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Induction of Response Genes Involved in The Antioxidants Defense System Against Wheat Steam Rust (*Puccinia graminis var. tritici*) by Lipopeptides Produced from Endophytic *Bacillus amyloliquefaciens* and *Bacillus subtilis* BMG03.

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ABSTRACT

Steam rust disease is one of the major wheat threats in the world, prevention of this plant pathogen accidence or reduction of its severity was of importance in this study. The isolated wheat lipopeptide producers' endophytic *Bacillus amyloliquefaciens* and *Bacillus subtilis* BMG03 an over co-producer of both lipopeptides plipastatin and surfactin were used for this purpose. The modified strain has been constructed from *B. subtilis* BMG01 (*B. subtilis* ATCC168 derivative) by the replacement of the P_{pps} weak native promoter with a constitutive one P_{repU} originated from the replication gene *repU* of *Staphylococcus aureus*. Five Egyptian wheat (*Triticum aestivum*) cultivars; Sk93, Yacara, Gemiza 5, Giza 157 and Giza 164 [sensitive cultivars] were infected by *Puccinia graminis* $_{var.}$ *tritici*. and treated with living cells and a microbial suspension containing plipastatin and surfactin mixture of tested bacterial strain and endophyticus *Bacillii*. Both treated and untreated seedlings have been estimated for Copper-Zinc Superoxide Dimutase (*SOD*) and Catalase (*CAT*) genes expressions by Real-Time PCR (RT-PCR) as responsible genes involved in the antioxidant defense system. The values of antioxidant enzymes showed elevated values on the plants with symptoms reduction, *SOD* has shown overexpression values about high two folds in treated and infected seedlings than in infected ones, while *CAT* expression values were about three folds more in treated and infected seedlings than in infected ones due to the relationship between the systemic resistance induction (SRI) with the involved biosynthetically substrates in antioxidant defense mechanisms.

INTRODUCTION

Wheat is the first crop known by humans and consider the most important and economic crop around the world. Wheat faces several diseases caused by fungi, bacteria, viruses, and insects. This huge attack on wheat requires different precautions as it may be due to crop and economic loss (Agrios 2005; Bennett and Wallsgrove 1994).

Wheat is one of the most important cereal crops which faces various plant diseases and the study use of biological alternatives in-place studies of chemicals treatment against

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plant pathogens became urgent with the use of beneficial bacterial endophytes, which can reduce the severity of symptoms by plant providing with benefits natural molecules and inducing the defense mechanisms (Benner 1993; Heath 2000).

Generally, plants protect themselves against pathogens, any other attack, or stress conditions by complex mechanisms such as secondary metabolites accumulation and production of reactive oxygen species (Agrios 2005; Benner 1993; Heath 2000; Bennett and Wallsgrove 1994), alterations the structure of cell wall (Castro and Fontes 2005), and synthesis of defense proteins (Ebrahim, Kalidindi and Singh 2011).

The reactive oxygen plant defense has three toxic major forms which are superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl free radical which can finally lead to the plant cells dying by oxidative destruction and therefore, by this action the plant can prevent the spreading of obligative pathogens (Heath 2000; Asada 2006; Gechev *et al.,* 2006).

The reactive oxygen defense can also act as signals that lead to the induction of another defense or resistant mechanisms (Dat *et al.,* 2000; Grant and Loake 2000). While, the major reactive oxygen defense scavenging enzymes include Catalases, Peroxidases, Glutathione reductase, Glutathione peroxidase, Ascorbate peroxidase, Dehydroascorbate reductase and Superoxide dismutase (Grant and Loake 2000; Mittler *et al.,* 2004).

Bacillus subtilis produce a wide range of active molecules by Non-Ribosomal Peptides System (NRPS) and/or polyketide synthases among these molecules are lipopeptides which include four known families till now: surfactins, fengycins or plipastatins, iturins and kurstakins. These molecules or compounds have antibacterial, antiviral, and antifungal activities against a wide range of plant pathogens (Peypoux 1999; Bonmatin 2003; Ongena *et al.,* 2007).

