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In vitro Pseudomonas putida BTP1-induced systemic resistance in grapevine rootstocks against Phylloxera (*Daktulosphaira vitifoliae*)

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Abstract: This study investigates the systemic resistance induced by *Pseudomonas putida* strain BTP1 against phylloxera using an *in vitro* model in Ruggeri (Ru140) and B41 roostocks. Significant differences were found with regard to matured females, fecundity and oviposition period between untreated and bacteria-treated plants in both rootstocks. Treated Ru140 rootstocks were more resistant than treated B41 ones. BTP1 impacted negatively on the ability of phylloxera to develop, indicating an increase in grapevine resistance and tolerance toward this pest in bacteria-treated plants. This is the first known study of biocontrol of phylloxera in grapevine rootstocks by non-pathogenic *P. putida* strain BTP1 *in vitro*.

1. Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae*) is a tiny aphid-like insect that feeds on grapevine (*Vitis vinifera* L.) roots and leaves, leading to stunted growth or death. It is considered the most destructive grapevine pest (Vidart *et al.*, 2013). In Syria, there are more than 70,000 ha of grapevine with an estimated 540,000 ton annual production (Statistics of Syrian Agriculture Ministry, 2011). However, phylloxera causes millions of dollars in losses in grapevine production annually. Grapevine phylloxera forms damaging root galls which are metabolically active organs suited to meet the nutritional requirements of phylloxera and support its generation with high reproductive rates, making this pest capable of destroying the root system of *V. vinifera* vines. Root injuries reduce the vines' ability to absorb nutrients and water, causing a decline in vigor and productivity. As a consequence, weakened plants probably become more susceptible to secondary infections by fungal diseases and other insects and are also vulnerable to environmental stresses (Granett *et al.*, 2001).

The use of resistant rootstocks is considered the most common and effective means to control phylloxera in the field. The vast majority of these rootstocks have been durably resistant for a long period. In Syria, the widely used resistant rootstocks are Ru140 (*V. rupestris* x *V. Berlandieri*), R99 (*V. rupestris* x *V. Berlandieri*), and 3309C (*V. riparia Michaux* x *V. rupestris*) and B41 (*V. vinifera* x *V. Berlandieri*) (Makee *et al.*, 2003). It is important to note that some rootstocks are more resistant than others to grapevine phylloxera. However, for yet unknown reasons, some rootstocks may lose their resistance to phylloxera. For example, AXR#1 (*Vitis vinifera* X *V. rupestris* Scheele hybrid) has failed to resist phylloxera in several parts of the world after many years of use (Granett *et al.*, 1983). Likewise, rootstock B41 has remained resistant in France while it is not resistant in Californian vineyards, therefore farmers have to replant their vineyards with the appropriate resistant rootstocks (Song and Granett, 1990; De Benedictis and Granett, 1993).

Plants have active defense mechanisms against pathogen attacks. A group of microorganisms referred to as plant growth-promoting rhizobacteria (PGPR) are able to reduce disease through the induction of systemic resistance (ISR) that renders the host plant more resistant to further pathogen ingress (Pieterse *et al.*, 2002). This phenomenon can occur in many plant species and was demonstrated to be effective against a broad spectrum of fungal, bacterial and viral diseases beside its effect on insect and nematode pests (Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001; Durrant and Dong, 2004; Verhagen *et al.*, 2010; Weller *et al.*, 2012). In addition to eliciting ISR against pathogens, protective effects of PGPR against insects have been noted (Zehnder *et al.*, 1997 a, b; Zehnder *et al.*, 2001; Kloepper *et al.*, 2004; Vijayasamundeeswari *et al.*, 2009; Valenzuela-Soto *et al.*, 2010). However, to our knowledge no studies have been carried out to assess *in vitro* the effects of PGPR on grapevine phylloxera. In this context, a non-pathogenic *Pseudomonas putida* BTP1 strain has shown enhancement of the level of resistance in cucumber, bean and tomato against the fungal pathogens *Pythium aphanidermatum* and *Botrytis*

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cinerea, respectively (Ongena *et al.*, 1999; Ongena *et al.*, 2004; Adam *et al.*, 2008). In a previous study performed on fresh roots from local grape variety Helwani (*V. vinifera*), we demonstrated the influence of *P. putida* BTP1 on reproduction and development of grapevine phylloxera (Adam *et al.*, 2012).

