

# Biological effects of three bacterial species on *Tetranychus urticae* (Acari: Tetranychidae) infesting eggplant under laboratory and greenhouse conditions

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## Original research

### ABSTRACT

This study evaluated the efficacy of three bacterial species. *Acinetobacter* sp. (concentration  $2.237 \times 10^8$  cfu/ml), *Bacillus subtilis* (concentration  $2.470 \times 10^8$  cfu/ml) and *Bacillus qassimus* (concentration  $3.320 \times 10^8$  cfu/ml) were tested against the two-spotted spider mite, *Tetranychus urticae*, infesting eggplants under laboratory and greenhouse conditions. In addition, we studied their side effects on the predatory mite, *Phytoseius plumifer*. The highest efficiency rate on *T. urticae* was observed with *Acinetobacter* sp. sprays. Three days after treatment, mite mortality reached 87.15 and 77.29 % under laboratory and greenhouse conditions, respectively. Moreover, the mortality rates were significantly lower (72.22 and 67.11 % and 70.74 and 65.19 %) using *B. subtilis* and *B. qassimus* under lab and greenhouse conditions, seven days after treatment. The predatory mite, *P. plumifer*, showed higher tolerance than *T. urticae* to the three bacterial preparations. The results of this study indicated that *Acinetobacter* sp. has a strong efficacy on *T. urticae* and slightly affected the predatory species herein tested. This study introduces a potentially new strategy for biological control of phytophagous mites.

**Keywords** *Tetranychus urticae*; *Phytoseius plumifer*; *Acinetobacter* sp.; *Bacillus subtilis*; *Bacillus qassimus*

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## Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most damaging pests worldwide, causing severe loss to a wide range of agricultural crops (Migeon and Dorkeld, 2009). *Tetranychus urticae* is also the most phytophagous species belonging to the family Tetranychidae (de Carvalho Ribeiro *et al.*, 2019) as it infests 3,877 host plants in both greenhouse and field crops (Migeon and Dorkeld, 2009). In most agricultural crops, the use of synthetic pesticides is the main method to control *T. urticae*. However, because environmental adverse effects of these pesticides, the development of pesticide resistance in the target pest, and potential impacts on biodiversity and Human health (Roobakkumar *et al.*, 2011; Dermauw *et al.*, 2013), alternative methods should be developed.

Biological control agents like predatory mites, parasitoids and bacterial pathogens play a key role in the natural regulation of many plant pests (Al-Azzazy, 2002, 2005; Attia *et al.*, 2013). As a group of important natural enemies of spider mites, pathogenic bacteria exhibit

diverse modes of action. These include parasitizing; competition for nutrients; production of toxins, antibiotics or enzymes; induce of systemic resistance of plants (Alsohim *et al.*, 2014; Alsohim and Fouly, 2015). Alternative crop protection strategies have been proposed based on the use of symbiotic bacteria (Christodoulou, 2011). In addition, many bacteria species are opportunistic pathogens that may be found in natural conditions, which may become pathogenic if conditions are favorable (Boucias and Pendland, 1998).