Recent studies have highlighted the ability of lipopeptides families in stimulating defense mechanisms in various plants; whereas fengycins or plipastatins from *Bacillus amyloliquefaciens* S499 have shown the ability to protect the fruit wounded against *Botrytis cinerea* causes gray mold disease in apple fruits (Ongena *et al.,* 2005).

Moreover, *B. subtilis* mixture strain M4 can produce a mixture of lipopeptides types from the surfactin, fengycin and iturin families which was capable to control the fungal disease incidence caused by *Pythium aphanidermatum* and *Colletotrichum lagenarium* in tomato and cucumber, respectively (Ongena *et al.,* 2005).

In addition, fengycins and iturins have shown a reduction of fusarium head blight in wheat (Athukorala, 2009), whereas the antiviral and stimulator ability of surfactin was proved by decreasing symptoms severity against ToMV virus in tomatoes (Hussein *et al.,* 2016).

On other hand, *Bacillus amyloliquefaciens* metabolites have been found to be antagonistic in the fungal growth limitation in wheat kernels and elimination of mycotoxin production. However, surfactins are amphiphilic cyclic compounds formed of seven amino acids linked to ß- hydroxyl fatty acid chain (C_{13} to C_{16}), while fengycins or plipastatins formed of ten amino acids linked to ß- hydroxyl fatty acid chain (C_{14} to C_{18}).

The aim of our investigation is the ability evaluation of lipopeptides types (surfactins, iturins and fengycins) produced by wheat endophyte and the strong mutant strain *Bacillus subtilis* BMG03 a derivative of strain ATCC168 on gradually stimulation of antioxidant defense mechanism in some Egyptian wheat cultivars and decrease the damaging effect which could be caused by hydrogen peroxide accumulation on wheat cells.

MATERIALS AND METHODS

1. Bacterial Strains, Plant Materials, and Cultivation Conditions:

Five Egyptian sensitive wheat cultivars; Sk93, Yacara, Gemiza 5, Giza 157 and Giza 164 were cultivated in mashed soil through plastic bags for each seedling under greenhouse conditions and regularly irrigated every week with sterilized water or treated by lipopeptides (bacillomycin, plipastatin and surfactin) bacterial mixture solution. Each plant group composite from three replicates of different tolerant and sensitive cultivars was chosen, and the seeds of each cultivar were surface pre-sterilized as described protocol (Snook *et al.,* 2009; Wen *et al.,* 2011).

The plant leaves were washed from the attached dust with distilled water, then by ethanol alcohol 70% and pre-sterilized with 5% sodium hypochlorite solution. After that, the seeds were rinsed with sterilized distilled water three times, then seeds were re-sterilized another time with ethanol alcohol 70% for and then washed with sterilized water many times, the seeds were soaked in 25 ml sterilized distilled water overnight to become soft easy to be mashed. For seed endophytic isolation, this mixture was homogenized and incubated through YPDA cultures medium (20g peptone, 10g yeast extract, 20g dextrose and 15g agar in 1-liter total volume, pH 6.5) and 1/10 869 [0.1g glucose, 0.5g Nacl, 1g tryptone, 0.5g yeast extract and 15g agar] in 1-liter total volume, pH 6.5 (Mergeay *et al.,* 1985).

For each bacterial cultivation in three replicates, one negative control (it was performed from surface-sterilized plant material) and positive control (100µl from the rinsed water), the incubation was made at 28°C until the appearance of colonies. Each resulting colony was cultured on LB solid plate separately at 28°C overnight till growth, physiological identification for strains followed by DNA extraction and 16srDNA isolation.

Bacillus subtilis BMG03 was the reference described as a recently constructed over-lipopeptide producer strain for plipastatin and surfactin by the replacement of the plipastatin native promoter by a constitutive one P_{repU} from strain *Bacillus subtilis* BMG01 which also was recently constructed by the insertion of an *sfp+* (4'-phosphopantetheinyl transferase) active gene in *B. subtilis* ATCC168. While *Bacillus subtilis* BMG03 has produced approximately 507 $mg.l^{-1}$ of plipastatin with 1162 $mg.l^{-1}$ of surfactin according to (Hussein and Fahim 2017).

2. Identification of Wheat Seeds Endophytic *Bacilli* Associated Bacteria:

The protocol of 16S rDNA amplifying was applied (Qihui 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.

