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The influence of a non-pathogenic *Pseudomonas putida* strain BTP1 on reproduction and development of grape phylloxera

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Abstract: Some non-pathogenic rhizobacteria called Plant Growth Promoting Rhizobacteria (PGPR) possess the capacity to induce defense mechanisms effective in plant against pathogens. The effect of *Pseudomonas putida* BTP1 on reproduction and development of phylloxera, which infested the roots of our local grape variety "Balady", was evaluated. Our results showed that the life table of grape phylloxera was different between treated and control plants. The percentage of matured females, developmental time, fecundity and oviposition period were reduced when plants were treated with bacteria. The results showed that the phylloxera resistance was influenced by root soaking duration in *P. putida* BTP1 suspension. The present study provides good information on the possibility of using *Pseudomonas putida* BTP1 to increase the resistance of grape to phylloxera.

1. Introduction

Grape phylloxera (*Daktulosphaira vitifoliae*), aphid-like gall-forming parasite, is an economically important homopteran pest of grape vines *Vitis vinifera* L. world wide. In Syria, there are more than 70,000 ha planted with grapevine and the annual production is about 540,000 t. Grape phylloxera annually causes millions of dollars in losses in grape production.

Grape phylloxera causes direct damage to grapevine by forming damaging root galls. The fleshy galls formed on immature roots, by swelling of the root cortex, are called "nodosities", while on mature roots they are called "tuberosities"; these latter are considered more damaging to the vine. These galls are metabolically active organs suited to match the nutritional requirements of phylloxera and can support populations with high reproductive rates, making this pest capable of destroying the root system of *V. vinifera* vines (Granett *et al.*, 2001). In most cases the swelling stops rootlet growth, and the affected portion dies. Root injuries reduce the vines' ability to absorb nutrients and water, causing decline in vigor and productivity. Weakened plants probably become more susceptible to secondary infections from fungal diseases and other insects, and to environmental stresses.

Since there is not an effective control method for grape phylloxera, sanitation and quarantine can be considered required procedures to prevent the spread of this insect pest. Insecticides and hot water dips are used as quarantine treatments (Granett *et al*., 2001). Makee *et al.* (2010) proposed that gamma irradiation could be economically very useful in quarantine treatments against phylloxera. However, once phylloxera is in a vineyard, the use of resistant rootstocks is the most common and effective means of managing phylloxera. It should be mentioned that some rootstocks were more resistant than others to grape phylloxera (Makee *et al*., 2003). Moreover, for unknown reasons, the resistance of some rootstocks may break down and farmers must replant vineyards (Granett *et al*., 1983; Song and Granett, 1990; De Benedictis and Granett, 1993). Because replanting is costly in money, time, and labor, additional ways to control this pest should be considered. All plants have active defense mechanisms against pathogen attacks. Some plant growth-promoting rhizobacteria (PGPR) are able to reduce disease through the stimulation of inducible plant defense mechanisms that render the host plant more resistant to further pathogen ingress. This induced systemic resistance (ISR) (Pieterse *et al*., 2002) can be the basis of integrated plant disease management strategies (Ramamoorthy *et al*., 2001; Zehnder *et al*., 2001; Saravanakumar *et al*., 2007).

Many studies in plants on PGPR against pathogens have been performed. However, only a few of them determined the protective effect of PGPR against insects (Zehnder *et al*., 1997 a, b; Zehnder *et al*., 2001; Kloepper *et al*., 2004; Vijayasamundeeswari *et al*., 2009; Valenzuela-Soto *et al*., 2010). A non-pathogenic *Pseudomonas putida* strain (BTP1) was shown to enhance the level

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of resistance in cucumber, bean and tomato against the fungal pathogens *Pythium aphanidermatum* and *Botrytis cinerea,* respectively (Ongena *et al*., 1999; Adam *et al*., 2008). These studies revealed that the disease-protective effect was associated with stimulation of defense mechanisms in host plant (Ongena *et al*., 2000, 2004; Adam *et al*., 2008).

The main objective of the present study was to evaluate the ability of strain *P. putida* BTP1 to protect grape roots against grape phylloxera. Thus, the effect of concentrations and root soaking duration in *P. putida* BTP1 suspension on percentage of matured females, developmental time, fecundity and oviposition period of local phylloxera strain were determined.

