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Systemic Resistance Induction of Tomato Plants against ToMV Virus by Surfactin Produced from *Bacillus subtilis* BMG02

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Abstract Tomato mosaic virus (ToMV) is a major disease of tomato (*Lycopersicon esculentum*) which can strongly reduce tomato yields. Different efforts have been made to control tomato virus infection. Among these efforts is the use of cyclic lipopeptides. The surfactin produced by *Bacillus subtilis* BMG02 single overproducer strain strongly reduced (ToMV) virus symptoms and was responsible for the elicitation of Induced Systemic Resistance (ISR) in tomato. Treatment with 1000 mg surfactin used as critical antivirial concentration showed a strong symptoms reduction with complete negative ELISA results . The infected seedlings showed the ideal severe symptoms with fully positive ELISA reaction, while both healthy and infected seedlings treated with surfactin showed the same effect of strong symptoms reduction with complete negative ELISA results thus confirming the antivirial activities of surfactin. The hydrogen peroxide (H_2O_2), salicylic acid (SA) and jasmonic acid (JA) pathways have been proven to be involved in the tomato plant defense responses by surfactin treatment, whereas relative gene expression of phenylalanine ammonia lyase (*PAL*) and β 1,3- glucanase 2 (*BGL*2) involved in SA and JA pathways, respectively increased in treated seedlings compared to infected ones. The healthy seedlings showed very low H_2O_2 levels compared to infected tomato seedlings. Moreover, high levels of H_2O_2 were detected in treated seedlings with the same behaviour *BGL*2 and *PAL* expression levels due to the relationship between the ISR involved substrates.

Keywords: *surfactin, ToMV, Salicylic Acid, jasmonic acid and Induced Systemic Resistance (ISR)*

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1. Introduction

Plant diseases are considered the most devastating factors in agriculture. Among plant diseases is tomato mosaic virus (ToMV) which has close serological relationship to tobacco mosaic virus (TMV) and is considered one of TMV strains [1]. The need to study biological alternatives has risen to face the environmental damage caused by the use of chemical substances against plant diseases [2,3]. Plant treatment with microbial components or beneficial endophytes can enhance induced systemic resistance (ISR) to resist against a wide range of pathogens. *Bacillus subtilis* produces a wide range of bioactive molecules among which lipopeptides; surfactin, iturin and fengycin families [4,5]. Surfactin is a cyclic lipopeptide composed of seven a-amino acids linked to β - hydroxy fatty acid and biosynthesized non-ribosomally by NRPS mechanism [6]. Salicylic acid (SA) plays an important role in the signaling pathway of ISR [7,8]. After infection, it have been reported that SA levels increase in the phloem before ISR occurs [9,10]. In transgenic plants expressing the bacterial nahG gene encoding naphthalene hydroxylase G cannot accumulate SA and are blocked by their ISR response [11,12]. The *PAL* enzyme is very important for plant defence and is considered one of the most studied enzymes in all secondary metabolisms. It acts upstream of SA biosynthesis and is implicated in defense pathways [13]. *PAL* initiates biochemical pathways leading to the SA formation. Also, pathogenesis-related proteins (PRs) are defined as plant proteins that are induced in pathological or related situations. There are several groups of these PRs; those grouped in JA dependent marker genes such as basic pathogenesis-related proteins *PR2* (basic *PR2*, *GluB* and *BGL2*) were activated in tomato and are responsible for the JA dependent defense pathway. *BGL2* is involved in plant development such as flower development, growth and seed germination and may help to degrade fungal cell wall [14]. In this work, we provide strong evidence for plant resistance-eliciting activity of surfactin lipopeptide by use of *Bacillus subtilis* 168 derivative surfactin-overproducing strain BMG02 [15].

2. Materials and Methods

2.1. Plant Material

Thirty tomato seedlings (*Lycopersicon esculentum*) of super marmand cultivar were cultivated in 1 kg of sterilized soil for each plant under green house conditions and irrigated regularly with one litter of sterilized water or surfactin treated solutions every week. Nine infected seedlings were used for virus propagation, nine seedlings were used for *In vitro* experiments and the other nine were used for *in vivo* experiments, while the control was made up of the last three seedlings.