PCR steps were performed at 94°C for one min as denaturation point and hybridization at 50°C within 30 sec, elongation at 72°C for one minute and a half, PCR was prepared for 35 cycles and the amplified 16S rDNA genes were separated on 1.2% agarose gel electrophoresis with expected product sizes of 1500 bp, gel excised bands were purified by ZymocleanTM Gel DNA recovery kit (Epigenetics company).

Purified 16S rDNA fragments were then ligated to pGEM-T Easy vector (Promega) and transformed into *E. coli* JM107 competent bacterial cells as described in pGEM- T Easy vector manual, while the success transformants colonies were grown on Ampicillin/IPTG/X-Gal LB media and incubated at 37°C for 24 hours until the types of white colonies appeared which were previously will choose 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, however, the sequences data for the cloned

genes were aligned in the GenBank database (Blast) and also aligned by online Needle software (Needleman and Wunsch 1970).

3. Lipopeptides Genes Detection and HPLC Productivity Determination:

In the presence or absence of lipopeptides genes, the degenerated primers detection for non-ribosomal peptides synthetases (NRPS) for lipopeptides families were applied to investigate the gene, however, the protocol of PCR was performed as described by (Tapi *et al.,* 2010; Hussein and Fahim 2017).

Moreover, the microbial fermentation was lanced in Landy modified medium for 48 h (stationary phase), and the bacterial cells were discarded from fermented media by centrifugation with 15.000 r.p.m for 10 min at 5°C. while, the lipopeptides extraction was made by passing 0.5 ml of supernatants samples through clean C_{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.

The spectrum of second derivatives of UV-visible and the retention time of each peak were used to identify the lipopeptides molecules (Waters integrated PDA 996 diode array detector: Millenniums Software according to (Fahim 2017).

4. Designed Experiments and Antagonistic Activities Against the Fungal Growth:

Seeds were sterilized by washing with ethanol solution 70 % for one min followed by washing many times with sterilized distilled water. For sample control, the seeds were grown in sterilized potting soil were irrigated with tap distilled water one time each week till seedling growth, whereas, treated seeds were dipped for 20 h in each prepared bacterial suspension (10^8 colony-forming units (CFU. g^{-1}).

After 15 days, the old plants were leaves infected with 10 ml drops of a pathogen spore *Puccinia graminis var. tritici* suspension at 10^6 spores ml^{-1} for both plants T_1 and T_2. Leaves samples were collected from control, T_1 and T_2 for RNA extraction.

The infected plants T_1 (modified *bacilli* treatment) and T_2 (endophytic *bacilli* treatment) were grown under sterilized conditions, each plant was sprayed and irrigated with 50 ml of prepared bacterial suspension every week, whereas five replicates of each wheat cultivars groups control, T_1 and T_2 (Ongena *et al.,* 2007).

5. RNA Extraction and Expression Quantification by RT-PCR Analysis:

The RNA isolation was performed with SV RNA isolation system (Promega). The cells are thawed on ice and then re-suspended by centrifugation at 12000 rpm, -9ºC for 2.5 min, the final supernatant was discarded and 50 µl of T10 E1 buffer solution was added to the collected pellet with 10 $mg.ml^{-1}$ lysozyme solution and then was incubated for 8 min at 25 ºC.

The equivalent of 250 µl of zirconium beads which were provided in the kit was added to the tubes, cells resuspended in 350 µl of RNA wiz (provided in the kit) were transferred into tubes with beads, the RNA wiz containing phenol, which allows fragilization of the cells and inhibits the RNases, the whole was homogenized 10 min by vortex to lyse the cells, the tubes were then centrifuged for 5 min at 4ºC and 12000 rpm.

A volume of 200 µL was obtained and 200 µl of chloroform was added, then the mix was agitated for 30 s and incubated for 8 min at 25 ºC, then tubes were centrifuged at 12000 rpm, 4ºC for five min, while the aqueous phase was obtained, and 0.5 equal volume of 100% pure ethanol was added and the whole was stirred vigorously.

The sample was loaded on the column provided by the kit to purify the RNA. After that, the treatment with DNase I was started, and the RNA dosage was measured. 2.5 volume of ethanol 95% was added to the RNA, which was stored at -80ºC (Fahim and Hussein 2016).

