Impact of Endophytic Produced Lipopeptides and Whitefly, (*Bemisia tabaci*)
Honeydew Kairomone Bacteria on Limitation of The Insect and Its Associated
Virus in Tomato

Sameh F. Fahim¹*, Walaa Hussein² and Hosam Awad³

1-Agricultural Microbiology and Biotechnology, Botany Department, Faculty of
Agriculture, Menoufia University, Shibin El-Kom 32514, Egypt.
2-Genetics and Cytology Department, Biotechnology Research Institute, National
Research Centre (Affiliation ID: 60014618), Dokki, Egypt.
3-Plant pathogenic, Agricultural Botany Department, Faculty of Agriculture, Menoufia
University, Shibin El-Kom 23514, Egypt.

E-mail*: sameh.shaded@agr.menofia.edu.eg

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ABSTRACT
The whitefly is one of the most damaging pests to economical tomato
crop in the world, it is considered the first plant virus’s transgenic insects.
Tomato mosaic virus (ToMV) is a major tomato viral disease transgenic by this
insect. Many efforts have been done for controlling this virus infection. Among
these many efforts was the whitefly biocontrol by using kairomonic bacteria and
the endophytic bacterial lipopeptides to induce plant resistance against viral
infection. The lipopeptides produced by *Bacillus subtilis* endophytic bacterial
strains isolated from tomatoes showed a strong reduction in the symptoms caused
by (ToMV) virus due to their responsibility for the elicitation of the Systemic
Resistance Induction (SRI) mechanism in tomato seedlings. The re-insertion of
these endophytic *Bacillus subtilis* BMG100 bacterial strains by tissue culture
technique has also a negative ELISA result with complete severe viral symptoms
reduction. The electron microscopical image confirmed the insertion and the
huge spreading of endophyticus within tissues. While the spraying of mechanical
or biological infected seedlings by the produced endophytic bacterial
lipopeptides suspension showed negative ELISA and significant symptoms
reduction by the same action happened on the endophyticus re-bacterized
seedlings, thus confirming the promising antiviral activities of produced
lipopeptides. However, the plant surfaces infected by whitefly and treated with
*Priestia endophytica* BMG103 and *Bacillus endophyticus* BMG104 kairomone
bacteria showed a big vanishment of whitefly stages on the opened field.
Moreover, the levels of relative gene expression of the enzyme phenylalanine
ammonia lyase (*PAL*) and the other β 1,3-glucanase 2 (*BGL2*) which they
involved in the promoting of induced resistance phenomenon in tomato seedlings
were elevated by lipopeptides treatment due to the relationship between the
systemic resistance induction (SRI) with salicylate and jasmoniate involved
substrates in defense mechanisms.
INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most widely produced and extensively consumed crops in the world, tomato production accounts for about five million hectares of harvested land area globally (FAO 2020). Tomato main source of income for many vegetable producers in semi-urban and most rural areas in all developing countries. Despite that, many challenges faced by this crop make its production less profitable in all developing countries around the world especially those in Africa. Egypt tops the list of producing countries in Africa by leading the continent with a total production of eighteen million tons (FAO 2020).

The silver leaf whitefly (*Bemisia argentifolii*) feeds on a diversity of plant species, it is considered the most dangerous insect to agricultural crops by damaging the vegetative photosynthetic part and releasing the viruses and honeydew during its phloem sap-feeding (Bellows *et al*., 1994).

The mycetoma’s of *Bemisia argentifolii* possesses obligative culturable and non-culturable closer endosymbionts bacteria which are transmitted during the female oviposition, one of these bacterial types at least is mildly pathogenic to nymphs or adults’ insects; Also, the pathogenic bacteria transmitted by hoverfly (*Episyrphus balteatus*) which attracted to the rich volatiles component honeydew and targeting the oviposition machines (Leroy *et al*., 2011).

However, the development of the non-chemical protocol for managing insects by invoking the parasitoids’ natural enemy is an innovative biological pest control strategy and considering high levels of resistance to insecticides development (Roopa *et al*., 2014).

