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Frateuria defendens reduces yellows disease symptoms in grapevine under field conditions

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Summary

Yellows diseases in grapevine, associated with the presence of different phytoplasmas, are a major problem for growers, with no environmentally friendly means of control. *Frateuria defendens* (*Frd*), a bacterium with endophytic traits, has been shown to reduce yellows symptoms in grapevine plantlets under laboratory conditions. The objective of this study was to test whether similar effects could be achieved under field conditions. A trial was conducted in a heavily infected vineyard in northern Israel for two consecutive years. A suspension of *Frd* cells ($10^8 \cdot mL^{-1}$) was applied bi-weekly by foliar spray on grapevines from bud burst to leaf senescence. *Frd* penetrated the leaves during the growing period but not during leaf senescence and could be detected in the leaves by PCR analysis up to 14 days post-spraying. The rate of yellows infection was lower in the treated grapevines compared to its increase in untreated grapevines and the yield of symptomatic plants was improved by 10 to 20 %. Taken together, the results suggest *Frd* acted as a biological control agent in vineyards under the experimental conditions tested.

K e y w o r d s : phytoplasma; stolbur; *Frateuria defendens*; grapevines; yellows disease; bio control agent.

Introduction

Grapevine diseases can be divided into those caused by pathogens that can be controlled by application of pesticides, and those associated with microorganisms that inhabit the inner vascular tissues and are therefore inaccessible and difficult to control (Bisztray *et al.* 2012, Constable and Bertaccini 2017, Gramaje *et al.* 2018, Laimer and Bertaccini 2019, Sicard *et al.* 2018). Among the former are foliar and berry diseases such as downy mildew and powdery mildew. The latter include xylem-residing pathogens such as fungal trunk pathogens, bacteria (e.g. *Xylella*) and viruses on one hand as well as phloem-inhabiting viruses and wall-less bacteria (e.g. phytoplasmas) on the other hand (Bisztray *et al.* 2012, Yadeta and Thomma 2013).

Species of the genus *Candidatus* Phytoplasma are associated with yellows disease in many important agricultural crops worldwide including grapevines (Bertaccini *et al.* 2014). The presence of these vector-borne, wall-less, obligatory pathogens in the phloem vessels of grapevines leads to major yield losses (Ember *et al.* 2018, Pavan *et al.* 2012). Chemical spraying against the leafhopper *Scaphoideus titanus*- the vector of Elm yellows group 16SrV phytoplasma, is efficient in controlling Flavescence dorée disease in grapevine (Pavan *et al.* 2012). Unfortunately, this method is not effective on Bois noir (BN) phytoplasma (Stolbur group 16SrXII) since the pathogen is vectored by the planthopper *Hyalesthes obsoletus* Signoret, which is an occasional visitor in the vineyard (Maixner 2007). Since 2013, several tests using nested PCR and q-PCR have shown that "stolbur" is the type of phytoplasma detected in Israeli vineyards (Dafni-Yelin *et al.* 2015, Zahavi *et al.* 2013).

Available strategies for controlling BN include the eradication of wild host plants, reduction of the vector population, and application of mechanical control measures on infected grapevines, such as stress-response induction, pollarding, pruning and uprooting (Belli *et al.* 2010, Langer *et al.* 2003, Zahavi *et al.* 2007). In addition, a reduction in phytoplasma titer and disease symptoms was observed following trunk injection of liquid antibiotics (mainly oxytetracycline), but the usage of antibiotics as a pest management strategy is expensive and prohibited in European countries (Bertaccini and Duduk 2010, Bertaccini *et al.* 2014). Nevertheless, because there are no efficient measures directed against the pathogen itself, the demand to increase the use of environmentally friendly solutions and reduce chemical applications calls for a different approach. Application of a bacterial bio control agent (bBCA) may provide a partial solution to these challenges, especially if the microorganism shares the same niche as the pathogen (Alabouvette *et al.* 2006). The bacterium *Frateuria defendens* (*Frd*; Rhodanobacteraceae),

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strain DHo^T (= NCCB 100648^T; = DLB^T = DSM 106169^T; NCBI accession number LFQR0000000; Lidor *et al.* 2019), previously reported as isolate X (Naor *et al.* 2015) or *Dyella*-like bacterium (Lahav *et al.* 2016), was isolated from the insect vector of BN (*Hyalesthes obsoletus* Signoret). It was found that *Frd* is able to penetrate various herbaceous and woody plant species, including grapevine, and resides in the plant vascular system (Iasur-Kruh *et al.* 2018, Lidor *et al.* 2018). *Frd* secretes different secondary metabolites that inhibit the growth of *Spiroplasma melliferum* (Naama-Amar *et al.* 2020). In addition, *Frd* is inhabiting the phloem of grapevines and reduces yellows symptoms in grapevine plantlets under laboratory conditions (Iasur-Kruh *et al.* 2018). These findings, supporting the potential use of *Frd* as a bacterial bBCA, have prompted this study to test the ability of the newly discovered bacterium to reduce the symptoms of grapevine yellows disease in a commercial vineyard.