The reverse transcription which reverses transcribed the RNA into cDNA was performed with Sensi FAST cDNA Synthesis Kit (BIOLINE), the primers used copper-zinc

superoxide dimutase (*SOD*) and catalase (*CAT*) responsible for inducing antioxidant systemic defense (ASD) was summarized in Table (1).

Table 1: Primers for genes responsible for inducing antioxidant defense system in wheat

Name	Primer sequence	Product size
***SOD* fwd**	5-AAACTCAACTTTACACTGATACAAACG-3	**781 bp**
***SOD* rev**	5-CCAAAAGTTCTTCCGCTTCC-3	
***CAT* fwd**	5-GTCCGGGTCGATGGTCTG-3	**1573 bp**
***CAT* rev**	5-TATGAGGAGCGGTTCGACTT-3	

On other hand, the expression quantification was made amplified of 15 µl fragment was mixed with 3 µl of 6x loading buffer provided with DNA HyperLadder^{TM} ladder from Fermentas and was migrated in 1.2 % agarose gel colored with GelRed, the gel was photographed under UV in GelDoc equipment (Fahim and Hussein 2016).

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 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_t (cycle threshold) values and the relative gene expression was quantified using the following formula, Whereas Efficiency= C_t untreated - C_t treated (Fahim and Hussein 2016).

Relative gene expression = Efficiency of tested gene / Efficiency of control.

RESULTS AND DISCUSSION

1. Identification of Wheat Seeds Endophytic *Bacilli* Associated Bacteria:

The dissimilar plant-associated bacterial colonies were isolated aerobically on YPDA medium from the five different wheat cultivars, two bacterial isolates were belonged morphologically to spore-forming long rode bacilli, as showed a positive result with Gram and spore staining, the other endophytic bacterial isolates, five of them belonged to short bacilli and three isolates were small cocci bacterial strains.

Moreover, all non-bacilli isolates revealed a negative result with gram and spore staining, in addition, that, several reports have discussed the relationship between endophytes diversity and plant resistance (Araújo *et al.,* 2002). however, the wheat-associated bacteria are important due to the importance of this plant species which have risen the important action of the analysis of these plants' endophytes as a beneficial microorganism (Reiter *et al.,* 2002).

Also, a lot of investigations have indicated that some of the endophytes could have the capability to excrete antimicrobial lipopeptides agents; such as surfactins, fengycins and iturins compounds, which are lipoheptapeptides 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).

On the other side, the amplified fragment of the 16S rDNA gene of the two endophytic *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 amyloliquefaciens* which named BMG06 and BMG07 according to the data summarized in Table (2).

Table 2: List of Blast nucleotide alignment of 16SrRNA partial gene of isolated *Bacillus* strains

Description	Identity %	Accession
Bacillus amyloliquefaciens strain DSM7 (F) 16S ribosomal RNA gene, partial sequence	99.00	**AY055225.1**
Bacillus amyloliquefaciens strain WB3 16S ribosomal RNA gene, partial sequence	99.00	**OL636032.1**
Bacillus velezensis strain AJAS1 16S ribosomal RNA gene, partial sequence	99.00	**MT459821.1**
Bacillus velezensis strain NWPZ-8 16S ribosomal RNA gene, partial sequence	99.00	**MT184820.1**
Bacillus amyloliquefaciens strain SX16NA 16S ribosomal RNA gene, partial sequence	99.00	**MT052665.1**
Bacillus velezensis strain VITSJDJ1 16S ribosomal RNA gene, partial sequence	99.00	**MN853992.1**
Bacillus amyloliquefaciens strain KC1 16S ribosomal RNA gene, partial sequence	99.00	**MH744622.2**
Bacillus amyloliquefaciens strain TRQ59A 16S ribosomal RNA gene, partial sequence	99.00	**MN133852.1**
Bacillus velezensis strain KACC_15879 16S ribosomal RNA gene, partial sequence	99.00	**MK235125.1**
Bacillus velezensis strain APBSDSB54 16S ribosomal RNA gene, partial sequence	99.00	**MG705600.1**
Bacillus amyloliquefaciens strain TRQ59 16S ribosomal RNA gene, partial sequence	99.00	**MK493701.1**
Bacillus amyloliquefaciens strain 16S rRNA gene, strain WS-1, partial sequence	99.00	**LN864483.1**
Bacillus amyloliquefaciens strain FB2 16S ribosomal RNA gene, partial sequence	99.00	**OM791713.1**
Bacillus amyloliquefaciens strain S4A2 16S ribosomal RNA gene, partial sequence	99.00	**OM341575.1**
Bacillus amyloliquefaciens strain JS518 16S ribosomal RNA gene, partial sequence	99.00	**KF983841.1**
Bacillus amyloliquefaciens strain KDP3 16S ribosomal RNA gene, partial sequence	99.00	**MZ396973.1**
Bacillus amyloliquefaciens strain TU11 16S ribosomal RNA gene, partial sequence	98.80	**MW504760.1**
Bacillus amyloliquefaciens strain VITKA1 16S ribosomal RNA gene, partial sequence	98.80	**MT826227.1**
Bacillus amyloliquefaciens strain S94 16S ribosomal RNA gene, partial sequence	98.80	**MT772149.1**
Bacillus amyloliquefaciens strain JT-P23 16S ribosomal RNA gene, partial sequence	98.80	**KP216613.1**

