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The relationship between grape phylloxera and *Fusarium* root infection

I. Idris,[1] **M.I.E. Arabi**

Department of Biotechnology, Atomic Energy Commission of Syria, P.O. Box 6091, Damascus, Syria.

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Abstract: Phyloxera seems to have key role in the fungal pathogen infection ratio while the fungal spread reduces the ability of pheloxera to reproduce. Intact roots of four-month-old grape plants were inoculated with phylloxera eggs in presence or absence of fungal pathogens. *Fusarium solani SY7* infection was detected in all plant parts when grapevine roots were infested with phylloxera. The spread ratio of *Fusarium solani SY7* increased from 74 to 100% of the infested plants with phylloxera. On the other hand, the phylloxera on *F. solani SY7* infected roots were developed more slowly, since the nymphs and tuberosities were significantly decreased 49% and 31% respectively. The total plant biomass decreased to 29% in the presence of both *F. solani SY 7* and phylloxera as compared to 9 and 17% in the presence of *F. solani SY7* or phylloxera, respectively. This study sheds light on the correlation between fungi, phylloxera and grapevine and could help in the application of integrated pest management (IPM) programs against grape phylloxera.

1. Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae Fitch*) is considered the most destructive grapevine insect pest in Syria where more than 70,000 ha of this crop, annually producing approximately 540,000 t of grapes, are planted (Makee *et al*., 2010). Grape phylloxera invaded Syria on nursery stock from bordering countries during the 1920s where it quickly spread in Syrian vineyards among the non-resistant local *Vitis vinifera* L. grape (Makee *et al*., 2003).

In Syria, the most commonly used resistant rootstocks are Ru140 (*V. rupestris* x *V. berlandieri*), R99 (*V. rupestris* x *V. berlandieri*), 3309C (*V. riparia Michaux* x *V. rupestris*) and 41 B (*V. vinifera* x *V. Berlandieri* Planchon). However, local phylloxera demonstrate an ability to develop and reproduce on all American rootstocks (Makee *et al*., 2010). In addition, the susceptibility of Ru140 rootstock was found to be higher than that of R99 and 3309C rootstocks (Makee *et al*., 2003). The rootstocks often allow limited growth and reproduction of phylloxera, but they may also reduce the growth vigor of grafted varieties because of incompatibility phenomena. Moreover, most Syrian grapevines are planted in areas of 300-500 mm rainfall, thus they are unirrigated and such conditions are inappropriate for resistant rootstocks (Makee *et al*., 2010).

Granett *et al*. (1996) reported that once a vineyard is infested with grape phylloxera it is expected to cease its production in about two to five years. Generally, spring phylloxera is found primarily on the small feeder roots that proliferate during root flush. However, during summer when the climate is dry and the top of the plant is growing, phylloxera is found extensively on the infected mature older roots. In winter, phylloxera is found on mature roots due to the disappearance of feeder roots (Omer *et al*., 1997). Once the phylloxera's proboscis is inserted into root bark cells, the parenchyma cells below the suberized outer layer, they inject saliva that, in turn, trigger the root cells to increase in size and number. These galls provide a feeding site from which stored nutrients such as sugars and amino acids can be extracted by developing phylloxera, which leads to a decrease in vine vigor and eventually destroys the roots causing vine death (Omer *et al*., 2002). The injured grapevines are usually attacked by secondary soil-borne pathogens at the insect feeding site (Omer and Granett, 2000). Granett *et al*. (1998) and Lotter *et al*. (1999) revealed that fungal infections associated with pylloxera galls are pathogenic and cause root death. In addition, fungal invasion of the roots at grape phylloxera feeding sites cause severe infections in grapevines. *Fusarium* is a large genus of filamentous fungi, and most *Fusarium* species are harmless saprobes and relatively abundant members of the soil microbial community (Domsch *et at*., 1980; Nwanma *et al*.,1993). This ecological habitat of the fungus implies that *Fusarium* could be a useful resource of extra cellular enzymes. The fungal isolate *F. solani SY7* was the best xylanase producer among tested isolates (Arabi *et al*., 2011; Bakri *et al*., 2012). Vine varieties susceptible to grape phylloxera were also highly susceptible to *F. oxysporum* (Omer *et al*., 1995; 1999). Granett *et al*.

