Bacillus atrophaeus HAB-5 secretion metabolites preventing occurrence of systemic diseases in tobacco plant

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Abstract Finding synthetic pesticide alternatives for health and a healthy environment has become a crucial issue for scientific research. A number of studies have reported efficacy of *Bacillus* species on promoting plant development, as well as protecting plants against pathogen invasion, especially pathogenic fungi and bacteria. However, little was known about *Bacillus* species in controlling viral diseases. In this study, *Bacillus atrophaeus* strain HAB-5, isolated from cotton field, Xinjiang, China efficiently promoted the growth of tobacco plants. According to the results, the treatment with the strain HAB-5 increased the expression of *NtEXP1* and *NtEXP2*. Then the *Tobacco mosaic virus* (TMV)/ *Nicotiana tobacco* system was employed to evaluate virus resistance induced by strain HAB-5. Tobacco leaves were treated with antimicrobial metabolites of strain HAB-5 (1 mg/mL), and 12 h later the treated leaves were challenged with TMV via rub-inoculation. The results showed that disease symptoms were

obviously compromised by tobacco leaves treated with strain HAB-5, and the viral accumulation level was reduced extensively. Moreover, it was found that the signaling regulatory gene (*NPR1*), defense genes (*PR-1a*, *PR-1b*, *Chia5*), and hypertensive response related genes (*Hsr203J*, *Hin1*) were up-regulated in plants treated with the metabolites. Altogether, these accumulated results strongly support strain HAB-5 to be a biological controlling agent against TMV.

Keywords *Bacillus atrophaeus* · TMV · Biocontrol

Introduction

Plant growth promoting rhizobacteria (PGPR), the beneficial bacteria having ability to enhance plant growth and control plant diseases by colonizing the roots have been well recognized for various beneficial effects in crop plants (Bodelier et al. 2000; Eric and Donohoe 2003; Nie et al. 2015; Ge et al. 2015). Their usage as biological control agent increases health and productivity of different plant species under various edaphic conditions (Kloepper et al. 2009; Chaudhry et al. 2012; Bharti et al. 2016; Devendra et al. 2016; Bharti et al. 2016); PGPR often resists phytopathogenic negative effects by inhibiting them (Leeman et al. 1996; Wang et al. 2009; Khan et al. 2012). Various studies has been carried out focusing on the identification of *Bacillus spp.* for their ability to promote plant growth and disease control (Emmert and Handelsman 1999;

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Kloepper et al. 2004; Niu et al. 2011; Piromyou et al. 2011). Various strains of *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, elicit significant reductions in the incidence severity of various diseases on a diversity of hosts (Zhang et al. 2004; Han et al. 2006; Raj et al. 2008; Raza et al. 2016). Therefore, several PGPR based products are recently available on commercial basis mostly containing *Bacillus* spp. Strains (Emmert and Handelsman 1999).

These PGPRs, commonly known biocontrol agents, are famous for producing variety of metabolites that can improve plant resistance against different crop pathogens, including lipopeptides, lytic enzymes, etc. (Worasatit et al. 1994; Ongena et al. 2007). It is interesting to note that different studies focused on harvesting these secreted compounds from culture suspension, cell filtrate and crude extract have been applied directly on plant as foliar treatment. For example, culture suspension of *Trichoderma harzianum* sprayed on leaves successfully reduced gray mold on fruits and foliage (Lo et al. 1997; Luca et al. 2016). Leaves sprayed with the spore suspension of *Bacillus subtilis* reduced different crop disease (Batchimeg and Dondov 2015). Zhang et al reported the efficacy of cell free supernatant of *B. cereus* on plant leaves to control *Sclerotinia sclerotiorum* (Zhang and Xue 2010). The foliar spray treatment of *Streptomyces* spp. was reported efficiently to control rice sheath blight pathogen (Yang et al. 2017). The crude extract metabolites from different strains of *B. amyloliquefaciens* were capable of reducing the severity of different diseases on plant (Ji et al. 2013).

Despite the successful use of *Bacillus* species, reports about *Bacillus atrophaeus* application are rare. We have reported that *B. atrophaeus* strain HAB-5 can control a broad spectrum of crop disease-causing agents (Rajaofera et al. 2017, 2019). *B. atrophaeus* are diverse in terms of plant protection playing important role in crop yield reducing risks. Furthermore, keeping in view the growing concerns over global food safety and environmental quality, efforts in increasing practical use of natural products need a better understanding and research.

