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Effect of two strains of Flavescence dorée phytoplasma on the survival and fecundity of the experimental leafhopper vector *Euscelidius variegatus* Kirschbaum

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Abstract

We have investigated the influence on longevity and fecundity of Flavescence dorée phytoplasma (FDP), the agent of a grapevine yellows disease, in the experimental vector *Euscelidius variegatus* Kirschbaum. Late instar nymphs were exposed to one or the other of two strains of FDP (FD92 and FD2000) by feeding on infected broad bean (*Vicia faba* L.) or on healthy broad bean or maize (*Zea mays* L.) for an acquisition access period of 13 days. Detection of FDP in individual insects was done with PCR assays and revealed that almost all exposed leafhoppers had acquired FDP, for both FD92 and FD2000 strains. FDP infection significantly reduced the life span of males and females (ANOVA of the quartiles of survival distribution and Weibull scale parameter). FDP-exposed females produced significantly fewer nymphs. The two FDP strains had similar effects on reduction of survival and fecundity of leafhoppers. There was no significant differences in longevity of *E. variegatus* males exposed to FD broad bean than held on healthy broad bean or maize, but female survival and fecundity were reduced when they fed on maize versus healthy broad bean.

Keywords: *Euscelidius variegatus*; Homoptera; Cicadellidae; Phytoplasma; Survival; Fecundity; PCR detection; Pathogenicity; Flavescence dorée

1. Introduction

Flavescence dorée (FD) is a serious yellows disease of grapevine (*Vitis vinifera* L.) in Europe. It is associated with Flavescence dorée phytoplasma (FDP) that belongs to the Elm Yellows group or 16SrV group (Daire et al., 1997; Lee et al., 1998; Seemüller et al., 1998) and is specifically transmitted by *Scaphoideus titanus* Ball (Homoptera: Cicadellidae) (Schvester et al., 1961). Several strains of FDP have been isolated from infected vineyards in Europe (Angelini et al., 2001; Daire et al., 1997; Desqué et al., 2004; Martini et al., 1999). Because of the difficulties of rearing *S. titanus* and maintaining phytoplasma sources in grapevines, *Euscelidius variegatus* Kirschbaum was used in the laboratory as an alternative and experimental vector to maintain FDP on broad bean (Caudwell et al., 1970, 1972). The multiplication of FDP in the body and the invasion of the different organs of this alternative vector have been described. It was shown that FD phytoplasmas multiplied in the guts about 2–3 weeks from the beginning of acquisition. Then they entered the haemocell and progressively invaded all main organs, except for the germinal organs. Infection of salivary glands, a key event of the ability of transmission to healthy plants, started on the fourth week after acquisition. Almost all of the leafhoppers were infective on the sixth week after the beginning of acquisition (Boudon-Padieu et al., 1989; Lefol et al., 1994; Lherminier et al., 1990). Prokaryotic plant pathogens may establish with their vectors various interactions ranging from beneficial

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to harmful (Purcell, 1982). In a previous study (Bressan et al., 2005), we observed that a population of the leafhopper *S. titanus* exposed to FDP by feeding on FD-infected broad bean had reduced adult life span, fecundity, and egg hatching rate in comparison to healthy *S. titanus*. In the present work, we studied the effect of FDP on life span of males and females and on fecundity of the vector *E. variegatus*. Two strains of FDP were used, called FD92 and FD2000. These strains are differently spread in France and they are identifiable on the basis of sequence of 16S rDNA gene and of the non-ribosomal FD9 DNA fragment (*Sec Y* gene) (Angelini et al., 2001; Desqué et al., 2004). The strain FD92 is indistinguishable from the Italian strain FD-D (Angelini et al., 2001; Martini et al., 2002).

2. Materials and methods

2.1. Leafhopper colonies

Colonies of healthy *E. variegatus* were maintained on maize (*Zea mays* L.) inside cubical cages (60 cm × 60 cm × 60 cm) according to Caudwell and Larrue (1977) in the insectary with controlled conditions (23 ± 1 °C, L16:D8). Longevity and fecundity tests were made in the same conditions of temperature and light.

