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The effect of *Bacillus* species on the response of common bean to *Tetranychus urticae* (Acari: Tetranychidae) infestation

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Abstract:

The alternative of chemical pesticides and bio-pesticides are now widely used to preserve the environment and prevent the spread of pests. In this study, two bacterial isolates well known for their ability to work as bio-control agent as well as plant growth promoting bacteria were tested as bio-pesticides against *Tetranychus urticae* (Koch.) (Acari: Tetranychidae). The foliar spray of common bean plants with the bacterial strains, phylogenetically relevant to *Lysinibacillus sphaericus* and *Bacillus amyloliquefaciens*, led to a significant decrease in *T. urticae* population by 37% after 3 days of treatment, a result that was supported by GC-MS analysis of the metabolites of both bacteria which indicated the presence of different phthalate derivatives as major constituents. On the other hand, the bacterial treatment led to a significant increase in total soluble carbohydrates, proteins and chlorophylls compared with the control indicating the ability of these isolates to alleviate the mites affect as a result of their plant growth promoting activity. Our results also, indicated the possibility of using these bacterial isolates as potential bio-control agents for *T. urticae*.

Introduction

The intensive use of pesticides helped the farmers for some extent to control; however, long-term damage has become more of its benefits as the use of pesticides affect human health (Senthil-Nathan, 2015) as well as the biodiversity in the environment (Pavela, 2015). In order to achieve the goals of sustainable agriculture, It became necessary to use biopesticides (Senthil-Nathan, 2015) that is developed from naturally occurring source and does not leave residues after use. Biopesticides could be microbial pesticide that contain microbe of specific action against the desired pest without negative effect on the treated crop. Microbial pesticides gains an increasing interest in order to achieve Integrated Crop Management (ICM) in an environmentally friendly way (Copping and Menn, 2000).

Mites such as *Tetranychus urticae* (Koch.) (Acari: Tetranychidae) are among the pests threating crop production (20-45%

of the yield might be lost depending on season as growth, chlorophyll contents and fruit size as well as quality are greatly affected in case of severe mites infection (Rhodes *et al.*, 2006 and Premalatha *et al.*, 2018). *T. urticae* has a high rate of fecundity and a short developmental time that can be as brief as one week at high temperatures of about 32°C. The long term intensive use of acaricides leads to the dominance of resistant population that could not be affected by chemical pesticides and that would be reflected on plant yield (Fraulo and Liburd, 2007).

Plant-Incorporated protectants biopesticides has been reported very recently (Pavela, 2015 and Premalatha *et al.*, 2018). Some fungal species, such as *Hirsutella*, have been used as a bio-control agent against mites (Burges, 2012), however, little is known about the use of bacterial pesticides against mites. The use of bacteria in sustainable agriculture is of particular special status for sustainable agriculture because some of these bacteria could have a duel role as a plant growth promoting bacteria beside its ability to control pests (Compant *et al.*, 2005).

Directly, the use of such plant growth promoting bacteria might provide plants with fixed nitrogen as the biological nitrogen fixation is not exclusive to rhizobia. Additionally, they might have the ability to make phosphate available to plants via phosphate solubilization. Siderophores as iron chelating agents has also been recognized as one of the direct benefits attained by these bacteria by which soil unavailable iron becomes available to the host plant (Pérez-Montaño *et al.*, 2014). Host plant growth and development is also regulated by phytohormones produced by endophytes such as auxins (Das *et al.*, 2013) and GA3 (Vessey, 2003) despite its scarcity. Indirectly, ACC (1-aminocyclo-propane-1-carboxylate) deaminase activity provided by the plant growth promoting bacteria decreases the level of elevated ethylene delaying senescence and restoring proper plant growth. In addition, such bacteria would raise the level of plant induced resistance to diseases and insect infections (Ramamoorthy *et al.*, 2001 and Li *et al.*, 2015). In this study, the effect of two types of local bacillus isolates has been evaluated as pesticides against *T. urticae* in a semi-field condition.