The current investigation is an effort to elucidate the concept of *B. atrophaeus* strain HAB-5 as a biocontrol resource. We have investigated strain HAB-5's ability to promote plant growth and evaluate the potential of crude metabolites extracts to control TMV on tobacco plant.

Materials and methods

Bacterial strain

The isolate designed as strain HAB-5 was obtained from a healthy cotton field in Xinjiang province, P.R China. After isolation, strain HAB-5 was examined for morphological, physiological and biochemical characteristics (Rajaofera et al. 2017, 2019).

Plant material, growth condition and TMV culture

Tobacco seeds were surface sterilized (2 min 70% ethanol, 5 min 5% calcium hypochlorite), rinsed several times with sterile water, and placed on Petri dishes containing half strength Murashige–Skoog (MS) medium containing 0.8% agar and 1.5% sucrose. The seeds were vernalized in dark for 72 h at 4 °C to break dormancy, and then germinated to pot culture containing autoclaved soil (vermiculite and Pindstrup substrate, in the ratio of 1:1). TMV was maintained by sap inoculations on *N. tabacum* cv. Samsun NN plants.

Assessment of plant growth promotion and plant inoculation

The ability of strain HAB-5 to promote plant growth was assessed. Tobacco seeds were surface sterilized as described above. The seedlings were grown till 3–4 leaves stage. Biological replicates of each treatment were used to minimize the influence of an individual plant results. Seedlings were transplanted individually into pots (with the same soil on which tobacco seeds were germinated) and grown.

Ten days after transplantation, bacterial strain was suspended in culture medium and incubated at 28 °C, 180 rpm for 24 h. After incubation, bacterial cells were harvested by centrifugation and re-suspended in distilled water, adjusted to 1×10^6 – 10^7 CFU/mL to maintain uniform cell density. The cell suspension (50 mL) was drenched in soil around each plant twice at weekly intervals. Plants only drenched with water were used as control. Experiments were carried out in three replicates and every replication contained 12 plants.

The experiment was terminated 60 days after inoculation. Plant length, fresh, dry weight of roots, shoots and chlorophyll content were measured from all harvested plants. Percent decrease or increase in each parameter was calculated with respective controls.

The data obtained was analyzed statistically for standard error.

Quantification of HAB-5 strain colonization in *N. tabacum* rhizosphere

The resistant of the strain HAB-5 to antibiotics was first determined. The strain was streaked onto LB agar plate containing different antibiotics including 50 mg/L of streptomycin, 100 mg/L of rifampicin, 100 mg/L of ampicillin and 200 mg/L of kanamycin. The antibiotic which had the positive result was used for further experimentation.

The abundance of the PGPR rhizosphere was then quantified. Total root systems and tightly-adhering soil were harvested at five time points 0, 15, 30, 45 and 60 dpi. The samples were thoroughly mixed and 1 g of mixture was suspended in 9 ml of sterile 0.85% NaCl and vortexed for 5 min. The suspensions of each sample were plated on LB agar medium with 50 mg/L of streptomycin and 200 mg/L of kanamycin according to a previous method (Zhou et al. 2015). In order to inhibit the fungal growth, 100 mg/L of cycloheximide was also used. Colony Forming Units (CFUs) counting (based on morphological shape of HAB-5) was carried out after incubation at 28 °C for 24 h.

Biological control of tobacco mosaic virus by HAB-5 treatment

First, the antimicrobial metabolite from strain HAB-5 was extracted. The organism was cultured in LB for 48 h at 28 °C in an incubator shaker at 180 rpm. The fermentation broth was centrifuged at 10,000 rpm for 10 min at 4 °C. *petroleum* ether were added into the cell free supernatant in 1:1 ratio and shaken vigorously. The organic phase was concentrated to dryness under vacuum using rotary evaporator. The crude extract was lyophilized and re-suspended with distilled water (1 mg/mL). The substance obtained from the non-inoculated culture medium with same procedure was used as a control.

The evaluation of strain HAB-5 efficacy against TMV reduction and infection ratio was investigated. Two different set of experiment were carried out in order to evaluate strain HAB-5 effect against TMV. In one set of experiment, plants drenched with the bacteria root inoculation were subjected of TMV inoculation. In the other set of experiment, the crude

extract antimicrobial metabolites from the strain were applied for foliar treatment and 12 h later the treated leaves were challenged with TMV via rub-inoculation. The effective treatment was used for further experiments.