2.2. Bacterial Strain

Bacillus subtilis BMG02 was recently constructed as a single surfactin producer strain by the interruption of the plipastatin operon using neomycin cassette for strain BMG01 which was constructed by the insertion of an sfp^+ (4'-phosphopantetheinyl transferase) active gene in *Bacillus subtilis* 168. This strain is capable of producing about 1758 $mg.L^{-1}$ of surfactin under the optimal production conditions according to Hussein and Fahim [15].

2.3. Surfactin Production, Extraction and Determination

The produced surfactin was extracted after microbial fermentation in Landy Modified medium in 5 L volume of three phase inverse fluidized bed bioreactor supplied by 0.06 S^{-1} of voulmetric oxygen transfer coefficient (k_La) with filling volume 25% at 30 ^oC for 48 h according to Fahim et al. [16]. The bacterial cells were removed from culture (stationary phase) by centrifugation at 15,000 rpm at 4°C for 20 min. The total yield was collected before analysis by high-performance liquid chromatography (HPLC) in C_{18} column (5 µm; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was acetonitrile – water - trifluoroacetic acid (80:20:0.5 [vol/vol/vol]) according to Fahim et al. [17].

2.4. Surfactin Purification, Concentration and Treatments Prepration

250 ml of extracted centrifuged crude media containing (HPLC) calculated surfactin was used as source of concentrated pure surfactin, the extracted surfactin was purified on large C_{18} Maxi-Clean cartridges (Alltech, Deerfield, IL) and eluted with 250 ml pure methanol (HPLC grade). The purified surfactin was completely dried by speed vaccum to be concentrated and redissolved in 25 ml of methanol. The eluted concentrated surfactin was usesd to prepare the concentration groups. The first concentration was adjusted with 2.5 ml of pure methanol as zero surfactin control and two voulmes of surfactin eluted pure methanol (7.5 and 15 ml) were added to the virus crude sap phosphate buffer solution by 10% methanol of pure surfactin additives concentration (zero, 500 and 1000 mg/L respectively), used as source of treatments *In vitro* experiments, while the centrifuged crude media containing surfactin quantitated by HPLC was diluted to 1 $g.L^{-1}$ by sterilized distilled water as source of treatments *in vivo* experiments. The surfactin treatment was carried out by irrigating every killogram of sterilized soil by dilution of about 570 ml of centrifugated culture media to reach about the critical surfactin concentration followed by spraying leaves with the same surfactin solution.

2.5. Isolation and Identification of (ToMV)

Sources of the virus isolates: Samples were obtained from naturally infected local cultivar tomato (*Lycopersicon esculentum*) plants. Leaf samples showing dark green mosaic or mottling, called «Calico» and aucuba symptoms doubted to be due to virus infection, were suspected in the subsequent experiments. Tomato mosaic virus (ToMV) was isolated from tomato plants suspected to be infected and showing the symptoms. Leaf samples were examined serologically by Double Antibody Sandwich - Enzyme linked immuno sorbent assay (DAS-ELISA) with specific antiserum for ToMV. These samples were homogenized in a sterilized mortar, after adding phosphate buffer (1: 5 w/v, 0.1 M, 0.1 ml, pH 7.2) [18] then the extracted sap was passed through a double layer of cheesecloth. The virus was purified biologically through the single local lesion technique as modified by El-Kammar et al. [19] to obtain the virus in a pure form. *Nicotiana glutinosa* L plants were used as a local lesion host. Single local lesion was isolated, grinded in phosphate buffer pH 7.2 and back inoculated onto *N. glutinosa* L. Finally the resulting local concentric lesions were singly back inoculated onto *Solanum tuberosum* potato plants. Inoculated potato plants were kept in the greenhouse conditions (25 ± 5°C) and used as a source of infection in the following experiments. Identification of isolated virus was based on host range studies, symptomatology, modes of transmission, inclusion bodies. Identity was further ensured by specific serological tests, electron microscopy and molecular studies in our lab.

2.6. Mechanical Transmission

Crude sap was extracted from fifty grames of infected tissues by using an electric homogenizer in phosphate buffer. The extracted sap was filtrated through double layers of cheesecloth and obtained sap was used to inoculate the tested plants. The leaves to be inoculated were first dusted with carborandum (600 mesh) and gently rubbed with a freshly prepared inoculum, using a cheese-cloth pad or by fore finger. Then the inoculated leaves were rinsed with water 15 min after inoculation. All inoculated plants as well as uninoculated ones (healthy control) were kept under greenhouse conditions and observed daily for 30 days and development of symptoms were recorded and examined serologically with specific antiserum.

