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# Acquisition efficiency of Flavescence dorée phytoplasma by *Scaphoideus titanus* Ball from infected tolerant or susceptible grapevine cultivars or experimental host plants

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## Summary

**The rate of Flavescence dorée phytoplasma (FDP) acquisition by the leafhopper vector *Scaphoideus titanus* Ball was tested under field and glass house conditions confining healthy reared nymphs on canes of FDP-infected grapevines or on FDP-infected cuttings collected in the field during the dormant season. Acquisition tests were performed using FD-tolerant (Merlot) or highly susceptible (Pinot blanc) grapevine cultivars, or alternatively using experimentally infected broadbean plants. Frequency of FDP acquisition by leafhoppers was evaluated using a polymerase chain reaction (PCR) assay. Different batches of insects were confined on the same infected source plants in the vineyard for acquisition access periods (AAP) of 7 d at a time at intervals of 15-20 d during spring and summer. When diseased Pinot blanc grapevines were used as source plants, acquisition by leafhoppers and transmission to healthy grapevines increased over summer, while almost no acquisition or transmission was observed when diseased Merlot grapevines were used as source plants. Tests conducted under controlled conditions confirmed that Merlot is a poorer source of FDP than Pinot blanc; the optimum FDP source for *S. titanus* was broadbean although this plant is not a natural host of the leafhopper. It is assumed that grapevine cultivars play an important role in influencing the proportion of FDP-infected leafhoppers in the vineyards and therefore influencing the rate of disease progress.**

K e y w o r d s : Flavescence dorée, varietal susceptibility, vector, acquisition, transmission, PCR detection.

## Introduction

Phytoplasmas are cell wall-less bacterial members of the class Mollicutes that are obligate parasites of plants and insect vectors. Phytoplasmas have been associated with hundreds of plant diseases, several of which have worldwide agricultural significance (McCoy *et al*. 1989). Flavescence dorée (FD) is probably the most serious phytoplasma disease of the grapevine (Grapevine yellows) in Europe. The agent is the Flaves-cence dorée phytoplasma (FDP) which is transmitted specifically by the insect vector *Scaphoideus titanus* Ball (= *S. littoralis* Ball) (Schvester *et al.* 1963) a specialist and monovoltine leafhopper on grapevines (Bonfils and Schvester 1960, Vidano 1964).

During the transmission process, insect vectors may acquire phytoplasma agents by feeding on infected source plants (horizontal transmission). Congenital transmission (vertical transmission) is uncommon although it has been reported in a few cases (Alma *et al.* 1997, Kawakita *et al.* 2000, Hanboonsong *et al.* 2002). Flavescence dorée phytoplasma (FDP) does not seem to have a high probability of being transmitted from one generation to another by congenital infection in *S. titanus* (Bressan *et al.* 2005). Nevertheless, all developmental feeding stages (nymphs and adults) may acquire FDP by feeding on FD-diseased grapevines (Schvester *et al.* 1969). Therefore the main sources of inoculum for vector acquisition are FDP-infected grapevines.

Early studies on transmission process of FDP by *S. titanus* conducted by Schvester *et al.* (1969) have reported minimum acquisition access period (AAP) on diseased grapevines of one week, although the probability of acquisition increased when the leafhoppers were confined for longer AAPs on host plants.

In this paper we studied the acquisition efficiency of FDP by *S. titanus* from diseased plants of FD-tolerant or -susceptible grapevine cultivars and we compared it with the ability of the vector to acquire FDP from infected broadbean, an experimental herbaceous host plant used to maintain phytoplasma strains in laboratory conditions (Caudwell *et al.* 1972).

## Material and Methods

I n s e c t s : *S. titanus* colonies were established according to Caudwell *et al.* (1970). Two-year-old egg-bearing grapevine wood was collected in organic vineyards at the end of winter, cut into pieces and kept in plastic bags in a cold room at 4 ºC. To allow egg hatching, 4-5 kg of wood pieces were put with potted healthy grapevines inside cubical cages (60 cm x 60 cm x 60 cm) in a climatic chamber (23 ± 1 ºC, photoperiod:16 h) or in a glasshouse with natural light and temperature about 25 ± 3 ºC. The wood pieces were placed over a layer of vermiculite and were periodically humidified in order to avoid egg dehydration. Grapevine plants were replaced about every 2 weeks. Egg hatching started about 30 d after transfer from the cold room to the rearing cages. Insects, nymphs or adults were periodically collected and used for the experiments. For field acquisition assays, the collected canes were maintained outdoors to synchronize the reared population of leafhoppers with the field popu-

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lation. They were transferred to the glasshouse in cages before the beginning of nymph emergence.