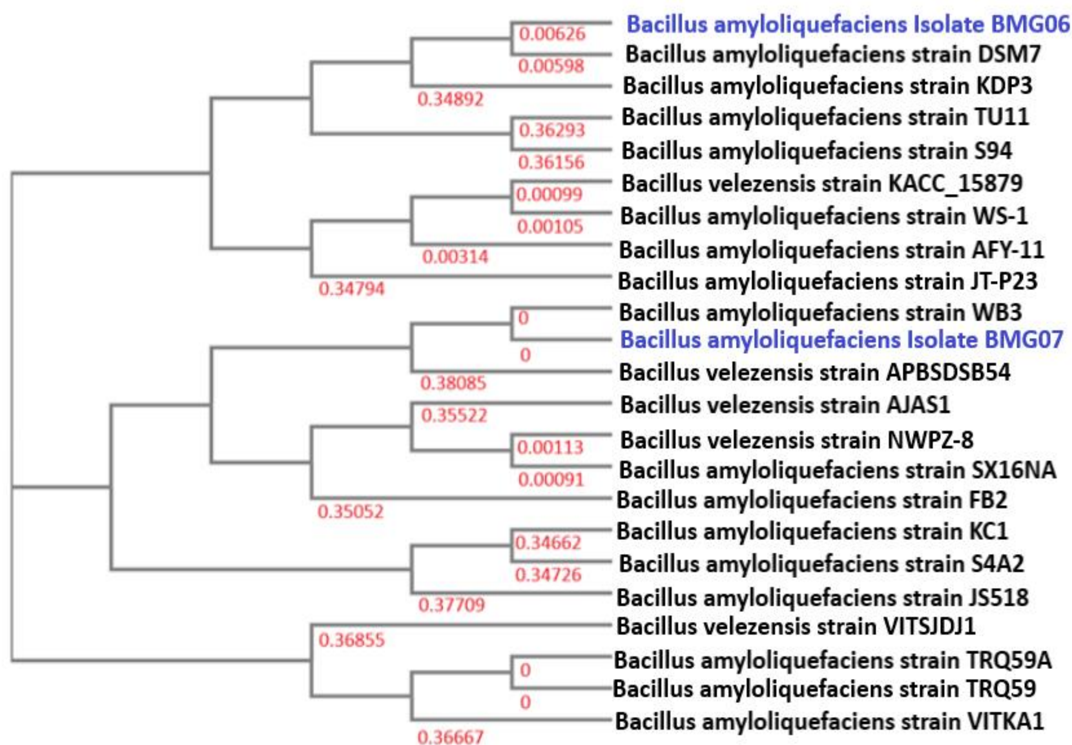

Fig. 1: Phylogenetic tree based on 16SrRNA partial gene sequence alignment of *Bacillus amyloliquefaciens* endophytic.

