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

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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1 FDP-titre in grapevines with different susceptibility to GYs

2

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

4

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12

13 **Keywords:** grapevine yellows, FD, susceptibility, Vitis vinifera, phytoplasma multiplication, real

14 time PCR quantification

15

16

17 **Abstract**

18

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

34

35 **Introduction**

36

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

41 sequence into more than 30 groups and within the '*Candidatus* Phytoplasma' genus (Firrao et al.,  
42 2004), (Zhao et al., 2010).

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

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

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

67 with growth reduction at the beginning of the vegetative season, while symptoms on Nebbiolo vines  
68 are milder, especially at the beginning of the vegetative season (Morone et al., 2001).  
69 In this study we describe a new approach to real time quantification of FD phytoplasma in  
70 grapevine which was used to determine a) relationships between cultivar susceptibility and  
71 pathogen concentration in the plant, b) relationships between pathogen presence/concentration and  
72 symptom severity, c) FD phytoplasma titre over the vegetative season in order to identify the best  
73 timing for sampling and detection.

74

75

## 76 **Materials and methods**

77

### 78 *Vineyard description*

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

88

### 89 *Establishment of a correct sampling procedure*

90 To establish a correct sampling procedure, five FD-infected Barbera and Nebbiolo plants were  
91 selected in the Cocconato vineyard at the growth stage 17 – 19 (12 to 16 leaves separated; Coombe,  
92 1995). Symptomatic and asymptomatic shoots were separately labeled on each plant and samples

4



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

97

#### 98 *Grapevine samples collection*

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

118

119 *Total DNA extraction and phytoplasma diagnosis*

120 Total DNA was extracted from 1.5 g of leaf veins following a phytoplasma enrichment protocol  
121 (Marzachi et al., 1999) and dissolved in 100 µl of sterile double distilled water (SDW). DNA  
122 concentration of extracts was quantified with a NanoDrop (ND-1000 Spectrophotometer, NanoDrop  
123 Technologies, Inc., Wilmington, DE, USA). Polymerase chain reaction (PCR) was employed for  
124 phytoplasma detection. Universal primer pair P1/P7 was used in direct PCRs as previously  
125 described (Deng & Hiruki, 1991; Schneider et al., 1995). Reaction products were diluted 1:40 in  
126 SDW and used as templates in nested reactions driven by primers R16(I)F1/R1 or R16(V)F1/R1 for  
127 the specific detection of 16S groups I and V, respectively (Lee et al., 1994). Amplicons were  
128 separated by electrophoresis in 1% agarose gels buffered in 0.5× TBE (90 mM Tris-borate, 2 mM  
129 EDTA) and visualized under UV-light after staining with ethidium bromide. To identify Bois Noir,  
130 a group 16SrXII (Stolbur group) phytoplasma infection, two µl aliquots of fragments amplified with  
131 R16(I)F1/R1 primers were digested for 2 h with 1U of endonuclease *MseI* (Invitrogen, Carlsbad,  
132 CA) at 37°C, according to the manufacturer's recommendations. Digestion products were separated  
133 in 5% polyacrylamide gels buffered in 1× TBE along with a 1 kb plus DNA size marker (Gibco  
134 BRL, Paisley, UK) and visualized by UV-light after staining with ethidium bromide. A Piemonte  
135 strain of FDP (FD-C), acquired from grapevine by *Scaphoideus titanus* and transmitted to *Vicia*  
136 *faba* L., a French strain of FDP (FD-D), kindly provided by Dr. E. Boudon-Padieu and graft-  
137 maintained in periwinkle in the collection of the Istituto di Virologia Vegetale, CNR, and a  
138 Sardinian strain of Stolbur phytoplasma from tomato (T2\_92), also maintained in periwinkle  
139 (Minucci & Boccardo, 1997), were used as reference isolates and positive controls in PCR and  
140 RFLP experiments. A healthy Barbera grapevine from *in vitro* propagation, maintained in an insect-  
141 proof greenhouse, was employed as negative control in PCR experiments.

