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FDP-titre in grapevines with different susceptibility to GYs

Flavescence dorée phytoplasma titre in field-infected Barbera and Nebbiolo grapevines

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Abstract

Flavescence dorée phytoplasma (FDP) titre in the two red grapevine cultivars Barbera and Nebbiolo was measured over the vegetative seasons of two consecutive years in two vineyards of Piemonte Region (north-western Italy), with a double absolute quantification of FDP cells and grapevine DNA in real time PCR. The relationships of pathogen concentration to cultivar susceptibility and symptom severity were investigated. FD titre was always higher in Barbera than in Nebbiolo infected vines, and this difference was significant at early and late summer samplings of 2008 and at early summer sampling of 2009. A seasonal trend in FD concentration (low in spring, high in early summer and intermediate in late summer) was conserved for Barbera and Nebbiolo in both years and vineyards. Considering both cultivars and years from both vineyards, a significant positive correlation between FD concentration and symptom severity was found in the spring samples. Regarding the FDP strains (-C or -D), no differences in pathogen titres were detected for both cultivars. Similarly, the presence of another grapevine yellows phytoplasma, Bois Noir, a subgroup 16SrXII-A phytoplasma, in mixed infection with FDP strains had no effect on FDP concentration. These results demonstrate for the first time that grapevine cvs with different susceptibility to FDP support different pathogen titres.

Introduction

Phytoplasmas are wall-less plant pathogenic bacteria of the class Mollicutes associated with diseases of numerous plant species (Bertaccini, 2007). They are phloem-limited and transmitted by phloem-sucking leaf-hoppers, plant-hoppers and psyllids (Marzachi et al., 2004), (Weintraub & Beanland, 2006). Phytoplasmas are classified on the basis of the highly conserved 16S rRNA

sequence into more than 30 groups and within the '*Candidatus* Phytoplasma' genus (Firrao et al., 2004), (Zhao et al., 2010).

Flavescence dorée (FD) is an epidemic, economically important, quarantine disease of grapevine in France, Italy and Spain (Boudon-Padieu, 2003). The disease is associated with a phytoplasma belonging to the 16SrV (Elm yellows) taxonomic group (Lee et al., 2000). FD epidemics are caused by phytoplasma strains belonging to three phylogenetic strain clusters, according to multilocus sequence analysis of *map*, *uvrB-degV* and *secY* loci (Arnaud et al., 2007). In Italy the disease is caused by phytoplasma isolates belonging to clusters 2 and 3, and in Piemonte (north-western Italy) FD strains within the highly variable cluster 3 (FD-C) are the most prevalent (Martini et al., 2002), although isolates of the less variable cluster 2 (FD-D) are also present. The leafhopper *Scaphoideus titanus* Ball is the specific vector of the different FDP strains to grapevine under natural conditions (Mori et al., 2002, Papura et al., 2009, Schvester et al., 1963). Recently, a role as potential vector of FDP from alternative hosts to grapevine has been suggested for the planthopper *Dictyophara europea* (L.) (Filippin et al., 2009).

FD-infected grapevines usually show symptoms the year after inoculation (Morone et al., 2007), although longer latencies have been reported (Osler et al., 2002). Yellowing, downward curling of leaves, fruit abortion, stunting, and lack of lignification of new shoots are among the most important symptoms (Caudwell, 1983, Caudwell, 1990). Infected grapes dramatically reduce their production, but following the first year of symptom expression, a spontaneous and cultivar-dependent remission of symptoms may occur, and asymptomatic plants usually do not contain detectable FD phytoplasma (Morone et al., 2007).

Control of the disease relies mainly on compulsory insecticide treatments to reduce vector population and roguing of infected plants. No resistance is available to FD, but cultivars with different susceptibility to the disease have been reported (Kuzmanovic et al., 2008). Barbera and Nebbiolo are two traditional and economically important grapevine cultivars of Piemonte for the production of red wines. Barbera is highly susceptible to FD and shows severe symptoms starting

with growth reduction at the beginning of the vegetative season, while symptoms on Nebbiolo vines are milder, especially at the beginning of the vegetative season (Morone et al., 2001).

In this study we describe a new approach to real time quantification of FD phytoplasma in grapevine which was used to determine a) relationships between cultivar susceptibility and pathogen concentration in the plant, b) relationships between pathogen presence/concentration and symptom severity, c) FD phytoplasma titre over the vegetative season in order to identify the best timing for sampling and detection.

Materials and methods

Vineyard description

Two vineyards located in Piemonte (north-western Italy) were chosen: Cocconato (Asti province) and Monteu Roero (Cuneo province). Barbera and Nebbiolo cvs are planted at the Cocconato vineyard in 1999 on SO4 rootstock. Nebbiolo cv was planted at Monteu Roero vineyard in 1998 also on SO4 rootstock. Both vineyards were subjected to conventional agronomic and phytosanitary practices. An electronic meteorological station was present in Cocconato between the neighbouring Barbera and Nebbiolo vineyards. For the Monteu Roero vineyard, climatic data were collected at a station located in a neighbouring municipality (Canale) in the same exposure and altitude conditions. Mean month temperature (°C) and number of days in each month with minimum temperature below 0°C (freezing days) were calculated from data collected at both stations.

Establishment of a correct sampling procedure

To establish a correct sampling procedure, five FD-infected Barbera and Nebbiolo plants were selected in the Cocconato vineyard at the growth stage 17 – 19 (12 to 16 leaves separated; Coombe, 1995). Symptomatic and asymptomatic shoots were separately labeled on each plant and samples

consisting of three basal, three median and three apical leaves (a total of nine leaves) were separately collected from each shoot. Total DNA was extracted from each sample, and the presence of FD phytoplasma was assessed by nested PCR as detailed below. FD phytoplasma titre was then measured in each sample as described below.