2.7. *In vitro* Experiments

The extracted sap which was prepared as source of virus inoculum and which gave a positive result with DAS-ELISA examination was divided into three voulmes (25, 75 and 150 ml) treated by three concentrations of purified surfactin (zero, 500 and 1000 $mg.L^{-1}$), each concentration was replicated three times. The concentration groups were used to re-inoculate three groups of three plants (A, B and C respectively) for each concentration as

a verification step of virus viability to ensure the absence of symptoms. All plant groups as well as non inoculated ones (control) were kept under greenhouse conditions and observed daily for 30 days, then development of symptoms were recorded and examined serologically with specific ToMV antiserum. The concentration which gave a fully negetive result with (DAS-ELISA) and absence of symbtoms was recorded as critical concentration of surfactin antiviral activity against ToMV virus.

2.8. *In vivo* Experiments

The field experiment was conducted with three plant groups in three replicates. The first group (D) was incoulated with ToMV virus and irrigated with water, the second group (E) non incoulated with virus and irrigated with the critical surfactin concentration, the third group (F) was combined between the virus inoculation and critical surfactin concentration irrigation. All plant groups as well as non inoculated ones (control) were kept under greenhouse and observed daily for 30 days and development of symptoms were recorded and examined serologically with specific antiserum for ToMV.

2.9. Serological Tests Assay (DAS – ELISA)

Enzyme linked immuunosorbent assay (DAS-ELISA) was used for rapid and very sensitive serological detection of alfalfa mosaic virus according to Clark and Adams [20]. HASA kits (completely ready to use) were supplied by SANOFI, Sante Animale, Paris, France. The use procedure as described in the kit's instruction is as follows: Polyethylene microtitre plates were coated with ToMV specific immuuno globulin (Ig_G) 100 pl / well, diluted in coating buffer at dilution (1/200) and incubated for 2 h at 37 °C. Then wells were washed 3 times, each for 3 minutes with phosphate buffer saline Tween 20 (PBST) pH 7.4. One gram of tested tissue was homogenized using electric homogenizer in 4m1 of grinding buffer (pH 7.4). 100 µl of test sample, and negative and positive control samples were added to duplicate wells. The plate was incubated at 4°C overnight, after that, the plate was washed 3 times, each for 3 minutes with PBST buffer, pH 7.4. 100 µl of anti-virus conjugate (diluted in conjugate buffer), were added to each well and incubated at 37 °C for 2 h, then the plate washed 3 times, each for 3 minutes with PBST buffer, pH 7.4. Finally a 100 pl of freshly prepared substrate [5 mg P- nitro phenyl phosphate dissolved in 5 m1 substrate buffer] were added to each well, and then the plate was incubated at 37 °C for 30 to 6o minutes. To stop the reaction 50 µl (3M, NaOH) was added to each well or the plate keep at (- 20 °C), the absorbance was measured at 405 A° using ELISA reader (EL, 800 universal microplate Reader) to determine the presence and quantity of the virus,, while the virus isolate was tested with its specific antiserum with an absorbance (at least three replicates). Those of control were considered positive by indirect EL1SA by using polyethylene microtitre plates technique as described by Lommel et al [21].

2.10. Hydrogen Peroxide Determination

Hydrogen peroxide (H_2O_2) was spectrophotometerically determined in tomato leaves crude sap by measuring the absorbance values of the ferric xylenol orange dye complex (Sigma) at 560 nm and calibrated with the H_2O_2 standard curve according to Peng et al. [22].

2.11. RNA Extraction and RT-PCR Analysis

RNA-later kit of Ambion RNA *later* ® (Applied Biosystems, Courtaboeuf, France) was used in RNA extraction. The reverse transcription was performed with Revert Aid First Strand cDNA Synthesis Kit (K1621, Fermentas). The reverse transcription products were used as templates for PCR analysis. Real Time-PCR were performed by adding 1X SYBR green Fluorescein Mix, 250 nM of each primer and 1 µl of cDNA in a final volume of 25 µl. The thermal cycling programme was: 95°C for 5 min, 35 cycles with 95°C for 20 seconds, annealing temperature according to primer tested for 45 seconds, 72°C for 40 seconds for 45 cycles. After amplification, the threshold cycles were calculated by a melt curve temperature. The Real time RT-PCR results were expressed as Ct (cycle threshold) values and relative gene expression was quantified using the following formula;

$$\textbf{Relative gene expression} = \frac{\text{Efficiency of tested gene}}{\text{Efficiency of control gene}}.$$

Whereas, Efficiency= Ct untreated - Ct treated.

