Blue EaLAMP—a specific and sensitive method for visual detection of genomic Erwinia amylovora DNA

ARTICLE *in* EUROPEAN JOURNAL OF PLANT PATHOLOGY · DECEMBER 2012

Impact Factor: 1.49 · DOI: 10.1007/s10658-012-0059-5

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Potent and Specific Bactericidal Effect of Juglone (5-Hydroxy-1,4-naphthoquinone) on the Fire Blight Pathogen *Erwinia amylovora*

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Supporting Information

ABSTRACT: A screening of plant quinones for inhibiting effects on the bacterial fire blight pathogen *Erwinia amylovora* was performed. The most active compound, juglone from walnuts, has a potent and specific bactericidal effect on *E. amylovora* and minimal inhibitory concentrations of only 2.5−10 μM, with stronger effects at lower, but still physiological, pH values. In vitro tests with juglone and inoculated flowers of apple (*Malus domestica*) showed an efficacy of 67% in preventing infection. In two years of field tests juglone had variable degrees of efficacy ranging from 40 to 82%, seemingly due to environmental conditions. A phytotoxic reaction to juglone, which is known for its allelopathic effect on plants, was restricted to browning of petals; later fruit russeting was not observed. Juglone is a promising candidate for the development of a new environmentally friendly plant protectant to replace the antibiotic streptomycin currently used in fire blight control.

KEYWORDS: *bactericides, fire blight control, naphthoquinones, pesticide development, plant quinones*

INTRODUCTION

The Gram-negative bacterium *Erwinia amylovora* is the causal agent of the fire blight disease of pome fruit. The primary infection mainly occurs via flowers, where the bacteria are distributed by pollinating insects, mainly honeybees. The infection spreads in the plant via the xylem and leads to progressive necrosis of infected shoots. There are no curative treatments, but trees may be saved by pruning before the infection reaches the stem. To date, the pathogen is present almost worldwide.[1] It causes severe losses of orchard plants and harvests in apple (*Malus* × *domestica*), pear (*Pyrus communis*), and quince (*Cydonia oblonga*) and is of substantial economic impact. Measures to restrict fire blight focus on avoidance of trading infected material and on spraying of active agents during bloom. Indications for such treatments are based on occurrences in previous years, ELISA and PCR testing, bacteria isolation, and a climatic prognosis system relying on integral thresholds for temperature and moisture.[2−4]

Measures that are successfully applied include biological approaches as well as chemical agents. The treatment of orchards during bloom with up to 600 g/ha streptomycin is the most widespread approach in horticultural practice.[1] Streptomycin is an aminoglycoside antibiotic from *Streptomyces griseus* and acts on prokaryotic ribosomes.[5,6] To some extent oxytetracycline and kasugamycin are also used.[7] However, due to environmental considerations and to prevent resistance development by ribosomal point mutations or acquisition genes for degrading enzymes in *E. amylovora*[8] or in human pathogens, such treatments are strongly regulated in many countries.[7] Other chemical control agents (bactericides, disinfectants) are in use or being tested.[7] A different strategy of chemical fire blight control is the induction of plant resistance leading to systemically acquired resistance (1,2,3-benzothiadiazole-7-carbothioic acid-*S*-methyl ester BTH, Bion; harpin protein of *E. amylovora*)[7,9,10] or to the formation of antimicrobial compounds by the plant itself (induced by calcium 3-oxido-4-propionyl-5-oxocyclohexene carboxylate, prohexadione-Ca, Regalis).[11,12] Biological control of fire blight focuses on the application of antagonistic microorganisms, which are also important measures for their low ecologic impact. Antagonistic bacteria that are commercially available are mainly *Bacillus subtilis*, *Pseudomonas fluorescens*, *Erwinia herbicola* (syn. *Pantoea agglomerans*), *Erwinia tasmaniensis*, and *Erwinia billingiae*. Nonpathogenic *E. amylovora* strains and bacteriophages of *E. amylovora* have also been studied.[13] The epiphytic yeast *Aureobasidium pullulans* (Blossom Protect) and other fungi are also used in biological fire blight control.[14]

Within a project on the development of new bactericides against the fire blight pathogen, plant secondary metabolites were screened for new antimicrobial substances that would also be degradable and environmentally acceptable. This screening focused on plant quinones.