Grapevine samples collection

Samples were collected in 2008 and 2009 only from symptomatic grapevines. Each sample consisted of three basal, three median and three apical leaves from one symptomatic shoot, for a total of nine leaves. In spring samplings, symptomatic shoots were severely stunted and dwarfed, and therefore the nine leaves were collected from two or three stunted shoots. Barbera samples were collected at Cocconato, while Nebbiolo samples were collected at both Cocconato and Monteu Roero, as not enough FD-infected plants were present at Cocconato. For sample collection, vineyards were surveyed three times a year: late spring (May – early June), early summer (July – early August) and late summer (September). During each survey, symptomatic grapes were labelled (Margaria et al., 2009) and scored for symptom severity. The same vines were sampled in both years provided that they were symptomatic. New symptomatic vines were sampled to replace recovered vines sampled in the previous year. In spring, symptom severity of each infected plant was rated on a scale of 1 to 3 according to the percentage of symptomatic shoots on the fruit bearing branch (1: up to 30%; 2: more than 30 % and up to 65%; 3: more than 65% and up to 100%). During early and late summer surveys, symptom severity of each infected grapevine was rated as A, B, C or D, according to the proportion of the plant canopy showing phytoplasma-specific symptoms (leaf downward rolling, sectorial leaf and vein reddening, bunch drying, premature leaf dropping, presence of brown pustules on canes, and, for late summer surveys, lack of cane lignification): A: plants lacking FD-specific symptoms, but not looking healthy; B: up to 30 % FD-specific symptoms; C: above 30 % and up to 60 %; D: above 60 %.

Total DNA extraction and phytoplasma diagnosis

Total DNA was extracted from 1.5 g of leaf veins following a phytoplasma enrichment protocol (Marzachi et al., 1999) and dissolved in 100 µl of sterile double distilled water (SDW). DNA concentration of extracts was quantified with a NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Wilmington, DE, USA). Polymerase chain reaction (PCR) was employed for phytoplasma detection. Universal primer pair P1/P7 was used in direct PCRs as previously described (Deng & Hiruki, 1991; Schneider et al., 1995). Reaction products were diluted 1:40 in SDW and used as templates in nested reactions driven by primers R16(I)F1/R1 or R16(V)F1/R1 for the specific detection of 16S groups I and V, respectively (Lee et al., 1994). Amplicons were separated by electrophoresis in 1% agarose gels buffered in 0.5× TBE (90 mM Tris-borate, 2 mM EDTA) and visualized under UV-light after staining with ethidium bromide. To identify Bois Noir, a group 16SrXII (Stolbur group) phytoplasma infection, two µl aliquots of fragments amplified with R16(I)F1/R1 primers were digested for 2 h with 1U of endonuclease *Mse*I (Invitrogen, Carlsbad, CA) at 37°C, according to the manufacturer's recommendations. Digestion products were separated in 5% polyacrylamide gels buffered in 1× TBE along with a 1 kb plus DNA size marker (Gibco BRL, Paisley, UK) and visualized by UV-light after staining with ethidium bromide. A Piemonte strain of FDP (FD-C), acquired from grapevine by *Scaphoideus titanus* and transmitted to *Vicia faba* L., a French strain of FDP (FD-D), kindly provided by Dr. E. Boudon-Padieu and graft-maintained in periwinkle in the collection of the Istituto di Virologia Vegetale, CNR, and a Sardinian strain of Stolbur phytoplasma from tomato (T2_92), also maintained in periwinkle (Minucci & Boccardo, 1997), were used as reference isolates and positive controls in PCR and RFLP experiments. A healthy Barbera grapevine from *in vitro* propagation, maintained in an insect-proof greenhouse, was employed as negative control in PCR experiments.

Characterization of Flavescence dorée phytoplasma isolates

Characterization of Flavescence dorée isolates was done by restriction analysis of the partial 16S-23S rDNA operon and *sec*Y gene. In the first case, fragments amplified with P1/P7 primer pair (Deng & Hiruki, 1991; Schneider et al., 1995) were diluted 1:40 with SDW, and re-amplified with primers M1/B6 as previously described (Martini et al., 1999). For the partial amplification of the *sec*Y gene, total grapevine DNAs were amplified in direct PCR with primers FDf2/ FD9r (Angelini et al., 2001), and then, following 1:40 dilution with SDW, amplified with primers FD9f3/FD9r2 (Martini et al., 2002). Amplicons were digested for 2 h with one unit of Taq*I* restriction endonuclease (Invitrogen) at 65°C. Digestion products were separated in 5% polyacrylamide gels in TBE along with a 1 kb plus DNA size marker (Gibco BRL, Paisley, UK) and visualized as detailed above.

Quantification of Flavescence dorée phytoplasma DNA

FD phytoplasma titre in infected grapevines was measured as number of FD phytoplasma cells per ng of grapevine DNA. To determine the number of FD phytoplasma cells, specific primers, targeting a portion of the *sec*Y gene of FD phytoplasma were designed using Primer3 software (Rozen & Skaletsky, 2000), and used in an absolute quantification real-time PCR-based assay. For the quantification of grapevine DNA, specific primers, targeting the 18S rDNA gene of grapevine were designed. The chosen primers were subjected to BLASTN analysis on GenBank through the NCBI database to test their specificity. To confirm the exclusive presence of the expected specific amplicon, 5 µL of real time PCR products were analyzed by electrophoresis in 1.5% agarose gel buffered in TBE. Standard curves for the absolute quantification of FD phytoplasma and grapevine DNA were obtained by dilution of plasmid p26SecYFD, containing the appropriate *sec*Y gene target sequence from FD-C phytoplasma, and of total DNA extracted from healthy grapevine grown from seed, respectively. Three quantities of p26SecYFD (0.5 ng, 5 pg, and 5 fg), were used, corresponding to $1.17 \cdot 10^8$, $1.17 \cdot 10^6$, $1.17 \cdot 10^3$ cells of FDP. For the absolute quantification of grapevine DNA, three quantities of total healthy grapevine DNA (5 ng, 0.05 ng, and 0.005 ng) were

used. Grapevine sample DNAs were diluted in SDW to a final concentration of 1 ng/µl, and used (5 µl) as template in real time assays together with Platinum SYBR Green qPCR Supermix UDG (Life Technologies, Carlsbad, CA, USA) and specific primer pairs at a final concentration of 300 nM, in a volume of 25 µL. The PCR was performed in 96-well plates in a BioRad iCycler (BioRad). Cycling conditions were as follows: one cycle at 95°C for 5 min, then 40 cycles at 95°C for 15 s, and 61°C for 1 min. For each sample, plant- and FDP-DNAs were amplified in the same plate with their corresponding reaction mixture, together with the three standard dilutions of healthy host DNA (Hg) and p26SecYFD. Each sample was run in triplicate in the same plate. For each PCR, water instead of DNA was used as negative control. Threshold levels, threshold cycles, and standard curves were automatically calculated by the BioRad iCycler software, version 3.06070. Per-well baseline cycles were determined automatically. Specificity of the reaction was tested by running a melting curve analysis of the amplicons following each quantification reaction.