[1] Corresponding author: ascientific@aec.org.sy

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(1998) also demonstrated that the two root types with *V. vinifera* parentage, Carignane and AXR#1, which are most susceptible to phylloxera feeding, are also susceptible to infection by *F. oxysporum* pathogen.

The present study therefore aims to investigate whether the presence or absence of phylloxera could enhance *F. solani SY7* fungal infection in the local Balady *V. vinifera* variety vine roots and the consequences of the interaction between the grape phylloxera and *F. solan SY7* on the vine. In this context, the interaction between root infection and the three *Fusarium* species root and root- feeding grape phylloxera were first investigated under controlled conditions, then the effects of fungal infection with and without the presence of phylloxera on grape vine vigor (plant biomass, roots, branches and leaves, internodes weights) were determined. The process included identification of the *Fusarium* species that invade the roots from grape phylloxera feeding sites.

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 according to the procedures mentioned by De Benedictis and Granett (1993). Fresh and healthy pieces of local grape variety (Makee *et al*., 2008) "Balady" roots, 4-7 mm in diameter and 5-7 cm long, were taken and washed with tap water. Each piece was wrapped with moist cotton wool at one end, and then 10 to 15 phylloxera eggs were placed on each piece. The infested root pieces were placed on a wet filter paper disk inside plastic Petri dishes (12 cm diameter and about 1 cm deep, three to four root pieces per dish). For ventilation purposes, the Petri dish lid was modified with a 1-1.5 cm cloth–screened hole. Dish edges were sealed with parafilm, kept in plastic boxes with tightly fitting lids and incubated at 25°C in the dark with 75% relative humidity. The root pieces were replaced when they desiccated rotted or the phylloxera became crowded.

Potted inoculation procedure

Before inoculation, three-day-old eggs (n =100) were removed from the colony and placed in 1.5 ml plastic tubes for surface sterilization. One ml of formaldehyde was added to the eggs and the tubes were gently shaken for 10 min. The sterilizing solution was removed from the mix and the eggs were extracted and placed on a sterile filter paper and dried for 5 min. They were then kept in a Petri dish, sealed with parafilm to prevent contamination and the escape of the phylloxera crawlers. Egg sterilization was carried out under sterile conditions (Makee *et al.*, 2003).

Fungal isolates

The fungal isolates were obtained from the plant pathology laboratory of the Atomic Energy Commission of Syria (Arabi *et al*., 2011) (Table 1). Host plant root samples infested with *Fusarium* were collected from different locations in Syria. Roots were sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, roots were dipped in 70% Ethanol for 1 min and then washed once with distilled water. Roots were cut into small slides under sterile conditions and transferred to Petri dishes containing potato dextrose agar (38g/L) (PDA, DIFCO, DETROIT, MI, USA) (Alazem, 2007). Thirteen mg/1 Kanamycin sulphyate were added after autoclaving and 10 days incubation at 23 ± 1°C in the dark to allow mycelia growth. All isolates were identified morphologically, according to Nelson *et al*. (1993). Emphasis was placed on selecting isolates that induced differential reactions on specific genotype, pathogencity and *in vitro* xylanase activity (Alazem, 2007; Arabi *et al*., 2011; Bakri *et al*., 2012). The above mentioned parameters lead to select of three monosporic isolates *F. culnorum SY*3*, F. solani SY*7 *and F. equisesti SY*24. The *Fusarium* isolates used in this study, their location, year of collection and xylanase production are listed in Table 1 (Arabi *et al*., 2011). The cultures were maintained on silica gel at 4°C until needed. Eighty "Balady" grape stem pieces were dipped in a solution of 2000 ppm IAA (Indol Butric Acid) for 2 min, then planted in plastic pots contacting sterile moistened soil. Finally, they transferred to 10-L plastics pots, after four months. The experiment was conducted in greenhouse, using a randomized complete block design with four replicates of four plants for each of the following treatments in each treatment.

