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Short Notes

Introducing the potential biological control agent *Frateuria defendens* into pot- and field-grown grapevines

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Abstract. Bois Noir is a major yellows disease in grapevine with no current means of control. The endophyte *Frateuria defendens (Frd)*, isolated from the insect vector of Bois Noir, is a potential biocontrol agent for this disease. The aim of this study was to determine an efficient way to introduce *Frd* into mature vines under field conditions. Shoots of young and field-grown vines were sprayed with *Frd* suspension supplemented with either Tween 20, BB5, DX, or Triton X-100 as surfactants. The presence of *Frd* was confirmed by PCR in sprayed leaves, and in leaves below and above the sprayed site. The results showed that cells of *Frd* penetrate and move inside the vine shoots under field conditions. Highest penetration rate was achieved when leaves were sprayed with 10^8 or 10^9 colony forming units per mL (CFU mL^{-1}) of *Frd* with addition of 0.1% Tween 20. The addition of a surfactant is essential to increase the proportion of shoots with *Frd*.

Keywords. Biological control, yellows disease, phytoplasma, endophytes.

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INTRODUCTION

Bacteria from the phloem-restricted genus '*Candidatus* Phytoplasma' (Mollicutes; hereafter referred to as phytoplasma) are cell wall-less prokaryotes, formerly called mycoplasma-like organisms (Doi *et al*., 1967). Members of this genus are obligate parasites that reside only in the phloem vessels of plants and the hemolymph of their insect vectors. They are pathogens associated with yellows diseases in more than a thousand plant species worldwide, posing major threats to many agricultural crops, including stone fruit trees, vegetables and grapevine (Marcone *et al*., 2014). "Bois noir" (BN) is one of the most important grapevine yellows diseases in Europe and in the countries around the Mediterranean Sea. This disease is associated with the presence of '*Candidatus* Phytoplasma solani', (Quaglino *et al*., 2013) which is classi-

fied in the ribosomal subgroup 16SrXII-A. These phytoplasmas are mainly vectored to grapevine (*Vitis vinifera*) from weeds by the polyphagous planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) (Sforza *et al*., 1998). However, *H. obsoletus* is considered an occasional grapevine feeder (Foissac and Maixner, 2013). In Europe, it completes its life cycle mainly on annual herbaceous species such as bindweed and nettle (Sforza *et al*., 1998, Maixner 2009), whereas in Israel, its preferred host plant is Abraham's balm (*Vitex agnus-castus;* Sharon *et al*., 2005). Control of BN has met with major difficulties because: i) insecticide applications are ineffective due to the erratic presence of the insect on grapevines; ii) other than by injection, the target site is hard to reach with bactericides; and iii) the relatively efficient application of antibiotics is prohibited in several areas of the world due to risks to the human health and the environment. These challenges, combined with growing public pressure for the application of "green products" and the emergence of pesticide resistance, call for the development of alternative plant disease-control strategies.

One such alternative is harnessing endophytes as biocontrol agents, especially if they share the same niche as the target pathogen (Compant *et al*., 2005, 2013). Because spontaneous recoveries from phytoplasmas have been reported in grapevine, a role of endophytes in control of these diseases has been previously suggested (Compant *et al*., 2013). Bulgari *et al*. (2011) showed differences in endophyte populations monitored in infected *vs*. healthy grapevines. Moreover, yellows disease symptoms or the phytoplasma have never been recorded in Abraham's balm, the host plant of *H. obsoletus* in Israel (Sharon *et al*., 2005, 2015). Besides the obvious requirement of efficiency against the disease agent, a potential biocontrol agent must be cultured, able to reach the target sites and survive for a reasonable period inside plant tissues (Compant *et al*., 2013).

Frateuria defendens (*Frd*; formerly referred as DLB), a bacterium with endophytic characteristics, was isolated from *H. obsoletus* (Iasur-Kruh *et al*., 2016; Lidor *et al*., 2019). Its genome was fully sequenced and deposited in GenBank (accession number LFQR00000000; Lahav *et al*., 2016). The potential of *Frd* as a biocontrol agent was suggested based on the following evidence: i) it reduces the symptoms of grapevine yellows under laboratory conditions (Iasur-Kruh *et al*., 2018); ii) it can penetrate plants via leaf stomata and reside in the vascular systems including phloem vessels (Lidor *et al*., 2018); and iii) it survives inside grapevine plantlets for up to 4 weeks (Iasur-Kruh *et al*., 2018).