Tomato mosaic plant virus (ToMV) is considered the major disease of tomato crop among all plant diseases which is usually transmuted by sap-sucking insects like whiteflies and can strongly reduce tomato crop yields, (ToMV) has a closely serological relationship to tobacco mosaic plant virus strain (TMV) (Fahim and Hussein 2016).

After viral infection salicylate biosynthesis plays an important role in the plant signaling pathway of systemic resistance induction (SRI). The phenylalanine ammonia-lyase (*PAL*) enzyme is one of the most studied enzymes in secondary metabolisms pathways. However, β 1,3-glucanase 2 (*BGL2*) enzyme levels were activated in tomato seedlings and are in charge of the jasmoniate which including on the defense mechanism, in addition, they act upstream of salicylate and jasmoniate biosynthesis are very implicated for plant defense pathway (Fahim and Hussein 2016).

However, calling the natural enemy is a genius technique in developing of insect’s bio-controlling strategy, moreover honeydew represented a favorable un-prey meal that stimulated the parasitoids explores (Hymenoptera) and predators’ implications (Wackers *et al*., 2008).

Furthermore, the whitefly parasitoid is attracted more to the main substances on the honeydew of trehalose and trehalulose. Thus, honeydew will serve as a meal and searching signal as a host-location kairomone for the predators and an oviposition stimulus parasitoid, the volatiles component secreted by the honeydew bacteria which enhanced kairomone attractivity for balteatus and hoverfly by ovipositional preferences and its driving for the prey location (Leroy *et al*., 2011).

In agricultural ecosystems, endophytic bacteria play an important key in plant disease control by their presenting a high adaptive ability and endospheric-competent (Larran *et al*., 2016). Besides that, the endophytic of the genus *Bacillus* can protect host plants from pathogenic infection, this protection is returned to the elicitation of plant resistance indication coupled with a mechanism of antagonism in the plant cellular constituents which
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provides a carefully prepared environment to stay protected from the abiotic stress or biotic invasion (Rajendran et al., 2015).

Very few investigations recorded the elicited phenomenon of induced resistance in plants by endophytic *Bacillus* genera as a response to the viral attack and its efficiency in reducing the disease severity which is difficult to control under greenhouse conditions (Sundin et al., 2016).

However, the plants' re-bacterization by systemic protection promoting endophytic bacterial strains could be sufficient as plant pathogens biocontrol agents replacing the use of agrochemicals, this trend being a promising and more economically viable alternative (Verma and Gange 2014).

In the same trends, the commercial inoculums include serenade, based on *B. subtilis* QST-713, which produces three types of lipopeptides and is effective against plant diseases (Marrone 2002).

As well as Bioshield™ which utilizes the spray-drying of a mixture of endospores with vegetative cells and lipopeptides from *B. subtilis* CPA-8, with effective control capabilities after six months of storage (Yáñez-Mendizábal et al., 2012).

Our team co-workers have also researched and developed effective forms of spraying lipopeptide (surfactin) single type produced from *B. subtilis* BMG02 against ToMV virus in tomato. Finally, this trend of using endophytic bacteria in the open field conditions is very safe and would reduce the number of sprayed agrochemical substances used to biocontrol many types of plant pathogens at the same time, which will be resulting in reducing the production costs and lowering the environmental toxicity to humans and animals.

**MATERIALS AND METHODS**

1. **Bacterial, Insects’ Stages, and Plant Materials:**

   Tomato super Marmande seedlings (*Lycopersicon esculentum*) were cultivated in sterilized soil in plastic bags groups under full isolation greenhouse conditions and irrigated regularly with sterilized water as control. The second seedlings group was infected by various whitefly (*Bemisia tabaci*) stages. The third seedlings group was infected by tomato mosaic virus (ToMV) crude sap, the seedlings were used for insects and virus propagation then *in-vitro* and *in-vivo* experiments and other additive seedlings for further investigations (Fahim and Hussein 2016).