2. Materials and Methods

Establishment of the phylloxera colony

Grape phylloxera was originally collected from field-infested roots of the local grape varieties in southern parts of Syria. The phylloxera colony was established following similar procedures to those mentioned by Makee *et al*. (2003). Fresh and healthy pieces of roots (4-7 mm in diameter and 5-7 cm long) of local grape cultivar Helwani (*V. vinifera*) were taken and washed with tap water. Each piece was wrapped with moist cotton wool around one end, and then 10 to 15 phylloxera eggs were placed on each piece. The infested root pieces were then placed on a wet filter paper disk inside a plastic Petri dish (12 cm diameter). Each dish had three to four root pieces. For ventilation purposes the Petri dish lid was modified with a 1-1.5 cm cloth-screened hole. The edges of the dishes were sealed with parafilm and they were kept in plastic boxes with tightly fitting lids and incubated at 25±1°C, 70±5% RH and 24 hr darkness. The root pieces were replaced when they desiccated, rotted or the phylloxera became crowded.

Microbial strains and inoculum preparation

P. putida strain BTP1, isolated from barley roots, was originally selected for its specific features regarding pyoverdine-mediated iron transport (Jacques *et al*., 1995; Ongena *et al*., 2002); it was maintained and prepared for use in the ISR assays as previously described by Ongena *et al*. (2002). For bioassays, two different concentrations (10^8 and $2x10^8$ CFU/ml) of bacterial suspension were prepared.

Effect of bacteria-treated roots on phylloxera

Fresh root pieces were soaked for 3 hr in solutions with various *P. putida* concentrations: 0 (roots were soaked in distilled water as a control), 10^8, and $2x10^8$ CFU/ml. All root pieces were then left to air-dry. For each concentration five root pieces were taken. Each root piece was infested with 50 newly-laid phylloxera eggs (<24 hr old) and then all root pieces were kept at 25±1°C, 70±5% RH and 24 hr darkness.

Evaluation procedure

A daily microscope inspection of all phylloxera stages on all root pieces was carried out. The number of eggs hatched, feeding nymphs and adults were detected to calculate the percentage of emerged mature females on each root piece at each concentration. Also, the mean developmental time (egg to egg) was determined. Fecundity (total number of eggs) of phylloxera was evaluated by randomly choosing five individuals of root-feeding phylloxera females on each root piece at each concentration. Thus, at each tested concentration 20 females were examined. All eggs laid by each female were observed and counted till the female's death. Additionally**,** the oviposition period (the time from the first laid egg to the natural death of individual ovipositing females) was recorded for the females chosen for the fecundity measurement.

Effect of root soaking duration in bacterial suspension on phylloxera

Fresh root pieces were soaked in solutions with various *P. putida* concentrations: 0, 10^8, and $2x10^8$ CFU/ml. At each tested concentration the root pieces were soaked for various periods: 0, 3, 5 and 15 hr. All root pieces were then left to air-dry. At each concentration and soaking duration, five root pieces were taken. Each root piece, at each concentration and soaking duration, was infested with 50 newly-laid phylloxera eggs (<24 hr old) and then all root pieces were kept at 25±1°C, 70±5% RH and 24 hr darkness.

The same evaluation procedure as described above was followed to determine the percentage of emerged mature females, mean developmental time, fecundity and the oviposition period of phylloxera.

Statistical analysis

All statistical analyses were performed using STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level (P = 0.05). Data were subjected to analysis of variance (ANOVA) for the determination of differences in means between tested plants at each dose. Differences between means were tested for significance using Tukey HSD test.

3. Results

Effect of bacterial treated roots on phylloxera

Table 1 shows that when root pieces were treated with *P. putida*, the percentage of emerged matured females was significantly affected compared to the control ($F=84.69$; $df=15, 64$; $P<0.05$). However, the percentage emerged of matured females was not significantly increased by increasing *P. putida* concentrations.

The result illustrates that the developmental time of phylloxera was significantly decreased by the application of *P. putida* ($F= 15$; $df = 2, 42$; $P < 0.05$). There was a significant reduction in developmental time as the concentration of *P. putida* was increased (Table 1).