The interaction of *Frd* with the grapevine plants is not clear. Genome-based analysis that uses computer models for predicting metabolic interactions between bacteria showed no evidence of common nutrient uptake of the bacterium with phytoplasmas, ruling out competition over metabolites in the shared niche (Iasur-Kruh *et al*., 2018). In contrast, compounds secreted by *Frd* inhibit *in vitro* growth of *Spiroplasma melliferum*, suggesting the presence of antibiosis activity (Iasur-Kruh *et al*., 2016). Nevertheless, to test the endophyte's efficiency in mature plants and under field conditions, its penetration into field-grown grapevines must be optimized. In the case of biocontrol agents, addition of wetting agents to the cell suspensions increases their cell adhesion to leaf tissues and improves cell spread on leaf surfaces by reducing the surface tension of the suspension solution (Ongena and Jacques, 2008; Wyss *et al*., 2004).

The aim of the present study was to determine an efficient way of introducing *Frd* into mature grapevines under field conditions.

MATERIALS AND METHODS

Plants and bacteria

Semi-field experiments were conducted on 6-week-old *ex vitro* grapevine plants cv. Chardonnay in 0.3 L capacity pots, grown under controlled conditions of 25°C and a 16/8 h light/dark photoperiod (Experiment 1). Field trials were conducted on table grape grapevines cv. Early Sweet in a 5-y-old commercial vineyard, ca. 30 d after bud burst, when the shoots were 20–30 cm long (Experiments 2 and 3).

Pure cultures of *Frd*, originally isolated from *H. obsoletus* in 2011 (Iasur-Kruh *et al*., 2016), was used in all the experiments. The strain type DHoT is deposited in both the Netherlands Culture Collection of Bacteria (NCCB; 100648T) and the German Collection of Microorganisms and Cell Cultures (DSM; 106169T) (Lidor *et al*., 2019). For experimental purposes, the bacterial suspension was prepared as described by Lidor *et al*. (2018) with slight modifications. Stock cultures of *Frd*, generally kept at -80°C in a 30% glycerol, were freshly streaked onto a sugar-rich medium [6.6% sucrose, 1% sorbitol, 0.2% Luria broth, 1.5% agar, (all w/v)] and cultured at 28°C. Liquid *Frd* cultures were prepared by inoculating 3 to 5 mL starter containing nutrient broth (NB, Difco) or Luria-Bertani medium (LB, Difco) with a single *Frd* colony from the agar plates (colony morphology: smooth, circular, 1.0–1.5 mm diam., with yellow-pigment; Lidor *et al*., 2019). Starters were cultivated on a shaker for ca. 24 h at 28°C and then used to inoculate broth at a ratio of 1:100 (v/v) in an Erlenmeyer flask. Cultures of *Frd* were cultivated to a concentration of $\approx 10^9$ cfu mL^{-1} (verified by

plating). To achieve the desired concentration for spraying, the suspensions were serially diluted with tap water to 10% and 1%, which was equivalent, respectively, to 10^8 and 10^7 cfu mL^{-1}. A surfactant was added to the final suspension before spraying.

Frateuria defendens *application*

To test for ability of *Frd* to migrate within plants, the upper or lower part of each shoot was covered and sealed with a polyethylene bag to prevent wetting with the bacterial suspension (Figure 1). For these experiments, the leaves from potted plants with 3–4 nodes (ca. 15 cm long) and the leaves from the upper part of shoots (ca. 15 cm long) from field-grown grapevines were used. The uncovered parts of each plant were sprayed with a hand sprayer to run-off with *Frd* suspension supplemented with a surfactant. Each shoot of the potted plants was sprayed with 2–3 mL, while 50 mL of *Frd* suspension was applied to each field grapevine. Non-sprayed plants served in the experiments as negative controls to confirm that *Frd* was not naturally present in the grapevines. After ca. 1 h, when the suspension on the sprayed leaves had completely dried, the polyethylene cover was carefully removed from the plants.