In Saudi Arabia, some works on the biological control of the date palm dust mite, *Oligonychus afrasiaticus* (McGregor), using two bacterial isolates of *Pseudomonas fluorescens* under laboratory conditions were developed (Alsohim and Fouly, 2015). However, no study to date has examined the potential of *Acinetobacter* sp., *B. subtilis* and *B. qassimus* on *T. urticae*. *Acinetobacter* sp., essentially found on plant roots, has been studied because of its effects on plant growth (Zamin *et al.*, 2011). Until 1996, the role of *Acinetobacter* in pest control had only been demonstrated in some species, such as the stable fly, *Stomoxys calcitrans* (Linnaeus), and the Greater Wax Moth Larvae, *Galleria mellonella* Linnaeus (Lal and Khanna, 1996). These bacteria are able to rapidly penetrate tissue barriers and disperse throughout the whole body of the host (Favia *et al.*, 2007; Crotti *et al.*, 2009). It was reported that some strains of the genus *Bacillus* produce crystalline proteins with miticide properties, by stopping mite feeding and consequently leading to death of the mite *Psoroptes cuniculi* (Delafond) (Dunstand-Guzmán *et al.*, 2015). The aims of the present study were to determine the effect of three bacterial species: *Acinetobacter* sp. (concentration  $2.237 \times 10^8$  cfu/ml), *B. subtilis* (concentration  $2.470 \times 10^8$  cfu/ml) and *B. qassimus* (concentration  $3.320 \times 10^8$  cfu/ml) on *T. urticae* in both lab and greenhouse conditions (eggplants). In addition, two application methods were studied (direct sprays on mite individuals and residual activity by spraying only the leaves). Furthermore, effects of three pathogenic bacterial species were also assessed on a phytoseiid predatory mite, *Phytoseius plumifer* (Canestrini & Fanzago), under laboratory and greenhouse conditions.

## Materials and methods

### Mite's colonies

The mite colonies (*T. urticae* and *P. plumifer*) were maintained at the Acarology laboratory of Qassim University. *Tetranychus urticae* specimens were collected in a conventional unsprayed eggplant (*Solanum melongena* L.) greenhouse in the Qassim region (26.3489° N, 43.7668° E), Saudi Arabia, and reared in the laboratory at  $30 \pm 2$  °C and  $50 \pm 5\%$  RH for three generations on eggplant seedlings before experiments. *Phytoseius plumifer* specimens were collected in unsprayed (for the previous 3 months) eggplant greenhouses in the Melida district, Qassim region, Saudi Arabia. For rearing, *P. plumifer* specimens were placed on detached lima bean leaves (*Phaseolus lunatus* L.) infested with *T. urticae*, placed upside-down on cotton wool in Petri dishes (4.5 cm in diameter  $\times$  1.5 cm high), moistened daily. To prevent the predators from escaping, the margin of bean leaves was covered with strips of wet cotton. Contaminated fresh leaves were added to provide a continuous food supply for *T. urticae*.

### Bacteria cultures

Bacteria were obtained from the biotechnology lab, Department of Plant Production and Protection, College of Agriculture and Veterinary Medicine, Qassim University, KSA. They were cultured in Luria-Bertani (LB) medium for 24 h at 26 °C. Afterwards, a single colony was re-cultured in LB medium overnight. All treatments were maintained in a media consisting of LB medium (10 g/L tryptone, 5 g/L NaCl and 5 g/L yeast) and incubated at  $27 \pm 1$  °C with shaking (250 rpm) overnight (Alsohim *et al.*, 2014). Optical density was measured using a standard spectrophotometer. The number of colony forming units (CFU) was calculated using the formula:  $\text{cfu/ml} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate}$ .

## Laboratory trials – residual effects on *T. urticae*

The host plant used in these bioassays was eggplant (*Solanum melongena* L.). Eggplants (three to seven-leaf stage) were first sprayed directly with 5 ml of the bacterial preparations of *Acinetobacter* sp. (concentration  $2.237 \times 10^8$  cfu/ml), *B. subtilis* (concentration  $2.470 \times 10^8$  cfu/ml) and *B. qassimus* (concentration  $3.320 \times 10^8$  cfu/ml) using a hand sprayer. In the control treatment, only water was sprayed. Randomly selected eggplant leaves were removed from the plants and let at air conditions during 30 minutes for drying. Leaf discs of 5 cm diameter were cut and placed on saturated cotton wool (0.5 cm thick) in covered Petri dishes (4.5 cm in diameter  $\times$  1.5 cm high). Ten adult females of *T. urticae* were added on each disc. To prevent *T. urticae* escape, a ring of Vaseline was placed around the leaves. Water was added daily to the Petri dishes to keep cotton moist. The Petri dishes were placed in transparent plastic boxes (40 x 25 x 14 cm), covered with lids and placed in an incubator at  $30 \pm 2$  °C,  $65 \pm 5\%$  RH and a photoperiod of 16 H light: 8 H dark. Dead mites within 24 h after their transfer were removed and not included in the statistical analyses. Leaf discs were checked daily and the number of dead mites recorded during seven days. Bacteria occurrence was confirmed by stereomicroscope (SD30, Olympus, Japan) examination of mite cadavers.