   Whitefly eggs, nymphs and adults were collected in groups due to their small sizes (about 100 units) from various infected tomato plants and then surface sterilized with ethanol 70% and household by sodium hypochlorite. The collected insects’ samples were well homogenized in sterile saline solution 0.9% and transferred into brain heart infusion pre-rich broth (Difco) for 12h, either whole the homogenized insects were plated on brain heart infusion agar (Sigma) and incubated aerobically at 30±2°C for complete enumeration (Davidson et al., 1994).

   The formed honeydews in infested nymphs tomato leaf were collected onto abaxial surface exposure of the sterile petri-plate lid. The collected nymph’s honeydew samples were sterile gathered and serially diluted, then honeydews samples were plated on brain heart infusion agar (Sigma) as a source for entomopathogenic bacterial isolates and then incubated aerobically at 30±2°C for complete enumeration for purified as single colony separation protocol (Thomas 2012).

   The previously isolated endophytic bacteria named *Bacillus subtilis* BMG100 was selected for our investigation due to its lipopeptides biocontrol activity in a tomato plant, the endophytic and plant pathogenic strains which were used to estimate the conditions of
antimicrobial tests were collected from the microbiology and plant pathology laboratory, botany department, faculty of agricultural, Menoufia University, Egypt (Hussein et al., 2018).

2. Identification of Entomo-Honeydew Associated Bacteria:

The protocol of 16S rDNA amplifying was applied (Teng 2006), and the genomic DNAs isolation was performed using Wizard ® Genomic DNA Purification Kit from Promega. PCR using the 16S rDNA bacterial universal primers 8F; 5’AGAGTTTGATCCTGGCTCAG’3 and 1492R 5’GGTTACCTTGTTACGACTT’3 was followed as standard protocols.

The PCR steps were performed at 94°C for 1 min as denaturation step and hybridization at 50°C within 30 sec, elongation at 72°C for one and half a minute, the PCR was performed for 35 cycles and the amplified 16S rDNA genes were separated on 1.2% agarose gel electrophoresis with expected product sizes of 600 bp, while the gel excised bands were purified by ZymocleanTM Gel DNA recovery kit (Epigenetics company).

The ligation step for the purified 16S rDNA fragments was done into pGEM-T Easy vector (Promega) and then the vector was transformed through competent cell type of E. coli JM107 as described in pGEM- T Easy vector manual.

The successful transformants colonies were grown on Ampicillin/IPTG/X-Gal LB solid medium and then incubated at 35±2°C for 18 hours until the appearance of white colonies which were chosen and purified by Mini-Prep Plasmid Purification Kit (Promega).

Plasmids were double digested by EcoRI restriction enzyme for 16S rDNA genes insertion verification into pGEM-T Easy vector. Cloned genes were then sequenced, and the obtained data were aligned in the GenBank database (Blast) online software (Hussein and Fahim 2017).

3. Bacterial Fermentation Conditions and Plants Re-Bacterization:

The strains used in this work were grown aerobically in Luria-Bertani (LB) medium. While the production of the lipopeptide was obtained in specific fermentation regimes in Landy modified Medium which was previously examined and mathematically calculated with a standard deviation percentage between 3.34 and 9.96 % for surfactin and kurstakin values and between 2.03 and 59.55 % for iturin and fengycin values. These conditions were tested in each Bacillus strain, the cultures were performed during 48 h of fermentation (stationary phase) at 30±2°C in a modified Landy MOPS medium with glutamic acid, the obtained results are means of three replicates with its standard deviations according to the protocol prepared by (Fahim 2017).

For re-bacterization of the isolated endophytic bacteria into the tomato, the protocol of tissue culture was applied, the tomato seeds and attached stems, leaves, or other plant surface dust was washed from samples by the distilled water, the seeds and plant fragments of 0.5 cm long used during tissues cultivation were pre-sterilization by using a solution of 70% C2H5OH and fowled by 5% NaOCl for no more than five minutes.

The seeds and plant fragments were re-sterilized again with 70% C2H5OH for 10s and well rinsed by sterilized tap water many times, then seeds and plant fragments were emergently incubated on tissue culture prepared media (pH 6.7) at 30±2°C with an endophytic cell suspension of Bacillus subtilis BMG100 and others insect associated Bacilli isolated from insects and honeydew and that population-adjusted at about of 10^8 CFU.ml^-1 at (OD600 = 0.5) (Kado and Heskett 1970).

