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Multiplication kinetics of Flavescence dorée phytoplasma in broad bean. Effect of phytoplasma strain and temperature

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Abstract The multiplication kinetics of the Flavescence dorée phytoplasma in broad bean after inoculation by the experimental vector *Euscelidius variegatus* was determined. The number of phytoplasma cells, measured by quantitative real-time PCR in the aerial parts of the plants, increased exponentially over the time. After 22 to 30 days post inoculation, when symptoms appeared, bacterial growth reached a stationary phase. Whatever the time following inoculation there were no statistical differences between numbers of phytoplasma cells in plants infected by Map-FD1 (FD-CAM05) and Map-FD2 (FD92 and FD-PEY05) genotype strains. On the contrary, temperature had an influence on Flavescence dorée phytoplasma multiplication which was nearly twice as fast in broad beans incubated at 25 °C than in broad beans incubated at 20 °C. At 25 °C, plants expressed symptoms 1 week earlier. In a context of climate change, the consequences of a global warming on the Flavescence dorée epidemics are discussed.

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Introduction

Phytoplasmas are a group of non-cultivated wall-less bacteria belonging to the class *Mollicutes* (Weisburg et al., 1989). They are responsible for hundreds of diseases on cultivated and wild plants worldwide (Lee et al., 2000). Restricted to the phloem sieve tubes, they are transmitted from plant to plant by phloem sap sucking leafhoppers and are graft transmissible (Weintraub and Beanland, 2006). The Flavescence dorée (FD) phytoplasma is associated with a severe grapevine yellows (Caudwell et al., 1971) which is efficiently transmitted by *Scaphoideus titanus* Ball (Schvester et al., 1963), a vine feeding leafhopper of American origin. First reported in the 1950s in Southwestern France (Caudwell 1957), the disease propagated in Southern France and Northern Italy in the 1980s, 90s (Boudon-Padieu 2002; Belli et al., 2010). In spite of its declaration as a quarantine disease and compulsory protection regulations (large-scale insecticide treatments, eradication of infected plants and protection of mother plants in nurseries), FD expanded in the occidental vineyards of Spain (Batlle et al. 1997) and Portugal (de Sousa et al., 2010), in the Balkans (Music et al., 2011; Duduk et al., 2004; Seljak, 2008) but also in further north regions in Switzerland (Schaerer et al., 2007), Austria

(Reisenzein and Steffek, 2011), Burgundy and Champagne (Moyse, 2005).

The FD phytoplasma belongs to the 16SrV taxonomic group, subgroups C and D (Martini et al., 1999; Lee et al., 2004). A Multi Locus Sequence Analysis study on FD phytoplasma collected in different French vineyards from 2003 to 2005 has shown the existence of two genetic clusters in vine plants. The Map-FD1 cluster (16SrV-C subgroup) displays some genetic variability, represents 17 % of the disease cases, and is mostly present in South-western France. The Map-FD2 (16SrV-D subgroup) is clonal in France, represents 83 % of the cases and is widely distributed in the French vineyards (Arnaud et al., 2007). A possible explanation for the predominance of Map-FD2 could be a better fitness of this strain in terms of multiplication in the plant. Indeed, higher and/or faster multiplication would lead to higher phytoplasma titres earlier in the season and probably a better diffusion in the plant. This would increase the probability of acquisition by the insect, speeding up the dynamics of its spread. In order to investigate this hypothesis, we compared the multiplication kinetics of Map-FD1 and Map-FD2 strains in an experimental host plant (broad bean) after transmission by the experimental vector *Euscelidius variegatus*.

Warming of the world climate is unequivocal as is now evident from observations of increases in global average of air and ocean temperatures (report of the Intergovernmental Panel on Climate Change, 2007). IPCC mentions a warming of the average surface temperature of 0.2 to 2 °C in Europe between 1970 and 2004. It foresees an increase of the average temperatures from 2 to 5 °C according to the scenarios for the next 100 years in Southern Europe. Global warming has an effect on the propagation of crop diseases worldwide by affecting the pathogens and their hosts plant or vector (Luck et al., 2011; Newton et al., 2011). The impact of the climate on phytoplasma diseases has been poorly studied and documented (Foissac and Wilson, 2009). A regional initiative, VitisCLIM project, aims in modelling epidemiology and economic impact of FD in Austrian viticulture under a climate change scenario (Steffek et al., 2011). But for such a modelling it is essential to feed the predictions with tools and data about the effects of warming on the FD phytoplasma and *S. titanus*. In this study, we investigated the effect of an increase of the temperature on the multiplication kinetics of the Map-FD2 predominant strain in broad bean plants.

Materials and methods

Phytoplasma strains

Phytoplasma strains FD92, FD-PEY05 and FD-CAM05 were transmitted to broad bean (*Vicia faba*, var Aquadulce) (Caudwell et al., 1970) by using *S. titanus* leafhoppers collected in 1992 and 2005 in FD-affected vineyards of South-west France. FD92 was collected in the locality of Ognoas (Landes), FD-PEY05 in Peyrière (Lot et Garonne) and FD-CAM05 in Saint Sulpice et Cameyrac (Gironde) (Papura et al., 2009). The strains have been maintained since this time by serial transmission from broad bean to broad bean using *E. variegatus* as an alternative leafhopper vector (Caudwell et al., 1972). Molecular typing based on *map* and 16S rRNA genes showed that FD-CAM05 belongs to the Map-FD1 genetic cluster (part of 16SrV-C subgroup) and that FD92 and FD-PEY05 belong to the Map-FD2 genetic cluster (16SrV-D subgroup) (Arnaud et al., 2007; Papura et al., 2009).

