Identification and Characterization of Menadione and Benzethonium Chloride as Potential Treatments of Pierce's Disease of Grapevines

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ABSTRACT

Xylella fastidiosa infects a wide range of plant hosts and causes Pierce's disease (PD) of grapevines. The type I multidrug resistance (MDR) efflux system is essential for pathogenicity and survival of bacterial pathogens in planta. *X. fastidiosa*, with a single MDR system, is significantly more vulnerable to inhibition by small-molecule treatments than most bacterial pathogens that typically carry redundant MDR systems. A high-throughput cell viability assay using a green fluorescent protein-marked strain of *X. fastidiosa* Temecula 1 was developed to screen two Prestwick combinatorial small-molecule libraries of drugs and phytochemicals (1,600 chemicals in total) approved by the Food and Drug Administration and European Medicines Agency for cell growth inhibition. The screens revealed 215

chemicals that inhibited bacterial growth by >50% at 50 μ M concentrations. Seven chemicals proved to lyse *X. fastidiosa* cells at 25 μ M, including four phytochemicals. Menadione (2-methyl-1,4-naphthoquinone, vitamin K) from the phytochemical library and benzethonium chloride (a topical disinfectant) from the chemical library both showed significant bactericidal activity against *X. fastidiosa*. Both menadione and benzethonium chloride foliar spray (15 and 5 mM, respectively) and soil drench (5 and 25 mM, respectively) treatments were equally effective in reducing PD symptoms by 54 to 59% and revealed that the effects of both chemical treatments became systemic. However, menadione was phytotoxic when applied as a foliar spray at effective concentrations, causing significant loss of photosynthetic capacity.

Xylella fastidiosa is a fastidious, insect-vectored, Gram-negative, xylem-limited bacterium with a broad host range across 300 different plant species in 63 different families (Rapicavoli et al. 2018a). *X. fastidiosa* causes several economically important diseases such as Pierce's disease (PD) of grapevines (*Vitis vinifera*), citrus variegated chlorosis, alfalfa dwarf, phony peach disease, leaf scorch of plum and almond, periwinkle wilt, and the recently emerging olive quick decline syndrome. *X. fastidiosa* strains are also associated with diseases in mulberry, pear, elm, sycamore, oak, maple, pecan, and coffee (Purcell and Hopkins 1996; Rapicavoli et al. 2018a). In grapevines, *X. fastidiosa* cells multiply and spread readily from the site of infection, leading to systemic colonization of the xylem in susceptible plants. Xylem occlusion results in marginally progressing leaf necrosis, desiccation of berries, and abnormal abscission of petioles, eventually resulting in death of the infected vines. Characteristic scorching of the leaves, although associated with water stress, typically occurs in bands, suggesting either toxin or pathogenicity effector activity (Goodwin et al. 1988; Stevenson et al. 2005; Thorne et al. 2006; Zhang et al. 2015). The economic consequences of PD to the California grape industry alone is estimated at \$100 million per year (Alston et al. 2015).

By contrast with most other phytopathogenic bacteria, *X. fastidiosa* lacks a type III secretion system as well as type III effectors that condition pathogenicity, virulence, or suppression of plant defenses. Instead, genes encoding type I, II, and V secretion systems and their effectors have been confirmed to determine

pathogenicity in the genome of *X. fastidiosa* (Chatterjee et al. 2008; Guilhabert and Kirkpatrick 2005; Rapicavoli et al. 2018a; Zhang et al. 2015). The type I secretion system is involved in processes such as drug resistance via multidrug resistance (MDR) efflux and secretion of proteases, hydrolases, and toxins (Delepelaire 2004; Zhang et al. 2015). In Gram-negative bacteria, the type I translocator channel is composed of two inner membrane protein components attached to a highly conserved protein, TolC, that traverses both the periplasm and outer membrane (Holland et al. 2005). Multidrug efflux has been functionally implicated in tolerating plant defenses in *Erwinia chrysanthemi* (Barabote et al. 2003), *E. amylovora* (Burse et al. 2004), *Agrobacterium tumefaciens* (Peng and Nester 2001), *Rhizobium etli* (Gonzales-Pasayo and Martinez-Romero 2000), and *Bradyrhizobium japonicum* (Krummenacher and Narberhaus 2000).

