) are involved in the oxidation of polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during the microbial invasion (Kolattukudy et al., 1992). Peroxidases (POD) are oxido-reductive enzymes that participate in the wall-building processes such as oxidation of phenols, suberization, and lignification of host plant cells during the defense reaction against pathogenic agents (Sommer et al., 1994). Moreover, Phenylalanine ammonia-lyase (PAL) is an essential enzyme in phenyl propanoid biosynthesis pathway leading to the synthesis of phytoalexins or phenols, which have defense functions in plants, such as fortification of plant cell wall. Both direct inhibition of the pathogen and induced resistance may be involved in the control of different bacterial diseases by *P. fluorescens* (Chitra et al., 2006; Lemanceau et al., 1992). In order to assess different enzymes related to ISR, two methods including spectrophotometry and native gel electrophoresis are usually used in different studies (Shanmugam & Kanoujia, 2011; Sundaramoorthy et al., 2012). Contrary to spectrophotometry, the expression of enzymes and the number of isoforms for each enzyme can be determined in native gel electrophoresis. Because there is a direct relationship between the number of isoforms and the level of enzyme activity, it is more possible to assess the inhibitory efficacy of different treatments. According to the results of spectrophotometry, three treatments including H8, 4812+H8 and H8+ASA were selected for electrophoresis. Of these three treatments, 4812+H8 and H8+ASA were found to have two isoforms of PPO and POD. Furthermore, intensity of PPO1 and POD1 was higher in H8+ASA than 4812+H8 (Figure 6). These results were compatible with *in vitro* tests because among these treatments, the most antibacterial activity against pathogen was found in H8+ASA, 4812+H8 and H8, respectively. In this study, real-time PCR showed significant reduction of *Ralstonia* population in tomato plants treated with all treatments and H8+ASA was the most effective treatment. In general, high positive correlation value 0.95488 was recorded between disease parameter AUDPC and *Ralstonia* population estimated by CFU g^{-1} plant tissue. Similar studies have already shown that pathogen population in plant tissues may decrease due to inhibitory activity of *B. subtilis* (Cao et al., 2011; Lemessa & Zeller, 2007).

Contrary to *in vitro* experiments, H8 showed relatively inhibitorier efficacy in greenhouse tests compared with other treatments showing that chemical inducers do

not have any synergistic activity with this PGPR against the pathogen under *in vivo* conditions. Although ASA significantly reduced the antibacterial activity of 4812 in *in vitro*, BABA was able to increase its antibacterial efficacy indicating that the synergistic/antagonistic *in vitro* effect of different plant defense inducers is variable to different PGPRs. Despite this fact that no additional biocontrol efficacy was observed in pot experiment due to combination with each other and/or chemical inducers, this study showed these methods can improve biochemical characteristics of a successful biocontrol *in vitro*. Additionally it became clear that the synergistic effect of plant defense inducers is a promising control method although it is variable to different *Bacillus* strains. In addition, this research showed that biocontrol efficacy of *Bacillus* strains is not quite affected by enzymatic activities, and other mechanisms are involved in their inhibitory activity against the pathogen *in vivo*. It is suggested to assess the inhibitory efficacy of different PGPRs and chemical inducers under different environmental and field conditions on different tomato cultivars.

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