Transmissions and sampling

Fourth-instar *E. variegatus* nymphs were placed for 1week on broadbeans infected by FD92, FD-CAM05 or FD-PEY05 strains. Plants used for acquisition were previously selected by quantitative real-time PCR for harbouring equivalent numbers of FD phytoplasma cells per g of midrib fresh weight. After phytoplasma acquisition, insects were placed for a latency period of 4 weeks on healthy broadbeans with a photoperiod of 16 h light, 8 h dark and temperatures of 22 °C during the day, 18 °C during the night. Then, they were transferred to small broadbean shoots (grown in the same conditions) by groups of six specimens during 7 days for FD phytoplasma transmission. The shoots issued from seedling had two to four leaves when the insects were transferred. At the end of the transmission, all the surviving insects were collected, weighted and stored at −20 °C. For each experiment condition, the total DNA of 20 insects randomly selected was extracted for further quantification. From the beginning of the transmission until the end of the experiment, plants were incubated in growth chambers under controlled conditions: L16:D8 photoperiod, 70 % humidity and constant temperature. Comparison of FD-CAM05 and FD92 strains (experiment 1) was performed at 20 °C constant, comparison of FD-

CAM05 and FD-PEY05 strains (experiment 2) was performed at 22 °C constant in the same growth chamber. Influence of the temperature on the multiplication of the phytoplasma strain FD-PEY05 (experiment 3) was measured in two growth chambers at 20 °C and 25 °C. On week 1, 2, 3, 4, and 5 from the beginning of the transmission, between four and seven plants were collected by cutting at the collar. The size of each plant was measured from the collar to the apex. Midribs, petioles and stem of the whole plant were dissected, pooled, weighed and total DNA was immediately extracted for further phytoplasma quantification. Each week, one plant incubated with healthy insects and grown in the same conditions was used as a negative control.

DNA extraction

Total DNA from each insect was extracted with the CTAB method as described in Maixner et al. (1995) by grinding individuals in 250 µl CTAB extraction buffer and re-suspending the final pellet in 40 µl. Total DNA from healthy leafhoppers was used as a negative control for each extraction series. Midribs, petioles and stem tissues of the whole plant were ground in CTAB buffer (1 g for 3 ml). One ml from the ground product was used for DNA extraction and the final pellet was re-suspended in 100 µl TE 1X. Total DNA from a healthy plant was also extracted in the same conditions for each extraction series. Concentrations of the nucleic acids were determined by measuring the absorbance at 260 nm with a spectrophotometer. Purity was assessed by calculating the ratio of the absorbance at 260 nm over the absorbance at 280 nm. Extracts were diluted in sterile distilled water to a final concentration of 50 ng/µl nucleic acids.

Construction of the standard

The *map* gene of FD phytoplasma was amplified using the primers FD9f5 and MAPr1 from the total DNA of a FD92 infected broad bean as described in Arnaud et al. (2007) with the high fidelity DNA polymerase "DyNAzyme EXT DNA" (Finnzyme). The 875 bp amplicon was purified using the Promega Wizard® Genomic (DNA Purification Kit) kit and cloned in the pGEM-T Easy® plasmid (Promega) following the instructions of the manufacturer. Plasmid was purified from *Escherichia coli* strain DH10B, using the Promega Wizard ® Plus Minipreps kit. Nucleotide sequence of the insert was verified. Plasmid concentration was measured by optical density. Ten-fold serial dilutions ranging from 10^8 to 1 copy of the recombinant plasmid per tube were prepared in nuclease free water (Abgene).

Quantitative real-time PCR

The *map* gene of the FD phytoplasma described in Arnaud et al. (2007) was chosen as a target for the amplification of the phytoplasma DNA. The gene was previously shown to be present in a single copy on the FD chromosome (Malembic-Maher et al., 2008) and sequences of different isolates of the 16SrV group were available in databases. Primers were designed using Beacon Designer™ 4.0 software on the basis of the *map* gene sequences alignment of 19 reference isolates representative of the 16SrV group. Strains and sequences were as reported in Arnaud et al. (2007). Accession numbers are AM384884 to AM384902. The chosen primers (mapF3+: 5′ CAATTAAATCATTAGATGAAATCG3′ and mapR5+: 5′TTCACTGAAATACAAATATAACC3′) led to the amplification of a 208 bp long fragment, from position 120 to 327 after the start codon of *map* gene. PCR assays were performed in a final volume of 25 µl with 12.5 µl Absolute™ QPCR SYBR® Green Fluroescein Mix (Abgene), primers at 250 nM and 5 µl DNA extract (at 50 ng/µl). Quantification was performed in 96-well PCR plates (Biorad) on ICycler and BioRad CFX96 Real-Time PCR Detection Systems with the following amplification cycles: 1 cycle at 95 °C for 15 min, 40 cycles of 94 °C for 15 s, 57 °C for 30 s, 66 °C for 30 s, then 58 cycles of 66 °C for 10 s with 0.5 °C/cycle increase and 20 °C hold. Threshold levels, threshold cycles (Ct) and standard curves were calculated automatically by the BioRad iCycler™ iQ 3.0 software and the CFX Manager™ 2.0 software. Polyvalence of the PCR for group 16SrV phytoplasmas was tested on periwinkle DNA extracts from strains FD 92, FD-PEY05, FD-CAM05, Palatinate grapevine yellows EY17-49 and EY38, Rubus stunt RuS, Elm yellows EY1 and Alder yellows ALY already described in Arnaud et al. (2007). Specificity of the PCR was tested on periwinkle DNA extracts from strains of the 16SrI, II, III, VI, VII, X and XII groups already described in Pelletier et al. (2009). Absolute

quantification of the FD phytoplasma in plant or insect samples was achieved by comparison with a dilution series from 10^8 to 10 copies of the standard plasmid which was performed in duplicates on the same plate as the samples to be quantified. The calibration curves were established by plotting the mean Ct of each standard dilution versus the logarithm of its concentration. For each kinetic experiment, all the samples from a same sampling time, healthy plant negative control and water negative control were tested on the same plate in duplicates. For each sample, the mean copy number of *map* gene per well was calculated between duplicates and was then expressed as a number of phytoplasma cells per whole host plant or insect.