Implementation of *in vitro* dual culture assay has been used to evaluate the phylloxera/grapevine interaction (Forneck *et al.*, 1996; Makee *et al.*, 2003; Vidart *et al.*, 2013). This method has several advantages for our designed experiments such as providing optimal conditions for phylloxera infestation, conducting experiments in small space, preventing the spreading of phylloxera and rhizobacteria, as well as reliable results in a relatively short period.

The present work aims to demonstrate the ISR-related protective effect triggered by *P. putida* BTP1 *in vitro* in Ru140 and B41 rootstocks against grapevine phylloxera. The percentage of mature females, fecundity and oviposition period of phylloxera were determined.

2. Materials and Methods

Establishment of the phylloxera colony

Grapevine phylloxera was originally collected from field-infested roots of the local grapevine 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 grapevine 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 h darkness. The root pieces were replaced when they desiccated, rotted or the phylloxera became crowded.

Microbial strain 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 the bioassays, BTP1 strain was grown in Erlenmeyer flasks (250 ml) containing 100 ml of Casamino Acids medium (CAA) for 24 h on a rotary shaker (150 r.p.m.) at 28ºC. Cells were removed by centrifugation at 16500 g for 15 min at 4ºC and washed in sterile NaCl (5 g l^{-1}). The final pellet was resuspended in an adequate volume of sterile distilled water to obtain a bacterial suspension at 10^8 CFU ml^{-1}.

In vitro culture of grapevine plants

For *in vitro* culture of grapevine plants, we used a protocol described by Makee *et al*. (2010). Wood cuttings having four to five nodes of Ru140 and B41 rootstocks were collected from the field while the buds were still dormant. All cuttings were washed in water, and then treated with gentamicine sulphate 160 mg l^{-1}. Thereafter, they were incubated in 0.5 g l^{-1} carbamate fungicide [Methyl-1-(butylcarbamoyl)-2-benzimidazole-Carbamate 50%] (Bell®) for 24 h, and then grown in sterilized water at 25±1°C under 16 h photoperiod (140-150 µmol m^{-2} s^{-1}) from daylight fluorescent tubes (Philips TLD 38/54). Shoots were grown in glass jars (1000 ml) when they became about 8 cm long; buds of 4 mm length were taken from the middle of each stem. These buds were dipped in a solution of 70% ethanol for 3 min, 1.5% commercial bleach for 15 min followed by 0.7% commercial bleach for 5 min (Charbaji and Nabulsi, 1999). After sterilization, they were washed three times with sterile water and planted in tubes containing 20 ml DSD1 medium (Da Silva and Doazan, 1995). The DSD1 media contains 100 mg l^{-1} NH_4NO_3, 1000 mg l^{-1} KNO_3, 180 mg l^{-1} $MgSO_4$. $7H_2O$, 100 mg l^{-1} KH_2PO_4, 500 mg l^{-1} Ca $(NH_3)4H_2O$, 27.5 mg l^{-1} $MnSO_4$.7H2O, 37.5 mg l^{-1} Na_2 EDTA, 0.025 mg l^{-1} $CuSO_4$ $5H_2O$, 0.025 mg l^{-1} $CaCl_2.6H_2O$, 1 mg l^{-1} H_3BO_3, 1 mg l^{-1} $ZnSO_4$ $7H_2O$, 27.5 mg l^{-1} Fe SO_4 7H2O, 10 mg l^{-1} Myoinositol, 1 mg l^{-1} Acid Nicotinic, 1 mg l^{-1} Thiamine, and 1 mg l^{-1} Pyrodoxine. The pH of the medium was adjusted to 6.4 before adding agar and it was then autoclaved at 116°C for 25 min. The tubes were closed using cellophane paper and the edge of the tubes was sealed with parafilm to avoid contamination. All tubes were then incubated as described above.