The MDR efflux system of *X. fastidiosa* is essential for both pathogenicity and survival of the PD pathogen in grapevines (Reddy et al. 2007). MDR efflux in *X. fastidiosa* is functionally dependent on *tolC*, encoding the outer membrane and periplasmic tunnel components, and *acrF*, encoding the inner membrane component of the type I system. *X. fastidiosa* strains have only single homologs of both *tolC* and *acrF*, in contrast to most plant pathogens, which have multiple and redundant *tolC* genes and MDR efflux system components. Knockout mutations of *tolC* rendered *X. fastidiosa* nonpathogenic and mutant strains could not be recovered from inoculated plants (Reddy et al. 2007). Knockout mutations of *acrF* reduced pathogenicity of the mutants (D. W. Gabriel lab, unpublished data).

During the course of the above investigations, it was evident that even wild-type *X. fastidiosa*, with its lone MDR efflux system, was much more sensitive to plant-derived antimicrobial chemicals than most other plant pathogens typically carrying multiple efflux systems (Reddy et al. 2007; Tegos et al. 2002). MDR efflux mutants have been useful in highly sensitive and quantitative screens for evaluating antimicrobial chemicals in diverse pathological systems with redundant type I systems (Tegos et al. 2011). The objective of

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this project was to exploit the increased vulnerability of *X. fastidiosa* to chemicals requiring MDR efflux to develop a high-throughput, sensitive, and quantitative live cell screen applicable to small-molecule combinatorial libraries for lead discovery and evaluation of chemicals with potential for use to control PD.

MATERIALS AND METHODS

Small-molecule combinatorial libraries. The Prestwick Chemical Library (1,120 small molecules, 95% drugs approved by Food and Drug Administration and European Medicines Agency) and the Prestwick Phytochemical Library (320 natural plant-derived chemicals) were purchased from Prestwick Chemical (Illkirch, Bas-Rhin, France) in dimethyl sulfoxide (DMSO) in a 96-well plate format (each chemical at 10 mM concentration).

Florescent (green fluorescent protein)-marked *X. fastidiosa* Temecula 1/pSZ90. *X. fastidiosa* PD strain Temecula 1 was grown at 28°C in PD3 medium, consisting of tryptone at 4 g liter⁻¹; soytone peptone at 2 g liter⁻¹; trisodium citrate, disodium succinate, MgSO₄·7H₂O, and KH₂PO₄ at 1 g liter⁻¹ each; K₂HPO₄ at 1.5 g liter⁻¹; and 0.1% (wt/vol) hemin chloride at 10 ml liter⁻¹. The pH was adjusted to 7.0 prior to adding soluble potato starch at 2 g liter⁻¹ and agar at 15 g liter⁻¹. The *P_{trp}::gfp* cassette was polymerase chain reaction (PCR) amplified from pUFZ75 (Zhang et al. 2009) using primers 5' GGG GTA CCG CTC TAA GAA GCT TGG CAA ATA TAC 3' and 5' GCG TCG ACC TAT TTG TAT AGT TCA TCC ATG CCA TG 3', and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Following sequence confirmation, the *P_{trp}::gfp* cassette was subcloned in the wide-host-range shuttle vector pBBR1MCS-5 (*Bordatella* replicon) (Kovach et al. 1995). The resulting plasmid pSZ90 was introduced into PD strain Temecula 1 as described by Zhang et al. (2015).

***X. fastidiosa* culture conditions and high-throughput assay conditions.** Starter cultures of green fluorescent protein (GFP)-marked *X. fastidiosa* (Temecula 1/pSZ90) were grown in PD3 medium plus gentamycin at 3 µg ml⁻¹ for 3 days and subsequently diluted. In initial experiments, 100, 150, and 300 µl of PD3 medium with or without Silwet L-77 at 200 ppm were inoculated from starter cultures into black polystyrene 96-well microtiter plates preloaded with medium with different inoculum densities of *X. fastidiosa* Temecula 1/pSZ90 and incubated with orbital shaking at 28°C at a speed of 120 revolutions/min. Silwet L-77 was added to enable detection of antimicrobial chemicals that might specifically affect the *X. fastidiosa* MDR efflux pump (Reddy et al. 2007). Optical density at 600 nm (OD_{600nm}) and GFP fluorescence (λ_{ex} = 485/20 nm and λ_{em} = 528/20 nm) data were recorded for 6 days using a BioTek Synergy HTX multimode reader (BioTek, Winooski, VT).