For disease severity evaluation, plant treatments were assigned into three groups included HAB-5 + TMV group (sprayed with the antimicrobial metabolite crude extract from the strain 12 h before inoculation with TMV), mock+ TMV group (sprayed with the extract products from LB 12 h before inoculation with TMV) and control group (only sprayed with water, and PBS was used in the place of TMV). All experiments were carried out with three replicates.

The virus inoculum was prepared in the tobacco juvenile. Leaf tissue was macerated in 10 mL of 50 mM potassium phosphate buffer (pH 7.0). The suspension was sprayed on 3–4 leaf stage tobacco seedlings. Disease severity symptoms (mosaic, leaf deformation and stunting) were observed at 7 days post inoculation (dpi) according previous method (Manidipa et al. 2013). The rating scale were 0 = no symptoms observed; 1 = light mottling and a few thin yellow veins; 2 = mottling and vein clearing unevenly distributed on the leaf; 3 = mottling, leaf distortion, and stunting; and 4 = severe mottling, leaf curling, and stunting.

Enzyme-linked immunosorbent assay (ELISA)

ELISA analysis was carried out to determine the relative level of coat protein (CP) in leaves. Samples were ground in a 50 mM carbonate buffer, loaded onto ELISA 96-well plates, coated with plastic wrap, and incubated at 40 °C overnight. After washing with a washing buffer (0.5% Tween 20 in PBS), the plates were blocked with 3% nonfat dry milk in PBS at 37 °C for 1 h, and washed five times. Next, the primary antibody rabbit anti-TMV immunoglobulin (Ig) (1.0 µg/mL) was incubated in the 96-well plates at 37 °C for 1 h and rinsed. The secondary antibody goat anti-rabbit IgG-HRP (1/10,000) was added to each well, then incubated at 37 °C for 1 h and washed. Finally 3, 3', 5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide were used as the substrate for developing at 37 °C for 15 min. The reaction was stopped by the addition of 50 µg of 1 M H₂SO₄. The plates were read in micro plate photometer.

Detection of hydrogen peroxide accumulation

To detect accumulation of hydrogen peroxide, leaf tissues were stained with 1 mg of diaminobenzidine (DAB, pH 3.8). Leaves were washed in distilled water and cleared with 95% (v/v) for 24 h. DAB staining visualizes hydrogen peroxide as dark brown precipitate under the light microscope.

Measurement of cell death

Experiment was carried out as describe earlier (Singh and Upadhyay 2014). Leaves incubated with TMV for 7 days were soaked in 0.05% Evans blue for 24 h, rinsed, dried, and then boiled in anhydrous alcohol and glycerol (9:1 V/V) for 30 min to remove the chlorophyll. A blue area represents cell death. The amount of cell death in tobacco leaves was also quantified. Stained leaves were added to 4 mL 1% SDS water solution for 3 days to extract Evans blue. The supernatant was collected after centrifugation at 10,000 rpm for 5 min and the absorbance was measured at 600 nm.

Nitric oxide production

According the previous method (Jia et al. 2016), the epidermis was peeled neatly from the surface of rosette leaves and then incubated in MES/KCl buffer (10 mM MES/KOH, 50 mM KCl, 100 μ M CaCl₂, pH 6.5) for 30 min in the light. Then, incubated in Tris/KCl buffer (10 mM Tris, 50 mM KCl, pH 7.2) containing 5 μ mol/LDAF-2DA (sigma) for 30 min in the dark at room temperature. Excess DAF-2DA was washed three times with fresh Tris/KCl buffer, the epidermis was placed in Tris/KCl buffer containing strain HAB-5 for 15 min, Tris/KCl buffer as contrast. NO production was photographed under fluorescence microscopy. The NO content in leaves was determined using Griess reagent (Beyotime Biotech) according to the manufacturer's instructions. Rosette leaves were sprayed with the antimicrobial metabolites from strain HAB-5 or products from inoculated LB, incubated for 15 min, weighed and quickly frozen in liquid nitrogen for subsequent NO content determination. The experiments were repeated at least three times for each treatment.

Evaluation of biophysical and biochemical parameters

The ion leakage of systemic leaf tissue was measured as described as earlier (Fan et al. 1997). Leaves were immersed in 10 mL and 0.4 mL mannitol solution at room temperature with 200 rpm shaking for 3 h, and the conductivity was measured. The total conductivity was determined after boiling leaves for 10 min. The conductivity due to leakage was expressed as the percentage of the initial conductivity versus the total conductivity.