Data analyses

For the analyses of phytoplasma concentration, raw data (expressed as log values) were used and the concentration of FDP in each plant was expressed as the difference between the logarithm of the number of FDP cells and the logarithm of the ng of grapevine DNA (DLog).

FDP titres in Barbera and Nebbiolo grapevines were analysed by Genstat 11th edition rel. 11.1 (VSN International Ltd, Hemel Hempstead, U.K.).

T-test and ANOVA were used to compare titre of FDP in samples, while the Mann-Whitney U test was used to compare symptom severity. Spearman's Rank correlation analysis was used to study relationships between symptom severity and FDP titre in spring, early and late summer samples of the two years.

Results

Symptom severity of infected grapevines and temperature parameters of the vineyards

The number of samples collected and symptom severity ratings of grapevine cultivars Barbera and Nebbiolo surveyed in vineyards during the spring, early summer and late summer of 2008 and 2009 are summarized in Table 1.

Overall, 59 Barbera samples were collected in the two surveys of 2008 (n = 41) and 2009 (n = 18). Details on the number of plants showing symptoms of the different categories in the two years are presented in Table 1. ~~During the spring surveys of the two years, 18 samples were collected, 15 in 2008 and three in 2009. During the early summer surveys, 24 samples were collected from symptomatic Barbera vines, 16 in 2008, and eight in 2009.~~ For early summer samplings, ~~I~~in 2008 most plants showed class B and C symptoms, and in 2009 most of them showed class D symptoms (Table 1). ~~During the late summer surveys, 17 samples were collected from symptomatic Barbera vines, ten in 2008, and seven in 2009.~~ For late summer samplings, ~~I~~in 2008, most of the plants showed class B and C symptoms, and in 2009 most of them showed class D symptoms (Table 1).

Overall, 60 samples were collected from Nebbiolo grapevines. As for the Barbera plants, details on the number of Nebbiolo vines showing symptoms of the different categories in the two years are presented in Table 1. ~~During the spring surveys of the two years, 8 samples were collected, five in 2008 and three in 2009. During the early summer surveys of the two years, 28 samples were collected, 15 in 2008 (11 from Monteu Roero and 4 from Cocconato, respectively), and 13 in 2009 (9 from Monteu Roero and 4 from Cocconato).~~ For early summer surveys, ~~I~~in 2008~~,~~ none of the infected plants showed symptoms of class D, and in 2009 none of the plants showed symptoms of class A (Table 1). ~~During the late summer surveys of the two years, 24 samples were collected from symptomatic vines, 14 in 2008 (10 from Monteu Roero and 4 from Cocconato), and 10 in 2009 (7 from Monteu Roero and 3 from Cocconato).~~ For late summer surveys, ~~I~~in 2008 most of the plants showed symptoms of classes B and C, and in 2009 about 50 % of the infected grapevines showed symptoms of class D (Table 1).

In the Cocconato vineyard, spring symptoms were more severe in Barbera than in Nebbiolo plants in both years (P= 0.991) (Table 2). FD symptoms in Barbera and Nebbiolo infected plants at Cocconato were more severe in 2009 than in 2008 (Barbera, early summer: P=0.999; late summer: P=0.996; Nebbiolo, early summer: P=0.975; late summer: P=0.990). In 2008, early summer symptoms at Cocconato were more severe on Barbera than on Nebbiolo (P=0.999). In the same year, early summer symptoms on Nebbiolo plants were more severe in Monteu Roero vineyard compared to Cocconato (P=0.999). The presence of BN phytoplasma in mixed infection with FDP in few plants had no effect on symptom expression on both Barbera and Nebbiolo (Supplementary table 1). Also FDP infection of the plant with -C or -D isolates did not influence symptom expression on both Barbera and Nebbiolo (Supplementary table 1).

In the Monteu Roero vineyard, mean winter (November to March) and spring (April and May) temperatures in 2008 were 4.8°C and 16.1°C, and in 2009 they were 4.1°C (winter) and 14.7°C (spring), with 63 (2008) and 79 (2009) freezing days during the winter time. In the Cocconato vineyard, mean winter and spring temperatures in 2008 were 6.7°C and 17.1°C, and in 2009 they were 5.6°C (winter) and 15.5°C (spring), with 41 and 49 freezing days recorded in 2008 and in 2009, respectively.

Phytoplasma detection and Flavescence dorée strain characterization

Table 3 reports the number of Barbera and Nebbiolo grapevines positive for the presence of FD and BN phytoplasmas alone or as mixed infection among the total samples collected in the surveys of 2008 and 2009. In spring surveys of 2008, almost all symptomatic Barbera samples were singly infected with FDP, and most Nebbiolo plants were doubly infected with FDP and BNP. In spring 2009, all symptomatic plants were singly infected with FDP, irrespective of cv identity and vineyard. In early summer surveys of 2008 and 2009, most symptomatic Barbera and Nebbiolo samples were infected with FDP only, although BNP and FDP-doubly infected grapes were found in both cvs and vineyards. In 2008 and 2009 late summer surveys, most symptomatic Barbera and

Nebbiolo vines were infected with FDP in single infection; few FDP and BNP mixed infections were also found in symptomatic plants sampled in late summer surveys of both years. BN phytoplasma was never detected as single infection in grapevines analyzed in any survey during each of the two years.

Overall, in the Cocconato vineyard, only FD-C type phytoplasmas (16S/secY; n = 17) were found in infected Nebbiolo plants sampled in the two years. FD-C phytoplasmas were detected in most Barbera samples collected in all surveys (n = 56), and three FD-D isolates were detected in 2008 surveys. FDP isolates from one Nebbiolo and six Barbera vines from the Cocconato vineyard could not be assigned to a type isolate. In the Monteu Roero vineyard, FD-C (n = 25) and -D (n = 11) phytoplasmas were both present in the surveys of 2008, and 2009. FDP isolates from six grapes from this vineyard could not be properly assessed.