4. Lipopeptides Genes Detection and HPLC Production Determination:

Lipopeptides genes detection by degenerated primers designed by Arthur and co-workers were lanced; degenerated primers for surfactin, fengycin, iturin, and kurstakin families were applied to investigate the gene absence or presence of NRPS, the PCR steps
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were performed as protocols described by (Needelman et al., 1970; Tapi et al., 2010; Hussein and Fahim 2017).

However, Microbial fermentation was lanced in Landy modified medium for 48 h (stationary phase), and the bacterial cells were discarded from centrifugate fermented media on 15,000 r.p.m for 10 min under cooling at 5°C. while, for lipopeptides extraction, 0.5 ml supernatants samples were passing through clean C\textsubscript{18} cartridges (protocol of Alltech, Fr).

The concentrations of lipopeptides families were determined by the spectrum of (HPLC) reverse phase, the produced lipopeptides were extracted after microbial batch fermentation. kurstakin produced strains, cells were collected and sonicated for 1 min at low temperature at 6 Watt (Ultrasonic processor, Cole-Parner Instruments, Illinois, USA).

The total yield was gathered before analysis by (HPLC) passing through a C\textsubscript{18} column (5.1 μm; 255 by 4.5 mm; VYDAC 218 TP, Hesperia, GE), while the second derivatives spectrum of UV-visible and the retention time of each peak was used to identify the lipopeptides molecules (Waters integrated PDA 996 diode array detector: Millenniums Software according to (Fahim 2017).

5. Greenhouse Bioassay, Insects’ Infection, And Mechanical Virus Transmission:

The ToMV virus isolates sources were collected from mosaicking symptoms and viable insects presented in infected tomato leaf by subsequent infection of host range experiments. The plant crude sap was extracted in phosphate buffer and filtrated through double layers of cheesecloth, for transmitting the virus into the plant, leaves were first dusted with carborundum 600 mesh, then the inoculated plant leaf samples were washed with sterilized tap water but not directly after inoculation.

All inoculated plants were kept under restricted conditions and observed daily for symptoms development. After 25 days from symptoms development, the crude sap of the infected leaf sample was re-collected and serologically examined by Double Antibody Sandwich - Enzyme-linked immune sorbent assay (DAS-ELISA) with viral specific ToMV antiserum.

The resulting concentric local lesions were singly back inoculated tomato plants under full isolate greenhouse conditions. Furth-more, the virus molecules were purified by employing the followed technique of the single local lesion as provisory described by (El-Kammar et al., 2016).

However, the tomato re-bacterized seeds or tissue cultured plants parts or other seedling types were sowed and cultivated in a sterilized mixture of clay, sand, and little of cattle manure with pH 7.0 and EC 0.8 dS.m\textsuperscript{-1}, the NPK formula was (5:15:20) and with a 10 h of photoperiod exposition in full isolated a greenhouse under closed saran at 30±2°C and 75% of relative humidity, and then the ten days old seedlings were spraying by suspensions of the two specific endophytic and entomopathogenic associated bacteria by 10 ml per plant, the original number of local lesions per leaflet was carried out on one week after inoculation by minimum. the experiments were performed on five replicates and also repeated five times (Lanna Filho, Souza, and Alves 2017).

6. RT-PCR Analysis and Scanning Electron Microscopy (SEM):

Ambien RNA later ® (Applied Biosystems, France) was used to determine the reverse SRI genes transcription. The Real Time-PCR was carried out by adding 1X SYBR Green Fluorescent Mix, 250 nmol of used primers and one μl of cDNA in a final volume of 25 μl, while the thermal cycling program was 95°C for five 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, RT-PCR results were expressed as C\textsubscript{i} (cycle threshold) values and the relative
Relative gene expression = Efficiency of tested gene / Efficiency of control.

