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Induction of Salicylic Acid in Cucumber Plants Against Cucumber Mosaic *Cucumovirus* Using Biotic Inducers

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ABSTRACT

Systemic Acquired Resistance (SAR) could be induced in cucumber plants using different individual of seven microbial isolates against Cucumber mosaic *Cucumovirus* (CMV). These isolates were *Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus circulans*, *Pseudomonas putida*, *Pseudomonas fluorescens* 2 and *Pseudomonas fluorescens* 8 as bacterial isolates and *Trichoderma harzianum* as fungal isolate. The occurrence of SAR was found by seed treatment with microbial liquid culture based on virus infectivity and the level of free and endogenous salicylic acid (SA), 14 days from CMV inoculation. Seven biotic inducers reduced CMV infectivity at range 16.6 to 39% and *T. harzianum* gave the highest percentage of reduction 39%. In addition, the level of total SA has been increased in treated plants, *B. subtilis* gave the highest level of SA 239.13 μg g^{-1} fresh weight (fwt) while, *B. circulans* gave the lowest level 70.1 μg g^{-1} fwt.

Key words: CMV-EG isolate, cucumber, salicylic acid, microbial isolates, systemic acquired resistance

INTRODUCTION

Cucumber (*Cucumis sativus*) is one of a major vegetable crops growing in Egypt. The loss of cucumber production was attributed to infection with different viruses. Cucumber mosaic virus (CMV) is a member of the family Bromoviridae that has a highly wide host range belonging to 100 families including more than 1200 plant species (Kaplan *et al*., 1997).

Biological control under greenhouse and field conditions can be achieved using different microorganisms to induce systemic resistance (ISR) against several plant diseases. As Wei *et al*. (1996) mentioned, ISR may be formed on one plant host using individual strains of plant growth-promoting rhizobacteria (PGPR) against multiple diseases. Ryu *et al*. (2007) observed that, the plant roots colonized by different bacterial rhizosphere had acquired plant growth promoters and systemic protection against a broad spectrum of plant pathogens. The role of the global regulator, GacS, in the rhizosphere of tobacco cv. Samsun colonized with *Pseudomonas chlororaphis* O6 was concluded as stimulating induced resistance against CMV and forced by growth promotion.

Ryals *et al.* (1996) mentioned that, the increasing in SA level as 50 and 70% in uninfected cucumber and tobacco tissues respectively was resulted as SA systemic translocation from the site of infection to the other parts of the plants.

Therefore, this study concerned with induction of systemic acquired resistance in cucumber plants using bacterial and fungal liquid cultures to control the virus infection and detection of systemic acquired resistance (SAR) by SA level in treated plants as signaling in plants defense.

MATERIALS AND METHODS

Virus isolate: Cucumber mosaic *Cucumovirus* (CMV-EG) isolate was obtained from the Virology Laboratory, Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt (Megahed *et al.*, 2012) and maintained in *Nicotiana glutinosa* as CMV propagative host.

Bacterial inducers: Six bacterial and one fungal isolates were obtained from Microbiological Resources Centre (MARCIN), Fac. of Agric. Ain Shams Univ., Cairo, Egypt.

The bacterial cultures were prepared via propagation of *B. subtilis*, *B. polymyxa*, *B. circulans* and *P. putida* on nutrient broth (Waksman, 1957), whereas *P. fluorescens* 2 and *P. fluorescens* 8 were propagated in King's B broth (King *et al.*, 1954). The suspensions of bacterial cells were adjusted at about mean density of 5×10^9 CFU mL^{-1} according to Raupach *et al.* (1996).

Fungal inducers: *T. harzianum* was propagated in potato dextrose broth, the conidial media suspension was adjusted at about mean density of 10^{10} spores mL^{-1} according to Chambers and Scott (1995) and Helmy and Maklad (2002).

Greenhouse experiment: The soil used in the experiments was prepared by mixing clay, sand and peat-moss by ratio (3:1:1, v:v:v) and divided into two parts. The first was autoclaved at 121°C for 1 h, while the second was used as non sterilized soil. Plastic pots (20 cm in diameter) were washed and air dried, then sterilized with clorox. The sterilized pots were filled with sterilized at rate 1.5 kg per each pot.