Materials and methods

1. **The bacterial isolates and *Tetranychus urticae* culture:**

Two bacterial isolates were used in this study. One of them has been isolated from the north coast of the Mediterranean Sea and molecularly identified as *Lysinibacillus* MFNC5 (accession no KT803879) with a close homology to *Lysinibacillus sphaericus* (Mowafy *et al.*, 2016). The second one was isolated from the nodules of common bean plants and has been identified as *Bacillus MAP3* (accession no MG214652) with a close homology to *Bacillus amyloliquefaciens*. Both strains were cultivated in 250 ml LB media and incubated at 28^{o}C for 2 days. The cells were collected by centrifugation at 5000 rpm for 10 min at 4^{o}C and then re-suspended in sterilized distilled water in order to adjust the CFU concentration of the used isolate using spectrophotometer at 600 nm. The O.D. was confirmed after suspension to be 1 in order to fix the number of cells in treatments via foliar application for both bacillus isolates used in this study considering that O.D 1 = 8 x 10^{8} cells (Anith *et al.*, 2004). The culture of *T. urticae* was obtained from a laboratory pure colony that was maintained on common bean leaves incubated in petri-dishes at 25±2^{o}C.

2. **The gas chromatography mass spectrometry analysis of the bacterial secondary metabolites:**

The *LB media* of both bacterial strains were exhaustively extracted using 1 L of ethyl acetate (4 x 250 mL). The organic layers were combined together, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. A sample of each crude extract was analyzed using the gas

chromatography mass spectrometry (GC-MS) in order to identify volatile organic metabolites. The GC-MS analysis was conducted at the Central Laboratory of the Ministry of Agriculture, Al-Bhooth Street, Giza, using an Agilent 6890 gas chromatograph equipped with an Agilent mass spectrometric column PAS-5ms (30 m x 0.32 mm x0.25 um film thickness). The bacterial extracts were injected under the following conditions. Helium was used as carrier gas at approximately 1.0 ml /min, pulsed splitless mode. The solvent delay was 3 min, and the injection size was 1.0 µl. The mass spectrophotometric detector was operated in electron impact ionization mode an ionizing energy of 70 eV scanning from *m/z* 50 to 500. The ion source temperature was 230°C and the quadrupole temperature was 150°C. The electron multiplier voltage (EM voltage) was maintained at 1250 V above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 60°C then elevated to 280°C at rate of 8°C/min and 10 min hold at 280°C the detector and injector temperature were set at 280 and 250°C, respectively. Wiley275 and NIST05 mass spectral databases were used in the identification of the separated peaks.

3. The semi-field experiment:

A homogeneous lot of apparent uniform common bean seeds were used in this experiment. Pure strains of seeds were obtained from the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. The experiment has been started in April 2017 in the experimental field of Faculty of Agriculture, Mansoura University. The used soil was a mixture of clay and sand (2:1 v/v). The pots used in this study were filled with 3 Kg of soil. Before cultivation, the soil was supplied with super phosphate fertilizer (1g/ each pot). Plants were inoculated with *T. urticae* obtained from large sensitive laboratory colony as mentioned previously. The required number of *T. urticae* was transferred from the colony to a 1.5 cm diameter common bean leaf disc that was then placed onto one leaf of the experimental plant.

The pots were divided into 4 groups including a control. The first and the second group were subjected for foliar spray by *Lysinibacillus* MFNC5 and *Bacillus MAP3* in which the O.D. of the bacterial suspension was kept 1 to justify the no of cells from each treatment. The third group was treated with Abmectin (Vertimec® 1.8% EC) with 40 cm^3/100 liter water. The last group was subjected to water foliar application to work as a negative control. The number of living mites was counted before treatment and (3, 7 & 14) days after treatment. The percentage of reduction was estimated according to the equation described before (Henderson and Tilton, 1955).

4. Estimation of photosynthetic pigments:

Chlorophyll a and chlorophyll b were determined at the flowering stages of plant growth using the spectrophotometric method as recommended by (Dye, 1962) for pigments as adopted by (Taylor and Achanzar, 1972)**.** A known fresh weight of plant leaves was cut and ground with 80 % acetone. After centrifugation, the supernatant absorbance was measured at 644 and 663 nm**.**

5. Estimation of total soluble carbohydrates:

A known volume of the dry leaf powdered tissue was submerged overnight in 10 ml 80 % (v/v) ethanol at 25°C with periodic shaking. After one day, the obtained ethanol mixture was filtered, made up to 20 ml and kept in the refrigerator (Vedder, 1915).Total soluble sugars (TSS) content was determined using the procedures described previously (Hansen and Møller, 1975). An aliquot of 0.1 ml of the alcoholic extract was added to 3 ml of freshly prepared anthrone and incubated in a boiling water bath for 10 min and the absorbance was obtained at 625 nm. The amounts of TSS in plant extracts were obtained using the standard curve of glucose.