Moreover, Images of the tomato phylloplane region were generated randomly. Images were scanned using Corel Draw 12 magnifications, the gold sputter-coated in an SCD 50 sputter (Balzers) and observed with an EVO 40 XVP scanning electron microscope (SEM) (Leo Electron Microscopy) as followed protocol adjusted by Lanna Filho (Lanna et al., 2017).

The 15 days endophyticus re-bacterized tomato seedlings were catted and divided into small parts and then fixed by using a modified Karnovsky buffer solution (1 mM CaCl₂ 0.1%, 0.25 mg.ml⁻¹ glutaraldehyde and 0.25 mg.ml⁻¹ paraformaldehyde in 50 mM sodium cacodylate buffer pH 7) for 20 h at 5°C, infiltrated with a glycerol cryoprotection solution for 20 min after being immersed in liquid nitrogen and cross-sectioned with a scalpel blade. The observed thin sections were transferred into a 0.1 mg.ml⁻¹ osmium tetroxide solution for one hour at 25°C and subsequently dehydrated by decimal graded acetone solution for 10 min (Lanna et al., 2017).

RESULTS AND DISCUSSION

1. Identification of Entomo-Honeydew Associated Bacteria:
The dissimilar entomo-honeydew-associated bacterial colonies were isolated aerobically on brain heart infusion agar medium from the insects and their honeydew. colonies usually appeared on agar plates within two days if insects will homogenize, suggesting that the associated bacteria were inside the insect. Two bacterial isolates belonged morphologically to spore-forming long rode bacilli, as showed a positive result with Gram and spore staining.

Of the other endophytic bacterial isolates, seven of them belonged to short bacilli and one isolate was small cocci, these isolates revealed a negative result with gram and spore staining. However, B. subtilis, B. licheniformis and B. endophyticus bacterial isolates in the latest investigations were found to produce long-chain oligosaccharides from sucrose (Davidson et al., 1994).

In the same inspection, a lot of investigations have referred to the endophytes which have the ability to produce antimicrobial agents; such as surfactins, fengycins and iturins compounds, which are lipopeptapeptides in cyclic form produced by several types of Bacillus strains with strong inhibitory activities against various phytopathogenic bacteria, fungi, viruses, and mycoplasma (Chae Gun Phae et al., 1990). In conclusion, the amplified fragment of the 16S rDNA gene of the two entomo-associated Bacilli isolates was 600 bp in length. while their sequences data were in comparison to those aligned in Blast databases, the results confirmed the belonging of the isolates to Bacillus endophyticus which are named BMG103 and BMG104 as shown data in Table (1).
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Table 1: List of Blast nucleotide alignment of 16SrRNA partial gene of isolated *Bacillus* strains

<table>
<thead>
<tr>
<th>Description</th>
<th>Identity %</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus endophyticus</em> strain 70 (BC7) 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>AY055225.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> strain A6 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>OL636032.1</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain XAAS.x29 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MT459821.1</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain MF126 16S ribosomal RNA partial gene, partial sequence</td>
<td>99.00</td>
<td>MT184820.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> 16S rRNA gene, isolate VrR14, partial sequence</td>
<td>99.00</td>
<td>MT052665.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> 16S rRNA gene, isolate VrL13, partial sequence</td>
<td>99.00</td>
<td>MN853992.1</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain BAB-6962 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MH744622.2</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain S2-11 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MN133852.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> strain YN14 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MK235125.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> strain 33 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MG705600.1</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain KLS23 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>MK493701.1</td>
</tr>
<tr>
<td><em>Bacillus endophyticus</em> strain S160(2) 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>LN864483.1</td>
</tr>
<tr>
<td><em>Priestia endophytica</em> strain JYW5 16S ribosomal RNA gene, partial sequence</td>
<td>99.00</td>
<td>OM791713.1</td>
</tr>
</tbody>
</table>

The sequence of 16S rRNA gene of BMG06 showed similarity to the *Priestia endophyticus* strain MF126 sequence (accession N°: MT184820.1) by (99%) Also, it has similarity to *Priestia endophyticus* strains KLS23 sequence (accession N°: MK493701.1) by the same percentage.