DNA extraction and PCR analyses

To test the presence of *Frd* in the treated plants, one leaf from the covered and one from the uncovered parts of the shoots were sampled 7 d post inoculation (dpi), together with one of the uncovered leaves from the non-sprayed control shoots. Each sampled leaf was washed under running water with a commercial detergent, then externally disinfected by a 15 sec dip in 70% ethanol followed by 2 min in 0.5% sodium hypochlorite solution, followed by three consecutive washes in sterile water supplemented with 0.1% (v/v) Tween 20. The covered leaves were not washed.

DNA was extracted from 300 mg samples of leaf tissue using the cetyltrimethylammonium bromide (CTAB) method according to Lodhi *et al.* (1994). For each sample, leaf tissue was ground and mixed in 4 mL of extraction buffer (20 mM sodium EDTA, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl and 2.0% w/v CTAB). DNA was extracted with isoamylalcohol: chloroform (1:24, v/v), precipitated in cold isopropanol and 10% (v/v) 3 M sodium acetate, and stored overnight at -20°C. The pellet was then washed in 70% ethanol and dissolved in 40 µL Tris-EDTA buffer.

Specific primers based on 16S rRNA (Iasur-Kruh *et al.*, 2016) were used to determine the presence of *Frd* by PCR (DLBF: 5'-CTCTGTGGGTGGCGAGTGGC-3', DLBR: 5'-ACCGTCAGTTCCGCCGGG-3'). The PCR mix of each tube (25 µL) contained 10 µL Apex Taq DNA Polymerase Master Mix (Genorama), 5 pmol of each primer, 12.5 µL double-distilled water, and 1 µL DNA template. The PCR consisted of 35 cycles of 94°C for 0.5 min, annealing at 63°C for 0.5 min and 72°C for 0.5 min, followed by a final step of 10 min at 72°C.

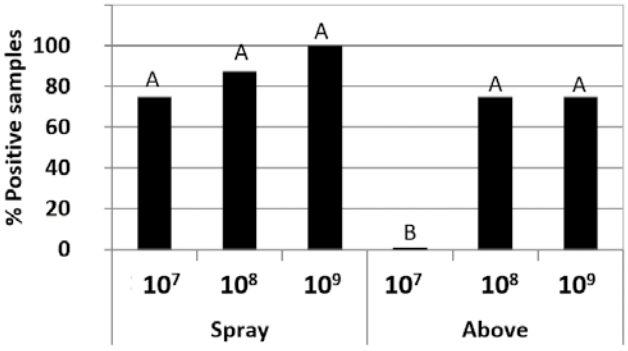

Figure 1. Mean proportions of grapevine tissue samples containing *Frateuria defendens* (*Frd*; determined by PCR) after spraying with various concentrations of bacterial suspension. *Frd* was monitored at and above spraying point of field grown grapevine plants 7 dpi (N = 8 per treatment, N = 6 per control). Since results showed statistically significant interactions between main effects (cell concentration of the sprayed suspension and point of inoculation) each variable is presented separately. Different letters indicate significant differences between categories (α = 0.05)

Experimental design and sampling

Experiment 1. To determine the most efficient surfactant, Tween 20 (TR1880-002; Tedia) and three commercially available surfactants commonly used in agricultural practice, BB5 (alkyl phenoxy polyethylene alcohol, Agrica CTS Ltd.), Triton X-100 (octyl phenyl polyether alcohol, Adama/Agan Ltd.) and DX (alkylaryl polyether alcohol, Adama/Agan Ltd.) were tested. Each surfactant was added to a 10^9 cfu mL^{-1} of the *Frd* suspension at concentrations that were chosen based on the respective manufacturers' recommendations for commercial usage (Table 1). For each treatment, the potted plants were divided into two groups: in the first group, the upper parts (three leaves) of seven plants was each covered and sealed with a plastic bag (Figure 1b); in the second group, the lower three leaves of seven plants were similarly covered (Figure 1a). The uncovered part of each plant shoot was sprayed with suspension of bacteria and surfactant. Control plants (n = 2) were not sprayed.