The sequenced data of the 16S rRNA gene of the strain BMG06 showed similarity to the *Bacillus amyloliquefaciens* strain DSM7 sequence (accession N°: AY055225.1) by (99%), and it also has the same similarity percentage to *Bacillus amyloliquefaciens* strains KDP3 sequence (accession N°: MZ396973.1). Also, the sequence of the second 16S rRNA gene of the isolated BMG07 has similarity to *Bacillus amyloliquefaciens* strain WB3

(accession N°: OL636032.1) sequence by (99%), while was similar to *Bacillus velezensis* strain APBSDSB54 (accession N°: MG705600.1) 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 endophytic *bacilli* isolates to the species of *Bacillus amyloliquefaciens* according to the phylogenic tree Fig (1).

From the presented phylogenetic tree drowned based on 16S rRNA alignment sequenced gene of wheat associated *Bacilli* isolates with other confirmed data of *Bacillus* strains concluded that all isolated *Bacillus* sp, in this study could be classified into two major clusters from the same end node as shown and summarized in Fig (1).

However, the first cluster includes the wheat endophytic associated *Bacillus* strain named BMG06, and the second cluster which includes wheat endophytic strain *Bacilli* BMG07 which appears to be related to the species of *Bacillus amyloliquefaciens*.

2. Detection of Produced Lipopeptides type and HPLC Quantification:

The non-ribosomal lipopeptides NRPL genes were detected by the designed degenerated primers technique in the two wheat endophytic isolates, three genes were amplified: Srf (surfactin-surfactin), Pps (plipastatin-fengycin), Myc (mycosubtilin-iturin) by designed degenerated primers, respectively.

The endophytic short rode bacilli and cocci forms strains had no fragments amplified with the three degenerated primers. While only the long rode *Bacillus amyloliquefaciens* endophytic strains were found to harbor the lipopeptides synthetases genes, these three strains were given laboratory names (BMG06 and BMG07) for further pursuit.

The observed fragment length from used degenerated primers for the standard lipopeptides producer, *Bacillus subtilis* BMG03 strain, and wheat-associated bacilli isolates were mentioned in Table (3).

Firstly, the strain BMG03 (*Bacillus subtilis* ATCC168 derivative) was recently used to produce plipastatin and surfactin types with a concentration of 507 $mg.l^{-1}$ and 1162 $mg.l^{-1}$, respectively as described before by (Hussein and Fahim 2017).

This latest strain was given two fragments sizes of 431 and 893 bp length for amplified surfactins and fengycins primers, respectively. While the endophytic strain *Bacillus amyloliquefaciens* BMG06 was given three fragments sizes of 425, 893 and 929 bp length for amplified surfactins, fengycins and iturins primers, respectively.

The endophytic strain *Bacillus amyloliquefaciens* BMG07 was also given three fragments' sizes of 431, 893 and 929 bp length for amplified surfactins, fengycin and iturins degenerated primers, respectively. As mentioned above, the use of plipastatin Pps primers could amplify fragment length by about 893 bp from all examined *Bacillus* strains belonging 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 wheat associated bacilli endophytes, these topics were confirmed before by (Tapi et al. 2010) who reported that the designed degenerated primers is helpful in achieving and screening various non-ribosomal peptides synthesis NRPS genes harbor by *Bacillus* spp.

This array is very promising and supported to achieve of a new non-ribosomal synthesis molecule and it really facilitates the detection of these types of molecules and provides the genetic potential knowledge of NRPL biosynthesis.

On the other side, the productivity quantification of lipopeptides by HPLC for each isolated endophytic *B. amyloliquefaciens* BMG06 and BMG07 were investigated as the details of the results are presented in Table (3), the production quantification of lipopeptides revealed the ability of the first isolate strain *B. amyloliquefaciens* BMG06 to co-produce three types of lipopeptides (surfactin, plipastatin and bacillomycin) which belonging to (surfactin, fengycin and iturin families) by 315, 321 and 512 $mg.l^{-1}$, respectively.

However, the second isolate strain *B. amyloliquefaciens* BMG07 showed no lipopeptides production although it showed the presence of lipopeptides synthetases genes, these obtained results indicated that not all the endophytic bacterial strains from wheat cultivars had the capability to lipopeptides production, we were found also that the lipopeptides harbored endophytic are naturally co-producers of more than two lipopeptides families; including surfactin as a principle with fengycin and iturin families or both of them which known as strong inducers of systemic resistance in a various plant (Fahim and Hussein 2016).