Also, the sequence of the second 16S rRNA gene of the isolated BMG104 has similarity to *Bacillus endophyticus* strain YN14 (accession N°: MK235125.1) sequence by (99%).

While was similar to *Bacillus endophyticus* strain VrL13 (accession N°: MN853992.1) sequence by the same percentage. In general, the previous data of 16S rRNA gene similarity refer to the strong probability of belonging to the two entomo-associated *bacilli* isolates to the species of *Priestia endophyticus* and *Bacillus endophyticus*, respectively according to the data presented in the phylogenetic tree and illustrated Figure (1).

![Fig. 1: Phylogenetic tree based on 16SrRNA partial gene sequence alignment of isolated entomo-associated *bacilli* strains.](image-url)

The phylogenetic tree based on the alignment of 16S rRNA gene sequence of tomato-associated *Bacilli* strains with *Priestia endophytica* and other *Bacillus endophyticus* strains indicated that all *Bacillus* sp could be classified into two major clusters from the same node as shown in Figure (1).
The first cluster includes the entomo-associated *Priesita endophytica* named BMG103, while the second cluster which includes the entomo-associated strain *Bacilli* BMG104 which appear to be related to the species of *Bacillus endophyticus*.

2. Lipopeptides Genes Detection and HPLC Production Determination:

The non-ribsosomal lipopeptides genes were detected by degenerated primers technique in the two entomo-associated isolates in addition to the recently endophytic isolated *B. subtilis* BMG100, four genes were amplified: *Srf* (surfactin-surfactin), *Pps* (plipastatin-fengycin), *Myc* (mycosubtilin-iturin) and *Kur* (kurstakin- kurstakin) by degenerated primers, respectively.

The entomo-associated short rode bacilli and cocci forms strains had no fragments amplified with the three degenerated primers. While only the long rode *Priesita endophytica* and *Bacillus endophyticus* strains were found to harbor the lipopeptides synthetases genes, these two strains were given laboratory names (BMG103 and BMG104) for further pursuit.

The observed fragment length from used degenerated primers for the standard lipopeptides producer *B. subtilis* BMG100 and whitefly associated bacilli isolates is mentioned in Table (2).

Firstly, the endophytic strain *B. subtilis* BMG100 was recently used to co-produce surfactin, mycosubtilin and plipastatin types with a concentration of 529 mg.l\(^{-1}\), 228 mg.l\(^{-1}\) and 198 mg.l\(^{-1}\), respectively as described before by Hussein *et al.* (2018).

The BMG100 strain was given three fragments’ sizes of 425, 419 and 893 bp length for amplified surfactins, iturin and fengycins primers, respectively. While the first entomo-associated strain *Priesita endophytica* BMG103 was given only one fragments size of 1162 bp length for amplified kurstakin primer.

While the second entomo-associated strain *Bacillus endophyticus* BMG104 was also given one fragments size of 431 bp length for amplified surfactins primer. As mentioned above, the use of plipastatin Pps primers could amplify fragment length about 893 bp from examined *B. subtilis* BMG100 strain belongs to plipastatin synthetases genes, which was confirmed before in *B. subtilis* ATCC 168 and *B. subtilis* ATCC 21332 strain by (Hussein and Fahim 2017).

In general, degenerated primers approach led to the achievement of NRPs genes presence in tomato associated bacilli, this trend was confirmed before by (Tapi *et al.* 2010) who reported that the use of degenerated primers is helpful in screening the various non-ribsosomal synthesis genes harbor by *Bacillus* spp. which supported to detect a new non-ribsosomal synthesis molecule and it facilitates the study and genetic potential knowledge of lipopeptides molecules biosynthesis.

On the other side, the productivity quantification of lipopeptides by HPLC of lipopeptides for isolated endophytic *B. subtilis* BMG100 and the other entomo-associated strain *P. endophytica* BMG103 and entomo-associated strain *B. endophyticus* BMG104 were investigated in Table (2).