142

143 *Characterization of Flavescence dorée phytoplasma isolates*

144 Characterization of Flavescence dorée isolates was done by restriction analysis of the partial 16S-  
145 23S rDNA operon and *secY* gene. In the first case, fragments amplified with P1/P7 primer pair  
146 (Deng & Hiruki, 1991; Schneider et al., 1995) were diluted 1:40 with SDW, and re-amplified with  
147 primers M1/B6 as previously described (Martini et al., 1999). For the partial amplification of the  
148 *secY* gene, total grapevine DNAs were amplified in direct PCR with primers FDf2/ FD9r (Angelini  
149 et al., 2001), and then, following 1:40 dilution with SDW, amplified with primers FD9f3/FD9r2  
150 (Martini et al., 2002). Amplicons were digested for 2 h with one unit of *TaqI* restriction  
151 endonuclease (Invitrogen) at 65°C. Digestion products were separated in 5% polyacrylamide gels in  
152 TBE along with a 1 kb plus DNA size marker (Gibco BRL, Paisley, UK) and visualized as detailed  
153 above.

154

155 | *Quantification of Flavescence dorée phytoplasma DNA*

156 FD phytoplasma titre in infected grapevines was measured as number of FD phytoplasma cells per  
157 ng of grapevine DNA. To determine the number of FD phytoplasma cells, specific primers,  
158 targeting a portion of the *secY* gene of FD phytoplasma were designed using Primer3 software  
159 (Rozen & Skaletsky, 2000), and used in an absolute quantification real-time PCR-based assay. For  
160 the quantification of grapevine DNA, specific primers, targeting the 18S rDNA gene of grapevine  
161 were designed. The chosen primers were subjected to BLASTN analysis on GenBank through the  
162 NCBI database to test their specificity. To confirm the exclusive presence of the expected specific  
163 amplicon, 5 µL of real time PCR products were analyzed by electrophoresis in 1.5% agarose gel  
164 buffered in TBE. Standard curves for the absolute quantification of FD phytoplasma and grapevine  
165 DNA were obtained by dilution of plasmid p26SecYFD, containing the appropriate *secY* gene  
166 target sequence from FD-C phytoplasma, and of total DNA extracted from healthy grapevine grown  
167 from seed, respectively. Three quantities of p26SecYFD (0.5 ng, 5 pg, and 5 fg), were used,  
168 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  
169 grapevine DNA, three quantities of total healthy grapevine DNA (5 ng, 0.05 ng, and 0.005 ng) were

7

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

182

### 183 *Data analyses*

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

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

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

193

## 194 **Results**

195

196 *Symptom severity of infected grapevines and temperature parameters of the vineyards*

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

200 Overall, 59 Barbera samples were collected in the two surveys of 2008 (n = 41) and 2009 (n = 18).

201 [Details on the number of plants showing symptoms of the different categories in the two years are](#)  
202 [presented in Table 1. During the spring surveys of the two years, 18 samples were collected, 15 in](#)  
203 [2008 and three in 2009. During the early summer surveys, 24 samples were collected from](#)  
204 [symptomatic Barbera vines, 16 in 2008, and eight in 2009. For early summer samplings, in 2008](#)  
205 most plants showed class B and C symptoms, and in 2009 most of them showed class D symptoms  
206 (Table 1). [During the late summer surveys, 17 samples were collected from symptomatic Barbera](#)  
207 [vines, ten in 2008, and seven in 2009. For late summer samplings, in 2008, most of the plants](#)  
208 showed class B and C symptoms, and in 2009 most of them showed class D symptoms (Table 1).