The sterilized seeds of *Cucumis sativus* cv. Beith Alpha were soaked for 60 min in 50 mL of each bacterial liquid culture and fungal culture. The treated seeds were cultivated in sterilized soil (5 plants/pot and 10 pot replicates for each biotic inducer. Seven days after planting, cotyledons were lightly rub-inoculated with CMV inoculum by spatula to all treatments. Healthy control plants were inoculated with sterile 0.1 M potassium phosphate buffer (PPB), pH 7.2. All treatments were conducted under greenhouse conditions at 29/22°C day/night temperatures until development symptoms (Wei *et al.*, 1996).

Percentage of virus infection: The percentage of virus infection was determined and calculated relative to infected control, 14 days from inoculation with CMV.

Quantification of total salicylic acid (SA): Free and endogenous of SA were measured at once in the treatments by a method according to Raskin *et al.* (1989), with one modification by Abo El-Nasr *et al.* (2004). One gram of frozen tissue was ground in 3 mL of 90% methanol and centrifuged at 6000 g for 15 min. The pellet was back extracted with 3 mL of 99.5% methanol and centrifuged as above. Methanol extracts were combined and then centrifuged at 1500 to 2000 g for 10 min. The supernatant was dried at 40°C under vacuum using rotary evaporator (Heidolph.). The dried extracts were then re-suspended in 3 mL of distilled water at 80°C and an equal volume of 0.2 M sodium acetate buffer, pH 4.5, containing 0.1 mg mL^{-1} β-glucosidase (22 unit/mg, Sigma)

was added, then the mixtures were incubated at 37°C overnight. After digestion, mixtures were acidified to pH 1 to 1.5 with HCl. SA was extracted by adding (1:2, v:v) of sample: cyclopentan/ethyl acetate/isopropanol (50:50:1). The organic extract was dried under nitrogen and analyzed by HPLC (SHIMADZO RF-10AXL Fluorescence, HPLC Lab., National Research Centre, Cairo, Egypt). One hundred microliters of each sample were injected into Dynamax 60A8 μm guord column (46 mm×1.5 cm) linked to 40°C. SA was separated with 23% v/v methanol in 20 mM sodium acetate buffer, pH 5.0 at a flow rate of 1.5 mL min^{-1}. SA level was determined as μg g^{-1} fresh weight using standard curve.

RESULTS

The microbial liquid culture refer to seven isolates consist of 6 bacterial and one fungal isolates were applied individually by cucumber seeds soaking to induce SAR against CMV infection under sterilized and non-sterilized soil. The induction of SAR by individual isolates in cucumber plants was biologically detected by percentage of CMV infection, as well as by biochemical quantification of free and endogenous salicylic acid.

Percentage of infection: The obtained results showed that seven biotic inducers reduced the CMV infection at range 16.6-39% under sterilized soil related to *B. subtilis* (31%), *B. polymyxa* (16.6%), *B. circulans* (30%), *P. putida* (21.4%), *P. fluorescens* 2 (29%), *P. fluorescens* 8 (21.4%) and *T. harzianum* (39%). While under the non-sterilized soil the reduction of CMV infection at range 0-46.5% by different percentage related to *B. subtilis* (46.5%), *B. polymyxa* (0%), *B. circulans* (25%), *P. putida* (0%), *P. fluorescens* 2 (41.5%), *P. fluorescens* 8 (16.6%) and *T. harzianum* (41.5%).

Quantification of total salicylic acid: The obtained results from quantification of total SA in induced cucumber plants in sterilized soil, Table 1 were agreed with percentage of infection, disease severity and virus concentration. It was observed that, the level of total SA has been increased in treated plants.

B. subtilis gave the highest level of SA (239.13 μg g^{-1} fwt.) followed by *T. harzianum* (214.75 μg g^{-1} fwt.) and *B. polymyxa* (200.78 μg g^{-1} fwt.), while *B. circulans* gave the lowest level (70.1 μg g^{-1} fwt.).