The production quantification of lipopeptides revealed the ability of the first isolate strain *P. endophytica* BMG103 to mono-produce types of lipopeptides (kurstakin) by 117 mg.l\(^{-1}\). However, the secede isolate strain *B. endophyticus* BMG104 also showed one type lipopeptides production as 471 mg.l\(^{-1}\) of surfactin.

These latest results proved that all the isolates from tomato cultivars or the insect sources had the ability to produce lipopeptides, it was also found that endophytic isolates are co-producers of three families of lipopeptides; surfactin, fengycin and iturin which are known as inducers of systemic resistance system in the plant (Fahim and Hussein 2016).

While the previous study showed that, *B. subtilis* 21332, *B. subtilis* BMG100, BMG101 and *B. amyloliquefaciens* FZB03 strains were thought to be lipopeptides co-
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producer, however, the primary lipopeptides production experimentations under investigation condition showed more type of lipopeptides production by the endophytic strains (Gancel et al., 2009; Hussein et al., 2018).

Table 2: The lipopeptides gene detected and the bacillus studied strains HPLC productivity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lipopeptides fragments detected</th>
<th>Lipopeptides HPLC productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surfactins primer</td>
<td>Fengycin primer</td>
</tr>
<tr>
<td><em>B. subtilis</em> BMG100</td>
<td>431 pb</td>
<td>893 pb</td>
</tr>
<tr>
<td><em>P. endophytica</em> BMG103</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. endophyticus</em> BMG104</td>
<td>431 pb</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detected

3. Greenhouse Bioassay, Insects’ Control, And Mechanical Virus Limitation:

Tomato seedlings infected by crude sap including ToMV virus imported from the infected plants showed the ideal symptoms within two weeks after infection and indicated as positive in ELISA reaction after mechanical or biological inoculation with ToMV virus, while some other seedlings showed very weak symptoms or no symptoms but also gave a positive ELISA reaction.

During greenhouse experiments, the biological infected seedlings showed the ideal symptoms with confirmed positive ELISA, while the re-bacterized seedlings with endophytic *B. subtilis* BMG100 showed evidence of a significant reduction in symptoms severity with complete negative ELISA results, these results were matching with those obtained by our team (Hussein et al., 2018).

Also, the re-bacterized seedlings with entomo-associated *P. endophyticus* BMG103 and *B. endophyticus* BMG104 showed moderated evident reduction in viral symptoms severity with still positive ELISA results as indicated for the presence of viral infection.

The same action happened by spraying mechanical infected seedlings with the produced *B. subtilis* BMG100 endophytic bacterial lipopeptides suspension which showed the same effect of significant viral symptoms reduction with complete negative ELISA, thus confirming the capability of produced lipopeptides as antiviral active agents.

While the plant surfaces infected by whitefly and treated with *P. endophyticus* BMG103 and *B. endophyticus* BMG104 kairomone bacteria had no action except those treated seedlings by *P. endophyticus* BMG103 which showed a significant limitation about 58% of whitefly of *B. tabaci* adults’ stages when treated by a high concentration of bacterial cell 10⁹ cells.ml⁻¹, this insecticidal phenomenon may be due to kurstakin lipopeptides effect produced by this strain (data not shown).

On the other hand, during the open-field experiment, it is remarkable that the plant surfaces formed honeydew seedlings and had been treated with *P. endophyticus* BMG103 and *B. endophyticus* BMG104 showed a big vanishment of whitefly various stages predation on the opened field.

4. RT-PCR Analysis and Scanning Electron Microscopy (SEM):

The recorded levels of phenylalanine ammonia lyase (*PAL*) and β 1,3-glucanase 2 (*BGL2*) relative gene expressions involved in the promoting of induced resistance phenomenon in tomato seedlings were elevated by lipopeptides treatment due to the relationship between the systemic resistance induction (SRI) with salicylate and jasmoniate involved substrates in defense mechanisms, the expression values were detected until 16 hours in both treated and untreated tomato seedlings were summarized in Table (3).
Table 3: The relative SRI genes expressions of treated tomato seedlings by endophytic bacilli