All primers used in the PCR are listed in Table 1. Four RNA samples were collected from both infected tomato seedling and treated with surfactin after 2, 4, 6 and 8 hours of tratment and each sample amplified in triplicate.

Table 1. Primers sequence used in ISR genes detection

Primers	Primers sequence	**GenBank Access No.**
PAL fwd	5' TTC AAG GCT ACT CTG GC'3	**M833314**
PAL rvs	5' CAA GCC ATT GTG GAG AT'3	
*BGL*2 fwd	5' CAC CAA CAT TCA CAT AAC AGA GGC'3	**M80680**
*BGL*2 rvs	**5' CAG GGC TGA TTT CAT TAC CAA C'3**	

3. Results

3.1. Surfactin Production and Quantification

The single surfactin producer strain BMG02 (*Bacillus subtilis* 168 derivative) was used to produce 14.614 litter of culture media with surfactin concentration about 1758 $mg.L^{-1}$ during 12 cycles (576 hours) of fermention in three phase inverse fluidized bed bioreactor illustrated in Table 2. 25 ml of purified surfactin (17.5 $mg.ml^{-1}$) were collected from 250 ml culture medium from the first fermentation cycle. This voulme was used to treat the virus crude sap *In vitro* experiment. On the other hand, the total collected culture medium was diluted to reach to 25.216 litters with final ciritical concentration 1000 $mg.L^{-1}$ of surfactin according to the antivirial activity results obtained from the *In vitro* experiments. 24 litters of the surfactin solution were used to irrigate the seedlings during *in vivo* experiments.

Table 2. Time table of surfactin production and quantification

TIME	Fermentation cycles (48 h)	Collected culture media (ml)	Surfactin concentration (mg.L^{-1})	Diluted culture media (ml) (1000 mg.L^{-1})
First week	1	1226 (- 250 ml used for purification)	1754	1712
	2	1217	1763	2146
	3	1223	1758	2145
Week yield	**3 Cycles (144 h)**	**3666**	*** 1758** ± 4.2	**6003**
Second week	4	1221	1739	2123
	5	1216	1762	2143
	6	1211	1744	2112
Week yield	**3 Cycles (144 h)**	**3648**	*** 1748** ± 3.8	**6378**
Third week	7	1219	1752	2135
	8	1216	1735	2110
	9	1218	1774	2124
Week yield	**3 Cycles (144 h)**	**3653**	*** 1754** ± 3.2	**6369**
Fourth week	10	1224	1754	2176
	11	1212	1783	2161
	12	1211	1758	2129
Week yield	**3 Cycles (144 h)**	**3647**	*** 1773** ± 4.4	**6466**
Total yield	**12 Cycles (576 h)**	**14614**	**1758**	**25216**

* Results are means of triplicate experiments ± standard deviation (SD).

3.2. Susceptibility of Tomato Plants to Virus Infection

Seedlings of inoculated tomato with the sap prepared from ToMV infected plants showed symptoms within two or three weeks post inoculation. The infected tomato plants showed virus symptoms and gave a positive reaction in ELISA test after mechanical inoculation with ToMV, while some others showed weak symptoms but gave a negative ELISA reaction (Table 3). During *in vitro* experiment, the infected seedlings with zero surfactin concentration (group A) showed the ideal severe symptoms with fully positive ELISA reaction, while the treatment with 500 mg (group B) showed evident reduction in symptoms severity. A strong symptoms reduction occured with complete negative ELISA results in case (group C) of 1000 mg surfactin concentration which was later used as critical antivirial concentration in our investigation. For the *in vivo* experiments, the untreated infected sedlings (group D) showed the ideal severe symptoms with fully positive ELISA reaction, while the treated non inoculated seedlings (group E) and the treated infected seedlings (group F) with surfactin critical concentration showed the same effect of strong symptoms reduction with complete negative ELISA results thus confirming the antivirial activities of surfactin.