209 Overall, 60 samples were collected from Nebbiolo grapevines. [As for the Barbera plants, details on](#)  
210 [the number of Nebbiolo vines showing symptoms of the different categories in the two years are](#)  
211 [presented in Table 1. During the spring surveys of the two years, 8 samples were collected, five in](#)  
212 [2008 and three in 2009. During the early summer surveys of the two years, 28 samples were](#)  
213 [collected, 15 in 2008 \(11 from Monteu Roero and 4 from Coconato, respectively\), and 13 in 2009](#)  
214 [\(9 from Monteu Roero and 4 from Coconato\). For early summer surveys, in 2008, none of the](#)  
215 infected plants showed symptoms of class D, and in 2009 none of the plants showed symptoms of  
216 class A (Table 1). [During the late summer surveys of the two years, 24 samples were collected from](#)  
217 [symptomatic vines, 14 in 2008 \(10 from Monteu Roero and 4 from Coconato\), and 10 in 2009 \(7](#)  
218 [from Monteu Roero and 3 from Coconato\). For late summer surveys, in 2008 most of the plants](#)  
219 showed symptoms of classes B and C, and in 2009 about 50 % of the infected grapevines showed  
220 symptoms of class D (Table 1).

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

231 In the Monteu Roero vineyard, mean winter (November to March) and spring (April and May)  
232 temperatures in 2008 were 4.8°C and 16.1°C, and in 2009 they were 4.1°C (winter) and 14.7°C  
233 (spring), with 63 (2008) and 79 (2009) freezing days during the winter time. In the Cocconato  
234 vineyard, mean winter and spring temperatures in 2008 were 6.7°C and 17.1°C, and in 2009 they  
235 were 5.6°C (winter) and 15.5°C (spring), with 41 and 49 freezing days recorded in 2008 and in  
236 2009, respectively.

237  
238 *Phytoplasma detection and Flavescence dorée strain characterization*

239 Table 3 reports the number of Barbera and Nebbiolo grapevines positive for the presence of FD and  
240 BN phytoplasmas alone or as mixed infection among the total samples collected in the surveys of  
241 2008 and 2009. In spring surveys of 2008, almost all symptomatic Barbera samples were singly  
242 infected with FDP, and most Nebbiolo plants were doubly infected with FDP and BNP. In spring  
243 2009, all symptomatic plants were singly infected with FDP, irrespective of cv identity and  
244 vineyard. In early summer surveys of 2008 and 2009, most symptomatic Barbera and Nebbiolo  
245 samples were infected with FDP only, although BNP and FDP-doubly infected grapes were found  
246 in both cvs and vineyards. In 2008 and 2009 late summer surveys, most symptomatic Barbera and

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

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

258

#### 259 *Quantification of Flavescence dorée phytoplasma*

260 The following primers were designed for the absolute quantification of FDP and grapevine DNA:

261 FdSecyFw/FdSecyRv (5' TGCCTTATGTTACTGCTTCT 3' / 5' TAATAATGATGGGGATTCAA  
262 3'), and Vitis18SF1/Vitis18SR1 (5' ATGATTAACAGGGACAGTCG3' /

263 5' GGTATCTGATCGTCTTCGAG3'). BLAST analysis of primers FdSecyFw/FdSecyRv, flanking  
264 a 185 bp region of the FD-70 isolate *secY* gene (accession number: AM238512), showed their  
265 specificity for the target gene of several "*Ca. P. vitis*" isolates and phytoplasmas belonging to other  
266 16Sr-V subgroups. These primers did not match any Stolbur or "*Ca. P. asteris*" related sequences,  
267 therefore preventing aspecific amplification from two phytoplasmas commonly infecting grapevine.

268 The FdSecyFw/FdSecyRv amplified a phytoplasma-specific amplicon of the expected size only  
269 from FD-C and FD-D infected periwinkle DNA, and gel electrophoresis confirmed the absence of  
270 nonspecific products (not shown). Melting curve analysis of the amplicon obtained with