In the present study, *Frd* was used as a bBCA in a BN-infected plot where its effects on the disease were studied in view of two possible modes of action: (i) preventative – where the bBCA prevents new infections; (ii) curative – where the presence of the bBCA enhances spontaneous recovery.

Material and Methods

Climate measurements: Climate measurements were retrieved from a regional weather station situated 500 m south of the vineyard. The collected data were processed by Excel software. Temperature (minimum and maximum) and relative humidity (RH) were monitored daily throughout the experiment.

Plot description: The experimental plot was located in northern Israel, 33°10'22.89"N, 35°47'52.23"E, 1045 m above sea level, inside a 60-ha vineyard (Fig. 1A and B). The growing season in the area is from early April to mid-November with no summer rains. The day/night temperature during the growing season ranges from 10 to 35 °C and day/night relative humidity (RH) ranges from 30 to 100 %. Wind velocity is generally 0 to 20 km·h^{-1}, although higher speeds are sometimes recorded.

The experiment was conducted in a 0.4-ha 'Chardonnay' grapevine plot planted in 1998. The grapevines were planted in rows (90-100 plants/row), 1.5 m between grapevines and 3 m between rows, spur pruned and trained to a bilateral cordon with vertical shoot positioning. The plot was infected with BN associated with stolbur type (16SrXII) phytoplasma (Zahavi *et al.* 2013). For the experiment, 5 rows, ca. 450 grapevines, were chosen with disease rates (*i.e.* percentage of symptomatic grapevines) of 38 to 40 % in 2014.

Disease assessment – monitoring new infections, recovery, and disease rate: From 2012 to 2016, each grapevine in the plot was monitored at harvest for yellows symptoms (Fig. 1B). New infection, recovery, and disease rate were calculated based on the history of disease symptoms of each plant. A grapevine was determined as newly infected if it was detected as symptomatic for the first time during the experiment. A grapevine was defined as recovered if no symptoms were present in a plant that had been defined as infected in the previous year. Disease rate was calculated as the ratio between the number of symptomatic grapevines and the total number of grapevines in each plot.

Experimental design: The experiment was conducted in the same plot during 2015 and 2016, where the same grapevines were sprayed with *Frd* in both years. The infection levels of the rows selected for the trial were as similar as possible, and each replicate was designed in the same manner. The plot was divided width-wise along

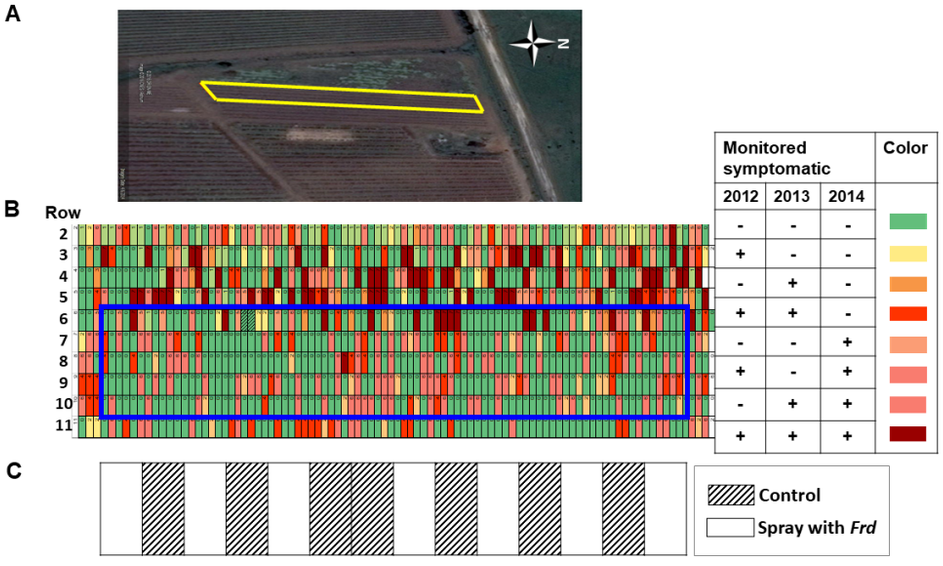

Fig. 1: Description of the experimental plot. **A**, location of the experimental plot inside the vineyard. **B**, disease ratings of grapevines in the 5 rows selected for the experiment (inside blue rectangle) and adjacent rows before trial onset. Each grapevine had been monitored for visual symptoms of yellows disease since 2012. Each tinted rectangle represents 1 grapevine. **C**, plan of the experimental design within the 5 rows. The plot was divided into 14 subplots, with 7 subplots treated with *Frd* (empty boxes) and the other 7 serving as controls (hatched boxes).