Data analysis

Mean values of numbers of phytoplasmas in whole insects collected at the end of the transmission period and mean values of numbers of phytoplasmas in the stem, petiole and midrib tissues of the whole plants collected at different times after inoculation were calculated for each experiment. Bacterial growth curves in plants were traced by plotting the logarithm of the mean numbers of cells as a function of the time. Growth kinetics were determined by calculating the mean growth rate constant of the exponential phase $k = (\ln N2 - \ln N1) / (t2 - t1)$ as described in Willey et al. (2008) where *N2* and *N1* correspond to the mean number of cells at time t2 and t1. The mean generation time which is the time required for the cell population to double was expressed as $g = \ln 2/k$. Comparison of the data and statistical significance were determined using the Wilcoxon rank sum test with the software R (Development Core Team, 2012) and the package R Commander (Fox, 2005). Coefficients of variation (CV) were calculated by dividing the standard deviation by the mean, multiplied by 100.

Results

Validation of the quantitative real-time PCR

A unique melting peak at 78 °C was observed after the real-time PCR on dilutions of the standard and on the DNA of broad beans infected by FD92, FD-PEY05 and FD-CAM05 strains (Fig. 1a). An identical peak was observed for periwinkles infected by FD92, FD-PEY05 and FD-CAM05, PGYA, PGYC, Rus, EY1, ALY strains with Ct ranging from 14 to 21. No peaks were observed with healthy broad beans and periwinkles. Small peaks could sometime be observed at 71 °C or 75 °C with periwinkles infected by phytoplasmas from other 16Sr groups; starting amplification curves were observed with high Ct ranging from 36.5 to 39 which might correspond to weak non-specific amplification.

Depending on the experiment, the standard was detected down to 100 or 10 copies of the plasmid whereas no signal was measured for one copy. Calibration lines had high R^2 between 0.98 and 1 and PCR efficiencies varied from 83 % to 104 %. Linearity was observed between 10^8 and 100 copies (Fig. 1b).

In order to evaluate the intra and inter-plate repeatability, six broad bean infected with various infection level were tested six times in six wells and repeated on two plates. The intra and inter-plate CVs of numbers of phytoplasma cells per well were below 10 % and 18 % respectively for broad beans with high quantities of phytoplasmas (around 10^6 per well). Broad beans with intermediate quantities of phytoplasmas (around 10^3–10^4 per well) showed intra and inter-plate CVs below 12 % and 10 % respectively. But the CVs of the weakly infected broad beans (between 10 and 50 phytoplasma cells per well) reached 55 %.

Effect of the strain on the phytoplasma multiplication kinetics

For the experiment 1 comparing FD92 (FD2) and FD-CAM05 (FD1) strains, 48.7 % and 49 % of *E. variegatus* respectively survived after transmission. Ninety per cent of the insects were found infected in both cases. The number of phytoplasma cells per insect was not statistically different between FD92 (mean $3.60 \times 10^7 \pm 2.57 \times 10^7$ SD, $n = 18$) and FD-CAM05 (mean $5.63 \times 10^7 \pm 7.74 \times 10^7$ SD, $n = 18$) strains according to the Wilcoxon rank sum test ($p = 1$). Figure 2a shows the evolution of the mean quantity of phytoplasma strains FD92 and FD-CAM05 in the whole infected plants and the evolution of the symptoms as a function of the time following the contact with *E. variegatus*. After 7 days post inoculation (dpi), phytoplasmas were detected in 66 % of the plants for both strains and after 14 dpi in 80 % and 100 % for FD-CAM05 and FD92 strains respectively. The later samplings

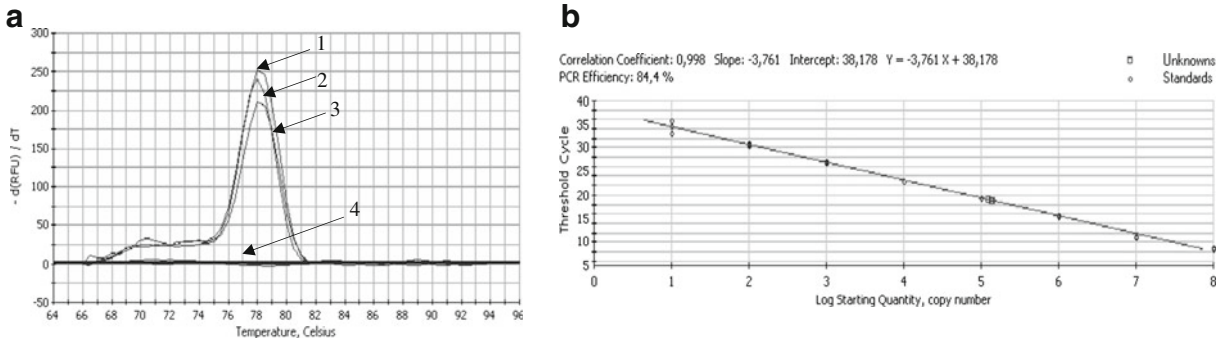

Fig. 1 **a** Examples of dissociation curves obtained after real-time PCR on the *map* gene cloned in the standard plasmid (1), from broad bean infected by FD92 strain (2), by FDCAM05 strain (3) and from healthy broad bean and water negative controls (4). **b** Example of calibration curve obtained by dilution series of the standard plasmid

were infected at 100 %. Each strain presented a classical bacterial growth curve with an exponential multiplication phase until 30 dpi, followed by a slowdown of the multiplication and a final stationary phase from 36 dpi to 43 dpi. Whatever the time following inoculation, the mean quantity of phytoplasma strain FD92 was not statistically different from the quantity of FD-CAM05 ($0.2<p<1$). Growth rates and generation times were very close for the two strains: $k=0.6$ day^{-1}, $G=27.7$ h for FD-CAM05 and $k=0.62$ day^{-1}, $G=26.8$ h for FD92. The first symptoms (slight twisting of the leaves) only appeared when bacterial multiplication reached the maximum at 30 dpi. The mean size, internodes number and total weight of stems, petioles and midribs of the plants were not statistically different between strains (data not shown). The expression of the symptoms after 30 dpi could not be differentiated (Fig. 2a).