Figures 1 A-I refers to the peaks obtained using HPLC; desired peak must be resulted in retention time similar to the retention time of the standard. These peaks were used to calculate total SA based on the area under peak.

Table 1: Quantification of total salicylic acid (SA) in cucumber plants

Treatments	No. of peak	Retention time	Area	Total SA (μg g^{-1} fwt.)
Standard SA	1	6.301	1293.02722	-
Infected control	3	6.374	359.61000	278.11
B. subtilis	3	6.384	309.20300	239.13
B. polymyxa	5	6.430	259.61900	200.78
B. circulans	23	6.656	45.32200	70.100
P. putida	19	6.495	148.26800	114.67
P. fluorescens 2	15	6.500	148.08000	114.52
P. fluorescens 8	1	6.495	185.19400	143.23
T. harzianum	11	6.486	277.68000	214.75

fwt.: Fresh weight

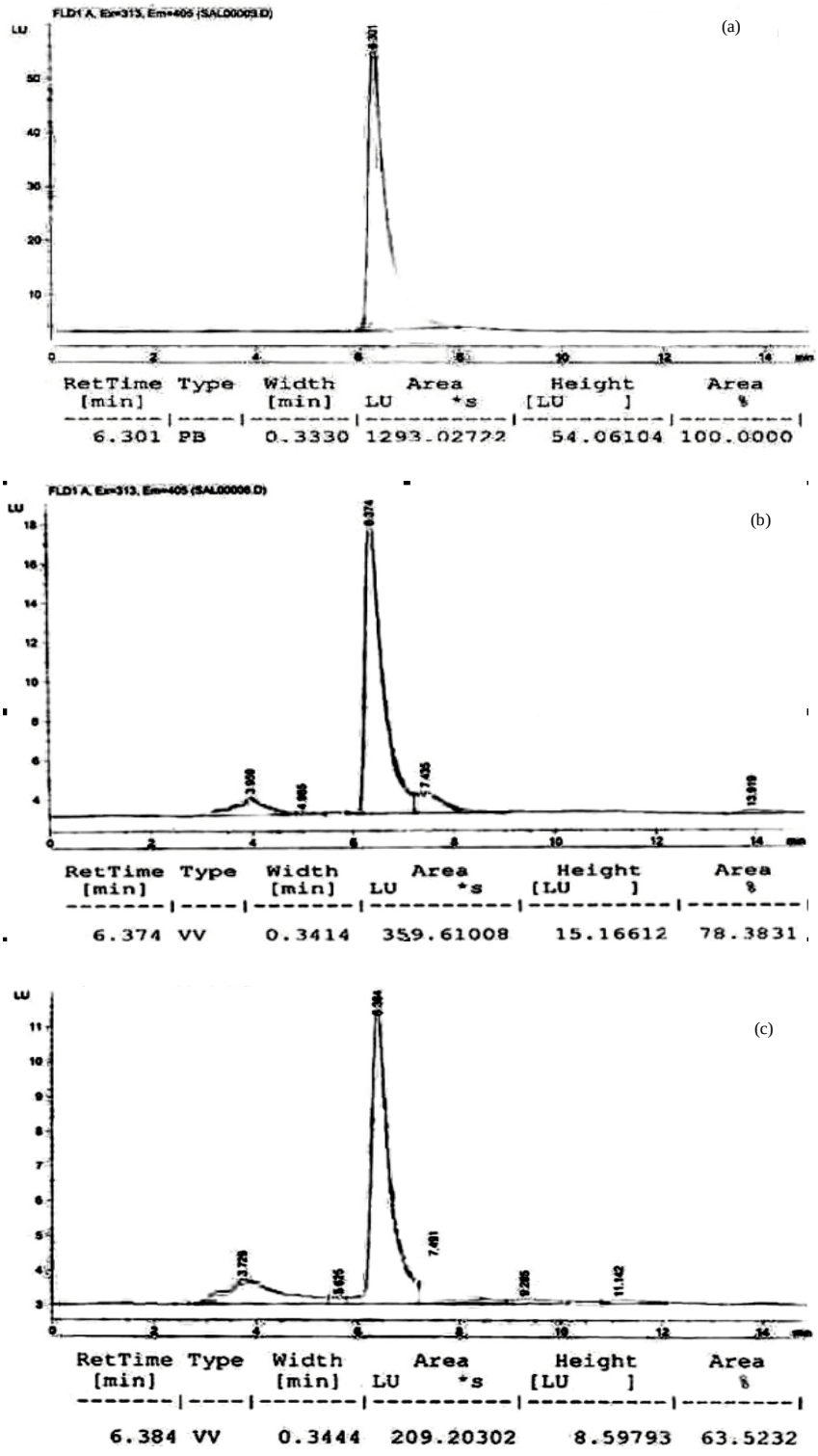

Fig. 1(a-i): Continue

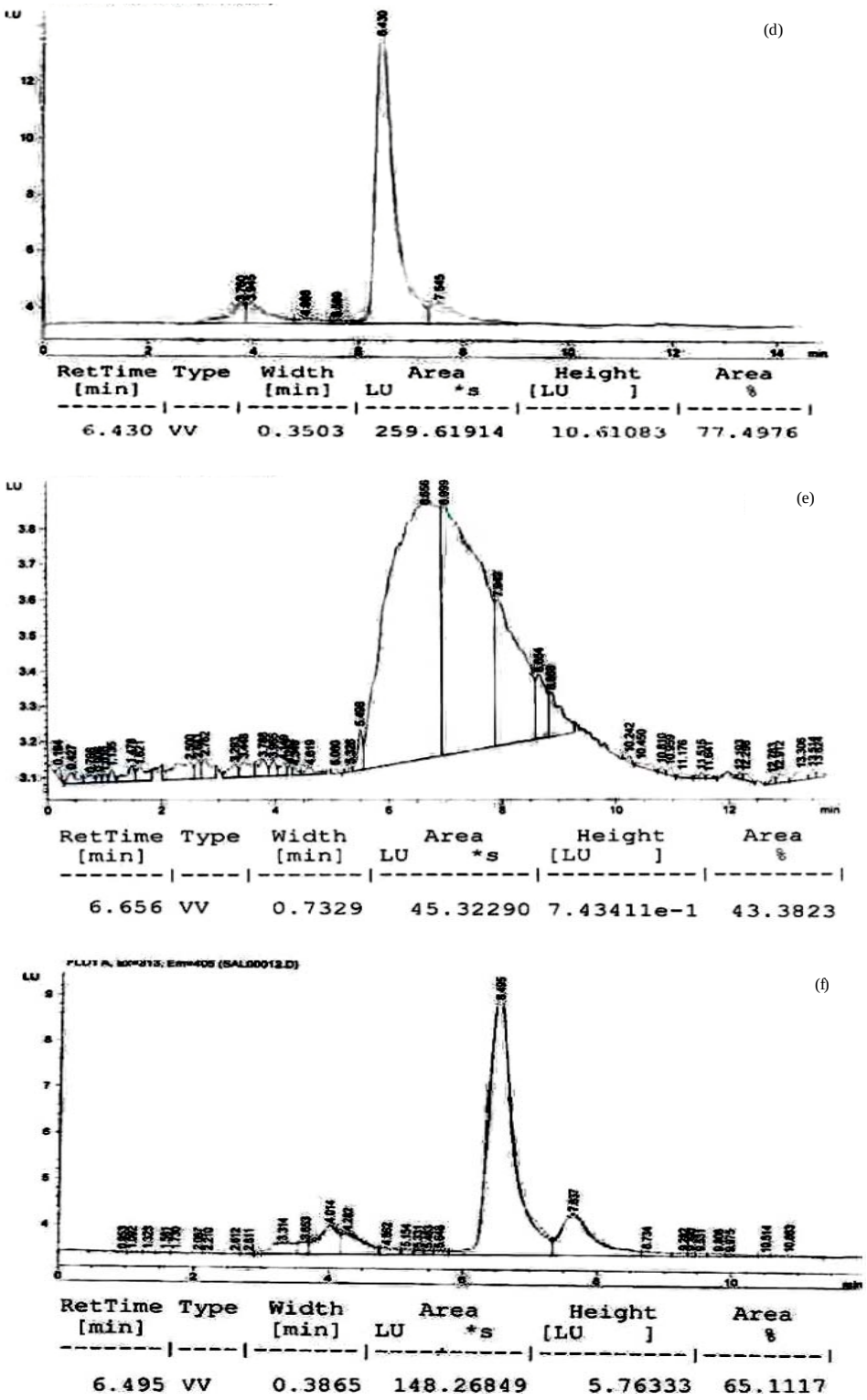

Fig. 1(a-i): Continue

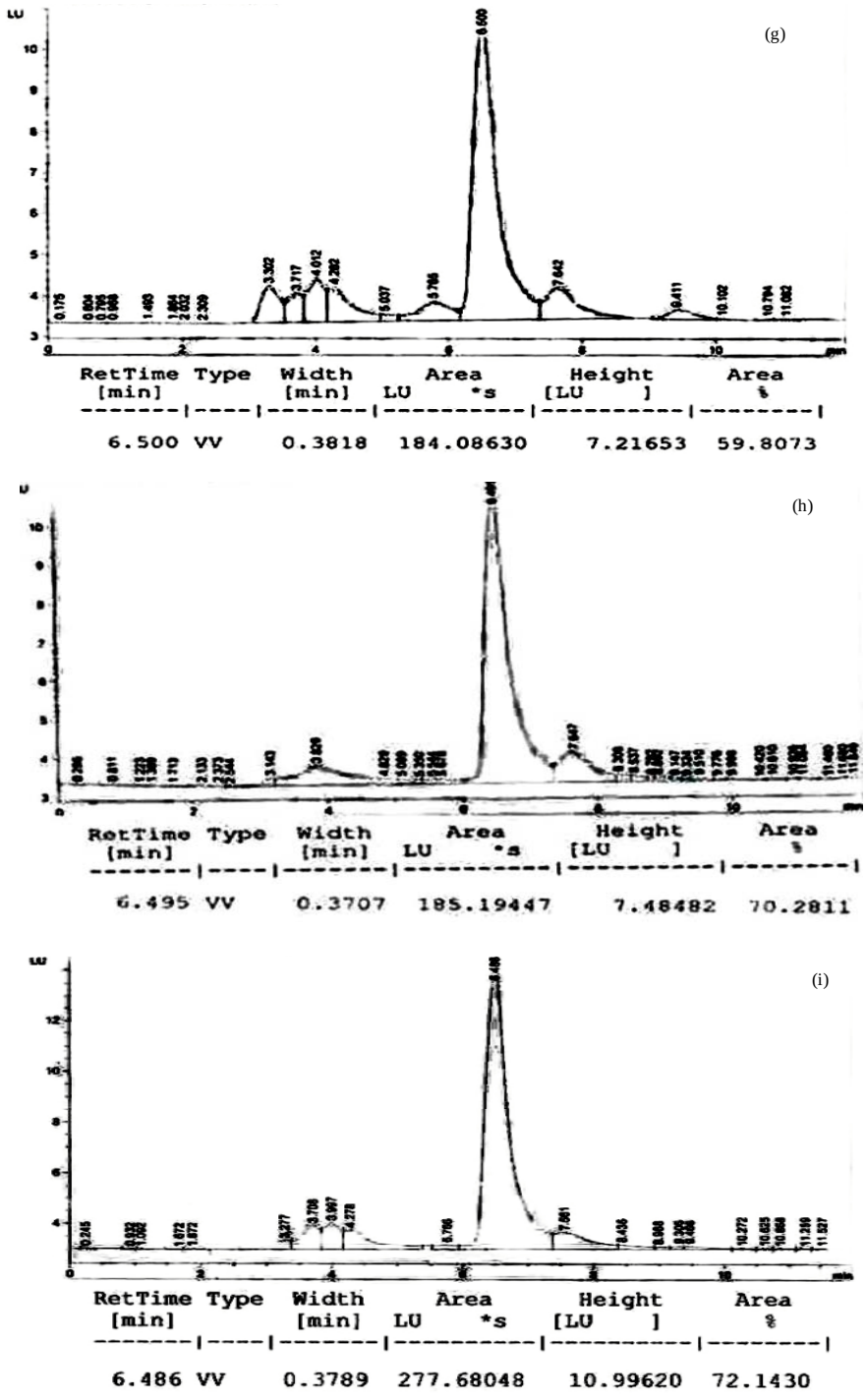