Table 1

Dates of *Frd* application by foliar sprays during the two-year experiment (dd·mm)

Year/Spray no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2015	14/4	29/4	13/5	31/5	10/6	24/6	7/7	21/7	9/8	19/8	17/9	24/9	1/10	15/10	1/11
2016	14/4	27/4	10/5	23/5	7/6	21/6	6/7	25/7	-	22/8	7/9	21/9	7/10	21/10	2/11

5 rows into 14 replicates, each consisting of 30 to 40 plants. Seven replicates (ca. 220 plants) were treated with *Frd* and seven (ca. 220 plants) served as controls (Fig. 1C). To assess the effect of *Frd* on disease rate and yield of symptomatic grapevines, the plants were sprayed with the bacterial suspension from bud burst to leaf senescence at 2-week intervals (Tab. 1). In 2015, control grapevines were untreated and in 2016, they were sprayed with water. In 2016, spraying was stopped 4 weeks before harvest and resumed after harvest. Before harvest, disease symptoms were visually inspected and assessed for each grapevine. In 2015, a similar number of treated (80) and control (70) grapevines from all rows were harvested. In 2016, all grapevines in 3 inner rows (150 grapevines from each treatment) were harvested separately for yield estimation.

Frd growth and field application: *Frd* cultures were newly prepared for each spraying from a starter according to Lidor *et al.* (2018). Inoculum of *Frd* was grown for 3 d at 28 °C to a concentration of ca. 5 to 8 x 10^9 CFU·mL^{-1}. A 10 % (v/v) suspension (0.5 L·$vine^{-1}$) supplemented with 0.1 % (v/v) Tween 20 as surfactant was prepared (Naor *et al.* 2019). The suspension was applied to the grapevines by foliar spraying using a Briggs & Stratton 6.5-HP motor spray, with spray pressure of 15 Atm, a 0.8-m handle, and 1.8-mm nozzle. In 2015, the spraying was done on foot, whereas in 2016, the spraying was performed while riding on a motor powered-mule. The grapevines were sprayed from the ventral side of the leaves.

Assessment of *Frd* penetration into vine and berries: Penetration of *Frd* into grapevines was assessed by both plating (only in 2015) and PCR analysis (2015 and 2016). For PCR analysis, DNA was extracted from 300 mg of petiole and blade base tissue or berries using the CTAB method according to Orenstein *et al.* (2003). The DNA was extracted from leaves of asymptomatic grapevines that were collected from the middle of the canopy of 2 grapevines from the 3 middle rows of each replicate (in total 14 samples per treatment). Each sample consisted of 2 (2015) or 4 (2016) leaves. In the first two applications in 2015, sampling was carried out two weeks post-spraying, with negative results. Therefore, in all subsequent sprayings, sampling was performed 6 to 8 d post inoculation (dpi) with *Frd* suspension. To determine how long *Frd* can be detected in the leaves, in addition to the regular sampling scheme, in 2015, leaves were also collected in other sampling dates, starting from 1 dpi to 15 dpi. To monitor the presence of *Frd* in the fruit, 8 berries (2 from each of 4 clusters) were sampled for DNA extraction from 5 sprayed and 5 control grapevines. In 2015, *Frd* was monitored in berries 7 d after the last spraying, which was 7 d prior to harvest. According to the results of 2015, the presence of *Frd* in berries was monitored twice in the second year (2016): 7 d and 1 d prior to harvest, which is equivalent to 3 and 4 weeks from last spraying, respectively. Before plating and DNA extraction, the leaves were thoroughly washed under running water with a commercial detergent, then externally disinfected by a 15 s dip in 70 % (v/v) ethanol and 2 min in 0.5 % (v/v) hypochlorite, followed by 3 consecutive washes in sterile water supplemented with 0.1 % Tween 20. For plating, 1000 mg of leaf blade were ground and 3 replicates of 100 µL were plated on nutrient agar plates supplemented with 100 mg·L^{-1} cycloheximide. Wash water was similarly plated as a control. *Frd* colonies were distinguished morphologically from other bacteria that grew on the plate. Suspicious colonies were also verified by PCR using specific primers for *Frd* according to Iasur-Kruh *et al.* (2016). Penetration rate was calculated as the numerical ratio of positive to total samples according to PCR analysis using specifically designed primers (Iasur-Kruh *et al.* 2016). Random PCR products were sequenced to confirm the PCR results.

Berries and must: For assessing the presence of *Frd* in the must, one day before harvest, 16 to 32 berries were sampled from both sides of each grapevine: 4 to 8 berries were collected at the far end of the cordons and 4 o 8 berries near the trunk. The samples were kept cool until pressing and analyzed on the same day in the laboratory. To assess yield quality, berry weight, degrees Brix (total soluble solids as a parameter for sugar content; °Bx), and pH of the must were recorded for each sample.