For the Prestwick library screens, different chemicals were initially screened at 50-µM concentrations in the preloaded medium and were seeded with starter cultures diluted to OD_{600nm} = 0.05 culture (150 µl well⁻¹). The outermost columns (provided as blanks in the Prestwick library format) were inoculated in the presence of either ampicillin at 1 µg µl⁻¹ + 1% DMSO (positive control, 100% lethality) or 1% DMSO (negative control, no lethality) treatments. To test whether the small-molecule-induced growth inhibition was due to perturbed outer-membrane barrier functions, Silwet L-77 at 200 ppm was added in PD3 medium in a duplicate plate with a similar layout. Plates were briefly centrifuged and incubated at 28°C. Bacterial growth was measured at 48 h after inoculation, both as (correlated) increase in OD (OD_{600nm}) and as GFP fluorescence (λ_{ex} = 485/20 nm and λ_{em} = 528/20 nm) (Steff et al. 2001). The experiments were repeated three times, initiated with different starter cultures and on different days.

The Z' factor (Zhang et al. 1999) was calculated according to the following formula: $Z' = 1 - [(3\sigma_{CK^-} + 3\sigma_{CK^+})/(\mu_{CK^-} - \mu_{CK^+})]$, where σ are standard deviations and μ are the average fluorescence read changes of the negative and positive controls over a period of

48 h. The average fluorescence read changes of negative control (μCK⁻) and the fluorescence read change of each chemical (f) were used to calculate the percent inhibitory activity according to the following equation: percent inhibition = $[1 - f/\mu_{CK^-}] \times 100$.

Phytotoxicity evaluation and photosynthesis measurements. Three leaf disc explants (10 mm in diameter) from young fully expanded grapevine leaves (*V. vinifera* 'Carignane') were floated on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) amended with different concentrations of menadione and benzethonium chloride. Three days after treatment, leaf pigments were extracted in 80% (vol/vol) acetone and total chlorophyll content was quantified as described by Arnon (1949). Photosynthetic CO₂ assimilation rates were measured according to Jain et al. (2012) in untreated healthy control, *X. fastidiosa*-inoculated, and chemical-treated grapevines with a portable photosynthesis gas exchange measurement system (LI-6400; LI-COR, Lincoln, NE) using the following parameters: 28°C leaf temperature, 21% O₂, light at 1,200 µmol m⁻² s⁻¹, and 75% relative humidity at 350 µbar CO₂ concentration. The data were collected for four fully expanded young leaves per plant.

***X. fastidiosa* pathogenicity assays.** Grapevines, approximately 90 cm tall in 3.8 liter pots, were inoculated with *X. fastidiosa* using the needle puncture method, as previously described (Zhang et al. 2015). Five-day-old cultures of *X. fastidiosa* Temecula 1 were grown in PD medium and resuspended to a final OD_{600nm} = 0.25 in SCP buffer containing trisodium citrate, disodium succinate, MgSO₄·7H₂O, and KH₂PO₄ at 1 g liter⁻¹ each and K₂HPO₄ at 1.5 g liter⁻¹, pH 7.0. A 10-µl droplet of bacterial suspension was applied in the middle of the internode and the bark tissue was punctured through the adhering droplet using a sterile tuberculin needle. Inoculations were made on alternating sides of four to five internodes, starting with the second internode from the base. Plant irrigation was withheld for at least 36 h prior to inoculation, facilitating the rapid intake of droplets of bacterial suspension into the xylem stream. Chemical controls, either soil drenches or spray treatments, were applied immediately following inoculation. The same chemical treatments were repeated twice later, at the end of 30- and 60-day periods. Disease severity was quantified by counting the total number of diseased leaves (symptomatic leaves, bare petioles, and bare nodes) and the data presented as percentage of total number of nodes (bare or with symptomatic or asymptomatic leaves attached).