Quantification of Flavescence dorée phytoplasma

The following primers were designed for the absolute quantification of FDP and grapevine DNA: FdSecyFw/FdSecyRv (5' TGCCTTATGTTACTGCTTCT 3'/ 5' TAATAATGATGGGGATTCAA 3'), and Vitis18SF1/Vitis18SR1 (5'ATGATTAACAGGGACAGTCG3'/ 5'GGTATCTGATCGTCTTCGAG3'). BLAST analysis of primers FdSecyFw/FdSecyRv, flanking a 185 bp region of the FD-70 isolate *sec*Y gene (accession number: AM238512), showed their specificity for the target gene of several "*Ca*. P. vitis" isolates and phytoplasmas belonging to other 16Sr-V subgroups. These primers did not match any Stolbur or "*Ca*. P. asteris" related sequences, therefore preventing aspecific amplification from two phytoplasmas commonly infecting grapevine. The FdSecyFw/FdSecyRv amplified a phytoplasma-specific amplicon of the expected size only from FD-C and FD-D infected periwinkle DNA, and gel electrophoresis confirmed the absence of nonspecific products (not shown). Melting curve analysis of the amplicon obtained with FdSecyFw/FdSecyRv showed a unique melting peak at 76.5°C (Fig. 1, Panel ~~B~~C). The Vitis18SF1/Vitis18SR1 primers amplified a grapevine-specific amplicon of the expected size (152

bp) and gel electrophoresis confirmed the absence of nonspecific products (not shown). Melting curve analysis of the amplicon showed a unique melting peak at 85.5°C (Fig. 1, Panel D). Serial dilutions of FDP plasmid DNA ranging from ~~2~~1.~~13~~17 x ~~10~~^{7}-10^{8} to ~~2~~1.~~13~~17 x 10^{3} copies of plasmid were tested in triplicate and the CT values were plotted against the copy number. Serial dilution of healthy grapevine DNA ranging from 50 ng to 0.05 ng were also tested in triplicate and the CT values were plotted against the DNA concentration. The linear correlation coefficient (*r*) between the CT and the logarithm of the DNA copy number was repeatedly greater than 0.995 for the amplification of both targets (Fig. 1, panels A and C). PCR efficiencies of both curves were constantly around 108% and 103% (plant and phytoplasma, respectively).

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Establishment of a correct sampling procedure

Results of this preliminary experiment are presented in Table 4. FD phytoplasma was detected by nested PCR in all samples from Barbera and Nebbiolo symptomatic shoots, confirming the infected status of the analyzed plants. FD phytoplasma was also detected in three samples from asymptomatic shoots of two Barbera plants, while it was never detected in asymptomatic shoots of infected Nebbiolo plants. FD phytoplasma titre was measured in all samples from symptomatic shoots of Barbera and in two nested PCR positive sample from the two symptomatic shoots of one Nebbiolo plant. FD phytoplasma titre in symptomatic shoots of the five Barbera plants ranged from about 39 to 1180 cells/ng grapevine DNA, and it was above the quantification threshold in one of the three nested PCR positive samples from asymptomatic Barbera shoots. In Barbera, the variance among FDP titre within shoots of each plant was lower than that among plants (Table 5) , therefore, collection of leaves from one symptomatic shoot per plant was set as sampling protocol for successive quantification experiments.

Flavescence dorée phytoplasma concentration in Barbera and Nebbiolo grapevines

Despite the low number of FD-D phytoplasma infected plants over the two years, tests indicated that no significant differences were recorded in the concentration of FD-C and -D phytoplasmas within each sampling of the different cvs (Table 6). Further tests also showed that the presence of BNP had no effect on FDP concentration (Table 7). FDP concentration in Barbera grapevines sampled in spring, early and late summer of 2008 and 2009 are illustrated in Fig. 2. Mean FDP concentration in Barbera samples was 242 cells/ng grapevine DNA in spring 2008, and 2 cells/ng grapevine DNA in spring 2009. In early summer samplings, FDP titre was 8,856 and 8,735 cells/ng grapevine DNA in 2008 and in 2009, respectively; in late summer, FDP concentration was 6,420 cells/ng grapevine DNA in 2008 and 671 in 2009. Due to significant differences in the variance, FDP titres in spring and early summer samplings were analyzed separately from those of late summer. Significant differences were recorded in FDP titre between sampling seasons and years. FDP concentration was consistently higher in 2008 than in 2009 (3.299 vs 2.456 DLog; N1 = 31; N2 = 10; S.E.D. = 0.2799; least significant difference at 0.05 level = 0.5672), and the difference was mainly due to phytoplasma titre in spring, since differences between FDP titre in 2008 and 2009 early summers were not significant (least significant difference at 0.05 level between two years: spring = 0.0618; early summer = 0.6108). FDP concentration was also significantly higher in 2008 late summer sampling compared to 2009 (P= 0.072). A seasonal trend in FDP concentration (low in spring, high in early summer and intermediate in late summer) was conserved in Barbera vines sampled in both years (Fig. 2).

FDP titre in Nebbiolo vines (Fig. 2) was significantly different according to year (2008 and 2009) and sampling season (spring, early and late summer), while it was similar in both vineyards (Table 8). Mean FDP titre in Nebbiolo vines was 134 cells/ng grapevine DNA in spring samples, 1,417 in early summer samples and 489 in late summer samples of 2008. In 2009, mean FDP titre in spring samples was 2 cells/ng grapevine DNA, in early summer 545 cells/ng grapevine DNA and in late summer samples 334. All data were then pooled to analyze differences between years and sampling seasons. Overall, in 2008, FDP in Nebbiolo was more abundant than in 2009 (2.837 vs 2.310 DLog;

N1 = 32; N2 = 26; S.E.D. = 0.1819; least significant difference at 0.05 level = 0.3651), and the difference was mainly due to the concentration of the spring samples, since differences between the remaining seasons were not significant (least significant different at 0.05 level between single seasonal samplings of the two years: spring = 1.0078; early summer = 0.5229; late summer = 0.5909). There was no difference in the FDP titre of early and late summer samples of the two years (least significant difference at 0.05 level between early and late summer 2008 = 0.5345 and between early and late summer 2009 = 0.5805), but a seasonal trend in FDP concentration (low in spring, high in early summer and intermediate in late summer) was conserved for Nebbiolo in both years and vineyards (Fig. 2).

FDP concentration was always higher in Barbera than in Nebbiolo infected vines, and this difference was significant at early and late summer samplings of 2008 (early summer: P=0.008; late summer: P=0.002) and at early summer sampling of 2009 ($P < 0.001$) (Fig. 2).