Table 3. Viral susceptibility and surfactin activity

In Vitro				*In Vivo*			
Group	**Replicates**	**ELISA**	**Symptoms**	**Group**	**Replicates**	**ELISA**	**Symptoms**
A	A1	+	+++	**D**	D1	+	+++
	A2	+	+++		D2	+	+++
	A3	+	+++		D3	+	+++
B	B1	-	+	**E**	E1	-	+
	B2	+	++		E2	-	+
	B3	+	++		E3	-	+
C	C1	-	+	**F**	F1	-	+
	C2	-	+		F2	-	+
	C3	-	+		F3	-	+

Abbreviation of symptoms and ELISA:
ELISA + Positive reaction - Negative reaction
Symptoms + weak symptoms ++ moderate symptoms +++ severe symptoms.

3.3. mRNA Expression Levels of *PAL* and *BGL2* Genes

The expression levels were measured at 0, 2, 4, 6, 8 and 10 hours in both treated tomato seedlings by surfactin and in infected seedlings by ToMV virus. Infected tomato seedlings showed expression levels of *BGL2* and *PAL* less than treated seedlings. The expression levels of *PAL* in treated seedlings was low at 0h (0.83 ± 0.13) and started to be induced at 2h (1.92 ± 0.17), *PAL* levels kept increasing to reach the highest expression at 6h (4.32 ± 0.19), then decreased at 8h (3.58 ± 0.14) and 10h (2.14 ± 0.15). Moreover, the infected seedlings showed the same pattern as those treated but with less ranges as shown in (Figure 1). *BGL2* pattern in the treated seedlings showed a rapid induction at 0h (2.69 ± 0.13) and continued increasing to reach the highest expression levels at 6h (7.98 ± 0.12), then decreased at 8h (4.87 ± 0.16) and 10 h (3.21 ± 0.18). A same pattern for *BGL2* was shown in infected seedlings but with less values. These presented results as means of triplicate experiments ± standard deviation (SD) revealed that the increase in expression of *BGL2* was highly induced, about two folds more than *PAL*, and in seedlings treated with surfactin than those infected with virus.

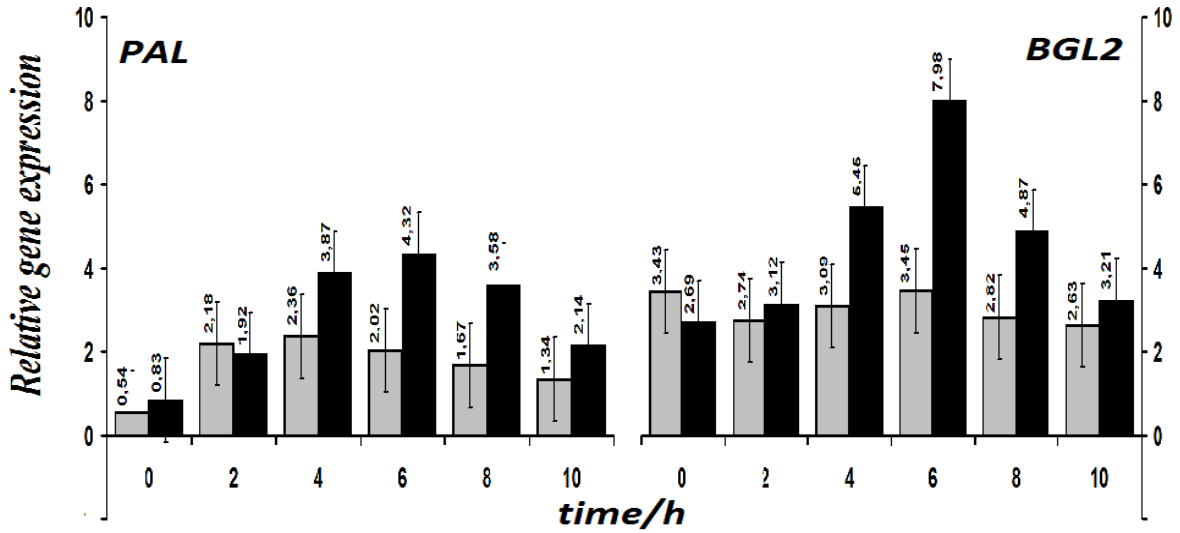

Figure 1. Relative genes expressions (*PAL* and *BGL2*) of the (SA and JA) pathways in infected and surfactin treated tomato seedlings (grey and black coulmns respectively)