There were significant differences in the mean number of laid eggs between all tested root pieces, regardless of concen-

Table 1 - Mean percentage of matured females, developmental time, fecundity and oviposition when phylloxera was reared on *P.putida*-treated grape root pieces

Oviposition period/d (Mean±SE)	Fecundity (Mean±SE)	Developmental time/d (Mean±SE)	% matured female (Mean±SE)	Bacterial Concentration CFU/ml
14.2±0.54 a	44.73±0.85 a	28.27±0.57 a	78.16±1.48 a	0
12.07±0.27 b	40.07±1.3 b	26.53±0.27 b	36.33±0.88 b	108
9.53±.53 c	34.2±1.3 c	24.53±0.54 c	32.03±1.49 b	2x108

Means, in a column, followed by the same letter are not significantly different at the $P < 0.05$ (Tukey HSD test).

tration (F = 19.9; df = 2, 42; P< 0.05). On untreated root pieces, the mean number of eggs was significantly higher than that on treated ones (Table 1). When phylloxera females were reared on *P. Putida*-treated root pieces, the mean number of eggs was markedly reduced by increasing *P. putida* concentration.

Table 1 shows that the oviposition period of phylloxera on untreated root pieces was significantly longer than that on treated ones, irrespective of the concentration of *P. putida* (F = 25.5; df = 2, 42; P < 0.05). There was a significant difference in the oviposition period between *P. Putida*-treated root pieces. The oviposition period on 10^8 CFU/ml-treated root pieces was significantly longer than that on $2x10^8$ CFU/ml-treated ones.

Effect of root soaking duration in bacterial suspension on phylloxera

There was a significant effect of the soaking duration in *P. putida* suspension on the percentage emerged of matured females, regardless of the concentration (F= 620.87; df= 11, 24; P<0.05) (Fig. 1). At each concentration, the percentage of emerged matured females on un-soaked root pieces was significantly higher than that on soaked ones, regardless of soaking duration. When the root pieces were soaked, the percentage of emerged matured females with 3 hr soaking duration significantly differed from that on 5 and 15 hr, whatever the *P. putida* concentration. At each *P. putida* concentration, there was no significant difference in the percentage of emerged matured females between 5 and 15 hr soaking duration (Fig. 1).

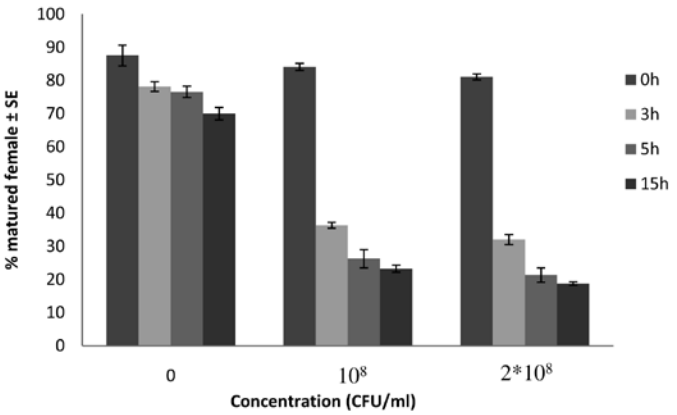

Fig. 1 - Effect of root soaking duration in bacterial suspension of percentage emerged matured females of phylloxera.

There was a significant effect of soaking in *P. putida* suspension on the mean developmental time, regardless of the soaking duration and concentration (F= 16; df= 11, 168; P< 0.05) (Fig. 2). At each concentration, the mean developmental time on un-soaked root pieces was significantly higher than that on soaked ones, regardless of soaking duration. The result illustrates that at $2x10^8$ CFU/ml the observed differences in mean development between soaking durations were not significant.

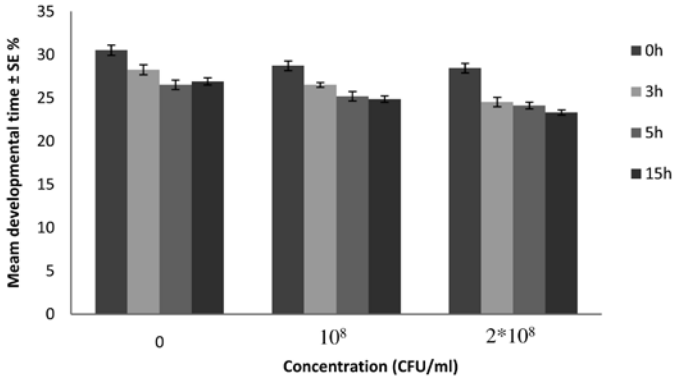

Fig. 2 - Effect of root soaking duration in bacterial suspension on mean developmental time of phylloxera.