The amount of chlorophyll present in the tobacco leaf was estimated. Fresh tissue (300 mg) was ground with 10 ml of 80% acetone. The homogenate was centrifuged at 3000 rpm for 15 min. Absorbance was measured at 645 nm and 663 nm. The chlorophyll content was determined according the previous method (Bashan et al. 2006).

Total sugar determined by improved procedure (Gerchacov and Hatcher 1972). 0.2 g of fresh tobacco leaf tissue was crushed in 80% methanol and centrifuged at 10,000 rpm for 10 min. The supernatant was incubated in water bath at 70 °C. Equal volume of 5% phenol and 5 ml of H₂SO₄ were added and absorbance was measured at 640 nm. A standard curve was prepared using different concentration of standard glucose solution. The estimation of total sugar content was done on background of the graph.

Total proline content was quantified by the acid-ninhydrin method (Bates et al. 1973). Leaf tissue (500 mg) was ground in 10 ml 3% sulfosalicylic acid. 2 mL of supernatant was mixed with equal volumes of acid-ninhydrin and acetic acid. The mixture was incubated at 100 °C for 1 h. Reaction was finished in an ice bath and extracted with 4 ml of toluene by using a vortex mixer for 15–20 s. Absorbance was measured at 520 nm.

The leaf malondialdehyde (MDA) content was determined following the protocol described previously (Qiu et al. 2008). 0.3 g of fresh leaf tissue was homogenized in 5 ml of 5% trichloroacetic acid and centrifuged at 8,000×g for 15 min. 1 mL supernatant was then mixed with 2.5 mL of thiobarbituric acid and heated at 100 °C in a water bath for 20 min and quick-chilled on ice. The mixture was centrifuged at 10,000 x g for 5 min. The absorbance was measured at 532 nm, 600 nm and 450 nm. The MDA content was determined as follow:

$$\text{MDA } (\mu\text{mol g}^{-1}) = 6.45(A_{532}-A_{600})-0.56A_{450}$$

RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was isolated from 100 mg leaf tissue of tobacco plants using RNA prep Pure Plant Kit Tiangen Biotech (Beijing, China Co., LTD) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized with the Fast Quant RT Super Mix Kit (Tiangen). Primers sets targeting genes related to development, signaling regulatory genes, defense genes, and hypersensitive response related genes were used for expression analysis (Table 1). SYBR Green Master Mix (Agilent technologies, Santa Clara, CA, USA) was used for qRT-PCR. *N. tabacum* elongation factor 1- α (EF1 α) was used as a constitutive reference transcript (Lu and Du 2012). The threshold (Ct) value for each gene was normalized against Ct for EF1 α . Relative expression level was analyzed using the delta-delta-Ct method (Livak and Schmittgen 2001). The following protocol was used for amplification: 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 40 s and 72 °C for 60 s.

Statistical analysis

The data were subjected to an analysis of variance using SPSS software (SPSS Inc., Illinois, U.S.A.). When a significant F test was obtained at $p < 0.05$, the separation of the treatment means was accomplished by Fisher's protected LSD.

Results

The strain HAB-5 promotes growth of tobacco plants

In greenhouse conditions up to 60 days, *B. atropis* strain HAB-5 exposed remarkable ability to improve growth of tobacco plant. Inoculated plant exhibited significant increase in fresh shoot weight, dry shoot weight, fresh root weight and dry root weight by 76.47%, 80.58%, 71.71%, 82.10% respectively compared with non-inoculated control plant. Noticeable differences in plant vigour and dissimilarity of leaf area were observed (Fig. 1b). The inoculated plant was distinctly taller than