RESULTS

Optimization of *X. fastidiosa* Temecula 1/pSZ90 culture conditions for a high-throughput bioassay to screen Prestwick small-molecule combinatorial libraries. To determine Temecula 1/pSZ90 growth characteristics in flat-bottom 96-well plates, 100, 150, and 300 µl of PD3 medium with or without Silwet L-77 at 200 ppm was inoculated with different initial inoculum densities from starter cultures and incubated with orbital shaking at 28°C. The 150-µl volume of seed culture per well was found to be optimal for consistent growth. Under these culture conditions, GFP fluorescence increased with OD and both correlated well with cell viability. All treatments adversely affecting bacterial viability (OD) also resulted in loss of GFP fluorescence due to protein degradation. Growth of the *ΔtolC* mutant strain was strongly suppressed by Silwet L-77 at 200 ppm, with a concomitant suppression of GFP accumulation. Similarly, antibiotic treatments that decreased cell viability also reduced GFP fluorescence (data not shown). Maximum growth was achieved within 48 h (Fig. 1).

Screening of Prestwick chemical and phytochemical combinatorial libraries for identification of small molecules affecting growth of *X. fastidiosa*. For the primary screen, plates were seeded with *X. fastidiosa* Temecula 1/pSZ90 cells with or without Silwet L-77 at 200 ppm and with each tested chemical loaded at a concentration of 50 µM, in two independent

experiments. The statistical parameter (Z') was used to evaluate the quality of the assays exactly as described (Zhang et al. 1999). The overall Z' scores for the Prestwick Chemical and Phytochemical libraries were 0.78 and 0.76, respectively; within the statistically “excellent” reproducibility range ($Z' > 0.75$) (Zhang et al. 1999).

Significant growth inhibition (>50%) of *X. fastidiosa* Temecula 1/pSZ90 was observed with 193 chemicals (Fig. 2; Supplementary Table S1). Among them, 91 chemicals exhibited growth inhibition between 90 to 100% as compared with controls and 30 chemicals exhibited 100% growth inhibition. Some chemicals (for example tetracycline, chlortetracycline, erythromycin, puromycin, rifampicin, and minocycline) have either antibiotic or major pharmaceutical uses. These were eliminated from further consideration because of anticipated regulatory concerns. Six chemicals (disulfiram, pinaverium bromide, methyl benzethonium chloride, benzethonium chloride, cephalothin sodium salt, and nitazoxanide) exhibited significant growth inhibition that appeared to be possibly enhanced by Silwet L-77 in the primary screen. However, further evaluation in dose-dependent secondary screens at concentrations of 25, 50, and 100 μM revealed no such enhancement (Supplementary Table S3), thus precluding any conclusions of significant effects on membrane barrier functions or TolC from any of the chemicals screened in these tests. Benzethonium chloride, a

commonly used topical disinfectant, provided 100% growth inhibition in three independently conducted secondary screens at all concentrations tested (25 to 100 μM) and was selected for evaluation against PD.

Similarly, the phytochemical library screen revealed significant growth inhibition (>50%) of *X. fastidiosa* Temecula 1/pSZ90 by 22 phytochemicals (Fig. 2; Supplementary Table S2). Six phytochemicals [menadione, 9-methoxyellipticine, olivacine, gossypol, 4,4'-(2,3 dimethyltetramethylene)dipyrrocatechol, and roseoflavin] exhibited >90% inhibition of bacterial growth. None of the 320 phytochemicals displayed evidence of enhanced inhibition in the presence of Silwet L-77 at 200 ppm. Menadione provided 100% growth inhibition in secondary screens at concentrations of 25, 50, and 100 μM , and was also selected for evaluation against PD.

Phytotoxicity evaluation of menadione and benzethonium chloride. Prior to conducting greenhouse experiments for PD control, phytotoxicity of menadione and benzethonium chloride was quantitatively measured by chlorophyll loss in leaf disc senescence assays (Fig. 3) to help determine appropriate or

Fig. 1. Optimal growth of *Xylella fastidiosa* Temecula 1/pSZ90 without contamination in the 96-well plate format was reproducibly achieved using the 150- μl culture volume per well, with the initial inoculation density of an optical density at 600 nm = 0.05. Under these culture conditions, green fluorescent protein (GFP) fluorescence and optical density were correlated with cell viability because any treatment adversely affecting cell viability also resulted in loss of GFP fluorescence due to protein degradation. Maximum growth was achieved within 48 h.