There was a significant effect of the soaking duration in *P. putida* suspension on mean number of eggs, regardless of the concentration (F= 112; df = 11, 168; P<0.05) (Fig. 3). At each concentration, mean number of eggs on soaked root pieces was significantly higher than that on un-soaked ones, regardless of soaking duration. At concentration 0 (root pieces soaked with distilled water), there was no significant difference in the mean number of eggs between 3 and 5 hr soaking duration. However, the mean number of eggs was significantly reduced with 15 hr soaking duration. At 10^8 and $2x10^8$ CFU/ml concentrations, the mean number of eggs on 3 hr soaking duration was significantly higher than that on 5 and 15 hr (Fig. 3).

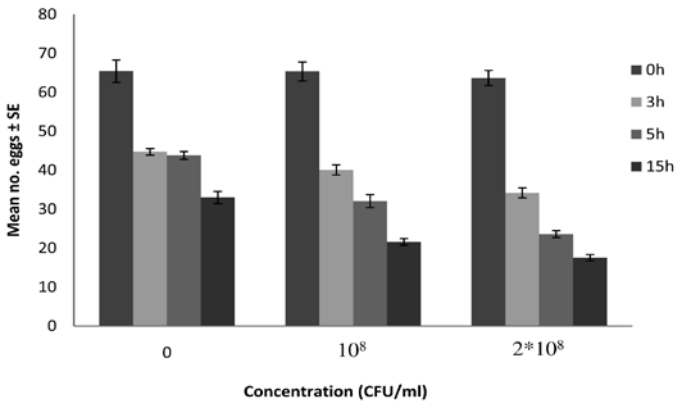

Fig. 3 - Effect of root soaking duration in bacterial suspension on mean number of eggs of phylloxera.

A significant effect of the soaking duration in *P. putida* suspension on mean oviposition period was found, regardless of the concentration (F= 46; df = 11, 168; P<0.05) (Fig. 4). At each concentration, the mean oviposition period on un-soaked root pieces was significantly higher than that on soaked ones, regardless of soaking duration. At both 0 and 10^8 CFU/ml, the mean oviposition period with 3 hr soaking duration was significantly higher than that with 15 hr. At 0 concentration, there was no significant difference in the mean oviposition period between on 3 and 5 hr soaking duration, while at 10^8 CFU/ml there was. At $2x10^8$ CFU/ml, there was no significant difference in the mean oviposition period between 3, 5 and 15 hr soaking duration.

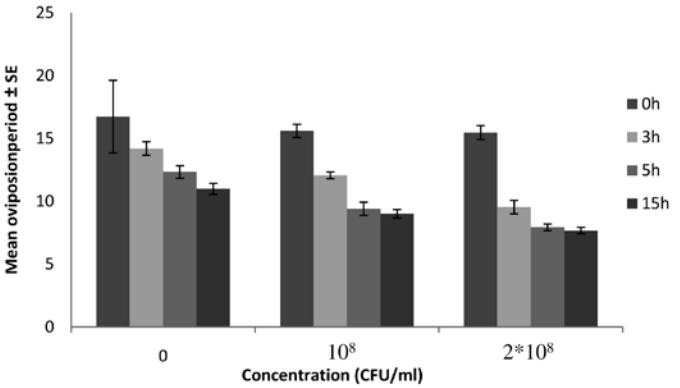

Fig. 4 - Effect of root soaking duration in bacterial suspension on mean oviposition period of phylloxera.

4. Discussion and Conclusions

Several studies reported the use of rhizobacteria as a biological control of pests (Racke and Sikora, 1992; Zehnder *et al*., 1997 a, b). To our knowledge, the application of rhizobacteria against phylloxera has not been investigated. Our study indicates that when *P. putida* BTP1-treated roots were infested by phylloxera eggs, the percentage of matured females was negatively influenced (Table 1). The result illustrates that the percentage of matured females was significantly decreased when the concentration and the soaking duration were increased (Fig. 1). The reduction of the number of matured females on the treated roots could be attributed to the inability of phylloxera to feed. Thus, nymphs fed on *P. putida* BTP1-treated roots showed antifeeding behaviour. When *Heliothis zeae* (Boddie) diet was contaminated with the bacterium *Pseudomonas maltophila,* a 60% reduction in adult emergence and high pupal and adult malformations were observed (Bong and Sikorowski, 1991). Moreover, when chestnut was treated with *Pseudomonas fluorescens*, 20% chestnut weevil mortality was recorded (Yaman *et al*., 1999). Therefore, the antifeeding behaviour of phylloxera nymphs on the PGPR treatments could be related to the reduction in the feeding stimulant.