Field acquisition assays: The experiments were carried out during 2002-2003 in a viticulture area of the Veneto Region (north-east Italy), where an epidemic of FDP started in the early 1990's (POSENATO and GIROLAMI 1994, MARTINI *et al.* 1999). For the whole experiment, 12 grapevines with FD symptoms were selected and used for field acquisition assays: a group of 4 plants in 2002 in a vineyard cultivated with cultivar Pinot blanc, a group of 4 plants in 2002 and another group of 4 plants in 2003 in a vineyard cultivated with Merlot.

Every 20 d from May 25th 2002 and every 15 d from June 4th 2003, three groups of about 300 healthy nymphs each, were confined with mesh cages on the canes of FD-affected grapevines. Each group of leafhoppers was allowed a 7 d-AAP, then leafhoppers were transferred to caged healthy grapevines in a glasshouse to complete the latency for 37 d from the beginning of AAP.

For transmission trials we used cylindrical plastic cages ventilated with dacron organdy windows to confine three insects per test plant for an inoculation access period (IAP) of 7 d inside the glasshouse. At the end of IAP, leafhoppers were removed from the plants, mortality was recorded and the surviving insects were stored in the deep-freeze (-80 °C) until they were tested for presence of FDP in their body using a nested-PCR assay. Test plants were sprayed with Imidacloprid, then transferred to an insect-proof glasshouse where they were checked for symptom expression.

Acquisition from FD-infected cuttings and experimental host plants: In March 2004 one- and two-year-old FD-infected dormant woody canes were collected from diseased Pinot blanc or Merlot vines. Wood cuttings of 15-30 cm, carrying 2-3 buds were planted in large pots after removal of the basal bud and maintained in a warm glasshouse to allow root and shoot formation. About 60 cuttings per cultivar were produced. Cuttings that developed FD symptoms were used for leafhopper acquisition for AAPs of 7 or 14 d. Additionally, FDP-infected broadbeans, experimentally produced by feeding transmission of *Euscelidius variegatus* Kirschbaum (CAUDWELL *et al.* 1972) were used to confine groups of *S. titanus* nymphs for an AAP of 7 or 14 d in a climatic chamber (23 ± 1 ºC, photoperiod: 16 h). Leafhoppers were transferred at the end of each AAP in cages with young healthy grapevines in pots and maintained for a post acquisition period of 10 d. Then the leafhoppers were individually stored in 1.5 ml tubes in the deep-freeze until detection of FDP in their body. Healthy *S. titanus* maintained in rearing cages were used as control. Moreover, all the FD symptomatic cuttings used as phytoplasma sources were tested with nested-PCR for FDP infection after each AAP.

Phytoplasma detection in insects and plants: The procedure described by GATINEAU *et al.* (2001) was used to extract nucleic acids from individual deep-frozen leafhoppers. DNA was extracted from grapevine leaf midveins or broadbean stems and petioles as described by DAIRE *et al.* (1997). A nested-PCR of the non-ribosomal DNA fragment FD9, which is specific for 16SrV-group phytoplasma (DAIRE *et al.* 1997, ANGELINI *et al.* 2001) was used, according to BOUDON-PADIEU *et al.* (2003). Five µl aliquots of final amplification products were submitted to electrophoresis in 1.2 % agarose gel, stained with Gelstar® (Cambrex) or ethidium bromide and visualised under UV light. Controls were used for each amplification assay. These were DNA-template extracted from FD-infected or healthy *E. variegatus* leafhoppers taken in laboratory-reared colonies (CAUDWELL *et al.* 1972).

Statistic analysis: Differences among treatments were tested for significance with Chi-square test using Statistica Software (STATSOFT 2004).