Yield measurement: To assess the effect of *Frd* on yield, the grapevines were harvested manually. For each grapevine, the number of clusters and total weight were determined.

Must fermentation: To monitor *Frd* in the must during fermentation, 5 replicates of 6 kg berries each were collected for each treatment. The samples were taken from the bulk of clusters harvested from each replicate, crushed, and filtered through a thin cloth. To ferment the must, 0.02 g·L^{-1} of commercial wine yeast was added and the must was kept at 18 °C. Sugar level was monitored daily until the end of the process using the hydrometer technique. The presence of *Frd* was monitored at the beginning, middle and end of fermentation by plating serial dilutions on nutrient agar. Colonies suspected as *Frd* were further analyzed by PCR.

Statistics: The rate of *Frd* presence in leaf samples was calculated from the total number of samples for each treatment: positive samples/(positive + negative samples). The quantity of *Frd* in the vine was calculated as CFU·g^{-1} fresh weight (FW) of leaf tissue. To compare *Frd* penetration

among different phenological stages, a two-way anova was performed for testing possible interactions between year and phenological stages, and between year and treatment effect on recovery, disease rate, yield and harvest parameters (*i.e.* kg·vine^{-1}, number of clusters·vine^{-1}, cluster weight, berry weight, pH and °Bx). Since neither phenology-by-year nor treatment-by-year interactions were seen, the results of the main treatments followed by Tukey or T-student post hoc tests ($\alpha = 0.05$) are presented. To compare the percentage of symptomatic grapevines between treatments in each year, Fisher exact test was performed. Nonlinear regression was performed using the Lorentzian peak equation because it describes well the relationship between dpi and *Frd* survival. The effect of spraying with *Frd* on yield was also determined by calculating the yield ratio between symptomatic and asymptomatic grapevines (symptomatic/asymptomatic) in each treatment. This way of calculation is based on the assumption that a ratio close to 1 resembles less difference between vine types (symptomatic vs asymptomatic), while a low ratio expresses a difference between them. Statistical analysis and significance post hoc tests were calculated using JMP software. The nonlinear regression was calculated by SigmaPlot software (Systat Sofware, Inc., 2011; Version 12.0).

Results

Climate conditions: In the 2-year trial, no rain was recorded during the 6-month growing period, and the climate in the experimental plot was characterized by sunny, hot and dry days and cool nights. On spraying dates, minimum temperature ranged from 5 to 20 °C and maximum temperature from 15 to 32 °C (Fig. S1A). RH ranged from 5 to 60 % minimum and 60 to 100 % maximum (Fig. S1B).

Frd presence in leaves: During the 2-year trial, 16 sprays were conducted at 2-week intervals from bud burst to leaf senescence (Fig. 2). To determine the presence of live *Frd* cells in the inner tissues of the leaves, in 2015, leaf tissue was plated in parallel to PCR analysis following surface sterilization. *Frd* was not detected in the leaves prior to spraying, detected in very low percentages in the leaf samples of control samples in 2015 (see Tab. S1) and was not detected in controlled samples in 2016. During these stages, the quantity of *Frd* ranged between 100-1000 CFU·g^{-1} FW of leaf tissue (Fig. 2A). Lower quantities were observed at the beginning of the growth period and at harvest. Higher quantity was monitored from flowering until harvest. Although plating express an estimate of the titer of live bacteria and PCR express both living and dead cells, both methods showed a similar pattern of penetration rates (Fig. 2B and C). The latter was therefore chosen as the method of choice for the rest of the study because of technical considerations. PCR analysis confirmed that in 2016, the presence of *Frd* in the leaves 7 dpi was significantly higher than in 2015 ($p = 0.02$ by ANOVA). Moreover, *Frd* was present in over 50 % of the leaf samples on 2 out of 15 spray dates in 2015, compared to 10 out of 15 dates in 2016. The presence of *Frd* in the leaves varied along the growing season. A high rate of *Frd*-positive leaf samples was seen before (65 %) and during (78 %) flowering and veraison (48.8 %). A lower rate was observed during harvest and leaf senescence. Samples at flowering differed significantly only from harvest samples. However, a trend of gradual decline was noticed starting at veraison continued to harvest, until no *Frd* could be detected in the senescing leaves in the fall (Fig. 3).