## Laboratory trials – direct effects on *P. plumifer*

To obtain same aged cohorts of eggs, fifty gravid females of *P. plumifer* taken from the stock culture were placed in the rearing units with about 1,000 spider mites as prey. These mite rearing units were maintained at  $30 \pm 2$  °C and  $50 \pm 5\%$  RH. After 24 hours, eggs laid by *P. plumifer* were transferred individually to new arenas, and the newly hatched larvae were supplied with *T. urticae* as prey; appropriate densities of prey nymphs (20 individuals of *T. urticae*) were added daily to the rearing units. Twenty adult females of the same age of *P. plumifer* were transferred on a lima bean leaf disc (5 cm. in diameter), placed on moist cotton in a Petri dish (Tree Tangle foot® Insect Barrier or a ribbon of moist cotton was put on the rim of leaf discs to prevent escape) and sprayed directly with 5 ml of *Acinetobacter* sp. (concentration  $2.237 \times 10^8$  cfu/ml), *B. subtilis* (concentration  $2.470 \times 10^8$  cfu/ml) and *B. qassimus* (concentration  $3.320 \times 10^8$  cfu/ml) using a hand sprayer. Three replicates were performed for each bacterial treatment and 20 adult females were considered per replicate. Female mortality was recorded 72 h after spraying. A control was included (females were sprayed with water only). Bacteria occurrence was confirmed by stereomicroscope examination of predatory mite cadavers.

## Greenhouse trial – effects on *T. urticae*

A greenhouse trial was conducted from March to April, 2018 at the Agricultural Research Station, College of Agriculture and Veterinary Medicine, Qassim University, Al-Mulida district at  $34-36 \pm 2$  °C and  $45-65 \pm 5\%$  RH. The experiment was conducted on young eggplant seedlings (at the seven leaf stage). Plants were cultivated in a mixture of vermiculite and soil in plastic pots (9.2 cm diameter top, 6.3 cm diameter base and 9 cm depth; capacity: 0.37 liter). About 800 individuals of *T. urticae* were collected from the stock culture and 50 adults were placed on each plant seedlings. Four plant seedlings were considered for each modality (three bacterial applications and control treated with water). Just after the infestation, bacterial pathogens were applied (with approximately 10 ml of bacterial suspension) using a hand sprayer at the following concentrations:  $2.237 \times 10^8$  cfu/ml,  $2.470 \times 10^8$  cfu/ml and  $3.320 \times 10^8$  cfu/ml for *Acinetobacter* sp., *B. subtilis* and *B. qassimus*, respectively. Observations were made, every day for seven days, by taking randomly 3 leaves, one from the top, one from middle and one from the bottom levels of each plant. All motile stages of *T. urticae* were counted on each leaf under a stereomicroscope and the average number of mites found on the three leaves (per plant) was considered as the number of mites/leaf/plant. Observations with stereomicroscope (SD30, Olympus, Japan) were carried out to detect bacteria on mite cadavers.

## Greenhouse trials – effects on *P. plumifer*

Under the same conditions as those mentioned above, 240 adult females of the same age of *P. plumifer* were transferred to eggplant seedlings infested with *T. urticae* as food source (20 females transferred per seedling; three seedlings/replicate for each treatment) and sprayed directly with 10 ml of *Acinetobacter* sp. (concentration  $2.237 \times 10^8$  cfu/ml), *B. subtilis* (concentration  $2.470 \times 10^8$  cfu/ml) and *B. qassimus* (concentration  $3.320 \times 10^8$  cfu/ml) using a hand sprayer. Female mortality was recorded 72 h after spraying. A control was included using the same number of females and sprayed by water only.