Flavescence dorée phytoplasma concentration and symptom severity

Considering both Barbera and Nebbiolo and both years from both vineyards, the spring samples showed a significant, positive correlation between FDP concentration and symptoms severity (~~r=0.412; S.E.=0.367; N=25; DF=23; t=1.89; *P*= 0.071~~Spearman's correlation coefficient r=0.412; adjusted for ties = 0.367; N=25; DF=23; t=1.89; P= 0.071), and so did the Barbera samples of 2008 (Spearman's correlation coefficient r=0.389; N=25; DF=23; t approximation 1.72 ; P= 0.099), but not in 2009~~, pooling both early and late summer samplings (r=0.389; S.E.=0.377; N=25; DF=23; t=1.72; *P*= 0.099)~~, and the Nebbiolo samples of 2009 (Spearman's correlation coefficient r=0.430; adjusted for ties r = 0.373; N=22; DF=20; t=1.98; P=0.087), but not in 2008~~, pooling both early and late summer samplings (r=0.448; S.E.=0.397; N=23; DF=21; t=1.98; *P*=0.061)~~. Early and late summer samples were analyzed together for correlations between FD concentration (as Log[FD]) and symptom severity as the two samplings were evaluated on the same severity scale. No other significant correlation was detected.

Overall, there was no highly significant correlation between FDP concentration and symptoms severity.

Discussion

Flavescence dorée infection in 2008 and 2009 in the two vineyards of Cocconato and Monteu Roero was caused mainly by Flavescence dorée type C phytoplasma (FD-C). This isolate is stably present in the area since the early outbreak of the epidemics in 1998, and it is the most represented one in FD-infected vines of Piemonte region (Marzachi et al., 2001). FD-D type phytoplasmas were detected sporadically in infected Barbera and Nebbiolo grapevines collected in spring, early and late summer of both years. Grapevines showing early symptoms were mostly infected with FD-C phytoplasma, as reported in other grapevine-growing areas (Angelini et al., 2006), and this isolate was detected throughout the vegetative season. Bois noir disease is also present in Piemonte vineyards (Marzachi et al., 2001), and BNP was found only in mixed infections with FDP in samples from the two vineyards. As we only sampled symptomatic grapevines in the two highly FD-infected locations, the presence of BNP in asymptomatic plants cannot be ruled out. ~~The FDP type (C or D) had no effect on symptom severity of the infected vines of both cultivars. Also, the presence of BNP in mixed infection, a rare occurrence, had no effect on symptom expression. Under these experimental conditions, a competition effect between BNP and FDP cannot be ruled out .~~

For two consecutive years, infected Barbera vines showed more severe spring symptoms than Nebbiolo ones. This observation coincides with the fact that Barbera is known as a highly FD-sensitive cv. Both cvs showed more severe symptoms in 2009 than in 2008, and in 2009 lower winter and spring mean temperatures and more freezing days were recorded at both localities than in 2008. Low spring temperatures have an immediate effect on grapevine in delaying bud burst

(Tomasi et al., 2007), but they also have a long term effect in reducing fruit set (%) and berry number per bunch in a cv-specific manner (Ebadi et al., 1995). Temperature stresses also affect $Ca2^{+}$ homeostasis in grapevine (Wang et al., 2004). Also, low temperatures reduce respiration rate in grapevine (Franck et al., 2011), thus reducing the production of H_2O_2 (Foyer et al., 2009), a molecule probably involved in defense response pathways to phytoplasmas (Musetti et al., 2007). We can speculate that more severe FD symptoms in both cvs in early and late summer assessment of 2009 may be an effect of a long-lasting stress of the plant caused by the occurrence of low winter/spring temperatures in that year. Moreover, in 2008, temperature probes recorded lower winter/spring mean temperatures and more freezing days in the Monteu Roero vineyard compared to Cocconato, and significantly more severe early summer symptoms were recorded in Nebbiolo infected vines in Monteu Roero than in Cocconato.

Quantification of FDP-DNA was obtained as the ratio of phytoplasma DNA per ng of grapevine DNA as previously described for "*Ca*. P. asteris" (Marzachi & Bosco, 2005). Real time based PCR assays for diagnosis and characterization of several phytoplasmas, including FDP, have been proposed (Angelini et al., 2007, Bianco et al., 2004, Galetto et al., 2005, Hren et al., 2007, Mehle et al., 2013, Pelletier et al., 2009), but this is one of the first attempts, together with the one of Prezelj and co-workers (2012), to quantify FDP in field-infected vines of different cvs. A double absolute quantification assay was developed and only samples with a threshold cycle value within that of the lowest standard concentration were considered. The quantification of less than 3 phytoplasma cells per ng plant DNA in three spring samples can still be considered reliable according to the MIQE guidelines (Bustin et al., 2009) as it exceeds by three times the most sensitive limit of detection. It is known that phytoplasmas have uneven distribution in the grapevine canopy, but a direct correlation between the presence of phytoplasma specific symptoms and FDP detection was clearly shown as all samples collected from symptomatic shoots of both cvs were positive for the presence of FDP, while all samples from asymptomatic Nebbiolo and most from asymptomatic Barbera were nested PCR negative. Similar results are reported for other grapevine varieties (Prezelj et al., 2012)

where FDP was mainly associated with symptomatic tissues, although it was also detected in some symptomless tissues of highly infected (and symptomatic) plants. Also, the differences between FDP titre in different symptomatic shoots from the same Barbera plant were lower than the differences among plants, therefore confirming that sampling from one symptomatic shoot can provide a reliable and representative evaluation of FDP titre in the infected parts of the plant. ~~We can suggest that FD-C and -D phytoplasmas multiply at the same level in grapevine, and that the presence of BNP has no influence on the accumulation of FDP in doubly infected plants. Very few quantitative information on plants co-infected by two phytoplasmas are available, however, mild and severe isolates of "*Ca.* P. asteris" show different multiplication rate and movement in the herbaceous host (Sinha, 1983) and, in the case of Ash yellows phytoplasma, strains that differ in aggressiveness have a different multiplication and/or movement following co-inoculation to the laboratory host periwinkle (Sinclair & Griffiths, 2000). In Apple proliferation (AP) infected apple trees, the ratio of mild to severe strains in the phytoplasma population determines the virulence of the infection and shifts in the population composition may alter virulence of multiple-strain accession (Seemueller et al., 2011).~~