The detection of *Frd* in leaves was correlated with time post-inoculation. The Lorenzian peak model showed a significant correlation. (df = 11, $p = 0.013$, $r^2 = 0.66$; $f(y) = 100/(1 + ((x - 3.46)/1.6)^2)$; Fig. 4A). Samples positive for

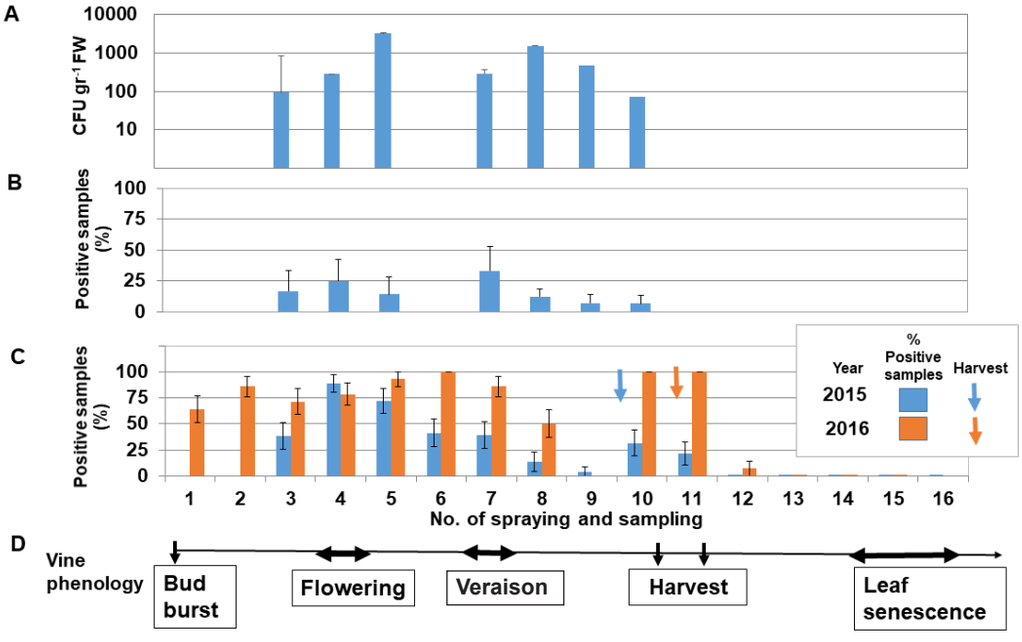

Fig. 2: Presence of *Frd* in vine leaves following each application during the 2-year experiment. The presence of *Frd* was quantified in the leaf tissue and is also expressed as the percentage of positive vs. total number of samples. **A**, number of *Frd* cells in leaf tissue as calculated by plating **B**, rates of penetration by plating. **C**, rates of penetration by PCR analysis. **D**, vine phenology stages along spraying dates. Bars represent standard error.

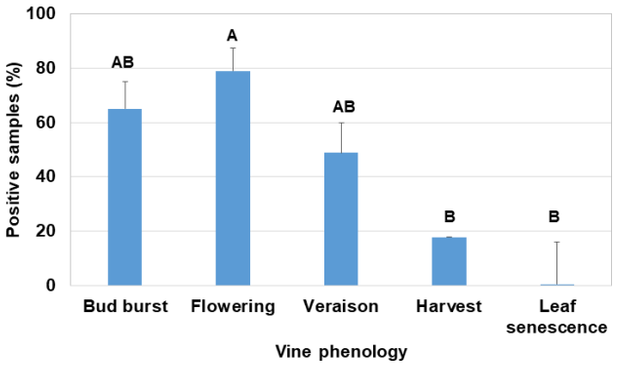

Fig. 3: *Frd* rate in vine leaves at various phenological stages as determined by PCR. The presence of *Frd* is expressed as the rate of positive samples. Bars represent standard errors. Different letters indicate significant differences between categories (ANOVA: p = 0.002; Tukey post hoc test: Q = 3.028, α = 0.05).

Frd were detected from 1 to 14 dpi. There was a gradual increase in *Frd* rate from 20 % at 1 dpi to 100 % at 4 dpi, suggesting that it takes time for the bacteria to penetrate the leaves. However, from day 5 onward, the percentage declined until the bacteria could not be detected 15 to 17 dpi. The amount of cells in the plant tissue ranged between 100-1000 CFU·g^{-1} FW (Fig. 4B).

Effect on disease rate: A vast survey conducted in 2012-2013 determined that the yellows disease in the experimental plot was associated with stolbur tuf-b phytoplasma (Dafny-Yelin *et al.* 2015). The phytoplasma type was re-confirmed in 2016 and 2017 using specific primers by PCR and q-PCR analyses respectively (Langer and Maixner 2004, Pelletier *et al.* 2009). The disease status of each plant was visually monitored for three years before the beginning of the trial. During that period, the disease rate increased from 1.3 % to 33.1 % (Fig. S2). Since the trial began, the percentage of symptomatic grapevines