For the experiment 2 comparing FDPEY-05 (FD2) and FD-CAM05 (FD1) strains, 68.7 % and 65 % of the insects respectively survived after transmission, among which 100 % were infected with FD-PEY05 and 90 % with FD-CAM05. The number of phytoplasma cells per insect was not statistically different between FD-PEY05 (mean $3.47\times10^7\pm4.8\times10^7$ SD, $n=18$) and FD-CAM05 (mean $2.70\times10^7\pm4.24\times10^7$ SD, $n=18$) ($p=0.73$). All the plants were found infected whatever the strain and the sampling time.

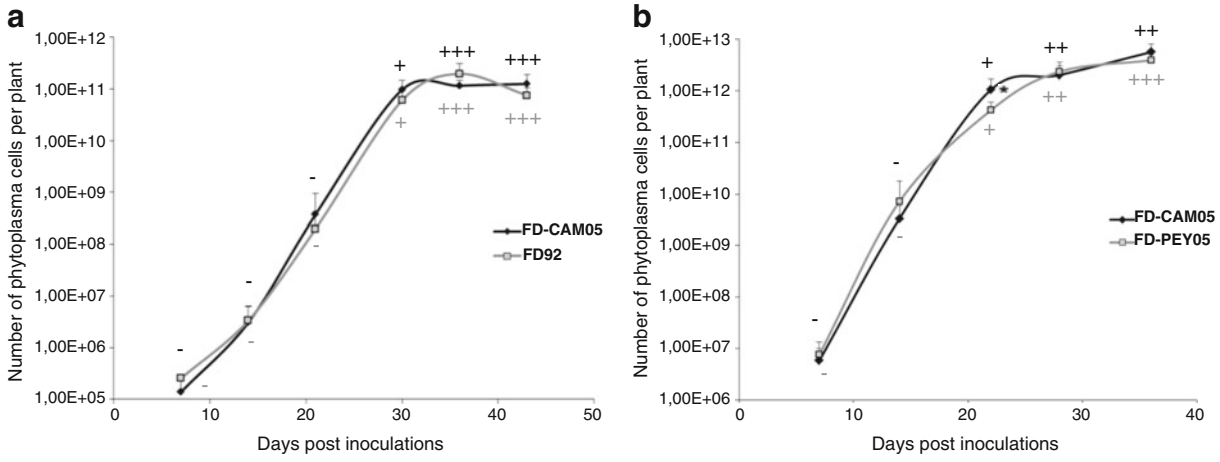

Fig. 2 Number of phytoplasma cells (mean SD) in aerial parts of broad beans and symptoms severity at different days post inoculations. a. Comparison between FD-CAM05 and FD92 strains. b. Comparison between FD-CAM05 and FD-PEY05 strains. − no symptoms, + slight deformation of apical leaves, ++ deformation and dwarfism of upper leaves, shortening of internodes, +++ deformation and dwarfism of leaves, shortening of internodes and yellowings. * indicates significant difference between means according to the Wilcoxon rank sum test ($p=0.02$)

The multiplication of both strains at 22 °C (Fig. 2b) was faster than in experiment 1 at 20 °C with the exponential growth phase ending at 22 dpi, coinciding with the observation of the first symptoms. As in the previous experiment, there were no statistical differences in the mean quantity of phytoplasma cells between strains FD-PEY05 and FD-CAM05 ($0.3<p<1$), except at 22 dpi where FD-CAM05 titres were slightly significantly superior to FD-PEY05 titres ($p=0.02$). The faster phytoplasma multiplication at 22 °C was illustrated by a growth rate higher and generation time lower than in experiment 1, but these constants were again very similar between Map-FD1 and Map-FD2 strains: $k=0.76$ day^{-1}, $G=18.4$ h for FD-CAM05 and $k=0.72$ day^{-1}, $G=17.2$ h for FD-PEY05.

Effect of the temperature on the phytoplasma multiplication kinetics

For the measurement of the phytoplasma multiplication (strain FD-PEY05) at 20 °C and at 25 °C, 74.3 % and 72.9 % of the insects respectively survived at the end of the transmission, among which 94 % and 100 % were infected respectively. The number of phytoplasma cells per insect was significantly higher at 20 °C ($7.91\times10^7\pm6.76\times10^7$ SD, $n=15$) than at 25 °C ($2.71\times10^7\pm3\times10^7$ SD, $n=17$) ($p=0.007$).

Eighty six percent of the plants were found infected at 8 and 16 dpi and 100 % for the following samplings. As shown in Fig. 3, the phytoplasma multiplication at 20 °C was slower than at 25 °C. Indeed, at 16 dpi, mean phytoplasma number was 100 times lower at 20 °C ($1.17\times10^8\pm1.21\times10^8$ SD) than at 25 °C ($1.16\times10^{10}\pm1.09\times10^{10}$ SD) with statistical significance ($p=0.002$). It was 4.5 times lower at 24 dpi ($3.63\times10^{10}\pm2.25\times10^{10}$ SD at 20 °C and $1.64\times10^{11}\pm8.35\times10^{10}$ SD at 25 °C) with statistical significance ($p=0.002$). It was illustrated by nearly twice higher growth rate ($k=1.2$ day^{-1}) and lower generation time ($G=14$ h) at 25 °C than at 20 °C ($k=0.65$ day^{-1} and $G=25.5$ h). The end of the exponential phase was positioned at 16 dpi at 25 °C and 1 week later at 20 °C. Like in the previous experiments, it perfectly coincided with the appearance of the symptoms which were 1 week delayed at 20 °C. After 24 dpi, the multiplication at 20 °C reached the same level that the one at 25 °C and no statistical difference could be found between the phytoplasma mean titres. But the appearance of the plant drastically differed. Indeed, as shown in Fig. 4, mean sizes of the plants at