In both cvs and in both years, the lowest FDP titre was observed in spring, the highest in early summer and an intermediate one at the end of the vegetative season. A continuous increase of FDP titre up to the end of the vegetative season was reported for other grapevine cvs in Slovenia (Prezelj et al., 2012). Seasonal fluctuations of phytoplasma populations in AP-infected apple trees have been measured with highest levels occurring from December to May (Baric et al., 2011). Despite the occurrence of more severe symptoms throughout the vegetative season of 2009 compared to 2008, at each sampling date, FDP titre was significantly higher in 2008 than in 2009, irrespective of the cv. This difference was most evident in the spring samplings, when as many as hundreds FDP cells were recorded in 2008 in contrast to only as few as 2 FDP cell per ng plant DNA in 2009. Since the lowest FDP titre was consistently measured in both cvs, both years and at both sites in spring, winter temperature together with plant dormancy and pruning, that eliminates most of the infected

plant parts, have a cooperative effect in reducing phytoplasma titre in the plant. Multiplication of pathogen resumed with the vegetative season and the differences in FDP titres between the two years were reduced, although FDP-specific symptom expression was more severe in 2009 than in 2008. We can speculate that colder winter/early spring climatic conditions have a long-lasting stress effect on the grapevines, increasing both their sensitivity to FD and symptom severity even in the presence of a low pathogen load. Also, the hypothesis that the pathogen may multiply less efficiently in the stressed plant, therefore reaching a low titre despite the severe symptoms, cannot be ruled out.

FDP titre in Barbera grapevines was always higher than in Nebbiolo. This is in good correlation with more severe symptoms expressed by FD-infected Barbera vines compared to Nebbiolo ones and provides the most likely explanation for the milder symptoms shown by FDP-infected Nebbiolo. Actually this latter cv sustains a low multiplication of the pathogen. Proteomic and transcriptomic analyses of FDP-infected Nebbiolo plants show that most proteins modulated during infection belong to the ‘‘cell rescue, defense and virulence’’ class (Margaria & Palmano, 2011a). On the other hand, preliminary studies on Barbera cv, show a lower presence of proteins of the ‘defense’ category compared to the total identified proteins (Margaria & Palmano, 2011b). Perhaps different responses of the two cvs to FDP may account for the different multiplication level of the pathogen, but this hypothesis remains untested.

The results indicate that the best time for sampling and detection of FDP infection is early summer, when phytoplasma titre is highest. However, our work, together with the one of Prezelj and co-workers (2012) provide evidence that FDP can be detected as early as at flowering season, or even before in the case of Barbera in our work. Quantitative real time PCR is a useful tool for the evaluation of phytoplasma multiplication and can be used when screening for resistance/tolerance. We have demonstrated that Barbera supports a higher FDP multiplication compared to Nebbiolo, and further studies are required to understand whether this has an effect on vector acquisition and

transmission efficiency, as already suggested for Pinot blanc and Merlot cultivars (Bressan et al., 2005).

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Captions for figures

Figure 1. A: Standard curve of Flavescence dorée phytoplasma DNA obtained by plotting threshold cycles (CT) values vs log of 2 x 10^7, 2 x 10^5, 2 x 10^3, and 2 x 10 copy number of p26SecYFD plasmid. ~~B~~C: Melting curve analysis of the amplicon obtained with primers FdSecyFw/FdSecyRv. ~~C~~B: Standard curve of plant DNA (*Vitis vinifera*) obtained by plotting threshold cycles (CT) values

633 vs log of 50, 5, 0.5, and 0.05 ng of healthy grapevine DNA. D: Melting curve analysis of the
634 amplicon obtained with primers Vitis18SF1/Vitis18SR1.
635
636 Figure 2. Mean Flavescence dorèe phytoplasma concentration (cells / ng grapevine DNA) and
637 standard error in Barbera and Nebbiolo plants sampled in spring, early and late summer surveys of
638 2008 and 2009 in Cocconato (C) and Monteu Roero (MR) vineyards.
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Table 1. Number of samples from Barbera and Nebbiolo grapevines showing phytoplasma-specific symptoms of different categories collected during spring, early and late summer surveys of 2008 and 2009. In spring, symptom severity of infected grapes was rated on a scale of 1 to 3 according to the percentage of symptomatic shoots on the fruit bearing branch (1: maximum 30%; 2: more than 30 % and up to 65%; 3: more than 65% and up to 100%). In early and late summer surveys, symptom severity of infected grapes was rated as A, B, C or D, according to the proportion of the plant canopy showing phytoplasma-specific symptoms (A: plants lacking FD-specific symptoms, but not looking healthy; B: up to 30 % FD-specific symptoms; C: above 30 % and up to 60 %; D: above 60 %).

Year	Cultivar	Spring			Early summer				Late summer			
		1	2	3	A	B	C	D	A	B	C	D
2008	Barbera	1	9	5	2	9	5	0	2	4	4	0
	Total plants	15			16				10			
2009	Barbera	2	1	0	0	1	2	5	0	1	1	5
	Total plants	3			8				7			
2008	Nebbiolo	2	1	2	4	6	5	0	3	6	5	0
	Total plants	5			15				14			
2009	Nebbiolo	2	1	0	0	2	4	7	0	2	3	5
	Total plants	3			13				10			

Table 2. Summary of significant to highly significant comparisons between FD symptom severities in Barbera (B) and Nebbiolo (N) grapevines in 2008 and 2009 at Cocconato (C) and Monteu Roero: (MR).

Season	Vineyard	Cv	Year/s	Comparison	N[a]	U[b]	*P*[c]
Spring[d]	C		2008+2009	B > N	17, 3	3.0	0.991
		B		2009 > 2008	15, 8	14.5	0.999
Early summer[e]	C	N		2009 > 2008	4, 4	0.0	0.986
			2008	B > N	15, 4	2.0	0.999
	MR	N		2009 > 2008	11, 9	23.5	0.975
		N	2008	MR > C	4, 11	0.0	0.999
Late summer[f]	C	B		2009 > 2008	9, 7	7.5	0.996
	MR	N		2009 > 2008	8, 7	9.0	0.990
	C	N	2008	Es[e] < Ls[f]	4, 4	0.0	0.986

[a] N: sample numbers; [b] U: value of U in the Mann-Whitney U test; [c] P: the exact probability (adjusted for ties) that one group is equal to, greater or smaller than the other group (according to the sign =, >, < specified in the table)~~;~~ [d] ~~Sp: spring;~~ [e] ~~Es: early summer;~~ [f] ~~Ls: late summer~~.