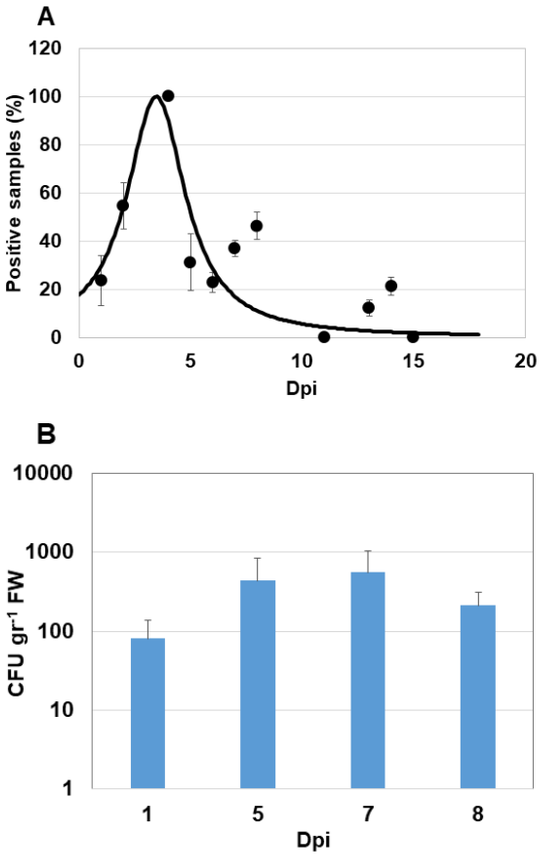

Fig. 4: *Frd* dynamics in leaves after spraying as determined by: A, PCR; B, plating. Dpi, days post inoculation. Bars represent standard errors.

in the control treatment further increased, to 42.8 % in 2015 and up to 57.8 % in 2016. However, the percentage of symptomatic plants from the sprayed grapevines was 41 % in 2015 and 45.7 % in 2016. This is an increase of

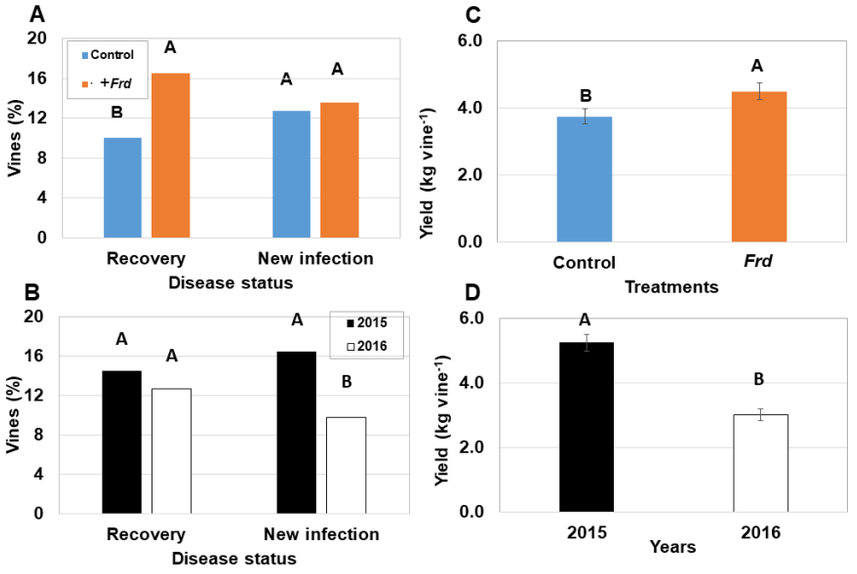

Fig. 5: Effect of *Frd* application on yellows disease in the vineyard. Data from 2015 and 2016 were combined because there was no interaction between year and treatment. **A**, the effect of *Frd* application on the rates of recovery (logistic regression, p = 0.014) and new infection (logistic regression, p = 0.72). **B**, the effect of years on rate of recovery (logistic regression, p = 0.42) and on the new infection (logistic regression, p = 0.012). **C**, the effect of Frd application on total yield of symptomatic vines (t-test, p = 0.14). **D**, the effect of years on yield of symptomatic vines (t-test, p = 0.014). Different letters indicate significant differences between treatments.

only 4.7% from 2015 to 2016 compared to ca. 15 % in the control group (p = 0.02). Statistical analysis of the effect of *Frd* spray and the rate of recovery or the rate of new infection revealed no interaction between years and disease status, and the results for both years were therefore pooled (Fig. 5A, B). The recovery rate of sprayed grapevines was 16.5 ± 1.8 %, which was significantly higher than the control grapevines (10.0 ± 1.9 %; t-test, p = 0.0114). However, spraying with *Frd* did not significantly affect the rate of new infections, which was 12.7 ± 1.9 % in sprayed grapevines and 13.6 ± 1.8 % in control grapevines.

Yield of symptomatic grapevines: The focus of this trial was the effect of *Frd* application on yellows symptoms. Therefore, only the effect of *Frd* application on the combined yield of 2015 and 2016 of symptomatic grapevines is presented (for yield of asymptomatic grapevines see appendix Tab. S2). In general, the yield of *Frd* treated grapevines was significantly higher than control grapevines (t-test, p = 0.014; Fig. 5C). The number of clusters in symptomatic *Frd*-treated grapevines was significantly higher (by 26 %), than in symptomatic untreated grapevines (t-test, p = 0.03; Tab. 2). Consequently, the yield of treated grapevines (4.5 ± 0.3 kg/vine) was 20 % higher than that of untreated grapevines (3.7 ± 0.2 kg·vine^{-1}).

