

Pattern of chrysanthemum yellows phytoplasma multiplication in three leafhopper vector species (Cicadellidae Deltocephalinae)

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Abstract

The titre of chrysanthemum yellows phytoplasma (CYP, 'Candidatus Phytoplasma asteris') in the three vector species *Euscelis incisus*, *Euscelidius variegatus* and *Macrostoteles quadripunctulatus* has been measured following controlled acquisition from infected source plants. Phytoplasma DNA was quantified in relation to insect DNA (genome units, GU, of phytoplasma DNA/ng of insect DNA) using a quantitative real time PCR procedure. The results provided evidence of phytoplasma multiplication in the three species although CYP multiplication over time in *M. quadripunctulatus* was faster than in *E. incisus* and *E. variegatus*. CYP titre was also highest in *M. quadripunctulatus*. This latter species showed the shortest latent period and the highest transmission efficiency.

Key words: 'Candidatus Phytoplasma asteris', *Euscelis incisus*, *Euscelidius variegatus*, *Macrostoteles quadripunctulatus*, multiplication, quantitative Real Time PCR.

Introduction

Phytoplasmas are parasites of both plants and insects and their life cycle depends on an intimate association with their hosts. The inter-relationships between the agent, the vector and the host are of great importance for understanding the epidemiology of the diseases associated with these pathogens. Phytoplasmas are transmitted in a persistent manner. During the latent period, they multiply in the midgut, haemocytes and salivary glands. Circulation of phytoplasmas through the insect vector body was described during the 1960s and 1970s. Multiplication of phytoplasmas in their vectors was suggested in early reports and, more recently, quantitative evidence of phytoplasma multiplication in the vector has been provided using ELISA, competitive PCR and dot-hybridization using DNA probes (reviewed in Marzachi *et al.*, 2004). An insect unable to sustain multiplication of a phytoplasma will not serve as a vector. On the other hand, multiplication of phytoplasmas in insects does not always result in infective vectors (Purcell *et al.*, 1981).

In this study we report on the multiplication of chrysanthemum yellows phytoplasma (CYP, 'Candidatus Phytoplasma asteris', ribosomal subgroup 16SrI-B) in the vectors *Euscelis incisus* Kirschbaum, *Euscelidius variegatus* Kirschbaum and *Macrostoteles quadripunctulatus* Kirschbaum following controlled acquisition. The fate of CYP in the leafhoppers has been monitored over time using a relative quantification method (phytoplasma genome units/ng of plant DNA) based on a quantitative real time PCR (qR-PCR) assay previously described (Marzachi and Bosco, 2005). Relative quantification is independent of DNA extraction yields, and data obtained from other phytoplasma-insect associa-

tions (when available) will be directly comparable when expressed as phytoplasma genome units per ng of host DNA.

Materials and methods

Chrysanthemum yellows phytoplasma (CYP, 16SrI-B), belonging to 'Candidatus Phytoplasma asteris' was used. Healthy colonies of *M. quadripunctulatus*, *E. variegatus* and *E. incisus*, vectors of CYP were maintained as previously described (Bosco *et al.*, 1997). Nymphs of *E. incisus*, *E. variegatus* and *M. quadripunctulatus* were caged for an acquisition access period (AAP) of 7 days on CYP-source daisies. After the AAP, surviving leafhoppers were transferred to healthy oat plants (immune to CYP). Insect were sampled at 10, 25, 40 and 55 days post acquisition (dpa) and their total DNA was extracted. To avoid differences due to insect gender, only females were used.

DNA was extracted from single CYP-exposed leafhoppers or healthy ones. Assays were done in 96-well plates in a BioRad iCycler (BioRad, U.S.A.), following the procedure described in Marzachi and Bosco (2005). CYP DNA was measured as fg of phytoplasma DNA/ng of insect DNA and then transformed into the number of CYP genome units (GU) per ng of vector DNA as in the original method. CYP and insect DNAs from the same sample were separately quantified in each plate. In each qR-PCR plate phytoplasma-free insects and water controls were also included. All the samples were run in triplicate. A one-way ANOVA of log-transformed raw data was performed to compare CYP quantities measured at different times.