Fig. 1(a-i): HPLC quantification of free and endogenous SA in induced cucumber plants, Arrow refer to the data for peak ranged in retention time range; (a) Standard SA, (b) Infected control, (c) *B. subtilis*, (d) *B. polymyxa*, (e) *B. circulans*, (f) *P. putida*, (g) *P. fluorescens* 2, (h) *P. fluorescens* 8 and (i) *T. harzianum*

DISCUSSION

The obtained results from quantification of free and endogenous SA using HPLC in induced cucumber plants in sterilized soil were agreed with percentage of CMV infection. The level of SA had been increased in treated plants, *B. subtilis* and *T. harzianum* have the highest level of SA (239.13 and 214.75 μg g^{-1} fwt.), respectively. While, *B. circulans* gave the lowest level (70.1 μg g^{-1} fwt.).

Many evidences suggest that SA is a SAR signal (Malamy *et al*., 1992; Vernooij *et al*., 1994). In tobacco, SA levels increased up to 180-fold after the formation of viral local lesion (Gaffney *et al*., 1993). It was found that free and bound SA is produced around the infection site, whereas only free SA was detected in distal region (Malamy *et al*., 1992).

Huang *et al*. (2006) mentioned that, SA was measured quantitatively *in situ Nicotiana tabacum* L. cv. Xanthi-nc leaves inoculated with Tobacco mosaic virus (TMV). The biosensor revealed accumulation of apoplastic SA before the visible appearance of hypersensitive response (HR) lesions. The same results were obtained by many authors (Mahmoud, 2003; Abo El-Nasr *et al*., 2004).

It has been claimed that SA act as the internal general resistance in plants and induces the expression of messenger RNA, which presumably direct the synthesis of the pathogenesis related (PR) proteins (Moffat, 1992).

Yalpani *et al*. (1991) showed an increase in endogenous salicylic acid in tobacco infected with TMV, which caused a hypersensitive response with systemic induction of PR proteins. SA has an extreme role as endogenous signal for host resistance against pathogens (Raskin, 1992).

CONCLUSION

SAR was accumulated and indicated in cucumber plants by different reduction in percentage of CMV infection and increasing of free and endogenous SA in all individual treatments of biotic inducers. Five biotic inducers; *B. subtilis*, *B. polymyxa*, *B. circulans*, *P. fluorescens* 2 and *T. harzianum* reduced the percentage of infection with different percentage 31, 16.6, 30, 29 and 39%, respectively. Where two isolates *P. putida* and *P. fluorescens* 8 have the same percentage of reduction 21.4%. In case of the non-sterilized soil, two isolates reached to 100% percentage of infection (*B. polymyxa* and *P. putida*), while other treatments have different reducing of infection, 46.5, 25, 41.5, 16.6 and 41.5% for *B. subtilis*, *B. circulans*, *P. fluorescens* 2, *P. fluorescens* 8 and *T. harzianum*, respectively.

On the other hands, *B. subtilis* gave the highest level of SA (239.13 μg g^{-1} fwt.) followed by *T. harzianum* (214.75 μg g^{-1} fwt.) and *B. polymyxa* (200.78 μg g^{-1} fwt.), while *B. circulans* gave the lowest level (70.1 μg g^{-1} fwt.).

Finally, there are two microbial isolates; *B. subtilis* and *T. harzianum*, able to induce highly levels of SAR in cucumber plants and reduction CMV infection.

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