Table 1 List of primers used in this study

Primer name	Primer ID	Sequences (5' to 3')	Amplicon size (bp)
NtEXP1-F	160323_010A03	ATTCAACAGGGTATGGTACA	175
NtEXP1-R	160323_010B03	GCATAATTTGGTGGACAA	
NtEXP2-F	160323_009E06	GGATGGCAGAGTGCTCAT	260
NtEXP2-R	160323_009F06	GCATAATTTGGTGGACAA	
NtEXP6-F	160323_010C03	CCCTGTGTGTTTATCGTCG	192
NtEXP6-R	160323_010D03	GTTTGCCAGTTCTGACCC	
Hsr203J-F	160323_009B05	AGCTATGAAAAAGGGGAAA	253
Hsr203J-R	160323_009C05	AACCATTAGAACGTGACAATC	
Hin1-F	160323_010B01	TGACTATTAGAAACCCCAACA	234
Hin1-R	160323_010C01	CTTCCATCTCATAAACCCCT	
PR-1a-F	160323_010D01	AACCTTTGACCTGGGACGAC	264
PR-1a-R	160323_010E01	CAACACGAACCGAGTTACGC	
PR-1b-F	160323_009D05	AATGTTAGTTATTCGTTCCG	338
PR-1b-R	160323_009E05	AGTAGAGCGGAGGTAAGTAA	
Chia5-F	160323_009H05	CAGGGCGGCACTGCTTCT	207
Chia5-R	160323_010F01	ATTCCATCGCTTCCACTAATA	
NPR1-F	160323_010G01	TTCGTCGCTACCGATAACAC	208
NPR1-R	160323_010H01	TTCTCGCTGACAAAACGCAC	
EF-1a-F	160323_009A06	ATCAATCCAGGTCATCATCA	142
EF-1a-R	160323_009B06	AAGTTCCTTACCAGAACGCC	

Fig. 1 Effectiveness of *B. atrophaeus* HAB-5 to promote tobacco plant growth. **a** Measurement of growth parameter on each treatment **(b)** Inoculation effect of HAB-5 on tobacco plant comparing with non-inoculated plant. **(c)** Effect of HAB-5 on plants root. Representative plants of each treatment were photographed 45 days post inoculation. **(d)** Rhizosphere colonization of HAB-5 in *N. tabacum*. Data represent the numbers of CFU/g of plants rhizosphere with standard deviation from three replicates. Experiments were carried with three replicates and all replication contained twelve plants

the non-inoculated plants by 45.76% and displayed much darker green leaves.

To clarify the mechanism of variation between non-inoculated and those inoculated with the strain HAB-5, gene expression analysis was performed. We have

selected two effectors, EXP1, EXP2 that are tobacco expansions encoded by *NtEXP1*, *NtEXP2* which function for loosen cell walls, regulating plant development, promoting cell division and extension, thereby promoting plant growth mediated by ethylene (Kuluev et al.

Fig. 2 Expression of expansin genes in leaves of different ages of *N. tabacum* cv. Samsun-NN in HAB-5 inoculated and non-inoculated plant. Plants leaves were harvested at indicated time

interval for RNA extraction. Values were presented as means ± SD from three independent measurements

Fig. 3 Severity of TMV mosaic symptom on tobacco leaves 7 days post TMV inoculation. HAB-5 + TMV: Leaves treated with the bioactive substance 12 h prior to TMV challenge; Mock+TMV: Leaves treated with extracted products from non-inoculated LB; Control: Leaves sprayed with water

2016). High expression levels were noticed in the strain HAB-5-inoculated plant. *NtEXP1* and *NtEXP2* genes showed parallel tendencies that expressed high level at 60 dpi in inoculated plant. In contrast both two genes showed a significant lower expression in non-inoculated plants (Fig. 2a and b).

To be an effective PGPR, bacteria must be able to colonize roots because they need to establish themselves in an adequate environment to produce beneficial effects. It was found that the strain HAB-5 was resistant to streptomycin and kanamycin. The colonization of strain HAB-5 of the tobacco plant soil was measured at different time points (Fig. 1d). The result showed that the amount of strain HAB-5 in soil was increased from 10^5 to 10^7 CFU per gram (0 h to 60 h) of rhizosphere soil in inoculated plants. In contrast, no HAB-5 strain was observed in non-inoculated soil. This indicates that strain HAB-5 successfully colonized the tobacco rhizosphere.

Metabolites of HAB-5 alleviate the virulence of TMV on tobacco plant

Two different applications were evaluated. Plants were amended with the bacteria suspension before virus inoculation. It was found at few days after virus inoculation; the bacteria non-inoculated plant was weak, faded and dead. The bacteria inoculated plant had vitality and did not display any disease symptom. Four weeks later, the bacteria inoculated plant showed disease symptom. Another set of experiment consist the application of crude extract metabolites from the strain as a foliar spray treatment

12 h before TMV inoculation. The antimicrobial-pretreated plant did not show any disease symptom except when the leaves were rubbed with the virus. The secretion metabolites from strain HAB-5 were found to inhibit systematic disease on tobacco plants.