Experimental design

Six-week-old grapevine plants were used to induced resistance; plantlets with two or three roots were selected. Due to the lack of phylloxera to infest the roots in the medium and to avoid the interaction between phylloxera and BTP_1, one root of each plants was pulled out of the medium but kept within the tube while the other root remained in the medium. The second root was treated with 1 ml of bacterial suspension (10^8 CFU ml^{-1}) of *P. putida* BTP1 on the root surface and inside the medium, or by distilled water for the control plantlets. The tubes were closed again as described above and incubated at 25°C under 16 h photoperiod. Seven days later, the second root was infested with sterile eggs of phylloxera according to Makee *et al*. (2003). Three-day-old eggs were taken from the colony and placed into 1.5 ml Eppendorf tubes for sterilization of the egg cuticle. One ml of formaldehyde (2.5%) was added to the eggs, gently shaken for 10 min, and left for 20 min. The sterilizing solution was then removed with a micro-pipette and the eggs were extracted and placed on sterile filter paper. The sterile eggs were gently transferred and spread on the non-inoculated roots of *in vitro* cultured plants by using a 10 ml sterile loop (Kendall, USA). For each rootstock, five treated and five untreated plantlets were infested with 25 surface-sterile phylloxera eggs. The tubes were resealed with parafilm to

prevent contamination and to avoid the escape of phylloxera crawlers, and were then incubated at 25±1°C under 16 hr photoperiod (140-150 µmol m^{-2} s^{-1}) from daylight fluorescent tubes (Philips TLD 38/54).

Evaluation procedure

Stereo microscope inspection was carried out daily on treated and untreated plantlets maintained in closed tubes to observe distribution of the eggs. The number of eggs hatched, feeding nymphs and adults were recorded to determine the mean developmental time (egg to egg) for each tested plant. Five random of root-feeding phylloxera females in each tube were inspected to determine the mean of oviposition period and the mean of fecundity (total number of eggs) of phylloxera. Thus, 25 females were examined on each plantlet. All eggs laid by each female were observed daily and counted till the female's death. Egg distribution during oviposition period (number of eggs per day), fecundity (total number of eggs) and female longevity were determined.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 program at 5% level (P= 0.05). Data were subjected to analysis of variance (ANOVA) for the determination of differences in means between tested plants of each treatment. Differences between means were tested for significance using Tukey HSD test.

3. Results

Effect of P. putida BTP1 on grapevine resistance against phylloxera

Percentage of matured females

The result showed significant difference in phylloxera egg numbers that were able to hatch and develop to reach adult stage (matured females) on both rootstocks Ru140 and B41 (F= 79.6; df=3, 16; P<0.001) (Fig. 1). *P. putida* BTP1-treated plants emerged significantly percentage decreased of matured females in both rootstocks comparing to control plants. However, there was no significant difference in the percentage of emerged matured females between treated plants of B41 rootstock and plants of rootstock Ru140 no treated (Fig. 1). The percentage of matured females of phylloxera on treated B41 was significantly greater (33%) than that on treated Ru140 (16%) (Fig. 1).

Fecundity

There was a significant difference in the mean of fecundity between Ru140 and B41 rootstocks (F= 140.8; df=3.96; P<0.001) (Fig. 2), with it resulting greater (19.4 eggs) in Ru140 than in B41 (13.7 eggs). When plants were treated with BTP1, the mean number of eggs laid significantly decreased in both rootstocks, pointing to a significant decrease in the mean of fecundity of phylloxera in both rootstocks. However the decrease in this parameter was greater in treated Ru140 (5.4 eggs) than treated B41 rootstocks (9.9 eggs) (Fig. 2).

Oviposition period

A significant difference was observed in the mean oviposition period of phylloxera between Ru140 and

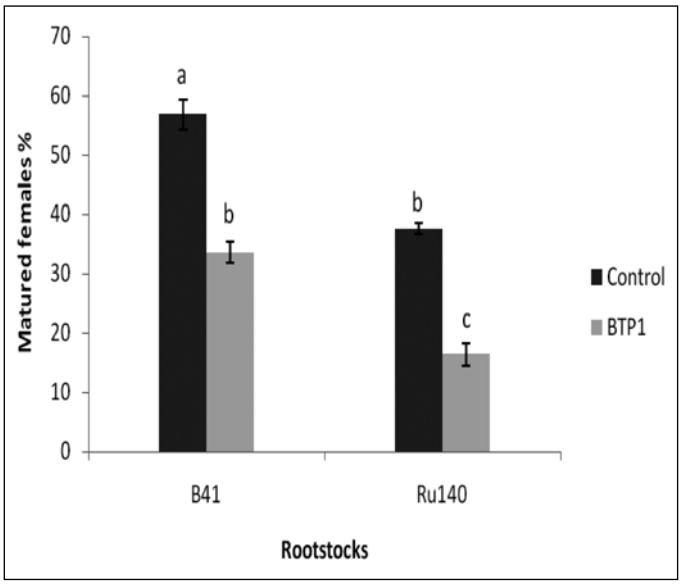

Fig. 1 - Effect of *P. putida* BTP1 on percentage of matured females of phylloxera *in vitro* in B41 and Ru140 rootstocks in comparison with control plants. Each column represents data from 25 samples. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at P<0.001).