The yield of asymptomatic grapevines was ca. 6 and 5.2 kg·vine^{-1}, respectively (Tab. S2). The yield of symptomatic grapevines decreased greatly in 2016 compared to 2015 due to a significant decrease in the number of clusters and cluster weight (Fig. 5D, Tab. 2); consequently, the difference between yields of asymptomatic and symptomatic control grapevines was nearly 100 % in 2016 (5.5 vs. 2.7 kg·vine^{-1}, respectively), compared to 20 % in 2015 (5.8 vs. 4.8 kg·vine^{-1}, respectively; see Supplementary Data). Another way of testing the effect of *Frd* on yield was to calculate the yield ratio between symptomatic and asymptomatic grapevines (symptomatic/asymptomatic). This rate in treated grapevines was 0.80, compared to 0.65 in control grapevines. This difference between treatments is another way of showing the positive effect of *Frd* spray on the yield of infected grapevines.

Cluster weight, berry size, °Bx and pH were measured just before harvest in each year. *Frd* did not affect any quality parameters of the must tested in both years in symptomatic plants (Tab. 2), although °Bx was significantly higher in asymptomatic treated plants compared to control (Tab. S2). The level of °Bx and pH was significantly higher in 2015 compared to 2016.

Frd in must during fermentation: The presence of *Frd* in berries was confirmed 3 weeks before harvest in 2015. Therefore, in 2016, spraying was stopped 4 weeks before harvest to prevent the presence of *Frd* cells in the must. Following this procedure, *Frd* was not detected in the berries or in the must during the 10-d fermentation process. In both years, *Frd* was not detected in the control samples.

Discussion

Modern agricultural practices stress the importance of biopesticides as an environmentally friendly alternative to chemical control (Nawaz *et al.* 2016). Many bacterial species and strains have been suggested as potential candidates as bio control agents, but relatively few have successfully passed the field trial barrier to become commercial products (Junaid *et al.* 2013). The current commercial products that are authorized in the EU in vineyard are mostly aiming to control fungal grapevine diseases (Zanzotto and Morroni 2016). They are based on seven bacterial species from three genera: *Bacillus*, *Pseudomonas* and *Streptomyces* (Velivelli *et al.* 2014). However, strains of *Bacillus* and *Pseudomonas*, are less likely to compete with phytoplasmas as they do not share the same habitat in the host plant (Compant *et al.* 2013). *Frd* makes a good bBCA candidate because of its abilities to penetrate plants, share the same niche as the pathogen and reduce yellows disease symptoms under laboratory conditions (Iasur-Kruh *et al.* 2018, Lidor *et al.* 2018, Naor *et al.* 2019). This is the first report on direct control under field conditions using *Frd* as a bBCA against phytoplasmas. The beneficial outcomes of *Frd* application in the vineyard were a reduction in disease level, an increase in recovery frequency, and an increase in the yield of symptomatic grapevines.

Frd penetration into the grapevines: The results suggested that *Frd* can penetrate not only young and tender leaves, as previously reported (Iasur-Kruh *et al.* 2018, Naor *et al.* 2019), but also mature leaves under the harsh hot and dry field conditions. Nevertheless, young leaves, at the stage of robust growth, correlate with

Table 2

Effect of applying *Frd* on yield parameters (mean ± standard error) of symptomatic vines at harvest between treatments and years. Yield quantity is expressed as cluster number and weight, yield quality is presented as berry weight, must soluble sugars (°Bx) and must acidity (pH). n - Number of tested vines for variable on the left. Different letters mean significant difference between treatments (higher case letters) or years (lower case letters)

Variable		Yield quantity				Yield quality			
No. of clusters		Cluster weigtht (g)	n	Berry	n	°Brix	pH	n	
Treatment	Control	34.3 ± 1.7 B	106.5 ± 4.7 A	82	1.5 ± 0.0 A	65	20.2 ± 0.22 A	3.3 ± 0.0 A	36
	+*Frd*	41.2 ± 1.9 A	107.4 ± 4.2 A	80	1.5 ± 0.0 A	54	20.3 ± 0.24 A	3.3 ± 0.1 A	27
Year	2015	43.4 ± 1.9 a	122.4 ± 4.1 a	61	1.5 ± 0.3 a	24	21.0 ± 0.3 a	3.5 ± 0.0 a	23
	2016	31.9 ± 1.4 b	91.6 ± 3.9 b	100	1.4 ± 0.0 a	97	19.5 ± 0.5 b	3.0 ± 0.0 b	40