Experiment 2. The two most efficient surfactants determined in Experiment 1 were further tested under

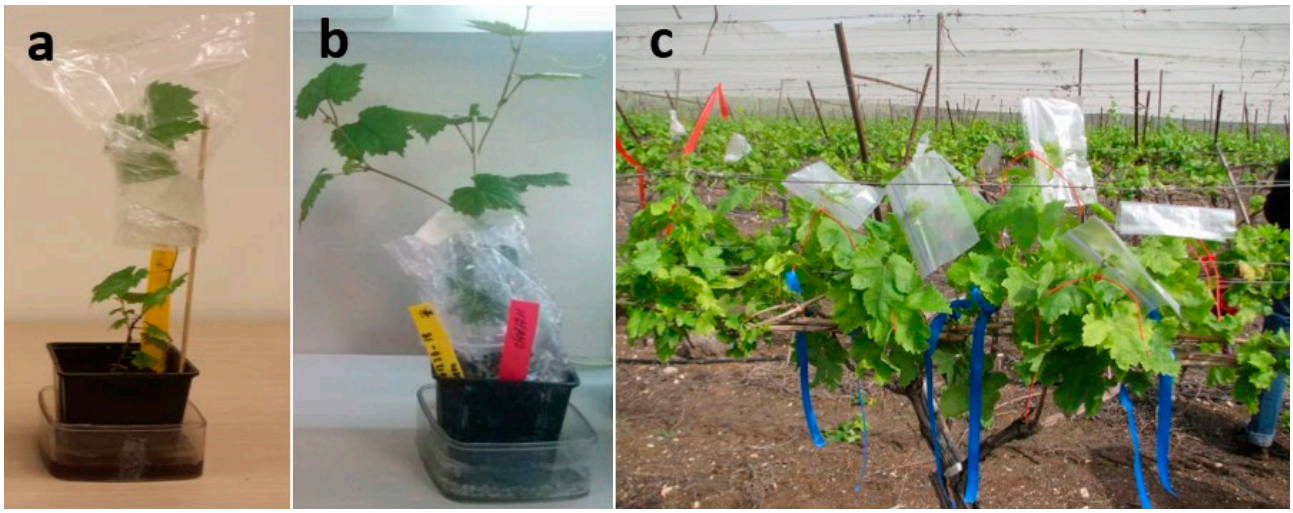

Figure 2. Spraying method. Shoots were partially covered and leaves were sampled 7 d post inoculation from uncovered and covered plant parts. Spray was applied on the shoots of: Lower (a) and upper (b) part of young plants in pots; (c) lower part of mature field grapevine (c).

field conditions. The upper part of each grapevine shoot was covered and sealed with a plastic bag (Figure 1c). Each surfactant was added to a 10^8 cfu mL^{-1} of *Frd* suspension which was sprayed on the uncovered parts of eight shoots.

Experiment 3. To determine the minimum bacterial concentration to give detectable *Frd* establishment in plant tissues in the field, the upper parts of the shoots of field-grown grapevines were covered (Figure 1c) and the uncovered parts were sprayed with *Frd* suspensions (treatments) at concentrations of 10^9, 10^8 or 10^7 cfu mL^{-1}. Tween 20 (0.1%) was added to each suspension as a surfactant. Each treatment was applied to eight shoots, and six marked non-sprayed shoots served as experimental controls.

Table 1. Mean proportions of grapevine tissue samples containing *Frateuria defendens* (*Frd*; determined by PCR) after spray application of bacterial suspensions on different leaf positions on potted grapevine plants 7 dpi (N = 7 per treatment). Main effects: surfactant type, and leaf position relative to sprayed site. Statistical significance was calculated separately for each main effect.

Treatment (%)	% Samples positive for *Frd*			
	On sprayed leaf	Above sprayed leaf	Below sprayed leaf	Main effect: surfactant ($P = 0.51$)
Tween 20 (0.1)	100	57	86	81 A[a]
BB5 (0.2)	100	29	86	71 A
DX (0.5)	100	57	57	71 A
Triton X-100 (0.03)	100	43	43	62 A
Main effect: leaf position ($P < 0.0001$)	100 a	46 b	68 a	

[a] Different letters indicate significant differences between categories ($\alpha = 0.05$): uppercase letters indicate differences between surfactants; lowercase letters indicate differences between leaf positions on the shoots.