Results

The relative quantification of CYp in *E. incisus* ranged between a minimum of 20 and a maximum of 120,000 GU/ng of leafhopper DNA at different times post-acquisition. One-way ANOVA applied to CYp titre in insects sampled at different dpa showed that titre significantly increased over time ($F = 16.408$; $P < 0.001$). CYp in *E. variegatus* ranged between 10 and 200,000 GU/ng of leafhopper DNA. CYp concentration increased over time in this species ($F = 31,927$; $P < 0.001$). CYp in *M. quadripunctulatus* ranged between 200 and 230,000 GU/ng of leafhopper DNA. CYp concentration increased over time also in this species ($F = 4,915$; $P = 0.016$). The mean quantities of CYp GU/ng of insect DNA at different dpa are shown in figure 1.

Discussion

We demonstrated the multiplication pattern of CYp in three vector species following controlled acquisition from infected *C. carinatum* plants. Increase in titre over time occurred in all three species. The comparison of the titre in the different leafhopper species shows that the multiplication pattern of the same phytoplasma in different vector species is different: *M. quadripunctulatus* supports the highest number of CYp cells per DNA unit and multiplication is much faster than in *E. incisus* and *E. variegatus*. The rate of multiplication is correlated with the incubation time in the insect, actually, latency in *M. quadripunctulatus* lasts 18 days, while in *E. variegatus* 30 days are required under the same conditions (Bosco *et al.*, 2007). A faster multiplication in the insect should result in an earlier invasion of the salivary glands and therefore, in a shorter latent period. It is interesting to note that, despite the different multiplication patterns in different leafhopper species, the final concentrations of CYp in the three species was about the same, suggesting that a saturation occurs. The most permissive species, *M. quadripunctulatus*, is also the most efficient vector (reaching a rate of 100% transmission compared to 75 and 82% for *E. incisus* and *E. variegatus*, respectively) (Bosco *et al.*, 2007).

The different CYp multiplication and transmission patterns in the vector species reflect the speed of development of the three species: *M. quadripunctulatus*, with the fastest development and the shortest life span, shows the fastest CYp multiplication and the shortest latent period, followed by *E. variegatus* and finally by *E. incisus*, which has the longest life cycle and longevity and shows the slowest phytoplasma multiplication. We suggest that different multiplication and transmission patterns are the result of interaction between vectors and pathogen. The phytoplasma must multiply and rapidly colonise the body of a short-lived vector to be spread in nature while speed of colonisation is not as important when the vector is long-living. Alternatively, a longer

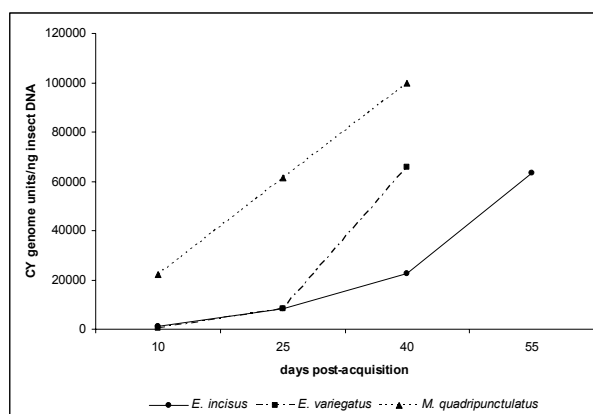


Figure 1. Mean quantities of CY cells per ng of insect DNA, estimated by qR-PCR at different dpa.

evolutionary interaction between *E. variegatus* and *E. incisus* and CYp, that allowed these vectors to become 'less susceptible' to phytoplasma infection, may explain the results.

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