## Results

Field acquisition assays: Results of transmission to grapevine seedlings after acquisition from field-grown Pinot blanc or Merlot, and FDP detection in individual insects are reported in Tab. 1. The rate of FDP acquisition and transmission increased over time when Pinot blanc grapevines were used as source plants; conversely, acquisition was less efficient for insects confined on Merlot grapevines. As a whole, FDP was transmitted to 12/92 (13 %) and 0/148 (0 %) seedlings by leafhoppers that were exposed for AAPs of one week to Pinot blanc and Merlot grapevines, respectively ($\chi^2$ = 20.32, d.f.=1, $P$<0.0001). The proportion of PCR-positive leafhoppers was 13/189 (7 %) and 1/160 (0.6 %) for Pinot blanc and Merlot vines, respectively ($\chi^2$ = 8.86, d.f. = 1, $P$ = 0.003). FDP was detected by PCR in the 12 symptomatic grapevines used as source of acquisition in 2002 and 2003 (results not shown).

Acquisition from FD-infected cuttings and broadbeans: In Tab. 2 the results of detection of FDP in *S. titanus* leafhoppers fed on infected sources for an AAP of 7 or 14 d are reported. Considering the proportion of PCR-positive insects, broadbeans were the most efficient source plants. Altogether, FDP was detected with PCR in 48/67 (71 %) of tested leafhoppers fed on broadbean, in 22/68 (32 %) of leafhoppers fed on cuttings of Pinot blanc and in 4/57 (7 %) of leafhoppers fed on cuttings of Merlot. Data for the different treatments: exposure to broadbean, Pinot blanc or Merlot, were significantly different by using Chi square test with $P$ values < 0.001.

Overall, the rate of FDP acquisition by *S. titanus* increased for all types of source plants when AAP was 14 d instead of 7 d. Old nymphs of *S. titanus* were able to acquire FDP from Merlot cuttings with an AAP of 14 d while acquisition did not occur with an AAP of 7 d.

## Discussion

Our data suggest that the efficiency of acquisition of FDP by *S. titanus* depends on the grapevine cultivar. FD-diseased Pinot blanc grapevines were a better source than FD-diseased Merlot for FDP acquisition by the leafhopper vector. The latter cultivar was a very poor source of FDP in both field and controlled conditions assays all along the growing season. In field tests we observed an incremental probability of acquisition with time when the susceptible

Table 1

Transmission of Flavescence dorée phytoplasma (FDP) to grapevine seedlings inoculated by *Scaphoideus titanus* that were exposed in the vineyard for an acquisition access period (AAP) of one week on FD-diseased grapevines of two different cultivars. PCR detection of FDP in individual leafhoppers after transmission. (see text for statistics)

| Year | Source | Beginning of AAP | Instar nymphs | (%) | Symptomatic/inoculated grapevines[a] rate | (%) | PCR on insects[b] rate | (%) |
|---|---|---|---|---|---|---|---|---|
| 2002 | Pinot blanc | May 25th | $L_1$ | 95 | 2/33 | (6) | 3/74 | (4) |
| | | | $L_2$ | 5 | | | | |
| | | | $L_1$ | 33 | | | | |
| | | June 15th | $L_2$ | 54 | 5/37 | (13.5) | 3/59 | (5) |
| | | | $L_3$ | 13 | | | | |
| | | | $L_3$ | 20 | | | | |
| | | July 6th | $L_4$ | 27 | 5/22 | (23) | 7/56 | (12.5) |
| | | | $L_5$ | 52 | | | | |
| 2002 | Merlot | July 12th | $L_4$ | 30 | 0/53 | (0) | 0/80 | (0) |
| | | | $L_5$ | 50 | | | | |
| | | | A | 20 | | | | |
| 2003 | Merlot | June 4th | $L_2$ | 33 | 0/35 | (0) | 1/39 | (2.5) |
| | | | $L_3$ | 66 | | | | |
| | | | $L_2$ | 10 | | | | |
| | | June 18th | $L_3$ | 25 | 0/32 | (0) | 0/40 | (0) |
| | | | $L_4$ | 30 | | | | |
| | | | $L_5$ | 35 | | | | |
| | | July 2nd | $L_5$ | 65 | 0/28 | (0) | 0/42 | (0) |
| | | | A | 35 | | | | |

[a] Three leafhoppers per seedling were used for transmission for an inoculation access period (IAP) of 7 d.
[b] number of PCR positive leafhoppers/total number of tested leafhoppers.