Consequently, the developmental time was altered leading to early emergence of matured females (Table 1). A comparison between *P. putida* BTP1-treated and untreated roots showed that the phylloxera development on treated roots was faster than that on untreated ones. Similar results were reported when cucumber beetles and American bollworm were fed on PGPR-treated cucumber plants and cotton bolls, respectively (Zehnder *et al*., 1997 a, b; Vijayasamundeeswari, 2009). Our current study indicates that the developmental time was significantly shorter on treated root pieces soaked for 15 hr (Fig. 2). Qingwen *et al*. (1988) detected a reduction of relative growth rate, consumption rate and digestibility of feed when *Heliothis armigera* fed on *P. gladioli*-treated cotton plants.

It is known that fecundity could be considered an essential factor in assessing the effect of *P. putida* BTP1on phylloxera. Phylloxera fecundity on *P. putida* BTP1-treated root pieces was distinctly lower than that on untreated ones. When phylloxera females were reared on treated roots they were unable to produce a normal number of eggs compared to the control, especially at high concentration (Table 1). Nevertheless, when soaking duration in *P. putida* BTP1 suspension was prolonged, a defective reproductive capacity was obtained (Fig. 3). The average number of eggs was 65.3, 40, 32 and 21.6 eggs on 10^8 CFU/ml-treated roots pieces soaked for 0, 3, 5 and 15 hr, respectively. While on $2x10^8$ CFU/ml-treated roots pieces soaked for 0, 3, 5 and 15 hr, the average number of eggs was 63.7, 34.2, 23.6 and 17.5 eggs, respectively. Inadequate nutrition and inability to establish good feeding sites could directly affect the number of eggs laid. Therefore, phylloxera resistance could be reflected in a strong relationship between poor feeding and the reduction of insect reproduction (Granett *et al*., 1983). Thus, phylloxera produced more eggs on untreated roots compared with treated ones.

Correspondingly, the oviposition period of phylloxera on treated roots was markedly shorter than that on untreated ones (Table 1). When the concentration of *P. putida* BTP1 was increased a great reduction in the oviposition period was obtained. Such reduction was noticeably increased by prolonging the root soaking duration in *P. putida* BTP1 (Fig. 4).

It is known that the resistance mechanisms of phylloxera could be related to several factors including: 1) reduction of phylloxera fitness (antibiosis); 2) decrease in plant attractiveness to phylloxera (antixenosis) (Granett *et al*., 2001). Zehnder *et al*. (2001) mentioned that the PGPR treatment led to alteration in the plant metabolic pathway which elicited the induction of plant defense compounds. Qingwen *et al.* (1998) reported that polyphenol and terpenoid content were increased with cotton treated with *P. gladioli.* The synthesis and formation of such materials in *P. putida* BTP1-treated roots would have a negative influence on phylloxera feeding and development.

The results illustrate that the soaking of grape roots with *P. putida* BTP1 suspension for 5 hr and 15 hr, regardless of concentration, led to a great reduction in matured females, developmental time, fecundity and oviposition

period. Such reduction might be attributed to increased bacterial cell concentration by prolonging the soaking duration. Therefore, roots pieces soaked with $2x10^8$ CFU/ml for 15 hr showed more resistance to phylloxera than lower concentrations and a shorter soaking duration.

These results provide essential information about the relationship between *P. putida* BTP1 and phylloxera resistance of *grape plants*. Phylloxera encounters serious difficulties in surviving, feeding and reproducing on *P. putida* BTP1-treated roots. *P. putida* BTP1 reduced the susceptibility of roots to phylloxera. Nevertheless, in future it is essential to determine for how long such effects could persist. Therefore, we acknowledge that these laboratory studies cannot simulate all conditions that exist in nature. Therefore, care must be taken when laboratory-based results are applied in nature.

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