Fig. 2. Growth inhibition (%) of green fluorescent protein-marked *Xylella fastidiosa* Temecula 1/pSZ90 after 48 h in the presence and absence of surfactant Silwet L-77 (200 ppm) across two Prestwick combinatorial small-molecule libraries (drugs and phytochemicals approved by the Food and Drug Administration and European Medicines Agency; 1,600 chemicals in total).

Fig. 3. Effect of menadione and benzethonium chloride treatments on total chlorophyll content in *Vitis vinifera* ‘Carignane’ grapevine leaf discs. Three leaf discs (10 mm in diameter) were floated in Murashige and Skoog medium at indicated concentrations for 18 h. Total chlorophyll was extracted overnight in 80% acetone and quantified. Means ($\pm$ standard deviation) marked with different letters are significantly different based on Tukey-Kramer’s mean separation ($P \leq 0.05$).

maximum treatment concentrations. Leaf disc explants from young, fully expanded *V. vinifera* grapevine leaves were floated on MS medium containing 25 to 100 mM menadione and benzethonium chloride, as indicated. Benzethonium chloride is a surfactant that caused leakage of chlorophyll pigments from the cut sites. However, benzethonium chloride treatment was not significantly detrimental to cellular membranes and chlorophyll integrity at lower concentrations. Grapevine leaves were found to be very sensitive to menadione treatment and resulted in nearly 40 to 45% loss in total chlorophyll content within 18 h at all concentrations tested as compared with water-treated control leaf discs. This response was not dose dependent. By contrast, benzethonium chloride-treated leaf discs exhibited a dose-dependent response in the chlorophyll degradation assay and, although no phytotoxicity was observed at 25 mM, an increasing loss in chlorophyll content was observed at 50-, 75-, and 100-mM treatments (to approximately 33% loss at 100 mM).

Menadione and benzethonium chloride were used at 15- and 25-mM concentrations, respectively, for foliar sprays and at 5- and 25-mM concentrations, respectively, for soil drenches in greenhouse experiments (Fig. 4). Sporadic patches of purple pigmentation and necrosis were evident following foliar sprays of menadione, particularly wherever the spray droplets

Fig. 4. Effect of menadione and benzethonium chloride treatments on Pierce's disease symptoms and on *Vitis vinifera* 'Carignane' grapevine leaves. **A** and **B**, Untreated controls inoculated with *Xylella fastidiosa* Temecula 1; **C** and **D**, menadione treatments, applied as a 5-mM soil drench (**C**) and 15-mM foliar spray (**D**); and **E** and **F**, benzethonium chloride treatments, applied as a 25-mM soil drench (**E**) and 25-mM foliar spray (**F**). Photos were taken 3 weeks postinoculation, showing lower leaves above the first inoculated internode.

accumulated on the leaf blade surface (Fig. 4D). Visible phytotoxicity was not evident with foliar sprays of benzethonium chloride (Fig. 4F) or with soil drench treatments using either chemical (Fig. 4C and E), indicating soil drenches to be the preferred application method.

Menadione and benzethonium chloride treatments delay the progression of PD symptoms in *V. vinifera* grapevines.

V. vinifera grapevines treated with 5 mM menadione or 25 mM benzethonium chloride for foliar sprays, and 15 mM menadione or 25 mM benzethonium chloride for soil drenches, all showed significantly reduced as well as delayed progression of PD symptoms when assessed over a 3-month period (Fig. 5). Following stem inoculations of untreated grapevines with *X. fastidiosa* Temecula 1, approximately 40% of the leaves showed PD symptoms at the end of a 1-month period, progressing to nearly 100% of the leaves either defoliating or symptomatic at the end of 3 months. By contrast, both menadione as well as benzethonium chloride limited the spread of PD symptoms to approximately 50% of the grapevine nodes at the end of 3 months, with limited defoliation and few bare petioles or nodes.

Effect of menadione and benzethonium chloride treatments on photosynthetic efficiency of *V. vinifera* grapevine leaves.