1 - Infection with phylloxera: roots of vines were infected with phylloxera eggs (50 eggs/root).
2 - Infection with *Fusarium spp*: vine roots were dipped in a fungal solution (5 x 10^4 spores/ml) containing an equal mix of spores (*F. culnorum SY*3*, F. solani SY*7*, F. equiseti SY*24) for 15 s, then dried for 30 min.
3 - Infection with *Fusarium spp* and phylloxera: vine roots were dipped in a fungal solution (5 x 10^4 spores/ml) containing an equal mix of spores (*F. culnorum SY*3 *, F. solani SY*7*, F. equiseti SY*24) for 15 s, dried for 30 min, and then infected randomly by phylloxera eggs.
4 - Plant control (free of *Fusarium spp.* and phylloxera): plants were transplanted into 10-L pots. Each experimental unit consisted of two plants. Pots were filled with sterile soil. The pots were all placed in a greenhouse at 25 ± 1°C (day) and 23±1°C (night) with 16-h

Table 1 - *Fusarium* isolates, location, year of collection and extra cellular xylanase production in solid state fermentation after five days of inoculation at 30°C

Isolate	Location of Syria	Year of collection	Xylanase (U/G)
F. culnorum *SY3*	North-West	2005	163.69
F. solani *SY7*	Middle-Region	2003	908.2
F. equiseti *SY24*	North-East	2005	122.43

daylight and 85-95% relative humidity. Plants were irrigated with water as needed.

A year later, the following parameters were measured: plant biomass, root weight, root number, internode weight. Five root pieces of each tested plant were sampled. Microscopic inspection was performed to determine the number of tuberosities and feeding nymphs for each tested plant.

Fungal inspection

Plant samples (roots, leaves and branches) were collected from each tested plant, surface sterilized for 3 min in 5% NaOCl, and rinsed twice in distilled water. Six disks to each roots, leaves and branches were transferred to Petri dishes containing PDA with 13 mg/L Kanamycin sulphyate added after autoclaving and incubated for 4 weeks at 23 + 1°C in the dark to allow mycelia growth. Mycelia edges of *F. culnorum SY*3, *F. solani SY*7, and *F. equiseti SY*24 were identified morphologically as describe by Nelson *et al.* (1993). The infection percentage of fungal ratio was calculated using the formula $R = F_e / Ft \times 100$, where R = the percentage of fungal ratio, F_e = number of disks that contain the fungi from roots, leaves and branches, and Ft = number of the total disks with or without fungi from roots, leaves and branches.

Statistical analysis was performed using the STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level ($P = 0.05$). Means were subjected to analysis of variance tested for significance using Tukey HSD test.

3. Results

Incidence of Fusarium spp infection in different parts of plant with or without phylloxera

The results demonstrated that fungal infection ratio in the whole plant was 74, 1.7 and 0% of *F. solani SY*7, *F. equiseti SY*24 and *F. culmorum SY*3, respectively. Furthermore, the ratio of infection with *F. solani SY*7 in treatment 4 increased 26% to reach 100% (Table 2).

Effect of different treatments on plant

The plant biomass (Fig. 1, I) decreased significantly in the presence of phylloxera and *Fusarium solani SY*7 (9 and 17%, respectively). This effect is more evidenced in the presence of fungi and phylloxera 29% ($F=2$; $df=1, 3$; $p<0.05$). Vine roots infection with *Fusarium spp*. had no effect on root weight (Fig. 1, II) compared with the control, while infection with phylloxera increased 36% significantly comparing to control ($F=2$; $df=1, 3$; $p<0.05$).

Branch and leaf weight (Fig. 1, III) decreased significantly ($F=2$; $df=1, 3$; $p<0.05$) compared to the control in the presence of phylloxera and *F. solani SY*7 (53% and 31%, respectively). This effect is more evidenced in the presence of phylloxera with infection of *F. solani SY*7 58%. The average internode weight (Fig.1, IV) in treated plants with both phylloxera and fungi increased considerably (50%) as compared to the control. However, no obvious correlation between phylloxera and fungi concerning the internode weights was shown in the experiment. In addition, the root number of vines exposed to the fungus was notably decreased but to a lesser degree compared to plants infested with phylloxera or both fungi and phylloxera ($F=3.6$; $df=1, 3$; $p<0.05$) (Fig. 2).