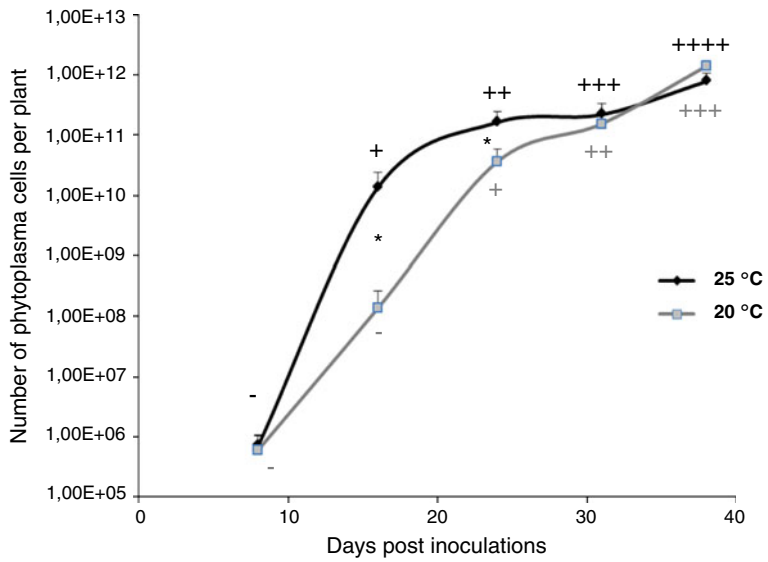

Fig. 3 Influence of the temperature on the number of phytoplasma cells (mean SD) strain FD-PEY05 in aerial parts of broad beans and on symptoms severity at different days post inoculations. − no symptoms, + slight deformation of apical leaves, ++ deformation and dwarfism of upper leaves, shortening of internodes, +++ deformation and dwarfism of leaves, shortening of internodes and yellowings, ++++ deformation and dwarfism of leaves, shortening of internodes, global yellowing and drying. * indicates significant difference between means according to the Wilcoxon rank sum test ($p=0.002$)

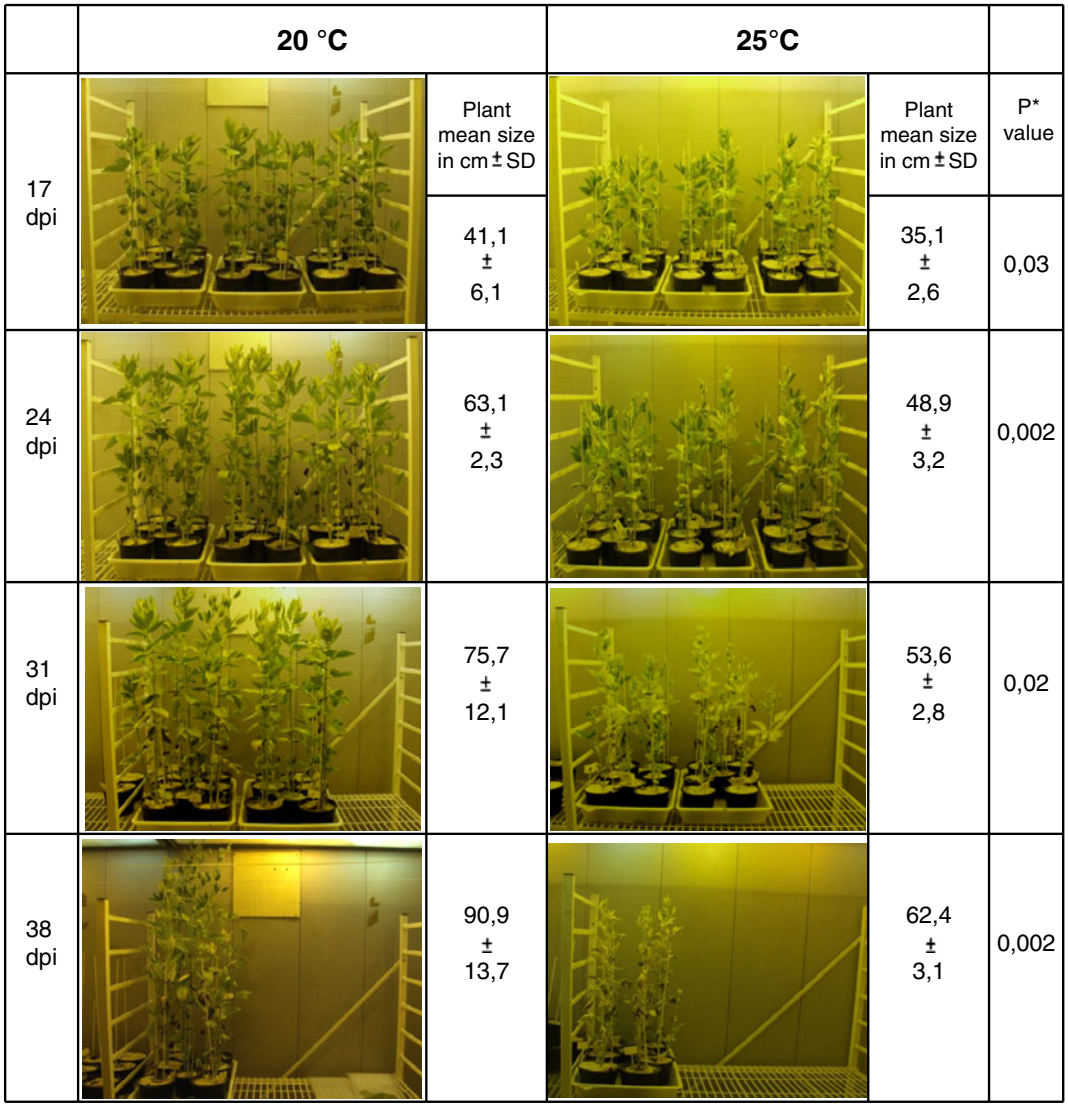

Fig. 4 Influence of the temperature on the growth of FD-PEY05 infected broad beans at different days post inoculations. * Statistical significance of size difference between 20 C and 25 C by using theWilcoxon rank sum test

24 dpi, 31 dpi and 38 dpi at 25 °C were statistically significantly smaller than at 20 °C ($0.002<p<0.02$). At 25 °C, plants begun to dry with a global yellowing after 31 dpi and they were almost dead after 38 dpi, whereas at 20 °C they stayed green at 31 dpi and did not decay until 38 dpi.