Gas exchange measurements were used to determine photosynthetic CO₂ assimilation rates of *V. vinifera* grapevines showing PD symptoms as well as those treated with menadione and benzethonium chloride (Fig. 6). The photosynthetic efficiency of untreated grapevine leaves showing PD symptoms was severely compromised as compared with *X. fastidiosa*-inoculated and treated leaves, and as well as healthy and untreated control plants. Foliar spray treatment of inoculated plants with 15 mM menadione resulted in an observably phytotoxic response and a significant 35.5% loss in photosynthetic efficiency. Benzethonium chloride (25 mM, soil drench) caused a 21.4% loss of photosynthetic rate. Grapevines inoculated with *X. fastidiosa* Temecula 1 and treated with 5 mM menadione (soil drench) and 25 mM benzethonium chloride (foliar spray) maintained adequate CO₂ assimilation rates, with only 15.2 and 12.3% reductions,

Fig. 5. Effect of menadione and benzethonium chloride treatments applied as soil drench or foliar spray on progression of Pierce's disease symptoms on *Vitis vinifera* 'Carignane' grapevines over a 3-month period. Chemical treatments were applied immediately after *Xylella fastidiosa* Temecula 1 inoculations and repeated twice at monthly intervals; dpi = days postinoculation. Bars represent average $\pm$ standard deviation for five replicates. Means ($\pm$ standard deviation) marked with different letters are significantly different based on Tukey-Kramer's mean separation ($P \leq 0.05$).

respectively, in photosynthetic gas exchange as compared with untreated and uninoculated plants.

DISCUSSION

In this study, a high-throughput screening assay for *X. fastidiosa* cell viability was developed using fluorescence and OD measurements. GFP-marked *X. fastidiosa* Temecula 1 was used to screen two Prestwick combinatorial small-molecule libraries (phytochemicals and drugs approved by the Food and Drug Administration and European Medicines Agency; 1,600 chemicals in total) for *X. fastidiosa* cell growth inhibition. None of the compounds from either library exhibited enhanced inhibition in the presence of Silwet L-77 at 200 ppm, indicating any effect on MDR efflux. Several of the chemicals identified as growth inhibitory or promoting cell lysis were eliminated from further consideration either for being cost prohibitive or for current pharmacological uses and therefore likely to face serious regulatory concerns. Based on primary and secondary screening data, menadione and benzethonium chloride were identified as promising lead discoveries for chemical control of PD.

Leaf disc senescence assays were used in an attempt to determine effective rate limits of the chemical treatments. Leaf disc assays that measure chlorophyll loss as a function of phytotoxicity are commonly used to assess abiotic stress conditions (Jain et al. 2012, 2018). Benzethonium chloride is a surfactant that might be expected to disrupt lipid membrane structure and cause chlorophyll degradation. Consistent with its detergent like action, benzethonium chloride caused chlorophyll degradation when applied to leaf discs in a dose-dependent manner (Fig. 3). Menadione also caused significant chlorophyll degradation in the grape leaf assays but the effect was not dose dependent. Menadione reduction by NAD(P)H cytochrome P450 oxidoreductase is required for sustained redox cycling and reactive oxygen species (ROS) generation (Gray et al. 2016). It seems likely that the available cellular NADPH pool was exhausted even at the lowest tested concentration of menadione (25 mM), thus limiting redox cycling and ROS generation at higher menadione concentrations.

Both menadione and benzethonium chloride were further evaluated as potential treatments for PD by both soil drench and spray applications and for phytotoxicity to grape leaves. Based on both chlorophyll degradation assays (Fig. 3) and photosynthesis measurements (Fig. 6), menadione was strongly phytotoxic when applied directly to grapevine leaves, consistent with the observed apoptotic cell death response in menadione-treated plant cells (Sirisha et al. 2014; Sun et al. 1999). When applied as soil drenches, both chemicals significantly reduced progression of PD symptoms (54 to 59% compared with untreated controls) over a period of 3 months, demonstrating that the effects of the chemical treatments were systemic and highly efficacious.