271 FdSecyFw/FdSecyRv showed a unique melting peak at 76.5°C (Fig. 1, Panel **BC**). The

272 Vitis18SF1/Vitis18SR1 primers amplified a grapevine-specific amplicon of the expected size (152

273 bp) and gel electrophoresis confirmed the absence of nonspecific products (not shown). Melting  
274 curve analysis of the amplicon showed a unique melting peak at 85.5°C (Fig. 1, Panel D). Serial  
275 dilutions of FDP plasmid DNA ranging from  $2.1 \times 10^7$  to  $2.1 \times 10^3$  copies of plasmid  
276 were tested in triplicate and the CT values were plotted against the copy number. Serial dilution of  
277 healthy grapevine DNA ranging from 50 ng to 0.05 ng were also tested in triplicate and the CT  
278 values were plotted against the DNA concentration. The linear correlation coefficient ( $r$ ) between  
279 the CT and the logarithm of the DNA copy number was repeatedly greater than 0.995 for the  
280 amplification of both targets (Fig. 1, panels A and C). PCR efficiencies of both curves were  
281 constantly around 108% and 103% (plant and phytoplasma, respectively).

282

#### 283 *Establishment of a correct sampling procedure*

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

296

#### 297 *Flavescence dorée phytoplasma concentration in Barbera and Nebbiolo grapevines*

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298 Despite the low number of FD-D phytoplasma infected plants over the two years, tests indicated  
299 that no significant differences were recorded in the concentration of FD-C and -D phytoplasmas  
300 within each sampling of the different cvs (Table 6). Further tests also showed that the presence of  
301 BNP had no effect on FDP concentration (Table 7). FDP concentration in Barbera grapevines  
302 sampled in spring, early and late summer of 2008 and 2009 are illustrated in Fig. 2. Mean FDP  
303 concentration in Barbera samples was 242 cells/ng grapevine DNA in spring 2008, and 2 cells/ng  
304 grapevine DNA in spring 2009. In early summer samplings, FDP titre was 8,856 and 8,735 cells/ng  
305 grapevine DNA in 2008 and in 2009, respectively; in late summer, FDP concentration was 6,420  
306 cells/ng grapevine DNA in 2008 and 671 in 2009. Due to significant differences in the variance,  
307 FDP titres in spring and early summer samplings were analyzed separately from those of late  
308 summer. Significant differences were recorded in FDP titre between sampling seasons and years.  
309 FDP concentration was consistently higher in 2008 than in 2009 (3.299 vs 2.456 DLog; N1 = 31;  
310 N2 = 10; S.E.D. = 0.2799; least significant difference at 0.05 level = 0.5672), and the difference  
311 was mainly due to phytoplasma titre in spring, since differences between FDP titre in 2008 and  
312 2009 early summers were not significant (least significant difference at 0.05 level between two  
313 years: spring = 0.0618; early summer = 0.6108). FDP concentration was also significantly higher in  
314 2008 late summer sampling compared to 2009 ( $P= 0.072$ ). A seasonal trend in FDP concentration  
315 (low in spring, high in early summer and intermediate in late summer) was conserved in Barbera  
316 vines sampled in both years (Fig. 2).

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

324 N1 = 32; N2 = 26; S.E.D. = 0.1819; least significant difference at 0.05 level = 0.3651), and the  
325 difference was mainly due to the concentration of the spring samples, since differences between the  
326 remaining seasons were not significant (least significant different at 0.05 level between single  
327 seasonal samplings of the two years: spring = 1.0078; early summer = 0.5229; late summer =  
328 0.5909). There was no difference in the FDP titre of early and late summer samples of the two years  
329 (least significant difference at 0.05 level between early and late summer 2008 = 0.5345 and between  
330 early and late summer 2009 = 0.5805), but a seasonal trend in FDP concentration (low in spring,  
331 high in early summer and intermediate in late summer) was conserved for Nebbiolo in both years  
332 and vineyards (Fig. 2).