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

Table 3: The lipopeptides gene detected and the bacillus studied strains HPLC productivity

Strain	Lipopeptides fragments detected			Lipopeptides HPLC productivity		
	Surfactins primer	**Fengycins primer**	**Iturins primer**	**Surfactin type**	**Fengycin type**	**Iturin types**
B. subtills BMG03	431 pb	893 pb	ND	1162 $mg.l^{-1}$	507 $mg.l^{-1}$	0.0 $mg.l^{-1}$
B. amyloliquefaciens BMG06	425 pb	893 pb	929 bp	315 $mg.l^{-1}$	321 $mg.l^{-1}$	512 $mg.l^{-1}$
B. amyloliquefaciens BMG07	431 pb	893 pb	929 bp	0.0 $mg.l^{-1}$	0.0 $mg.l^{-1}$	0.0 $mg.l^{-1}$

3. Amplification and Expression Levels Stimulation of SOD and CAT Genes:

Two genes; superoxide dismutase and catalase responsible for inducing an antioxidant defense system were amplified with the expected sizes of 781 and 1573 bp, respectively. Expression levels were then measured after the first treatment by the Real-Time PCR technique.

The relative genes expressions levels were estimated according to the equation detailed previously. Relative genes expressions were measured at 0, 2, 4, 6, 8 and 10 hours by comparison of controls wheat seedlings (data not shown).

Generally, in T_1 seedlings, expression of *SOD* and *CAT* showed levels less than T_2 seedlings, the expression levels of *SOD* in T_2 increased gradually with time as follows; it was low values at zero hours by (0.76) and begin to induce after two hours by (2.37), and then 4h (4.70), 6h (6.28) till reaching the highest expression at 8h (7.8) as present in Fig (2).

The levels of *SOD* in T_1 showed the same pattern of expression levels in T_2 seedlings but with about two folds fewer values. On the other side, the levels of *CAT* pattern in T_2 seedlings showed a rapid induction at 2h (3.40) and still rose to the highest expression values at six hours by (9.34), then decreased at 8h (5.68). The same pattern for *CAT* was shown in T_1 seedlings but with about two folds and half, fewer values summarized in Fig (3).

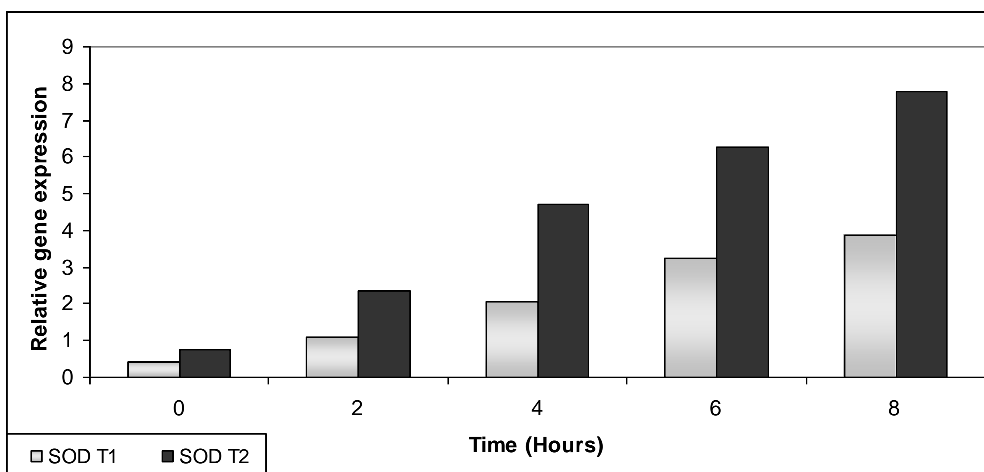

Fig. 2: The *SOD* relative genes expression; T_1: (modified *bacilli B. subtilis* BMG03) grey and T_2: (endophytic *bacilli B. amyloliquefaciens* BMG06) for treated infected seedlings.