## Statistical analysis

The reduction percentages of the population density of *T. urticae* were calculated using the equation proposed by Henderson & Tilton (1955):

Mortality % =  $1 - (n \text{ in C before treatment} \times n \text{ in T after treatment} / n \text{ in C after treatment} \times n \text{ in T before treatment}) \times 100$ . Where: n = pest mite population, T= treated, C = control

Statistical differences in mortality mean rates between the treatments were assessed using ANOVA followed by Duncan's Multiple Range test (DMRT).

## Results and Discussion

### Laboratory efficacy on *T. urticae*

*Bacillus subtilis* and *B. qassimus* caused similar reductions in mite populations, seven days post treatment (72.22 and 70.74%, respectively), whereas mortality was significantly higher when mites were sprayed with *Acinetobacter* sp. The same trend was observed during the successive days. *Acinetobacter* sp. reached the highest effect on *T. urticae* three days after treatment (mite mortality of 87.15%) (Table 1). It thus seems that *Acinetobacter* sp. caused significantly higher mortality on the two- spotted spider mites infesting eggplant plants than *B. subtilis* and *B. qassimus*.

### Greenhouse efficacy on *T. urticae*

The number of mobile stages was counted every day for seven days to monitor the accumulative reduction of mites' population. The mortality rates increase till the day 3 for *Acinetobacter* sp treatment, whereas for the *B. subtilis* and *B. qassimus* treatments, an increase in mortality rate

**Table 1** Corrected mortality (in % calculated with Henderson-Tilton equation) of the spider mite, *Tetranychus urticae* treated with three pathogenic bacterial species: *Acinetobacter* sp., *Bacillus subtilis* and *Bacillus qassimus* at 30 °C and 65% RH under laboratory conditions.

Treatment	Days after treatment							P-Value
	1	2	3	4	5	6	7	
<i>Acinetobacter</i> sp. $2.237 \times 10^8$ cfu/ml	24.29 Aa	52.44 Ab	87.15 Ac	86.69 Ac	86.15 Ac	85.15 Ac	84.11 Ac	0.0096
<i>B. subtilis</i> $2.470 \times 10^8$ cfu/ml	12.06 Ba	42.35 Bb	65.79 Bc	66.38 Bc	70.88 Bc	71.01 Bc	72.22 Bc	0.0086
<i>B. qassimus</i> $3.320 \times 10^8$ cfu/ml	10.89 Ba	41.11 Bb	62.92 Bc	65.11 Bc	68.19 Bc	69.00 Bc	70.74 Bc	0.0072
P value	0.0092	0.0068	0.0077	0.0053	0.0045	0.0078	0.009	

The capital letter denotes significant differences within the same column and small letter denotes the significant differences within the same row at  $P < 0.01$ .

was observed till the day 7. Statistical analysis demonstrated that the reduction percentages of *T. urticae* populations were significantly different among the three treatments ( $P < 0.001$ ). Results indicate that the application of *Acinetobacter* sp. might be considered a promising control agent against *T. urticae* as it caused a population reduction of 77.29% during a three days' period following the application. *B. subtilis* and *B. qassimus* caused remarkable less reduction compared to *Acinetobacter* sp. (67.11% for *B. subtilis* and 65.19% *B. qassimus*, seven days after the treatment) (Table 2).