Effects of F. solani SY7 infection on nymph and tuberosity numbers

The numbers of nymphs and tuberosities in vines infested with phylloxera decreased significantly ($F=85.3$; $df=1.1$; $p<0.05$), ($F20=$; $df=1.1$; $p<0.05$) in comparison with vines infested with both phylloxera and fungi. In addition, nymphs and tuberosities were reduced by 49 and 31%, respectively, compared to the control plants (Table 3).

4. Discussion and Conclusions

Phylloxera seems to have key a role in the fungal pathogen infection ratio and, in contrast, the fungal spread reduces the ability of pheloxera to reproduce. The high infection ratio of *F. solani SY7* (74%) may attribute to its ability to spread in the phloem parenchyma through a special mechanism which allows the fungi to infect the roots (Omer *et al*., 1999). Phylloxera can serve as a vector and transport fungal propagates from infected to healthy roots (Omer *et al*., 2000). Therefore, our data suggest that the injury caused by phylloxera may give benefit to *F. solani*

Table 2 - Incidence of *Fusarium spp.* infection in different parts of plant with or without phylloxera

Treatment	*F. solani SY7 %*			*F. equiseti SY24 %*			*F. culnorum SY3 %*		
	Roots	Branches	Leaves	Roots	Branches	Leaves	Roots	Branches	Leaves
Phylloxera	0	0	0	0	0	0	0	0	0
Fusarium mix	72	77	75	0	0	0	0	0	0
Phylloxera + *Fusarium* mix	100	100	100	5	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0

Data represents the infection percentage of *Fusarium spp.* ratio in roots, branches and leaves in each treatment.

Fig. 1 - Effect of different treatments on plant biomass (I), root (II), branches + leaves weights (III) and internode weights (IV).

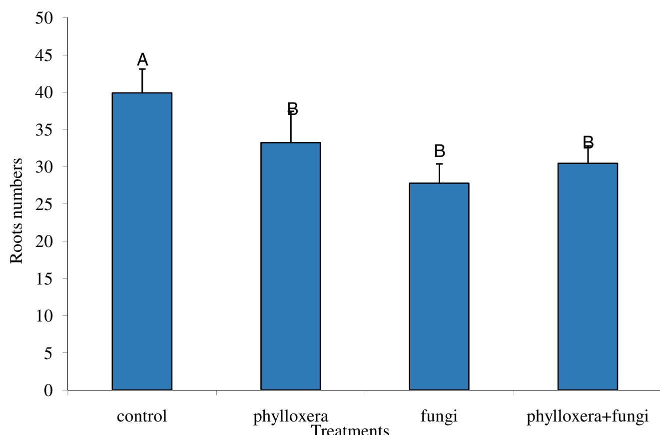

Fig. 2 - Effects presents of phylloxera and fungi on roots numbers

SY7 to spread throughout the entire plant 100%. However, the other two fungi (*F. culmorum SY24* and *F. equiseti SY3*) species may not possess this mechanism to spread in the phloem parenchyma and eventually they are not able to infect the vine despite the presence of phylloxera which may be attributed either to the lack of proper growth condition in vine or to the presence of immune response in the plant which prevents the growing of the later fungi species (Omer *et al*., 1999; Fossen, 2002).

To confirm the effect of *F. solani SY7* and phylloxera on vines, we established an experiment to evaluate five parameters (the weights of plant biomass, root internodes, roots, branches and leaves and root numbers in presence or absence of phylloxera compared to control plants). From these biological parameters, we can estimate the relationship between *F. solani SY7* and phylloxera. Omer *et al.*

Table 3 - Effects of *F. solani SY7* infection on numbers of nymphs and tuberosities

Treatments	Nymphs number (SE)	Tuberosities number (SE)
Phylloxera	25 ± 1 a	4.9 ± 0.9 a
F. solani SY7	0	0
Phylloxera + *F. solani* SY7	12.9 ± 0.9 b	3.4 ± 0.5 b
Control	0	0

Data represents nymphs number mean in tested roots of 16 plants in each treatment. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test. Means followed by different letters (columns) are significantly different at $P < 0.05$.