6- Estimation of total proteins:

The method of protein extraction was adopted by (Scarponi and Perucci, 1986). A known weight of fresh plant tissue was cut into small pieces and homogenized in five volumes of chilled acetone using a homogenizer for one minute followed by sonication. The crude homogenate was filtered and the residue was used for determination of protein content after re-suspension in 50 mM tris-HCl buffer pH 9. Protein content was determined spectrophotometrically according to the method adopted by (Bradford, 1976). Bovine serum albumin was used as standard in this experiment. Data were analyzed by one way analysis of varience (ANOVA), and the means were separated using Duncan's Multiple Range Test (Snedecor, 1980).

Results and Discussion

1. The gas chromatography mass spectrometry analysis of the bacterial secondary metabolites:

The organic volatile constituents (Figure 1 and Table 1) of the ethyl acetate extract were detected using the GC-MS technique.

The GC-MS profile of Lysinibacillus MFNC5 indicates the presence of thirteen organic compounds belonging to several classes including 2,5-diketopiperazine alkaloids, long chain saturated fatty acids, phthalate esters in addition to other organic compounds. Furthermore, twenty two volatile compounds were elucidated from the GC-MS analysis of Bacillus amyloliquefaciens indicating the presence of 2,5-diketopiperazine alkaloids (2,5-DKPs), piperidone alkaloids, pyrimidine alkaloids, long chain saturated fatty acids and phthalate esters .

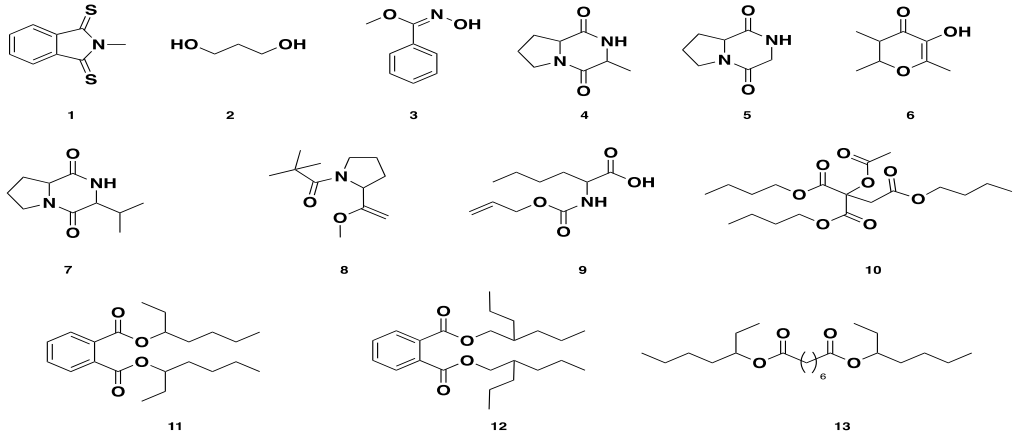

Figure (1): The structure of identified compounds 1-13.

Table (1) : Identified volatile compounds from *Lysinibacillus* MF5 via GC-MS analysis.

Peak	Compound	Formula	R. T	Peak area %	Quality
1	2-methylisoindoline- 1,3-dithione	$C_{10}H_9NS_2$	3.13	3.30	83
2	Propane 1,3- diol	$C_3H_8O_2$	3.91	7.59	72
3	Methyl (Z)-N-hydroxybenzamide	$C_8H_9NO_2$	5.08	0.80	83
4	3-methylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	25.58	0.30	52
5	Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_7H_{10}N_2O_2$	25.93	1.05	95
6	3-Hydroxy-2,5,6-trimethyl-4-H-pyran-4-one	$C_8H_{10}O_3$	26.88	0.12	47
7	3-isopropylhexahydropyrrolol[1,2-a]pyrazine-1,4-dione	$C_{10}H_{16}N_2O_2$	27.40	0.41	64
8	1-(2-(1-ethoxyvinyl)pyrrolidin-1-yl)-2,2-dimethylpropan-1-one	$C_{13}H_{24}NO_2$	29.37	0.71	53
9	2(((allyloxy)carbonyl)amino) hexanoic acid	$C_{10}H_{18}NO_4$	33.82	3.20	47
10	Tributyl 2-acetoxypropane- 1,2,3-tricaboxylate	$C_{20}H_{34}O_8$	34.96	0.3	64
11	Di(octan-4-yl) phalate	$C_{22}H_{34}O_4$	38.70	0.22	91
12	Bis(2-propylpentyl)phthalate	$C_{24}H_{38}O_4$	39.66	69.16	90
13	Bis(2-ethylhexyl) decanedioate	$C_{26}H_{50}O_4$	43.12	0.21	84