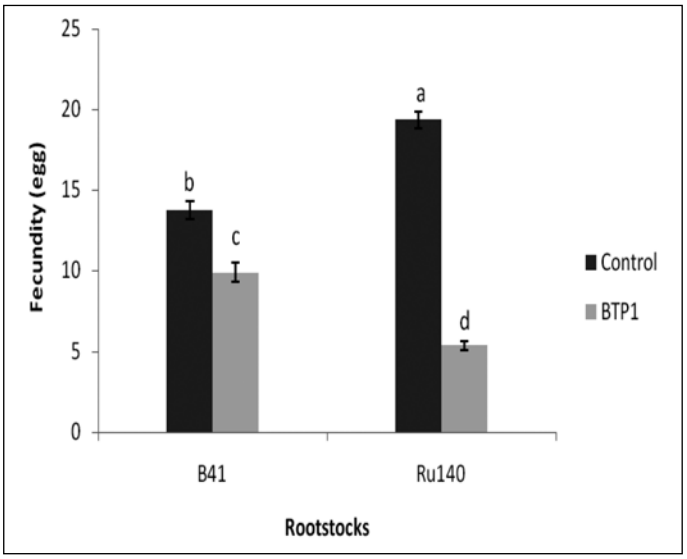

Fig. 2 - Effect of *P. putida* BTP1 on fecundity of phylloxera *in vitro* in B41 and Ru140 rootstocks in comparison with control plants. Each column represent data from 25 samples. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at P<0.001).

B41 rootstocks (F= 38; df=3.96; P<0.001) (Fig. 3). The oviposition period was 7 and 6 days in Ru140 and B41, respectively. However, when plants were treated with *P. putida* BTP1, the oviposition period decreased in a significant way only in Ru140. No significant differences were observed in the mean oviposition period between treated and untreated B41 plants (Fig. 3). The mean oviposition period of phylloxera on treated B41 was significant: lasting one day longer than treated Ru140 (6 and 5 days respectively) (Fig. 3).

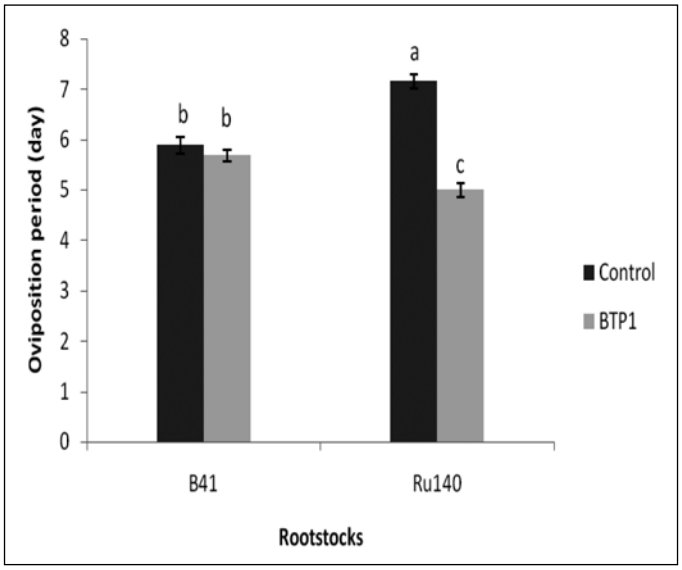

Fig. 3 - Effect of *P. putida* BTP1 on oviposition period of phylloxera *in vitro* in B41 and Ru140 rootstocks in comparison with control plants. Each column represent data from 25 samples. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at P<0.001).