Discussion

In the present study, the multiplication kinetics of FD phytoplasma in aerial parts of the experimental host plant *V. faba* has been determined. The number of phytoplasmas in the whole plants sampled at the same time point showed a high variability. Differences up to 100 times were recorded during the exponential phase, which were reduced to a maximum of 10 times when the growth reached the stationary phase. A high variability between plants (up to 50 times) was also observed when measuring the growth of chrysanthemum yellows phytoplasma in *Chrysantemum carinatum* (Saracco et al., 2006). In the present work, the variability due to experiments was minimized by working under highly controlled and reproducible inoculations

and incubations conditions: plants had all the same age and size at the beginning of the inoculation, the percentage of infected insects was high (between 90 and 100 %) and constant between plants. Insects were placed by groups of six during 7 days in order to homogenize the quantity of phytoplasma injected into plants. The variability was not due to the real-time PCR method because we showed that the intra-plate coefficient of variation was less than 12 %, except for very low concentrations. Some variability can be explained by differences in terms of size, weight, hydration or nucleic acid content of each plant, but variations of these parameters never exceeded two times (data not shown). As already suggested in Saracco et al. (2006), such differences could mainly be due to different rates of phytoplasma multiplication during the exponential growth, due to individual differences in plant response to phytoplasma infection.

When the mean numbers of phytoplasmas were plotted against time, a classical bacterial growth curve was observed, with an exponential phase of growth (linear in semi-log), a slowdown of the growth and a stationary phase. In experiments 1 and 2, we could not find any difference between the multiplication kinetics of Map-FD1 and Map-FD2 genotype strains in the experimental plant host *V. faba*. The numbers of phytoplasma cells measured at each sampling date were statistically similar, leading to a near superimposition of bacterial growth curves and equivalent generation times and growth rates. Both strain genotypes also reached equivalent titres in the experimental insect vector *E. variegatus.* Thus, Map-FD2 strains isolated from the field at different periods (1992 and 2005 for FD92 and FDPEY-05 respectively) do not show a better fitness than Map-FD1 strains in term of multiplication in the plant and in the insect.

It is difficult to anticipate if these results can be extrapolated to the natural grapevine host because there are few data about FD multiplication kinetics in this plant. Bosco and Marzachì (2011) have shown that the phytoplasma titre can vary from two phytoplasma cells per ng of plant DNA in the spring to 1.5×10^4 cells per ng in the late summer in the FD sensitive cultivar Barbera. Comparisons of Map-FD1 and Map-FD2 kinetics in grapevine will have to be performed in the field and in controlled conditions of inoculation, in order to measure their multiplication but also their migration in the plant, because the dynamics of the epidemic can also be influenced by the ability of the pathogen to move within its host. However, field observations on Map-FD1 or Map-FD2 strain infected vineyards could not evidence any difference in symptom expression, repartition, and incidence in the plot (unpublished data). Furthermore, both Map-FD1 and Map-FD2 strains can be efficiently transmitted by the natural vector *S. titanus* (Papura et al., 2009) and they have similar effects on the longevity and fecundity of the experimental vector *E. variegatus* (Bressan et al., 2005). As a conclusion, the predominance of Map-FD2 strain in French vineyards may not to be due to a better fitness in terms of multiplication in its hosts and in terms of transmission by the vector. Papura et al. (2009) also showed that there is no significant relationship between the genetic structure of French *S. titanus* populations and the distribution of FD phytoplasma strains they carry, concluding that there was no phenomenon of a specific large diffusion of Map-FD2 by insect subpopulations. They suggested that the prevalence and the wide spread of Map-FD2 strain in the vineyards may be the consequence of extensive diffusion of this genotype through commercial exchange of infected grapevine material. In a previous study, we showed that the clonal Map-FD2 was detected in the first outbreaks and in some nurseries between 1994 and 2005 in France, Spain, Italy and Switzerland (Salar et al., 2009). It is in favour of a large diffusion of Map-FD2 by planting material in the 90's and 2000 in South-western Europe followed by a local dispersion by the insect vector.

In contrast, we showed that phytoplasma multiplication in *V. faba* is influenced by the temperature. Indeed, in experiment 3, multiplication rate of the Map-FD2 strain FD-PEY05 at 25 °C was significantly higher than at 20 °C with a generation time nearly twice shorter: 14 h instead of 26 h. The effect of the temperature was also observed in experiment 2 in which generation times were shorter at 22 °C than in the experiment 1 conducted at 20 °C (18 h and 27 h respectively). But at the stationary phase, numbers of phytoplasmas reached the same levels. As a matter of comparison, we estimated that the multiplication rate of chrysanthemum yellows phytoplasma in aerial parts of *C. carinatum* observed by Saracco et al. (2006) was a little slower, but in the same order of magnitude with an approximate generation time of 24 h at 25 °C. First results from Galetto et al. (2011) also showed the positive influence of the temperature on phytoplasma multiplication: FD phytoplasma titres in aerial parts of

broad beans were significantly higher at 30 dpi when plants were incubated at higher temperature (18 °C min-22 °C max and 22 °Cmin-26 °C max). But the temperature was not the only variable parameter since the CO2 level was also increased in the experiment.