Comparable results were obtained by Alsohim and Fouly (2015), who stated that the direct application of *Bacillus thuringiensis* and *Pseudomonas fluorescens* on the date palm dust mite, *O. afrasiaticus*, caused a mite mortality of 93.8% and 85.8%, respectively. Moreover, Hall *et al.* (1971) mentioned that the  $\beta$ -exotoxin fraction of *B. thuringiensis* caused high mortality in adult mites of the citrus red mite, *Panonychus citri* (McGregor). Eggs and immature stages were similarly affected. The findings of Hornsey *et al.* (2013) and Andrea *et al.* (2019) are also similar to the present results, in suggesting that the pathogenic bacteria *Acinetobacter* has high virulence potential on *Galleria mellonella*. In the present study, we can hypothesize that the high mortality caused by *Acinetobacter* sp., *B. subtilis* and *B. qassimus* could possibly provide solutions for pests' resistance to pesticides and decrease pesticide use. However, further studies on field efficacy have to be conducted for defining the application conditions allowing a successful biocontrol of *T. urticae*. Growers in the Qassim region, Saudi Arabia, where this pest causes high losses (up to 90% on eggplant crops), spend as much as \$100 per acre to manage this pest. It would thus also be interesting to analyze the cost of bacterial applications compared to other solutions.

## Symptoms of *T. urticae* with a bacterial infection

Bacterial pathogens invade their hosts mostly through the mouth and alimentary canal. Less often, they enter through peritremes and wounds in the integument (Poinar and Poinar, 1998). Bacterial infections may be classified as toxemia, when bacteria are confined in the alimentary canal where they produce toxins, bacteremia, when bacteria multiply in the hemolymph of the mite without producing toxins (apparently unaffected by the mite immune system), or septicemia, when bacteria multiply in the hemocoel, producing toxins and killing the mite (Tanada and Kaya, 1993). In the present study, many of these factors might have contributed to the mortality of the two-spotted spider mite. Diagnostic features considered as general characteristics of bacterial infection were recorded in the current study: distinct color changes (dark brown color with a black tinge), degeneration of internal tissues, discontinuation of

**Table 2** Corrected mortality (in % calculated with Henderson-Tilton equation) of the spider mite, *Tetranychus urticae* treated with three pathogenic bacterial species: *Acinetobacter* sp., *Bacillus subtilis* and *Bacillus qassimus* under eggplant greenhouse conditions.

Treatment	Days after treatment							P value
	1	2	3	4	5	6	7	
<i>Acinetobacter</i> sp. 2.237×10 <sup>8</sup> cfu/ml	29.89 Aa	45.95 Ab	77.29 Ac	75.19 Ac	74.99 Ac	74.82 Ac	73.80 Ac	0.0088
<i>B. subtilis</i> 2.470×10 <sup>8</sup> cfu/ml	10.28 Ba	24.31 Bb	41.06 Bc	55.48 Bd	61.36 Bd	64.77 Bd	67.11 Bd	0.0071
<i>B. qassimus</i> 3.320×10 <sup>8</sup> cfu/ml	9.14 Ca	23.55 Cb	40.25 Cc	54.97 Cd	60.08 Cd	63.07 Cd	65.19 Cd	0.009
P value	0.0048	0.0066	0.0079	0.0094	0.0041	0.0056	0.0073	

The capital letter denotes significant differences within the same column and small letter denotes the significant differences within the same row at  $P < 0.01$ .





**Figure 1** Pictures of dead mite individuals after spray with the pathogenic bacteria: A – *Acinetobacter* sp.; B – *B. subtilis* and C – *B. qassimus*

feeding, inability to move, weakening of the outer skin, females failing to oviposit, excretion of diarrhoea-like faeces, occasionally causing mites to become glued to the substrate, vomiting and cadavers becoming black due to the bacterial infection (Figure 1). In another study (Wilson *et al.*, 2002) reported that bacterial hemolysins are exotoxins that attack blood cell membranes and cause cell rupture and body swelling. These hemolysins may also be involved in the pathogenicity of the three bacterial species on *T. urticae*, especially given the rapid mortality observed.

We can hypothesize that *Acinetobacter* sp. was found to be a better bacterial agent under greenhouse and lab experiments, because of its higher colonizing capacity in small-bodied mites by providing very strong adhesion to the cuticle (Van der Geest *et al.*, 2000). Future studies would be needed to understand interactions between mites and these bacterial pathogens.