(1995) demonstrated that greenhouse experiments with effects of fungal infection on grapevine vigor after pruning at week 13 showed that damage was significantly greater in vines infested by phylloxera, *F. solani* and *Pythium ultimum* than the damage in vines infested with phylloxera alone. Total biomass was reduced by 16% in vines infested with phylloxera and by 24% in vines infested by phylloxera and *F. solani* (Omer *et al*., 1995). In our study it was found that the total biomass was reduced by 9% in vines infested with phylloxera and by 29% in vines infested with phylloxera and *F. solani SY7*, compared to the controls. The differences between *F. solani SY7* and *F. solani* (Omer *et al*., 1995) may be attributed to the disparity of aggressiveness of the isolates. Therefore, the infection with phylloxera and fungi together caused a synergetic effect where the plant biomass tend to decrease in presence of phylolllxera or fungi. This can be attributed to the damage in the vine roots caused by either phylloxera or fungi which cause dysfunction of the root as they should be in the normal conditions.

As both phylloxera and fungi affected the roots directly therefore, more work focused on the roots. The root weight and internodes increased significantly by 36 and 63% comparing to the control, in the presence of phylloxera and fungi respectively. This increase is normal in the presence of phylloxera due to the tuberosities and nodosities formed by the phylloxera. However, fungi alone did not cause changes in the root weight (Omer *et al*., 1995).

Our results demonstrated that the significantly growth of infested vines with phylloxera alone or with a combination of phylloxera and *F. solani SY7* may attribute to the rapid growth observed after potting. Moreover phylloxera may require more time necessary to establish feeding sites (Omer *et al*., 1995).

Additionally, a reduction of 50%, 30% respectively was observed in branch and leave weight and in the number of roots in the presence of phylloxera or both phylloxera and fungi compared to control, is attributed to roots death.Therefore, *F. solani SY7* infection can spread radially causing necrosis in the parenchyma and phloem. Ultimately the infection kills the roots and eventually the plant (Omer *et al*., 1999). Similarly Fossen (2002) found that the virulence isolates in present of *V. vinifera* on vine roots led to proportion of the root circumference that became necrotic in a 5-week period. Some isolates were highly virulent, causing up to 80% necrosis while other isolates were negligibly virulent (Fossen, 2002).

It has been shown in other plant-herbivore-pathogen systems that co-occurrence can, through direct interaction or changes in host's susceptibility, affect the performance of the pest or the pathogen (Karban *et al*., 1987; Hatcher., 1995). Omer *et al*. (2002) reported that the ability of grape phylloxera to exploit grape roots increased in the absence of fungal pathogens and indicated that phylloxera on infected roots developed more slowly, and had substantially reduced survival and reproduction rates. Therefore our results revealed the presence of fungi reduced the ability of phyloxera to form tuberosities by 31%. Our data also demonstrated that the total root weight decreased in presence of fungi with the Phylloxera-infested vine roots which were also reflected by a decrease in the number of nymphs.

Therefore our assays demonstrated that phylloxera on *F. solani SY7* infected roots were developed more slowly, since the nymphs and tuberosities were significantly decreased by 49% and 31% respectively. The reproduction and feeding activities of phylloxera were significantly decreased in the presence of fungal infection, consequently this result is in agreement with Omer *et al*.(2000). Furthermore the ability of *F. solani SY7* to spread within the plant parts increased when grapevine roots were infested with phylloxera grape.

These results provide interesting piece of information about the relationship between *F. solani SY7* pathogens and phylloxera. However these results are to be proved in the field. The present study provides preliminary information that could help in application of integrating pest management (IPM) program against grape phylloxera.

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