~~;~~ [d] ~~Sp: spring;~~ [e] ~~Es: early summer;~~ [f] ~~Ls: late summer.~~

Table 3. Number of Barbera (B) and Nebbiolo (N) grapevines PCR positive for the presence of Flavescence dorée phytoplasma (FDP) as either single or mixed infection with Bois Noir phytoplasma (BNP+FDP) among the total samples collected from vineyards in Cocconato and Monteu Roero during spring (Sp), early (Es) and late summer (Ls) growing seasons of 2008 and 2009.

Year	Vineyard	Cv	FDP (Positive/ total sampled)			BNP+FDP (Positive/total sampled)		
			Sp	Es	Ls	Sp	Es	Ls
2008	Cocconato	B	14/15	15/16	8/10	1/15	1/16	2/10
		N	0	3/4	2/4	1/1	1/4	2/4
	Monteu Roero	N	1/4	7/11	9/10	3/4	4/11	1/10
2009	Cocconato	B	3/3	8/8	6/7	0	0/8	1/7
		N	2/2	4/4	3/3	0	0	0
	Monteu Roero	N	1/1	8/9	6/7	0	1/9	1/7

Table 4. Number of symptomatic and asymptomatic shoots from the five Barbera and five Nebbiolo FD-infected grapevines from which samples were collected for successive diagnostic direct (P1/P7 primers) and nested (R16(V)F1/R1 primers) PCRs and FDP quantification. Only samples with a threshold cycle value within that of the lowest standard concentration were considered for FDP quantification. Titre of FDP is expressed as phytoplasma cells/ng plant DNA. SE: standard error.

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Cultivar	Symptoms	Shoots (n)	Direct PCR (positive / tested)	Nested PCR (positive / tested)	qPCR (positive / tested)	FDP titer (mean)	SE
Barbera	Symptomatic	11	9/11	11/11	11/11	345.1	103.94
	Asymptomatic	9	1/9	3/9	1/9	9.6	na
Nebbiolo	Symptomatic	7	1/7	7/7	2/7	45.3	5.39
	Asymptomatic	13	0/13	0/13	0/13	na	na

Table 5. Mean FDP titer (expressed as the difference between the logarithm of the number of FDP cells and the logarithm of the ng of grapevine DNA, DLog) in symptomatic shoots of the five Barbera plants, variance (Var) among FDP titres within shoots of each plant and among plants (General, among plants).

Plant	Mean FDP titer in shoots (DLog)	Var
1	2.178167	0.07131606
2	2.1135	0.0772245
3	2.850333	0.09827222
4	2.134833	0.0981245
5	2.7865	0.00098272
General, among plants	2.412667	0.16141599

Table 6. Analysis of variance (ANOVA) for grouping FD-C and FD-D phytoplasma titres of Barbera (B) and Nebbiolo (N) plants sampled at different times in the two vineyards. C, MR: Cocconato and Monteu Roero vineyards, respectively. Es, Ls: early and late summer samplings. Log[FD-C], LOG[FD-D]: Log of FD-C or -D phytoplasma concentration. N_C, N_D: number of samples infected by FD-C or FD-D phytoplasmas. *F* pr: F probability. LSD: least significant difference. N.S.: not significant.

Cv	Vineyard	Year	Season	LOG[FD-C] (mean)	N_C	LOG[FD-D] (mean)	N_D	*F* pr	LSD	Comment
B	C	2008	Es	4.0242	14	3.640	1	0.436	1.034	N.S.
			Ls	3.8911	7	3.785	1	0.714	0.6746	N. S.
N	MR	2008	Es	3.2713	4	2.7967	3	0.206	0.8409	N.S.
		2009	Es	2.7845	6	3.5642	2	0.315	1.740	N.S.
			Ls	3.0948	5	2.551	1	0.397	1.594	N.S.

Table 7. Analysis of variance (ANOVA) for grouping FDP tires in FD singly infected and FD and BN doubly infected Barbera (B) and Nebbiolo (N) plants sampled at different times in the two vineyards. C, MR: Cocconato and Monteu Roero vineyards, respectively. $Log[FD]_{FD}$, $Log[FD]_{FD+BN}$: Log of FD phytoplasma concentration. N_{FD}, N_{FD+BN}: number of samples infected only by FD or by FD and BN in mixed infection. Sp, Es, Ls: spring, early and late summer samplings. F pr: *F* probability. LSD: least significant difference. N.S.: not significant.

Cv	Vineyard	Year	Season	$LOG[FD]_{FD}$ (mean)	N_{FD}	$LOG[FD]_{FD+BN}$ (mean)	N_{FD+BN}	F pr	LSD	Comment
B	C	2008	Sp	2.384	14	2.370	1	0.989	2.204	N.S.
			Es	4.0242	14	3.1773	1	0.100	1.034	N.S.
		2009	Ls	2.935	6	2.179	1	0.603	3.498	N.S.
N	C+MR[a]	2008+2009	Sp	0.820	4	2.366	2	0.165	2.531	N.S.
	C+MR[b]	2008	Es	3.2644	7	3.3878	4	0.651	0.5955	N.S.

[a], [b]: according to corresponding symbol in Table 8.

Table 8. Analysis of variance (ANOVA) for grouping FDP titres from infected Nebbiolo plants collected in the two vineyards. Plants infected with FDP (FD-C) of FDP and BNP (FD-C+BN) in double infection were analysed separately. Sp, Es: spring and early summer samplings. $LOG[FD]_{CC}$, $LOG[FD\text{-}D]_{MR}$: Log of FDP titre in plants collected at Cocconato or Monteu Roero vineyards, respectively. F pr: *F* probability. LSD: least significant difference. N.S.: not significant.

Infection	Year	Season	$LOG[FD]_{CC}$ (mean)	N_{CC}	$LOG[FD]_{MR}$ (mean)	N_{MR}	F pr	LSD	Comment
FD-C	2008+2009[a]	Sp	0.349	2	1.291	2	0.549	5.681	N.S.
	2008[b]	Es	3.255	3	3.271	4	0.962	0.826	N.S.
FD-C+BN	2008[b]	Es	2.942	1	3.536	3	0.386	2.324	N.S.