3.4. Hydrogen Peroxide Accumulation Levels

The accumulation levels of H_2O_2 were measured at 0, 2, 4, 6, 8 and 10 hours in healthy, infected and surfactin treated (1000 $mg.L^{-1}$) tomato seedlings. The healthy seedlings showed very low H_2O_2 levels compared to the treated seedling which gave higher levels than infected tomato seedlings with the same pattren of *BGL2* and *PAL* expression levels due to the relationship between the ISR involved substrates (SA and JA) with accumulation of H_2O_2 (Figure 2). The concentrations of H_2O_2 ranged between 0.08 to 0.11 ± 0.03 $\mu mol.g^{-1}$ in the healthy seedlings. In infected and treated seedlings, the concentrations showed continued increase to reach the maximum accumulation at 6h (0.33 ± 0.02 and 0.46 ± 0.03 $\mu mol.g^{-1}$ respectively), then decreased. Moreover, the infected seedlings showed the same pattern as those treated but with less ranges. The results presented as means of triplicate experiments ± standard deviation (SD) revealed that the increase in accumulation levels of H_2O_2 was in parallel with increase of *PAL* and *BGL2* expression levels and the levels in treated seedlings with surfactin were one and half folds more than those infected with virus.

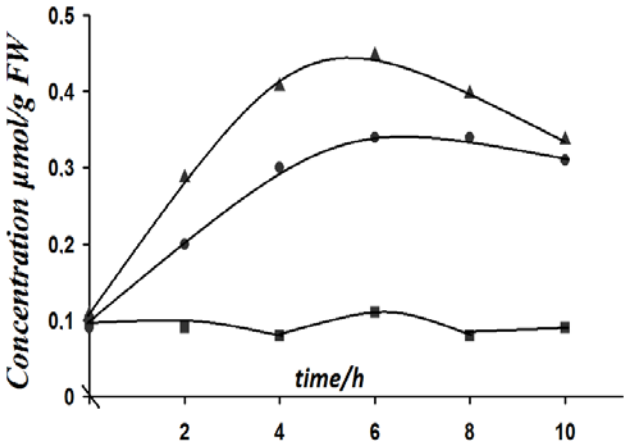

Figure 2. Hydrogen peroxide H_2O_2 concentrations $\mu mol.g^{-1}$ fresh weight in healthy (■), infected (●) and surfactin treated (▲) tomato seedlings

4. Discussion

In this work we proved the antiviral and stimulator ability of surfactin as plant defense reponse by decreasing symptoms severity against ToMV virus and by activated the expression of two genes *PAL* and *BGL2* involved in SA and JA pathways, respectively. The antiviral activities of surfactin may be due to destabilization process of the lipid bilayer in virus membrane which is facilitated by the three-dimensional form of the surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane [23]. The treatment by surfactin may result in the accumulation of plant phenolics derived from phenylpropanoid metabolism with a significant accumulation of mRNA coding for *PAL*. These phenolics, due to the repression of virus multiplication, appear in the strong symptoms reduction with complete negative ELISA results. These results gave the surfactin lipopeptide importance and highlighted their role, especially since none of the pathogen associated molecular patterns identified in the interaction of pathogenic microorganisms with eukaryotic cells resembles the structure of surfactin [24]. Moreover, surfactin is considered not toxic for plant cells and should act by a different way to activate the inducible defence responses [25,26]. In contrast to syringopeptins and syringomycins lipopeptides produced by *Pseudomonas syringae* (phytopathogenic bacterium), they share a similar global structure with *Bacillus* lipopeptides but they act as virulence factors by forming pores in plant plasma membranes and inducing cell death [27]. On the other hand, *PAL* and *BGL2* genes which are common marker genes functioning in the SA and JA biosynthetic pathways play an important role in plant resistance [28] and their expressions were reported after fungal infection in tomato as mentioned by Ahmad and Eveillard [29]. Moreover, the (SA) pathway has also been considered to be involved in the tomato plant defense responses to the herbivore and the accumulation levels of H_2O_2 was also in parallel with increasing of ISR involved substrates as reported by Peng et al. [22]. Interestingly,

surfactin considered as one of lipopeptide families are promising compounds that should be considered more in the fields of plant resistance induction and biocontrol in addition to their multiple roles in environment, agriculture and pharmaceutical applications.

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