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

#### 337 *Flavescence dorée phytoplasma concentration and symptom severity*

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

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

352

353

#### 354 **Discussion**

355

356 Flavescence dorée infection in 2008 and 2009 in the two vineyards of Cocconato and Monteu Roero  
357 was caused mainly by Flavescence dorée type C phytoplasma (FD-C). This isolate is stably present  
358 in the area since the early outbreak of the epidemics in 1998, and it is the most represented one in  
359 FD-infected vines of Piemonte region (Marzachi et al., 2001). FD-D type phytoplasmas were  
360 detected sporadically in infected Barbera and Nebbiolo grapevines collected in spring, early and late  
361 summer of both years. Grapevines showing early symptoms were mostly infected with FD-C  
362 phytoplasma, as reported in other grapevine-growing areas (Angelini et al., 2006), and this isolate  
363 was detected throughout the vegetative season. Bois noir disease is also present in Piemonte  
364 vineyards (Marzachi et al., 2001), and BNP was found only in mixed infections with FDP in  
365 samples from the two vineyards. As we only sampled symptomatic grapevines in the two highly  
366 FD-infected locations, the presence of BNP in asymptomatic plants cannot be ruled out.

367 ~~The FDP type ( C or D) had no effect on symptom severity of the infected vines of both cultivars.~~  
368 ~~Also, the presence of BNP in mixed infection, a rare occurrence, had no effect on symptom~~  
369 ~~expression. Under these experimental conditions, a competition effect between BNP and FDP~~  
370 ~~cannot be ruled out.~~

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

376 (Tomasi et al., 2007), but they also have a long term effect in reducing fruit set (%) and berry  
377 number per bunch in a cv-specific manner (Ebadi et al., 1995). Temperature stresses also affect  
378  $\text{Ca}^{2+}$  homeostasis in grapevine (Wang et al., 2004). Also, low temperatures reduce respiration rate  
379 in grapevine (Franck et al., 2011), thus reducing the production of  $\text{H}_2\text{O}_2$  (Foyer et al., 2009), a  
380 molecule probably involved in defense response pathways to phytoplasmas (Musetti et al., 2007).  
381 We can speculate that more severe FD symptoms in both cvs in early and late summer assessment  
382 of 2009 may be an effect of a long-lasting stress of the plant caused by the occurrence of low  
383 winter/spring temperatures in that year. Moreover, in 2008, temperature probes recorded lower  
384 winter/spring mean temperatures and more freezing days in the Monteu Roero vineyard compared  
385 to Cocconato, and significantly more severe early summer symptoms were recorded in Nebbiolo  
386 infected vines in Monteu Roero than in Cocconato.

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

402 [where FDP was mainly associated with symptomatic tissues, although it was also detected in some](#)  
403 [symptomless tissues of highly infected \(and symptomatic\) plants.](#) Also, the differences between  
404 FDP titre in different symptomatic shoots from the same Barbera plant were lower than the  
405 differences among plants, therefore confirming that sampling from one symptomatic shoot can  
406 provide a reliable and representative evaluation of FDP titre in the infected parts of the plant.

407 ~~We can suggest that FD-C and -D phytoplasmas multiply at the same level in grapevine, and that~~  
408 ~~the presence of BNP has no influence on the accumulation of FDP in doubly infected plants. Very~~  
409 ~~few quantitative information on plants co-infected by two phytoplasmas are available, however,~~  
410 ~~mild and severe isolates of “Ca. P. asteris” show different multiplication rate and movement in the~~  
411 ~~herbaceous host (Sinha, 1983) and, in the case of Ash yellows phytoplasma, strains that differ in~~  
412 ~~aggressiveness have a different multiplication and/or movement following co-inoculation to the~~  
413 ~~laboratory host periwinkle (Sinclair & Griffiths, 2000). In Apple proliferation (AP)-infected apple~~  
414 ~~trees, the ratio of mild to severe strains in the phytoplasma population determines the virulence of~~  
415 ~~the infection and shifts in the population composition may alter virulence of multiple strain~~  
416 ~~accession (Seemueller et al., 2011).~~

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

428 plant parts, have a cooperative effect in reducing phytoplasma titre in the plant. Multiplication of  
429 pathogen resumed with the vegetative season and the differences in FDP titres between the two  
430 years were reduced, although FDP-specific symptom expression was more severe in 2009 than in  
431 2008. We can speculate that colder winter/early spring climatic conditions have a long-lasting stress  
432 effect on the grapevines, increasing both their sensitivity to FD and symptom severity even in the  
433 presence of a low pathogen load. Also, the hypothesis that the pathogen may multiply less  
434 efficiently in the stressed plant, therefore reaching a low titre despite the severe symptoms, cannot  
435 be ruled out.