4. Discussion and Conclusions

A recent study carried out on healthy pieces of roots of local grapevine cultivar Helwani showed the influence of non-pathogenic *P. putida* BTP1 on reproduction and development of grapevine phylloxera (Adam *et al.*, 2012). However, in the present work, the aim was to investigate the ability of this bacteria to induce systemic resistance in two grapevine rootstocks against phylloxera by using *in vitro* cultured plants. For our module this approach provided a strict separation condition between the inducer (bacteria) and the pathogen or pest (phylloxera) to induce systemic resistance (Ongena *et al.*, 2002; Bakker *et al.*, 2007). In agreement with a previous study (Ongena *et al.*, 2002), the bacteria did not migrate through the plants, suggesting the observed decrease in the life cycle of phylloxera was due to induction of systemic resistance in the host plant.

The present study confirmed that *P. putida* BTP1 had a protective effect on Ru140 and B41 rootstocks against phylloxera. The means of fecundity and oviposition period and emerged mature female percentage decreased significantly in both BTP1-treated rootstocks in comparison with control plants. These results are consistent with similar previous studies that demonstrated the ability of some strains of PGPR to induce systemic resistance in tomato against whitefly, where the percentage of matured females decreased in treated plants (Hanafi *et al.*, 2007; Valenzuela-Soto *et al.*, 2010). In addition, similar results were reported when cucumber beetles and American bollworm fed on PGPR-treated cucumber plants and cotton bolls, respectively (Zehnder *et al.*, 1997) (a, b; Vijayasamundeeswari *et al.*, 2009). Other studies also indicated that changes in the feeding behavior of Leaffolder and decreases in the weight of larvae and pupae were observed in rice plants treated with rhizobacteria (Radjacommare, 2002).

On the other hand, our results showed that there was a significant difference in reproduction and development of grapevine phylloxera between BTP1-treated B41 and Ru140 rootstocks. In comparison, the percentage of matured females and the means of fecundity and oviposition period decreased significantly by up to 50%, 45% and 12% respectively in treated Ru140 rootstock versus treated B41 rootstock (Figs. 1, 2 and 3). This is consistent with results of previous studies indicating the presence of a type of gradient from the resistant plant to sensitive plant (Granett *et al.*, 1983; Makee *et al.*, 2010). These results show that phylloxera laid a large number of eggs on sensitive varieties, more than on resistant varieties. It is believed that poor nutrition or the inability to colonize good locations for feeding could directly affect the number of eggs and ultimately the ratio of hatching. Thus, the resistance of grapevine to phylloxera could be a reflection of the strong relationship between poor nutrition and a decline in the productivity of the insect (Granett *et al.*, 1983). In addition, the mechanism of defense in these rootstocks may be due to toxic effects against phylloxera, such as the accumulation of some phenolic compounds in the cells of resistant plants leading to an increase in the death rate (Omer *et al.*, 1999). Other workers illustrated that there is a positive relationship between resistance/susceptibility characteristics against aphids and flavonoid glycoside content (Quercetin and Isorhamentin) of cowpea lines as these compounds possess a good inhibitory rate for aphid reproduction (Lattanzio *et al.*, 2000). Therefore, the resistance of Ru140 and B41 rootstocks to phylloxera may be attributed to an ability to produce such toxic phenolic compounds.

In conclusion, understanding the mechanisms of defense induced by some strains of PGPR in plants is very important to develop systemic resistance in plants. The current study provides evidence that *P. putida* strain BTP1 has the ability to stimulate a systemic resistance in grapevine rootstocks against phylloxera. We suggest that *P. putida* BTP1 treatment leads to an alteration in the plant's metabolic pathway eliciting the induction of plant defense compounds. These substances would have a negative influence on phylloxera feeding and development in treated plants. However, more research in the field must be done before implementing this technique on a large scale.

To our knowledge, this work is the first study interested in biocontrol of phylloxera in grapevine by PGPR strains *in vitro*. Furthermore, this investigation supplies important information about the possibility of implementing this strain to stimulate systemic resistance against plant pests. Moreover, this study illustrates the effectiveness of using *in vitro* dual culture in evaluating the phylloxera/grapevine and grapevine/rhizobacteria interactions. In fact, this testing system could be considered a very promising tool to: I) examine the phylloxera resistance of newly developed rootstocks; II) prevent the spread of phylloxera; III) study phylloxera genetic variation, biology and control method; and IV) study the mechanisms of defense induced in plants by rhizobacteria against pests.

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