Disease severity was quantified 7 days after TMV inoculation. Mock treated plants showed severe mosaic symptoms on the surface area of systemic leaves as compared to the healthy control plant which had a 0-scale disease rating. Infected plants showed severe mosaic symptom on surface area of systemic leaves. Plants pretreated with the antimicrobial metabolites exhibited significant tolerance to TMV infection, only showed 0–1 scale (Fig. 3).

Intense reduction of chl a, chl b and total chlorophyll content were noticed in TMV mock treated plants by 63.15%, 62.91% and 63% respectively, which was ameliorated by 41.84%, 41.43% and 41.59% respectively in pretreated leaves (Fig. 4e and f). Pretreated leaves showed a significant (50.94%) reduction in membrane ion leakage (Fig. 4b), MDA by 50.75% and proline by 27.63% as compared with mock treated leaves (Fig. 4a and c). An increased sugar content of 36.59% was noticed in pretreated leaves comparing to the control, while it was reduced by 94.89% in TMV mock treated leaves compared to those pretreated with the antimicrobial metabolites (Fig. 4d).

Both ROS accumulation and cell death are greatly attenuated in tobacco leaves treated with metabolites of HAB-5/TMV

The relationship between systemic resistance, cell death and H_2O_2 in the tobacco leaves was investigated. DAB staining revealed that H_2O_2 production was concentrated in the whole leaf of mock treated plants; it was nil in healthy control plant leaves and slightly appeared in pretreated-with-antimicrobial leaves (Fig. 5a). Leaves were also stained with Evan blue for cell death determination (Fig. 5b). No visible cell death was observed in control healthy leaves. A slight effect was found in pretreated leaves. While mock treated leaves had a 14.9-fold increase in stained cells (Fig. 5c). This data indicate that strain HAB-5 could inhibit ROS accumulation and cell death in leaves during TMV infection. It can be concluded that there is a mechanistic connection between H_2O_2 accumulation and cell death. Low H_2O_2 accumulation was fundamental and adequate for inhibiting cell death in tobacco leaves.

Fig. 4 Biophysical and biochemical characters response in systemic leaves of *N. tabacum* cv. Samsun-NN. Revealing the improved plant efficiency in treated laves comparing with untreated

Metabolites of HAB-5 induced nitric oxide production in tobacco leaves

It has been shown that nitric oxide (NO) plays important roles in immune regulation and is recognized as important signaling messenger involved in plant immunity. After spraying the leaves with 1 mg/mL of antimicrobial metabolites, the NO content in leaves was increased up to 2.32 nmol/g, whereas it was 1.11 nmol/g in the mock treated leaves (Fig. 6). This result showed that strain

leaves. The data was calculated at 7 day post TMV inoculation. Different letters indicate significant differences between treatments (Duncan's multiple range test $P < 0.05$)

HAB-5 induced NO production in epidermal cells of tobacco. It is suggested that the NO stimulation on pretreated leaves contributed to the activation of defense responses in tobacco plant against TMV.

Gene expression on tobacco leaves and ELISA analysis

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to investigate the role of HAB-5 in patterns genes included *PR-1a*, *PR-1b* and *Chia5*

Fig. 5 Determination of H_2O_2 and cell death on *N. tabacum* cv. Samsun-NN leaves. **a** High accumulation of H_2O_2 (represented by brown color in leaf) was detected in untreated leaves, followed by TMV infection pretreated leaves. No H_2O_2 accumulation was observed in control leaves. **b** Evan blue staining of tobacco leaf tissues treated with HAB-5/TMV and Mock/TMV. At 12 h post-spray with HAB-5 at 1 mg/mL or water (Mock), the leaves were challenged with TMV via rub-inoculation. The blue areas on leaves represent cell death. **c** Quantification of cell death in leaf tissues treated with HAB-5/TMV and Mock/TMV via detecting the absorbance of Evans blue from treated leaves indicated above. Experiment were carried out 7 days after TMV inoculation. Values means $\pm$ SD from triplicate independent measurements