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

446 The results indicate that the best time for sampling and detection of FDP infection is early summer,  
447 when phytoplasma titre is highest. [However, our work, together with the one of Prezelj and co-  
448 workers \(2012\) provide evidence that FDP can be detected as early as at flowering season, or even  
449 before in the case of Barbera in our work.](#) Quantitative real time PCR is a useful tool for the  
450 evaluation of phytoplasma multiplication and can be used when screening for resistance/tolerance.

451 We have demonstrated that Barbera supports a higher FDP multiplication compared to Nebbiolo,  
452 and further studies are required to understand whether this has an effect on vector acquisition and

453 transmission efficiency, as already suggested for Pinot blanc and Merlot cultivars (Bressan et al.,  
454 2005).

455

456

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458

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### 627 **Captions for figures**

628

- 629 Figure 1. A: Standard curve of Flavescence dorée phytoplasma DNA obtained by plotting threshold  
630 cycles (CT) values vs log of  $2 \times 10^7$ ,  $2 \times 10^5$ ,  $2 \times 10^3$ , and  $2 \times 10$  copy number of p26SecYFD  
631 plasmid. **BC**: Melting curve analysis of the amplicon obtained with primers FdSecyFw/FdSecyRv.  
632 **EB**: 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 dorée 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 <sup>a</sup>	U <sup>b</sup>	P <sup>c</sup>
Spring <sup>d</sup>	C	B	2008+2009	B > N	17, 3	3.0	0.991
Early summer <sup>e</sup>	C	N	2008	B > N	4, 4	0.0	0.986
	MR	N	2008	MR > C	4, 11	0.0	0.999
Late summer <sup>f</sup>	C	B	2008	Es <sup>e</sup> < Ls <sup>f</sup>	9, 7	7.5	0.996
	C	N	2008	Es <sup>e</sup> < Ls <sup>f</sup>	4, 4	0.0	0.986

<sup>a</sup>N: sample numbers; <sup>b</sup>U: value of U in the Mann-Whitney U test; <sup>c</sup>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); <sup>d</sup>Sp: spring; <sup>e</sup>Es: early summer; <sup>f</sup>Ls: late summer.

; <sup>d</sup>Sp: spring; <sup>e</sup>Es: early summer; <sup>f</sup>Ls: late summer.



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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			BNP+FDP		
			(Positive/ total sampled)			(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).

<b>Plant</b>	<b>Mean FDP titer in shoots (DLog)</b>	<b>Var</b>
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: Coconato 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	0.43 6	1.034	N.S.
			Ls	3.8911	7	3.785	1	0.71 4	0.6746	N. S.
		2008	Es	3.2713	4	2.7967	3	0.20 6	0.8409	N.S.
N	MR	2009	Es	2.7845	6	3.5642	2	0.31 5	1.740	N.S.
			Ls	3.0948	5	2.551	1	0.39 7	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.  $\text{Log}[\text{FD}]_{\text{FD}}$ ,  $\text{Log}[\text{FD}]_{\text{FD+BN}}$ : Log of FD phytoplasma concentration.  $N_{\text{FD}}$ ,  $N_{\text{FD+BN}}$ : 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	$\text{LOG}[\text{FD}]_{\text{FD}}$ (mean)	$N_{\text{FD}}$	$\text{LOG}[\text{FD}]_{\text{FD+BN}}$ (mean)	$N_{\text{FD+BN}}$	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 <sup>a</sup>	2008+2009	Sp	0.820	4	2.366	2	0.165	2.531	N.S.
	C+MR <sup>b</sup>	2008	Es	3.2644	7	3.3878	4	0.651	$\frac{0.595}{5}$	N.S.