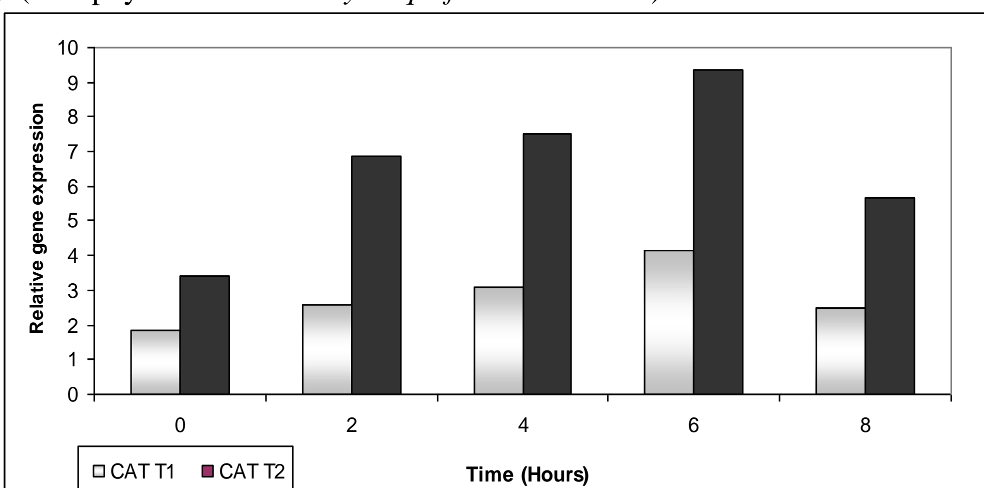

Fig. 3: The *CAT* relative genes expression; T_1: (modified *bacilli B. subtilis* BMG03) in grey and T_2: (endophytic *bacilli B. amyloliquefaciens* BMG06) for treated infected seedlings.

Notably, the big reduction of steam rust symptoms in T_2 seedlings and its antioxidant genes expressions which were three folds more than T_1 and Five times more than control, these results proved the efficacy of endophytic strain *B. amyloliquefaciens* BMG06 (surfactin, plipastatin and bacillomycin co-producer strain) in biocontrol of wheat cultivars against *Puccinia graminis var. tritici* by compression of modified strain *B. subtilis* BMG03 (over surfactin, plipastatin co-producer strain).

However, surfactins and plipastatins differ in their biological activities despite their similar structures whereas, plipastatin play a strong role in plant disease reduction as an antifungal (Ongena, Jacques, *et al.,* 2005).

The combination between plipastatins and surfactins in biocontrol is more efficient than each one separately, whereas surfactin are considered strong hemolytic, antibacterial, antiviral, and antitumor compounds which can be affected the lipid structure of biological membranes and anchor into lipid layers and facilitate the role of plipastatins (Peypoux *et al.,* 1999; Heerklotz and Seelig 2001; Deleu *et al.,* 2003).

Moreover, the preparation of 5 mM of lipopeptides concentration as surfactin has been reported by (Ongena *et al.,* 2005) in its efficiency in the induction of systemic resistance system (ISR) assays and without any toxic effect on plant health and plipastatins can also interact with lipid bilayers and change the cell membrane permeability by alternate its structure (Deleu 2005).

Also, Ali and co-workers have reported that superoxide dismutase (*SOD*) and catalase (*CAT*) have been found to play important role in the tolerance mechanism of some local wheat cultivars to the abiotic stresses (Ali *et al.*, 2017).

Conclusion

The uses of *Bacillus* lipopeptides are displaying a good stimulator for antioxidants defense system and could be an antifungal activator against *Puccinia graminis var. tritici* causes steam rust diseases in some Egyptian wheat cultivars.

These results proved the efficacy of the two *Bacillus* strains in reducing the severe symptoms, especially the endophytic strain *B. amyloliquefaciens* BMG06 (surfactin, plipastatin and bacillomycin co-lipopeptides producer strain) in biocontrol of wheat cultivars against *Puccinia graminis var. tritici* by compression of modified strain *B. subtilis* BMG03 (over surfactin, plipastatin co-lipopeptides producer strain) against the studied plant disease.

Moreover, this topic became promising and necessary for the protocol using beneficial microorganisms' or assonated bacterial endophytes, which can produce the benefits lipopeptides in plants and reduce diseases severity, it can also promote the plant growth, and induce various plant defense mechanisms.

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