## Effect of three bacterial species on the predatory mite *Phytoseius plumifer*

The three pathogenic bacteria studied have no different significant effects on *P. plumifer* (Table 3). The corrected mortality percentages were 15.48, 13.56 and 12.87% under laboratory conditions and 11.17, 10.28 and 10.11% under greenhouse conditions after one week of exposure. The pathological manifestations in *P. plumifer* recorded during this study are as follows: the predators became very pale and so thin that they became translucent. Those results, in general, agree with the studies carried out by other authors (i.e. Hess and Hoy, 1982). Some adult females were plumb and had a cream-color to pink rectal plug that extruded from their posterior end and occasionally caused mites to become glued to the substrate.

## Conclusion

The present results show that although *Acinetobacter* sp., *B. subtilis* and *B. qassimus* were harmful to *T. urticae*, they were relatively harmless to the predatory mite *P. plumifer*. We hypothesize that the three pathogenic bacteria studied might affect *T. urticae* via ingestion, via contact while crawling on sprayed leaves or by direct reception of spray droplets. Higher activity of pathogenic bacteria towards *T. urticae* can be explained by the ability of pathogenic bacteria to penetrate their thin body wall, inter-skeletal membranes or through body openings. In the case of *P. plumifer*, the thicker exoskeleton (Duso and Fontana, 2002; Zannou *et al.*, 2007) may prevent penetration of pathogenic bacteria into body tissues. Furthermore, predatory mites usually do not eat on plants, which can have limit bacteria entrance (McMurtry *et al.*, 2013).

**Table 3** Corrected mortality (in % calculated with Henderson-Tilton equation) of the predatory mite *Phytoseius plumifer* treated with three pathogenic bacterial species: *Acinetobacter* sp., *Bacillus subtilis* and *Bacillus qassimus* under laboratory and greenhouse conditions.

Treatment/ Concentrate	Days after treatment							P-Value
	Under laboratory conditions at 30±2°C and 50±5% RH							
	1	2	3	4	5	6	7	
<i>Acinetobacter</i> sp. 2.237×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	1.00Aa	4.51Ab	8.23Ac	12.89Ad	15.48Ae	0.0094
<i>B. subtilis</i> 2.470×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	0.00Aa	3.20Ab	7.40Ac	10.33Ad	13.56Ae	0.0089
<i>B. qassimus</i> 3.320×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	0.00Aa	4.74Ab	7.83Ac	10.82Ad	12.87Ae	0.0052
	Under greenhouse conditions at 34-36°C and 45-65% RH							
<i>Acinetobacter</i> sp. 2.237×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	0.00Aa	2.19Aa	7.87Ab	10.28Ac	12.17Ad	0.0076
<i>B. subtilis</i> 2.470×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	0.00Aa	2.54 Aa	6.38 Ab	9.45Ac	10.28Ac	0.0044
<i>B. qassimus</i> 3.320×10 <sup>8</sup> cfu/ml	0.00Aa	0.00Aa	0.00Aa	2.00Aa	7.26Ab	9.66Ab	10.11Ac	0.0062

The capital letter denotes significant differences within the same column and small letter denotes significant differences within the same row at P<0.01.

Many vegetable crop growers throughout the world are now exploring bacterial potential applicability due to ongoing pesticide restrictions. The results of the present study indicated that although all three bacterial species lead to significant mortality to *T. urticae*, the pathogenic bacteria of *Acinetobacter* sp. seems to be the most promising candidate for biocontrol of the two-spotted spider mite. Furthermore, high selectivity towards *T. urticae* versus *P. plumifer* was remarked and can be considered as a positive aspect for integrated pest management (IPM). Future research should, however, be carried out for using more bacterial species, especially species that are similar to *Acinetobacter* and to determine how the predators can be affected if fed with infested preys. This may help in developing an IPM program that can minimize environmental pollution. This study is a preliminary step for future work which will help policy decision makers meet agricultural product safety standards and maintain ecological balance.

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