<sup>a</sup>, <sup>b</sup>: 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) or FDP and BNP (FD-C+BN) in double infection were analysed separately. Sp, Es: spring and early summer samplings.

LOG[FD]<sub>CC</sub>, LOG[FD-D]<sub>MR</sub>: 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] <sub>CC</sub> (mean)	N <sub>CC</sub>	LOG[FD] <sub>MR</sub> (mean)	N <sub>MR</sub>	F pr	LSD	Comment
FD-C	2008+2009 <sup>a</sup>	Sp	0.349	2	1.291	2	0.549	5.681	N.S.
	2008 <sup>b</sup>	Es	3.255	3	3.271	4	0.962	0.826	N.S.
FD-C+BN	2008 <sup>b</sup>	Es	2.942	1	3.536	3	0.386	2.324	N.S.

<sup>a</sup>, <sup>b</sup>: Results allowing groupings as reported in Table 7 at corresponding symbols

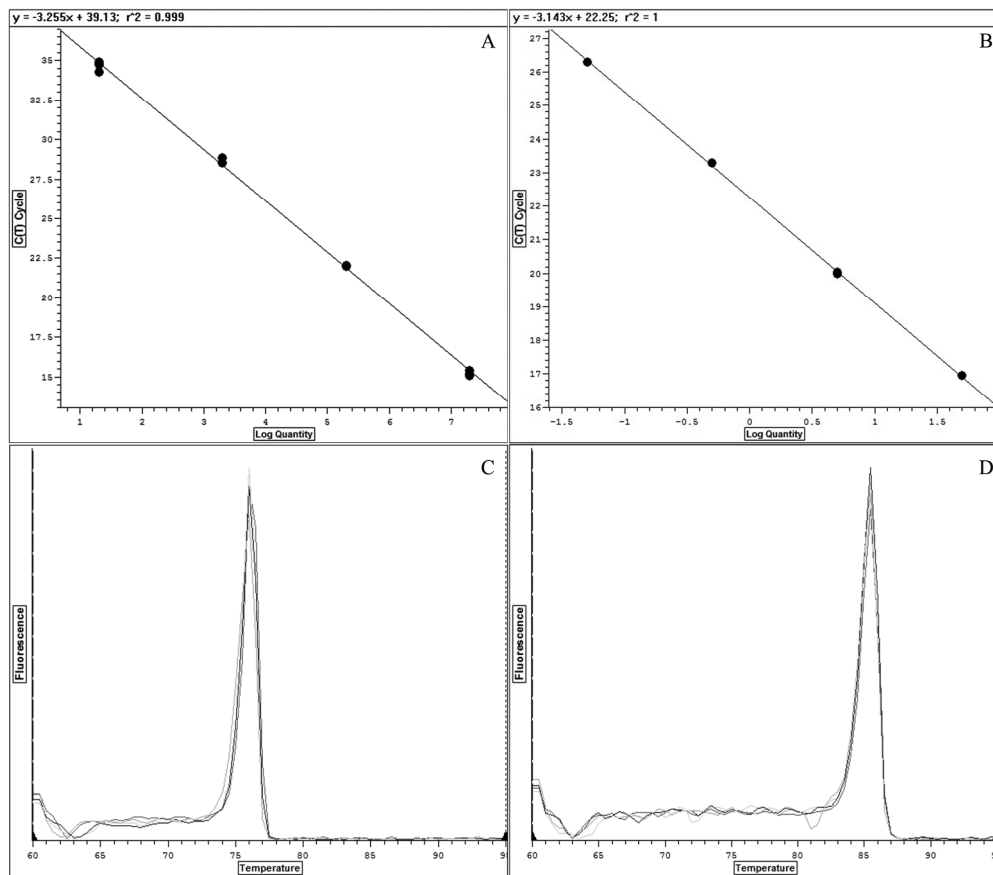


Figure 1  
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