Liposoluble compounds such as menadione influence both the integrity and fluidity of bacterial membranes (Pretto et al. 2004) and have been shown to enhance antibiotic activity via increased cell membrane permeability (Andrade et al. 2017), while also affecting bacterial efflux systems (Tintino et al. 2018). At low concentrations, menadione acts as a plant defense activator by generating ROS at multiple cellular sites (Gray et al. 2016), thus enhancing both local and systemic resistance to pathogens. Menadione pretreatments triggered redox-dependent defense gene expression in oilseed rape (*Brassica napus*), resulting in effective disease resistance against stem canker caused by *Leptosphaeria maculans* (Borges et al. 2003). Likewise, enhanced biosynthesis and accumulation of antifungal phytoalexins was observed in menadione-pretreated banana (*Musa acuminata*) plants infected with Panama disease pathogen *Fusarium oxysporum* f. sp. *cubense* (Borges et al. 2004). Menadione pretreatments suppressed gray mold (*Botrytis cinera*) symptoms on tomato leaves through reduced conidia germination and mycelial growth (Hong et al. 2016).

Xylem parenchyma cells provide H_2O_2 that is necessary for the lignification of differentiating xylem vessels (Ros Barcelo 2005), thereby exposing the xylem-limited *X. fastidiosa* to an extremely oxidative environment at steady-state levels within the xylem sap. Therefore, in addition to being directly antimicrobial, it is highly likely that menadione induces the oxidative burden, including H_2O_2 (Gray et al. 2016), to highly antimicrobial levels. Further, H_2O_2 compromises *X. fastidiosa* OxyR, an oxidative stress regulatory protein that is essential for surface attachment, cell-to-cell aggregation, and biofilm maturation (Wang et al. 2017). Successful xylem colonization by *X. fastidiosa* is dependent upon delaying immune recognition and elicitation of a host-mediated oxidative burst (Rapicavoli et al. 2018b).

Benzethonium chloride is a synthetic quaternary ammonium salt commonly used as a topical disinfectant in the food industry and in antiseptic preparations. Benzethonium chloride exhibits broad-spectrum microbicidal activity against bacteria and viruses (Sickbert-Bennett et al. 2005), human tumor cells (Yip et al. 2006), and pathogenic free-living amoebae *Acanthamoeba lugdunensis* and *A. quina* (Lukáč et al. 2013). Siles et al. (2013) identified benzethonium chloride as a lead compound in a small-molecule screen for inhibition of *Candida albicans* biofilm formation.

X. fastidiosa causes some of the most serious plant diseases worldwide and in a wide variety of hosts, including (i) PD of grapevines in the United States (*X. fastidiosa* subsp. *fastidiosa*), (ii) citrus variegated chlorosis in sweet orange and coffee and leaf scorch in Brazil (*X. fastidiosa* subsp. *pauca*), (iii) leaf scorch of oleander (*X. fastidiosa* subsp. *sandyi*), (iv) leaf scorch of mulberry and blueberry in the United States (*X. fastidiosa* subsp. *morus*), and (v) leaf scorch diseases in North America on a wide range of tree species such as almond, peach, and oak (*X. fastidiosa* subsp. *multiplex*) (Almeida and Nunney 2015; Nunney et al. 2014; Rapicavoli et al. 2018a), and more recently associated with quick decline of olive trees in Italy (*X. fastidiosa* subsp. *pauca*) (Saponari et al. 2017). Both menadione and benzethonium chloride used as soil drenches caused systemic effects on *X. fastidiosa* in *V. vinifera*

Fig. 6. Effect of menadione and benzethonium chloride applied as soil drench or foliar spray on photosynthetic CO₂ assimilation rates (leaf CO₂ concentration at 350 μbar) in *Xylella fastidiosa* Temecula 1-inoculated *Vitis vinifera* 'Carignane' grapevine leaves. Chemical treatments were applied immediately after inoculations. Gas exchange data were collected from four leaves from each plant one month postinoculation. Means (± standard deviation) marked with different letters are significantly different based on Tukey-Kramer's mean separation ($P \leq 0.05$).

grapevines, and reduced PD symptoms and disease progression by approximately 50%. There is no reason to believe that either chemical would not also have systemic effects and potential applications as soil drench treatments for citrus, coffee, olive, or blueberry. Menadione is used in animal feed and pet foods as a vitamin K substitute and the cost of both chemicals is relatively low. One of the major limiting factors to growing *V. vinifera* grape in Florida is PD, and these two potential chemical control treatments should be considered for field trials to control *Xylella* diseases of several crops in addition to grapevines.

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