In the experiment 3, 20 °C constant was chosen because it corresponds to the average temperature from the beginning of June until the end of August between 1997 and 2007 in two representative vineyards of Gironde, south-west France (unpublished data, personal communication from Armand J.M.). The 25 °C constant condition was determined by adding 2 °C to the average temperature in the same vineyards, during the summer 2003 which was characterized by a strong heat wave in south-west France. It corresponds to the more extreme scenario of the IPCC which foresees an increasing of the average temperatures around 5 °C for the next 100 years in southern Europe. In such a context of increasing of the temperature, one can try to make predictions about FD epidemics in the vineyards. The next spring following inoculation by insects, phytoplasmas move up from roots to developing shoots with an irregular distribution (Boudon-Padieu 2002). Higher temperature might induce a quicker multiplication of the phytoplasma which will reach higher titers earlier in the summer. It might also trigger a better distribution in the grapevine because it was shown that phytoplasma distribution in plants gets more uniform in time (Lherminier et al., 1994; Wei et al., 2004; Saracco et al., 2006). Higher concentrations and even distribution of the bacteria should favour the probability of acquisition by *S. titanus* feeding on the plants. It might increase the proportion of infectious insects in the plot and speed up the dynamics of the epidemic. Furthermore, Chuche and Thiéry (2009) have shown that an increase of the temperature during the winter induces an asynchrony between egg hatching of *S. titanus* and budburst of *Vitis vinifera*: larvae will appear later in the season, and leaves earlier. Thus, young leafhopper will be in contact with higher titres of phytoplasmas which might also increase the disease spread. But this effect could be restrained by other parameters. Indeed, an increase in temperature can slow down the phytoplasma multiplication kinetics in the vector. Galetto et al. (2011) showed that the bacteria multiply more slowly in *S. titanus* in conditions of higher temperature and CO2. Interestingly, we also observed a negative influence of the temperature on phytoplasma multiplication in the insect *E. variegatus* incubated only 1 week at 25 °C during the transmission period of experiment 3, carried significantly lower titres than the ones incubated at 20 °C. Temperature can also have negative effects on *S. titanus* populations. Chuche and Thiéry (2009) suggest that the asynchrony between egg hatching and plant development induced by mild winters might have severe impact on larval survival because they will have to feed on older leaves with poorer nutritional value. Furthermore it was demonstrated that populations of *S. titanus* which eggs were incubated at 20 °C were affected in their sex-ratio dynamics of hatching with a weaker protandry (weaker ratio of males) than eggs exposed to 5 °C (Chuche and Thiéry, 2012). According to Chuche and Thiéry (2012), a poor degree of protandry could have dramatic consequences on the population dynamics by reducing mating success and inducing inbreeding. So, winter warming might disadvantage the populations from southern Europe, whereas longer and hotter summers might favour the spreading of *S. titanus* to northern vineyards. *S. titanus* has recently been observed in the vineyards of Champagne in France, Austria and Hungary (Der et al., 2007; Reisenzein and Steffek, 2011).

As a conclusion, foreseeing the influence of global warming on the spread of FD disease is very complex because many factors must be studied and different effects may oppose or compensate. All the data collected in the studies mentioned above should be implemented in an integrated manner in modelling studies as such as the project VitisCLIM (Steffek et al., 2011).

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References

Arnaud, G., Malembic-Maher, S., Salar, P., Bonnet, P., Maixner, M., Marcone, C., et al. (2007). Multilocus sequence typing confirms the close genetic inter-relatedness between three distinct flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology, 73*, 4001–4010.

Batlle, A., Lavina, A., Kuszala, C., Clair, D., Larrue, J., & Boudon-Padieu, E. (1997). Detection of flavescence doree phytoplasma in grapevine in northern Spain. *Vitis, 36*, 211–212.

Belli, G., Bianco, P. A., & Conti, M. (2010). Grapevine yellows in Italy: past, present and future. *Journal of Plant Pathology, 92*, 303–326.

Bosco, D. & Marzachì, C. (2011). Flavescence doree in the cv Barbera and Nebbiolo: recovery incidence and susceptibility to the pathogen. *Protezione delle Colture*, 21–23.

Boudon-Padieu, E. (2002). Flavescence dorée of the grapevine: knowledge and new developments in epidemiology, etiology and diagnosis. *ATTI Giornate Fitopatologiche, 1*, 15–34.

Bressan, A., Clair, D., Sémétey, O., & Boudon-Padieu, E. (2005). Effect of two strains of Flavescence dorée phytoplasma on the survival and fecundity of the experimental leafhopper vector *Euscelidius variegatus* Kirschbaum. *Journal of Invertebrate Pathology, 89*, 144–149.

Caudwell, A. (1957). Deux années d'étude sur la flavescence dorée, nouvelle maladie grave de la vigne. *Annales d'amélioration des plantes, 4*, 359–363.

Caudwell, A., Kuszala, C., Bachelier, J. C., & Larrue, J. (1970). Transmission de la Flavescence dorée de la vigne aux plantes herbacées par l'allongement du temps d'utilisation de la cicadelle Scaphoideus littoralis Ball et l'étude de sa survie sur un grand nombre d'espèces végétales. *Annales de Phytopathologie, 2*, 415–428.

Caudwell, A., Gianotti, J., Kuszala, C., & Larrue, J. (1971). Etude du rôle de particules de type "Mycoplasme" dans l'étiologie de la Flavescence dorée de la vigne. Examen cytologique des plantes malades et des cicadelles infectieuses. *Annales de Phytopathologie, 3*, 107–123.

Caudwell, A., Kuszala, C., Larrue, J. & Bachelier, J. C. (1972). Transmission de la Flavescence dorée de la fève à la fève par des cicadelles des genres Euscelis et Euscelidius. Intervention possible de ces insectes dans l'épidémiologie du Bois noir en Bourgogne. Annales de Phytopathologie, N° hors série, 181–189.

Chuche, J., & Thiéry, D. (2009). Cold winter temperatures condition the egg-hatching dynamics of a grape disease vector. *Naturwissenschaften, 96*, 827–834.

Chuche, J., & Thiéry, D. (2012). Egg incubation temperature differently affects female and male hatching dynamics and larval fitness in a leafhopper. *Ecology and Evolution, 2*, 1–8.

de Sousa, E., Casati, P., Cardoso, F., Baltazar, C., Durante, G., Quaglino, F., et al. (2010). Flavescence doree phytoplasma affecting grapevine (Vitis vinifera) newly reported in Portugal. *Plant Pathology, 59*, 398–398.