(Pontier et al. 1994); *NPR1* gene (Pontier et al. 1994); Hypersensitive response (HR) markers, *Hin1* and *Hsr203J* (Mou et al. 2003; Kim and Zhang 2004). Total RNA was isolated from the tobacco leaf tissues at different time intervals from 0 h to 96 h post treatment (hpt). The amount of the *PR-1b* and *NPR1* genes transcript peaked at 96 hpt. Expression of *PR-1a* was increased at 72 h and overexpressed in pretreated leaves. Transcripts of the *Chia5* gene reached its maximum at 48 h and gradually increased with time (Fig. 7c). Expression of *Hsr203J* gene was increased with the time and high level was found at 96 h (Fig. 7a). Similarly, the *Hin1* gene also showed high levels of expression at 96 hpt (Fig. 7b). By contrast, in mock treated leaves, the transcription of *PR-1a* and *PR-1b* genes was not detected (Fig. 7e and f) and was very low in

NPR1 and *Chia5* (Fig. 7c, d). Increased and over-expression of these genes confirm the success of strain HAB-5 in enhancing TMV resistance.

ELISA analysis was performed to quantify the amount of TMV CP. The healthy control plants did not show any virus accumulation; while virus accumulation in TMV mock treated plants was found to be approximately 3-fold higher than antimicrobial metabolite treated plants (Table 2). Based on the plant symptoms and virus accumulation, this result confirms that strain HAB-5 reduced the severity of TMV disease.

Table 2 Enzyme linked immune sorbent assay (ELISA) detection of TMV accumulation in rub-inoculated leaves at 7 dpt.

Fig. 6 Treatment with HAB-5 greatly increases the production of NO in tobacco leaves. **a** Microscopy observation of NO production (green) in tobacco leaves treated with HAB-5. **b** Determination of NO content in tobacco leaves treated with HAB-5 using Griess reagent. Mean values $\pm$ SD from three independent measurements

Discussion

In this study, the mechanism used by *Bacillus* spp. to stimulate plant growth is still elusive (Schmidt and Delaney 2010; Lyngwi et al. 2016). Strain HAB-5 cell suspension supported growth up to 60 days post inoculation under greenhouse conditions and increased tobacco plant growth parameters. Gene expression investigations on the interaction with *Bacillus* sp. still provide an avenue for understanding the molecular mechanisms convoluted in enhancing plant growth and increasing yield.

Biological control by using PGPR mechanisms emerged long time ago but unfortunately chemical control has proved to be irreplaceable. One of the major limitations to their success is because they are adapted for the soil ecological conditions and the mechanisms for disease control agents are found to be associated with plant roots. It is known that a wide range of soil microbial species secrete different kinds of antimicrobial compounds. Among these, surfactin and fengycin lipopeptides of *Bacillus subtilis* were reported as elicitors of induced systemic resistance in plants (Ongena et al. 2007, 2008). Research has shown that lipopeptides

antibiotics of *Bacillus* spp. are involved in the suppression of Fusarium wilt in tomato (Abdallah et al. 2017).

As previously reported, except for root drenching, foliar application is one of the efficient methods for foliar disease control. Studies have focused on harvesting these compounds from the culture suspension, culture supernatant and crude extract sprayed on plants for disease control (Lo et al. 1997; Ji et al. 2013; Yang et al. 2017). The present study provides evidence that crude extract metabolites of efficacy from the PGPR strain HAB-5 in reducing the virulence of TMV on tobacco plant. Based on visible symptoms, the crude extract metabolites extracted from the strain protect plant from TMV. It was found that the crude extract metabolites can inhibit the systemic disease on tobacco plant. ELISA analysis also showed the ability of the strain to decrease (by approximately 3-fold) virus accumulation in pretreated leaves. These data confirm successful protection by the strain HAB-5 against TMV and consolidate findings that crude extract can be used for diseases control (Ji et al. 2013).

To further clarify antimicrobial-metabolite mechanisms which will benefit in controlling plant diseases, physiological, biochemical, anatomical changes between the treatments were assessed. First, chlorophyll is important in photosynthesis for plant bioproductivity. In the present study, significant increases in chlorophyll content were observed in strain HAB-5 treatment compared to the control. Similar to other biocontrol methods (Mou et al. 2003), foliar treatment helped plants to maintain chlorophyll during TMV infection. Then, the content of MDA and ion leakage were reduced in pretreated leaves. It has been reported that MDA was induced during stress on plants leading to enhanced membrane peroxidation disease tolerance mechanisms (Bashan et al. 2006; Li et al. 2013). In addition, the molecular mechanisms of sugar in disease remain largely unknown. We found that the sugar content was increased in treated plants. The possible explanation is high sugar content helps to reduce disease in plants. This finding suggested the ex vivo treatment maneuvered the ion leakage sugar, proline, chlorophyll, and malondialdehyde to protect plant cells integrity leading to induced TMV tolerance.