2. Effect of the used bacterial isolates on *Tetranychus urticae* population and on the response of common bean to *Tetranychus urticae* infection:

The data represented in Figure (2) showed the foliar spray with both bacterial isolates led to a significant decrease in the number of *T. urticae* individuals/ plant leaf by almost 37% after 3 days of treatment, however, the reduction percent was 89% in case of Abamectin. The number of individuals after 7 days was still significantly lower than that of the control in response to bacterial treatments although the reduction percent was reduced to at least 15.25% after 7 days of treatment compared with 86% in case of Abamectin treatment. After 14 days, there was almost no significant difference between water treatment and bacterial treatments at the time which Abamectin treatment still able to significantly decrease individual populations (70%).

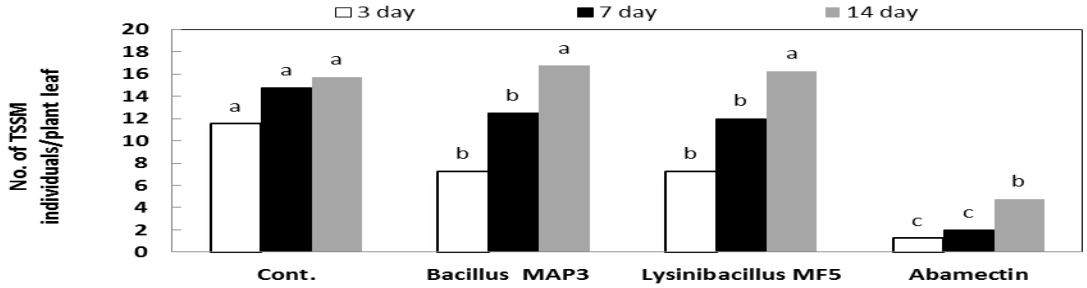

Figure (2): The effect of the foliar spray of both bacterial isolates in addition to the insecticide Abamectin to act as a positive control and water (Cont.) to represent negative control on the number of *Tetranychus urticae* individuals/ plant leaf.

The data represented in Figure (3) shows that the amounts of total carbohydrates and total proteins as a response to bacterial treatments were significantly more than that of the negative control. The same was observed in response to Abamectin treatment and there was no significant difference compared to bacterial treatments. The total chlorophyll was significantly higher in response to both bacterial treatments compared with water treatment and Abamectin.

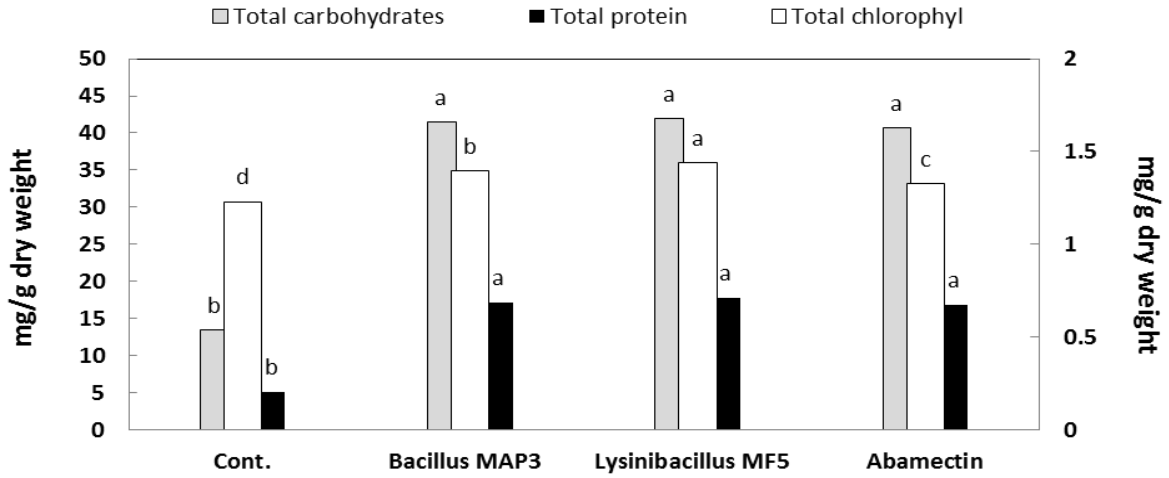

Figure (3): The effect of the foliar spray of both bacterial isolates in addition to the insecticide Abamectin to act as a positive control and water (Cont.) to represent negative control on plant total soluble carbohydrates, total protein and total chlorophylls. The values of total chlorophylls are represented according to the scale in the secondary axe.