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>Gene</th>
<th>0 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal treated</td>
<td>PAL</td>
<td>3.42</td>
<td>3.67</td>
<td>3.45</td>
<td>3.28</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td>BGL2</td>
<td>6.60</td>
<td>6.46</td>
<td>6.37</td>
<td>6.25</td>
<td>6.38</td>
</tr>
<tr>
<td>Infected control</td>
<td>PAL</td>
<td>1.07</td>
<td>2.24</td>
<td>1.63</td>
<td>1.18</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>BGL2</td>
<td>2.13</td>
<td>4.58</td>
<td>2.96</td>
<td>2.23</td>
<td>2.19</td>
</tr>
<tr>
<td>Infected treated</td>
<td>PAL</td>
<td>3.76</td>
<td>3.89</td>
<td>3.47</td>
<td>3.34</td>
<td>3.41</td>
</tr>
<tr>
<td></td>
<td>BGL2</td>
<td>6.81</td>
<td>6.87</td>
<td>6.54</td>
<td>6.41</td>
<td>6.59</td>
</tr>
</tbody>
</table>

The expression values after mechanical transmission of ToMV virus were elevated directly after treatment and kept raising to reach the highest levels of expression at 6h after that the values were decreased gradually for both SRI examined genes, differences between the obtained expression values of PAL and BGL2 for the post-viral infection reaction were calculated every 2 hours as a base of normal control and also summarized in Table (3).

The presented data revealed that the increase in the PAL and BGL2 expression values were approximately three and six-folds, respectively of nontreated seedlings which were highly induced in the presence or absence of viral infection, while the levels of BGL2 were about two folds more than PAL expiration values. While the obtained data were matched with those obtained by our teamwork (Fahim and Hussein 2016).

Whereas the pattern of PAL and BGL2 SRI genes showed the same rapid induction from zero hours and continued increasing to reach the maximum expression levels after eight hours then little decreased after that stabilization had happened. Moreover, the genes of PAL and BGL2 are very mutual as marker genes indicators involved in the plant defense pathways which played a necessary role in plant systemic resistance mechanism, while the damaging effect which could be caused by hydrogen peroxide accumulation on tomato cells accumulation levels of H₂O₂ was also in parallel with SRI involved biosynthetic substrates augmentation (Yalpani et al., 1994).

However, the electron microscopy scanning photo for the tomato seedlings’ abaxial foliar surface (super-marmande cultivar) treated from seeds or by tissue culture technique with endophytic B. subtilis BMG100 were showed by magnification bar of 20 μm (Fig 2).

![Fig 2: Electron microscopy scanning for the normal tomato seedlings (right), treated from seeds (middle) or by tissue culture technique (left) with endophytic B. subtilis BMG100.](image_url)

The endophytic colonialization was a heavy presence around the plant sub-stomatal chambers after tissue culture re-bacterization by comparing with those re-bacterized from seeds which showed moderated bacterial colonialization of adjacent epithelial cells. These results confirmed the efficiency of the tissue culture technique for reinsertion of the isolated endophytic bacteria, the same foundation was observed by (Lanna Filho, Souza, and Alves 2017).
Impact of Endophytic Produced Lipopeptides

Conclusion

The use of promising endophytic beneficial bacteria which have a biological control activity is gaining popularity in agriculture as a protocol concern about, safe cultivation, post-harvest control and food security. Further exploration discussed the ability to re-employ endophytic bacteria, but the role of these bacteria on the plant systemic defense induction is still limited. In our study, we have focused on the control of the ToMV viral infectious disease which is related to the phenomenon of the whitefly biological transmission limitation and another side by the induction of resistance elicited by the associated tomato endophytic bacteria. However, the confirmed evidence for the presence of endophytic bacterial aggregates through plants tissue was observed by electron microscopic photography. Thus, our investigation referred to the ability of endophytic *Bacilli* to modulate the systemic defense response against viral and insectile attacks. While the development of the expected bio-pesticide product that consists of *Bacilli* endophytic bacterial strains to target a most important tomato disease.

REFERENCES


Impact of Endophytic Produced Lipopeptides