Statistical analyses

Descriptive statistics, analysis of variance and Fischer LSD tests were performed with JMP software. Because the non-sprayed shoots in all experiments served as negative controls to confirm that no spray had drifted (*Frd* not present), they were not included in the statistical analyses. The statistical analysis of Experiment 1 showed no significant interactions between the main effects (data not shown). However, in Experiment 3 the statistical analysis showed a significant interaction between cell concentration of sprayed suspension and leaf position, (Figure 2).

RESULTS AND DISCUSSION

Frateuria defendens has been reported as a potential biocontrol agent against phytoplasmas and other phloem-restricted pathogens (Lidor *et al.*, 2018). Because *in planta Frd* occupies the same niche as the target organism and penetrates via plant leaves (Iasur-Kruh *et al.*, 2018; Lidor *et al.*, 2018), it could serve as control against phloem residing pathogens. The presence of *Frd* in leaves of field-grown grapevines 7 dpi was confirmed by PCR (Table 1, Figure 1). In contrast, *Frd* was not detected in

control plants in Experiments 1 or 2, confirming that the bacterium did not naturally inhabit the grapevines. This also indicated that the bacterium was present in the leaf samples solely from its penetration following applications on the leaves.

To promote the potential of *Frd* for application to grapevines, three types of surfactant that are commercially available and commonly used in agricultural practice were compared to Tween 20. When young plants were sprayed, there was no significant difference among the different surfactants, although Tween 20 gave the best results (Table 1). Adding Tween 20 or BB5 to the solution resulted in a better penetration of *Frd* into leaves, as was shown by the greater percentage of positive of leaves below the spraying point scompared to DX and Triton X-100 (Table 1). Therefore, Tween 20 and BB5 were further tested under field conditions. In this experiment, *Frd* was detected in 63% of the samples from leaves above the spraying points, 7 d post-Tween 20 application, whereas all the samples from the BB5 treatment were negative. Therefore, Tween 20 was used in the experiment to optimize bacterial concentration in the spraying solutions (Experiment 3).

Frd has been shown to penetrate perennial and annual plant species of various plant families (Lidor *et al*., 2018), where GFP-labeled bacteria were observed in the vascular systems. The results from the present study broadened these observations by showing that *Frd* can move inside grapevines both upward and downward from original application points in young plants (Table 1). The ability of *Frd* to penetrate the leaves of mature grapevines and move one node upward in the plants was also demonstrated under field conditions (Figure 2).

Spraying is the best method for introducing *Frd* in the plant tissues (Iasur-Kruh *et al*., 2018, Lidor *et al*., 2018). Since spraying intervals in agricultural practice range from several days to weeks, the mobility of the biocontrol agent inside treated plants is critical for its colonization of the non-sprayed plant parts that grow between spray applications. The fact that *Frd* cells penetrate and move along grapevine shoots increases the bacterium's potential as a biocontrol agent (Table 1, Figure 2).

The cell concentration in the applied suspensions possibly affected *Frd*'s ability to penetrate leaves under field conditions. In non-sprayed leaves of treated plants (above the spraying point), *Frd* was detected 7 dpi only when its concentration in suspension was 10^8 cfu mL^{-1} or greater. This indicates that penetration rate and survival are both concentration-dependent (Figure 2). Since laboratory assessment of growth rate showed that *Frd* accumulates in the growth medium to 10^9–10^{10} cfu mL^{-1} within 3–4 d (Lidor *et al*., 2018), it is possible to dilute the growth culture 10- and 100-fold without affecting the rate of penetration into the plant tissues.

The fact that *Frd* reduced yellows symptoms in grapevine plantlets (Iasur-Kruh *et al*., 2018) combined with the results of the present study lead to the hypothesis that the bacterium is a potential biocontrol agent. The results indicate that in order to verify the efficacy of *Frd* to reduce symptoms of yellows disease in grapevine under field conditions, foliar spray should be applied at 7 d intervals with bacterial suspensions of 10^8 cfu mL^{-1} supplemented with 0.1% Tween 20.

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