[a], [b]: Results allowing groupings as reported in Table 7 at corresponding symbols

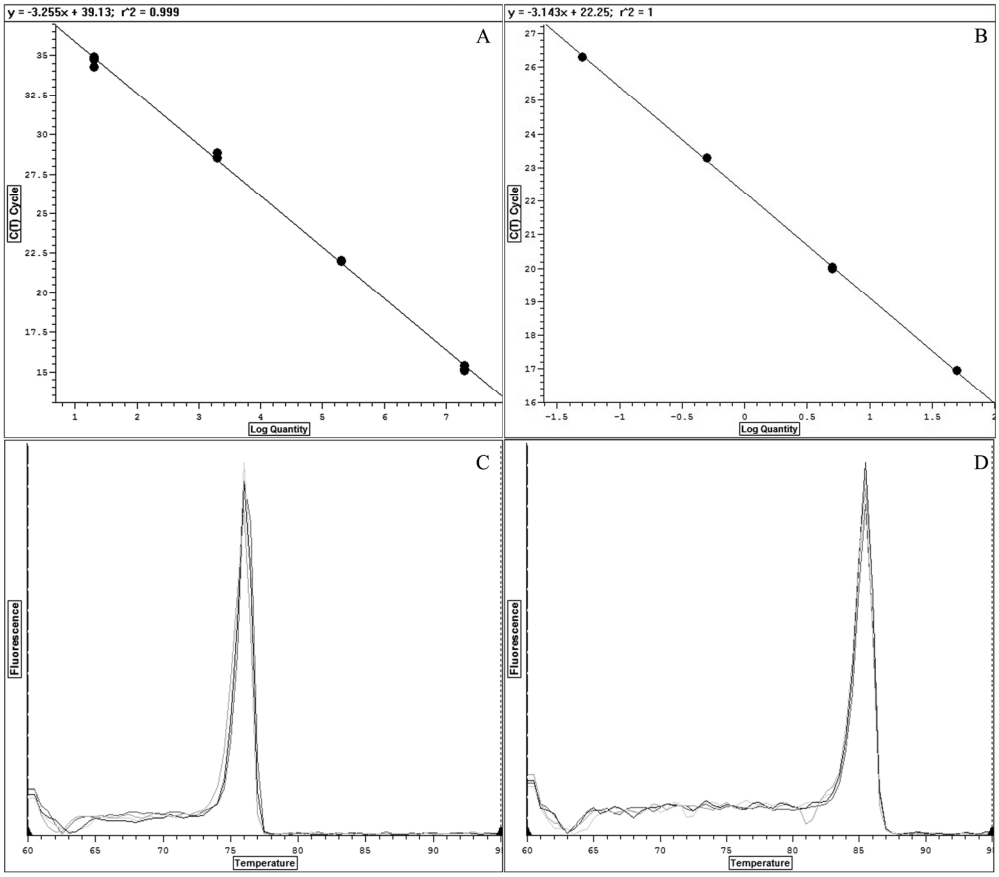
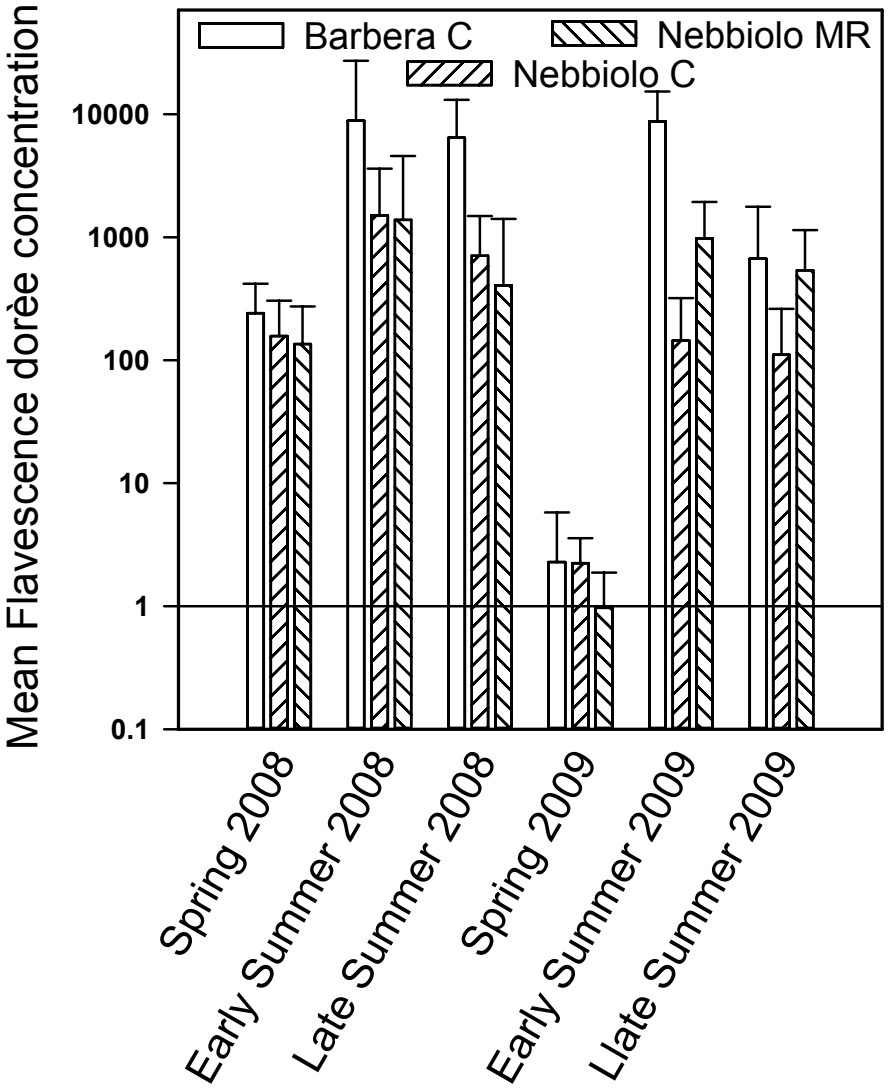


Figure 1
205x181mm (300 x 300 DPI)


Barbera C
Nebbiolo MR
Nebbiolo C
Mean Flavescence dorèe concentration
10000
1000
100
10
1
0.1
Spring 2008
Early Summer 2008
Late Summer 2008
Spring 2009
Early Summer 2009
Llate Summer 2009