2.2. Maintenance and source of phytoplasma strains

The phytoplasma strains FD92 and FD2000 (16SrV group) were transmitted to broad bean according to Caudwell et al. (1970) in 1992 and 2000, respectively, using wild *S. titanus* leafhoppers collected in FD-infected vineyards in France. They have been maintained since in the greenhouse by uninterrupted serial cycles of broad bean-to-broad bean transmission, using *E. variegatus* (Boudon-Padieu et al., 1989; Caudwell and Larrue, 1977).

2.3. Longevity and fecundity assays

Late instar nymphs of *E. variegatus* were collected from rearing cages with a mouth aspirator and confined on FD-infected broad beans (strains FD92 or FD2000) for an acquisition access period (AAP) of 13 days, or on healthy broad bean or maize plants as control for the same period. These constituted four different treatments. At the end of the AAP, four groups (replications) of 52 (26 males and 26 females) leafhoppers from each treatment were confined each on one maize plant at the four-leaf stage caged into a plastic cylinder (27 cm height and 11 cm diameter) closed at the bottom with a sponge pad and ventilated with five lateral apertures covered with nylon mesh.

To test fecundity, each group of leafhoppers was transferred every week to a new maize plant at the four-leaf stage and this was repeated for the whole life of the insects. Exposed plants bearing eggs were maintained in a cubical cage for an additional week after the removal of insects. Then (before hatching onset) stems and leaves of each plant were carefully observed with a magnifier and the parts bearing eggs were cut into pieces and put into large Petri dishes (2 cm height and 14 cm diameter) for incubation with humid filter paper for another week. Then (21 days from egg deposition) the number of hatched nymphs in each Petri dish was counted.

To test survival, the cages were checked twice a week and dead leafhoppers were removed, sexed, and placed separately in 1.5 ml tubes and stored at −80 °C. The experiment was continued until all leafhoppers were dead.

2.4. PCR detection of phytoplasma DNA in insects and FDP strain characterization

The procedure described by Gatineau et al. (2001) was used to extract DNA from insects. Individual deep-frozen leafhoppers were crushed in 400 µl of extraction buffer (2% cethyltrimethylammonium bromide, 100 mM Tris–HCl, 10 mM EDTA, pH 8, 1.4 M NaCl, and 0.1% 2-mercaptoethanol). The brei was heated for 15 min at 65 °C in a water bath. After addition of an equal volume of chloroform, followed by centrifugation, the aqueous phase was collected. The nucleic acid pellet obtained after isopropanol precipitation and centrifugation was washed with 70% ethanol, dried in vacuum, and dissolved in 25 µl of 10 mM Tris, 1 mM EDTA, pH 8. The amplification with nested-PCR of the non-ribosomal DNA fragment FD9, which is specific for 16SrV-group phytoplasmas (Angelini et al., 2001; Daire et al., 1997) was used, according to Clair et al. (2003). The first pair of primers was FD9f/r and the second pair was FD9f3b/r2. The 20 µl amplification mixture contained 0.375 µM each primer, 0.150 µM each dNTPs, 1 µM $MgCl_2$, *Taq* buffer (Appligene), and 1 U/100 µl of *Taq* DNA polymerase (Appligene). Five to 10 ng of template DNA was added for the first amplification. A predenaturation step at 92 °C for 90 s was performed, followed by 30 cycles with denaturation at 92 °C for 45 s, annealing at 57 °C for 45 s, and elongation at 72 °C for 105 s. One microliter of the diluted (1/100) amplification product was amplified for 35 cycles with the second pair of primers in the same conditions. For each batch of amplification we used FD92-infected *E. variegatus* leafhopper DNA as positive control and healthy *E. variegatus* DNA as negative control. Two negative controls were used for each series of 24 specimen tested in PCR. Five microliters of the final product was submitted to electrophoresis in 1.2% agarose gel, stained with Gelstar (Cambrex), and visualized under UV light. The identification of FD strains was done by RFLP analysis of PCR products after digestion with *Alu*I (Desqué et al., 2004).