In this study, the used bacterial isolates, with phylogenetic relevance to *Lysinibacillus sphaericus* and *Bacillus amyloliquefaciens*, were selected due to their significant effect on several pests. *Lysinibacillus sphaericus* has been regarded as a plant growth promoting bacteria as well as its ability to act as a bio-control agent against several phyto-pathogenic fungi (Naureen *et al.*, 2017). It also could be used as insect pathogen (Berry, 2012). The GC-MS analysis of *Lysinibacillus* MF5 isolate indicated the presence of divers volatile compounds. Among them, the bis (2-propylpentyl) phthalate (12), which represents the major component with 69.16% in addition to its derivative di (octan-4-yl) phthalate (11) that represents 0.22% of the total extract composition. Both compounds and their analogues were found to exhibit several biological activities including antibacterial and antilarval activities (Qi *et al.*, 2009). The observed decrease in *T. urticae* population as a result of *Lysinibacillus* spraying might be attributed to the production of the afromentioned compounds as well as the reported effect of the surface layer protein "S-layer" (Allievi *et al.*, 2014). The reported ability of *Lysinibacillus* to promote plant growth has been observed in the value of the detected metabolites, protein and carbohydrate, and chlorophylls treated with the bacterial isolate while infested with *T. urticae* in comparison with that of the untreated mite-infected plants.

Additionally, *Bacillus amyloliquefaciens* has been regarded as a bio-control agent against phytopathogenic fungi (Danielsson *et al.*, 2007) and bacteria (Wulff *et al.*, 2002). It also has been found to enhance and increase ornamental hosta resistance to insects (Li *et al.*, 2015). It also has been regarded as a plant growth promoting bacteria (Idris *et al.*, 2007 and Nautiyal *et al.*, 2013). The GC-MS analysis performed on our *Bacillus* MAP3 isolate showed the presence of two phthalates derivatives, bis (2- ethylhexyl) phthalate (BEHP) as the major chemical constituent with (81.25%) of the total ethyl acetate extract, along with another regioisomer, phthalic acid, di(oct-3-yl) ester with 0.12%. Both compounds are well-known to exhibit antibacterial and ant larval properties (Qi *et al.*, 2009). The observed decrease in *T. urticae* population might be directly attributed to the effect of the afro-mentioned metabolites of both bacterial strains that have been detected by GC-MS analysis particularly (2-propylpentyl) phthalate and di(octan-4-yl) phthalate for *Lysinibacillus* MF5 and bis(2-ethylhexyl) phthalate (BEHP) and phthalic acid that have been detected to *Bacillus* MAP3.

The activity against *T. urticae* might also be attributed to the surface layer protein "S-layer" as evidenced previously for *Lysinibacillus sphaericus* (Allievi *et al.*, 2014) or indirectly, it might be due to the increase in induced systemic resistance due to the volatile organic compounds produced by *Bacillus* (Farag *et al.*, 2013). The observed increase in carbohydrates, proteins and chlorophylls for plants treated with bacteria, might be attributed to the effect of the used bacterial isolate on plant growth and metabolism. These isolates, for their effect on plant, have been termed as plant growth promoting bacteria (PGPB). They promote plant growth either directly by phytohormones production such as IAA and GA3 (Egamberdieva and Lugtenberg, 2014), ACC deaminase activity that would decrease the level of ethylene in plant tissue to alleviate stress and delay senescence (Penrose and Glick, 2003), siderophore production to accumulate and make iron availability to plant (Bashan and De-Bashan, 2005) and phosphate solubilization to make phosphate in the available form to plant (Rodríguez *et al.*, 2006) or indirectly via pathogen control.

It is concluded that the data of this study are considered as a result that describing the use of bacterial pesticides in *T. urticae* management. Up to our knowledge, it might be the first work to describe such interaction although the results gave the

impression that the bacterial pesticide used in this study need to be formulated and developed to be more efficient against *T. urticae*.

Conflict of Interest

The present study was performed in absence of any conflict of interest.

Acknowlegement

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