The hypersensitive response is a mechanism used by plants to prevent the propagation of pathogen infection, and commonly results in the formation of necrotic lesions (Qiu et al. 2008). It is characterized by the appearance of

Fig. 7 Comparison of expression level of disease defense-related genes between tobacco leaves treated with HAB-5 + TMV and Mock+TMV at different time points. HAB-5 + TMV and Mock + TMV represent *N. tabacum* plants pretreated with 1 mg/mL of antimicrobial metabolites and extracted products from non-inoculated LB, respectively, and challenged with TMV at 12hpt.

The samples were collected at a series of time points as indicated, and total RNA was extracted and subjected for qRT-PCR detection. EF1a was determined as internal reference gene to normalize the data. Values were presented as means $\pm$ SD from three independent replicates

localized cell death (Pontier et al. 2004; Wang et al. 2012). In this study, cell death in leaves were correlated with the amount of virus present in leaves (Fig. 6). The antimicrobial pretreated leaves were able to maintain the viability of host cells against TMV. In addition, reduction of H_2O_2 triggered by strain HAB-5 treatment may play a role in the enhancement of plant resistance. This suggests that the low

level of H_2O_2 accumulation in tobacco leaves may insufficiently cause cell death. This finding provide new information on incompatible interactions between TMV infection and plant hosts.

Depressed proline and higher ROS accumulation in plants usually contributes to the ability of the bacteria to maintain ROS balance in accordance with plant

Table 2 Enzyme linked immune sorbent assay (ELISA) detection of TMV accumulation in rub-inoculated leaves at 7 dpt

Treatments	TMV disease severity	ELIZA TMV detection (450nm)
Treatments	TMV disease severity	ELIZA TMV detection (450nm)
HAB-5 + TMV	1.25b	0.87b
Mock + TMV	3.75a	1.86a

requirements (Elsayed et al. 2013). The need for proline in maintaining the elevated ROS level has been reported earlier (Apostol and Heinsteins 1989; Kumar et al. 2016). Interestingly, our results showed lower proline and ROS accumulation in pretreated plants. It suggests that the antimicrobial-metabolite induced systemic resistance via proline and ROS in epidermal plant cells was activated in an independent manner.

NO is an important second messenger involved in plant immunity, acting as a signaling molecule in plant/pathogen interactions (Savicka and Škute 2010). It was observed that NO content in rosette leaves of tobacco increased following strain HAB-5 antimicrobial treatment (Fig. 7). This finding suggests that NO may contribute to the induction of defense response in plant leaves.

The expression of plant defense-related genes usually occurs quickly after plant treatments. PR proteins were found in genotypes of tobacco infected with tobacco mosaic virus (Hare and Cress 1997; Singh et al. 2017). NPR1 is known to regulate SAR (Systemic acquired resistance) and Induced systemic resistance (ISR) resistance pathways (Pontier et al. 2004). We pretreated the leaves with antimicrobial metabolites 12 h before TMV inoculation, and we noticed that plant immune associated genes were stimulated. *Hsr203J* and *Hin1* reported as HR-related genes exhibit rapid, high level, specific activation in response to HR-inducing bacteria (Pontier et al. 1994; Delledonne et al. 1998). Unexpectedly, evidence was not presented in the present study that expressions of those genes were noticed at late time point (96 hpt). The use of different pathogen species, different plant cultivar, different bacteria, different treatments from a different genetic background, or plant encoded functions can modulate their action. Similarly, recent reports also indicate the induction of *Hsr203J* in response to *hrp* mutants of *Pseudomonas. syringae* pv. *tabaci* WF4 and *P. syringae* pv. *syringae* at late time points (Park and Kloepper 2000; Raupach et al. 1996; Pontier et al. 1994).

Overall, results conclude that effective biological control not only depends on the organisms but also on the control

strategy. We found that foliar application of a crude extract for controlling diseases was effective for foliar treatment and proved to be potential biocontrol treatment against TMV used as a bio-pesticide. This aspect will be a new instrument in improving the application of bioactive natural products for plant protection.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

Human participants and/or animals This article does not contain any studies with human or animals subjects performed by any of the authors.

Informed consent Not applicable.

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