an increase penetration rate, which declines as the leaves age and towards plant dormancy. This result suggests that plant phenological stage affect the penetration and/or the establishment of the bacteria in the leaf tissue. This is different from evergreen trees where population abundance of endophytes was higher in mature leaves compared to young leaves (Yadav *et al.* 2011, Arnold and Herre 2003). The low cell concentration of *Frd* in the plant tissue, the strong decline compared to the original inoculum in the spray solution and the short survival period imply that although *Frd* has endophytic characteristics, grapevine is not its favored habitat. Accordingly, repeated sprays are necessary to maintain a constant presence of *Frd* inside the grapevines. However, it must be tested whether the number of applications can be reduced to the affective period from bud burst to veraison. This action should probably be adjusted to the specific factors, such as local environmental conditions, grapevine variety, and insect vector etiology.

In Israel, the flight periods of the insect vector *Hyalesthes obsoletus* Signoret, are in late spring and autumn (Klein 2001, Sharon 2005). Although plants can theoretically be infected twice during their growing period, the chances are higher in the fall, when a higher percentage of specimens harbor phytoplasmas (Sharon *et al.* 2015). The decline in *Frd* penetration rates during the last period of grapevine growth suggests that it may be difficult to prevent new infection during the second flight period. Indeed, in this study, the rate of new infections was not affected by *Frd* application. This is therefore an interesting question to address under European conditions, where the vector's single flight period occurs during June-July, when the leaves are young and tender (Maixner 2007).

Frd effect on recovery, disease rate, and yield: Spraying the same grapevines with *Frd* for 2 consecutive years seemed to have a significant positive effect on disease rate and recovery in a heavily infected plot as well as on yield. The bacteria survived inside the plant for several days before their elimination, suggesting low to no multiplication. Therefore, it can be speculated that the effect of *Frd* does not depend on its increase in population size but rather on the antibiotic metabolites that it secretes (Iasur-Kruh *et al.* 2016, Naama-Amar *et al.* 2020), This possibility needs further investigation. Spontaneous recovery from yellows disease is a known phenomenon in grapevines, apples and apricots (Laimer and Bertaccini 2019). In recovered grapevines, the symptoms disappear and the yield production is similar to that of asymptomatic grapevines (Zahavi *et al.* 2013). Following *Frd* application, the percentage of recovered grapevines increased by ca. 65 % in treated grapevines, suggesting that *Frd* plays a role in the plant's defense response. Recently, Naama-Amar et al. (2020) showed a reduction in phytoplasma titer and yellows symptoms in periwinkles, by applying chemical analogs of metabolites secreted by *Frd* (4-Quinolinecarboxaldehyde and 5-Hydroxymethyl-2-furaldehyde). Landi *et al.* (2019), using sensitive detection methods, showed that phytoplasma was present in the roots of recovered grapevines in lower titer compared to symptomatic plants. Moreover, they suggested that plant defense system is involved. Hence, if these metabolites are also secreted by *Frd* in grapevines under field conditions, they may positively affect the recovery from yellows disease *i.e.* symptom remission without elimination of the pathogen as shown by Naama-Amar *et al.* (2020). Another explanation for enhanced recovery is the induction of the plant defense system by biological products, as reported by Romanazzi *et al.* (2009); it took 18 applications of Kendal, Olivis (different mixture of glutathione and oligosaccharide) or Bion (benzothiadiazole) to increase the recovery by 2- to 2.5-folds of the natural rate of 23 or 37 %, suggesting that continuous application is needed to obtain a positive effect. In the present study, the natural rate of recovery was lower than that reported by Romanazzi *et al.* (2009), but increased following 11 sprayings with positive *Frd* penetration.

Conclusions

This is the first field trial with *Frd* to control yellows disease in grapevine. The results of this trial emphasize the previously reported characteristics of *Frd* and generally reflect both advantages and disadvantages of the bacterium as a potential bio control agent. On the up side, the bacterium reaches the target site, survives for two weeks, increases percent of recovery and amount of yield, does not induce any detectable phytotoxicity, and disappears without a trace from the berries and must. On the down side, continuous spraying is needed to reduce disease symptoms.

Unfavorable habitat in the plant limits bacteria's survival to short periods (Nega 2014). This would explain why application of *Frd* had a relatively low impact on the disease, although it was applied at a high concentration. A direct evolution technique to select a *Frd* strain with a better survival rate inside the plant tissue may improve its effect. Another problem with applying bio control agents is inconsistent results (Alabouvette *et al.* 2006). Nevertheless, under the above limitations, a positive effect was observed with respect to disease level and yield of infected grapevines. The effect of BCAs in general is lower than that of chemical pesticides (Nega 2014). However, in the absence of effective control measures, improving the yield of symptomatic grapevines by 20 % and blocking the disease's dispersion in the vineyard should be weighed against the cost of application.

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