Der, Z., Koczor, S., Zsolnai, B., Ember, I., Koelber, M., Bertaccini, A., et al. (2007). Scaphoideus titanus identified in Hungary. *Bulletin of Insectology, 60*, 199–200.

Duduk, B., Botti, S., Ivanovic, M., Krstic, B., Dukic, N., & Bertaccini, A. (2004). Identification of phytoplasmas associated with grapevine yellows in Serbia. *Journal of Phytopathology, 152*, 575–579.

Foissac, X. & Wilson, M. R. (2009). Current and possible future distributions of phytoplasma diseases and their vectors. In P. G. Weintraub & P. Jones (Eds.), *Phytoplasmas: genomes, plant hosts and vectors* (pp. 309–324) CABI.)

Fox, J. (2005). The R commander: a basic-statistics graphical user interface to R. *Journal of Statistical Software*, 14.

Galetto, L., Marzachì, C., Marques, R., Graziano, C., & Bosco, D. (2011). Effects of temperature and CO(2) on phytoplasma multiplication pattern in vector and plant. *Bulletin of Insectology, 64*, S151–S152.

Lee, I. M., Davis, R. E., & Gundersen-Rindal, D. E. (2000). Phytoplasma: phytopathogenic mollicutes. *Annual Review of Microbiology, 54*, 221–255.

Lee, I. M., Martini, M., Marcone, C., & Zhu, S. F. (2004). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of 'Candidatus Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *International Journal of Systematic and Evolutionary Microbiology, 54*, 337–347.

Lherminier, J., Courtois, M., & Caudwell, A. (1994). Determination of the distribution and multiplication sites of flavescence-doree mycoplasma-like organisms in the host-plant vicia-faba by Elisa and immunocytochemistry. *Physiological and Molecular Plant Pathology, 45*, 125–138.

Luck, J., Spackman, M., Freeman, A., Trebicki, P., Griffiths, W., Finlay, K., et al. (2011). Climate change and diseases of food crops. *Plant Pathology, 60*, 113–121.

Maixner, M., Ahrens, U., & Seemüller, E. (1995). Detection of the german grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology, 101*, 241–250.

Malembic-Maher, S., Constable, F., Cimerman, A., Arnaud, G., Carle, P., Foissac, X., et al. (2008). A chromosome map of the Flavescence doree phytoplasma. *Microbiology, 154*, 1454–1463.

Martini, M., Murari, E., Mori, N., & Bertaccini, A. (1999). Identification and epidemic distribution of two flavescence doree-related phytoplasmas in Veneto (Italy). *Plant Disease, 83*, 925–930.

Moyse, S. (2005). Flavescence doree in Bourgogne. Survey of young plantations. Phytoma, 33.

Music, M. S., Skoric, D., Haluska, I., Krizanac, I., Plavec, J., & Mikec, I. (2011). First report of flavescence doree-related phytoplasma affecting grapevines in croatia. *Plant Disease, 95*, 353–353.

Newton, A. C., Johnson, S. N., & Gregory, P. J. (2011). Implications of climate change for diseases, crop yields and food security. *Euphytica, 179*, 3–18.

Papura, D., Delmotte, F., Giresse, X., Salar, P., Danet, J. L., Van Helden, M., et al. (2009). Comparing the spatial genetic structures of the Flavescence doree phytoplasma and its leafhopper vector Scaphoideus titanus. *Infection, Genetics and Evolution, 9*, 867–876.

Pelletier, C., Salar, P., Gillet, J., Cloquemin, G., Very, P., Foissac, X., et al. (2009). Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis, 48*, 87–95.

Reisenzein, H., & Steffek, R. (2011). First outbreaks of grapevine 'flavescence doree' in Austrian viticulture. *Bulletin of Insectology, 64*, S223–S224.

Salar, P., Clair, D., Foissac, X., Boudon Padieu, E. & Malembic-Maher, S. (2009, September). Comparison of prevalence,

geographic distribution and biological properties of two flavescence dorée phytoplasma strains. Paper presented at the 16e meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Dijon, France. Le Progrès Agricole et Viticole, Hors Serie, 137–138

Saracco, P., Bosco, D., Veratti, F., & Marzachì, C. (2006). Quantification over time of chrysanthemum yellows phytoplasma (16Sr-I) in leaves and roots of the host plant Chrysanthemum carinatum (Schousboe) following inoculation with its insect vector. *Physiological and Molecular Plant Pathology, 67*, 212–219.

Schaerer, S., Johnston, H., Gugerli, P., Linder, C., Shaub, L., & Colombi, L. (2007). "Flavescence doree" in Switzerland: spread of the disease in canton of Ticino and of its insect vector, now also in cantons of Vaud and Geneva. *Bulletin of Insectology, 60*, 375–376.

Schvester, D., Carle, P., & Moutous, G. (1963). Transmission de la flavescence dorée de la vigne par Scaphoideus littoralis Ball. *Annales des Epiphyties, 14*, 175–198.

Seljak, G. (2008). Distribution of Scaphoideus titanus in Slovenia: its new significance after the first occurrence of grapevine "flavescence doree". *Bulletin of Insectology, 61*, 201–202.

Steffek, R., Reisenzein, H., Strauss, G., et al. (2011). VitisCLIM, a project modelling epidemiology and economic impact of grapevine 'flavescence doree' phytoplasma in Austrian viticulture under a climate change scenario. *Bulletin of Insectology, 64*, S191–S192.

Wei, W., Kakizawa, S., Suzuki, S., et al. (2004). In planta dynamic analysis of onion yellows phytoplasma using localized inoculation by insect transmission. *Phytopathology, 94*, 244–250.

Weintraub, P. G., & Beanland, L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology, 51*, 91–111.

Weisburg, W. G., Tully, J. G., Rose, D. L., et al. (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of Bacteriology, 171*, 6455–6467.

Willey, J. M., Sherwood, L. M. & Woolverton, C. J. (2008). Prescott, Harley, and Klein's, Microbiology, 7 edn: Mc Graw Hill International Edition.