Table 2

Rate and percentage of Flavescence dorée phytoplasma (FDP) acquisition by late instar nymphs of *Scaphoideus titanus* that were exposed for different acquisition access periods (AAP) on FD-diseased cuttings of two grapevine cultivars or on FDP-infected broadbeans (see text for statistics)

| Source | AAP (d)[a] | PCR positive insects Rate | (%) |
|---|---|---|---|
| Pinot blanc | 7 | 7/26 | 27 |
| | 14 | 15/42 | 36 |
| Merlot | 7 | 0/25 | 0 |
| | 14 | 4/32 | 12.5 |
| Broadbean | 7 | 17/29 | 58 |
| | 14 | 31/38 | 81.5 |
| Control[b] | - | 0/15 | 0 |

[a] Fourth-fifth instar nymphs were confined on the plants.
[b] Control was *Scaphoideus titanus* leafhoppers reared on healthy grapevines.

variety Pinot blanc was used as source of inoculum. As the development of reared and field populations were synchronized, we used for acquisition tests insects at about the same developmental stages as those present in the field. Therefore, during the first acquisition period very young nymphs were available for feeding on diseased plants. The latter developmental stages are probably not as efficient in acquiring the pathogen as late-instar nymphs or young adults (Purcell 1982). This might be associated with the feeding behavior of the early developmental stages. According to Carle and Moutous (1965), first and second instar nymphs of *S. titanus* limit the depth of feed-probing to young vascular tissues or to non-vascular tissues more often than older nymphs.

The multiplication and increasing concentration of FDP with time in the phloem of infected grapevines, might also explain an incremental probability of acquisition of the pathogen. We observed an incremental severity of symptom expression in grapevines used as sources of acquisition, even though there was no direct evidence for a parallel incremental titer of the pathogen since the PCR assay applied at the end of each acquisition period did not provide quantitative information. In fact, the time distance between acquisition by the first groups of nymphs (25th May) and by the third groups (6th July) was of 40 d, during which the concentration of viable phytoplasma in infected tissues might have increased and/or the volume of infected tissue on diseased plants was augmented by shoot growth (Kuske and Kirkpatrick 1992, Lherminier *et al.* 1994).

The observed limited ability of *S. titanus* to acquire FDP from Merlot grapevines is probably associated with the lower

titer of viable pathogens in this host plant. Actually, this grapevine cultivar is tolerant to FDP and symptoms are generally limited to a few canes. Conversely, in Pinot blanc, a highly susceptible grapevine cultivar, symptoms are generally present on most of the canes of infected grapevines (BRESSAN, unpubl.). It might be possible that the feeding behavior of *S. titanus* leafhoppers is different on the two cultivars.

Acquisition from diseased cuttings seemed to occur more efficiently than acquisition from field-diseased grapevines, although the two grapevine cultivars had the same differences in providing FDP for vector acquisition under controlled and field conditions. Moreover, although broadbean is not a natural host for *S. titanus*, acquisition of FDP from infected broadbeans was extremely efficient and comparable to that of other phytoplasmas associated to yellows disease of herbaceous host plants and vectored by other auchenorrhyncha species (MURRAL *et al.* 1996, BOSCO *et al.* 1997). Hence, we can assume that grapevine cultivars influence in an important manner the acquisition efficiency and therefore the proportion of infected leafhoppers in vineyards, and eventually the rate of disease progress.

If the crop is a principal source of inoculum, cultivars that are poor hosts for vector acquisition should decrease the infection rate (PURCELL 1982). We observed (BRESSAN, unpubl.) that natural populations of *S. titanus* collected in persistently FD-infected vineyards cultivated with Merlot grapevines had a very low proportion of FDP-infected leafhoppers, if compared to the proportion of infected leafhoppers observed during FD epidemic on several cultivars (BOUDON-PADIEU *et al.* 1989 and unpubl. data).

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