MATERIALS AND METHODS

Chemicals. Test compounds were purchased from Merck (Darmstadt, Germany) (1,4-benzoquinone, **1**), Sigma-Aldrich (Taufkirchen, Germany) (duroquinone, **2**; juglone, **3**; lawsone, **8**; menadion, **5**; plumbagin, **4**; naphthazarin, **6**; shikonin, **7**), and Alfa-Aesar (Ward Hill, MA, USA) (bilawsone, **9**). Mushroom tyrosinase was purchased from Biochemika (Düsseldorf, Germany); other chemicals were from Sigma-Aldrich.

Received: April 24, 2012
Revised: November 14, 2012
Accepted: November 20, 2012
Published: November 20, 2012

Bacterial Strains. *B. subtilis* NCTC 10315, *E. amylovora* 295/93, *E. billingiae* 6830, *Erwinia piriflorinigrans* 5888, *Erwinia pyrifoliae* 4171, *E. tasmaniensis* 37/10, *Escherichia coli* EcB, EcB 2201, and XL-Blue, *P. agglomerans* 599, *Pectobacterium carotovorum* NCPB 312, and *P. fluorescens* A 506 were obtained from the AGES (Austrian Agency for Health and Food Safety, Vienna, Austria). All other strains of *E. amylovora* were kindly provided by K. Richter, Institute for Resistance Research and Stress Tolerance (JKI), Quedlinburg, Germany.

Assay for Inhibiting Activity of Compounds in Suspension Culture. Assays were performed by testing the inhibition of growth of *E. amylovora* 295/93 in suspension culture in King's B medium (20 g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 10 mL/L glycerol, pH 7.2). The highest concentration of test compound tested was 1 mM in most cases; standard was 0.1 mM. An overnight culture was diluted with King's B medium to OD_{600} of 0.2 and 2 mL; each was transferred to individual photometric cuvettes. Test compounds were dissolved as 100, 10, or 1 mM stock solutions in ethanol or dimethyl sulfoxide (DMSO). Aliquots from these were used to apply the compounds to the assay in their respective end concentrations (up to 5% v/v); solvent control assays were always run in parallel. The cuvettes were sealed with parafilm, and the initial OD_{600} was measured. Then the cuvettes were incubated at 28 °C and 200 rpm, and OD_{600} values were taken every 2 h (0, 2, 4, 6, 8 h) and at 15 or 24 h. All tests were performed at least in triplicate. For analysis of the pH dependence of juglone activity, King's B medium was adjusted to various pH values between 4 and 8 with 1 M HCl or 1 M NaOH before sterilization. An inducing medium simulating infection conditions was used according to the method of Wei et al.[15] (0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.5 mM MES, 350 mM sorbitol (instead of 175 mM mannitol), 5 mM $(NH_4)_2SO_4$, and the pH was adjusted to 7.0. Minimal inhibitory concentrations (MIC) were determined by serial tests with variable concentrations of the test compound in parallel to solvent controls; the MIC of this compound is the lowest concentration found to completely inhibit bacterial growth in this suspension culture after overnight incubation.

In Vitro Flower Inoculation Assay. Single-flower inoculation assays with juglone were performed according to an assay developed by Pusey[16] and adapted by Marschall (personal communication). In early spring dormant potted apple trees (cv. Gala grafted on rootstock M9) were transferred to a greenhouse at 15 °C, later at 22 °C. Flowering occurred approximately 3 weeks later, and flowers at the "balloon stage" (day before opening) were collected. Series of 20 flowers each were put with their peduncles in 2 mL cups with sterile 10% sucrose solution, and these were incubated in transparent plastic boxes at 28 °C for 24 h to induce flower opening. In parallel, two strains of *E. amylovora* (295/93 and 763) had been grown on King's B medium agar plates and then were transferred to sterile 0.05 M Na/KP_i buffer, pH 7.0, with 0.03% Tween 20 and were adjusted to 1×10^7 colony forming units (CFU)/mL; both strains were mixed in equal proportions. For flower inoculation, 1 μL of the mixed 1×10^7 CFU/mL was distributed on the five styles of each flower. After 2 h of incubation, spraying with the test compounds and control treatments were performed. The test compound solution of juglone, water as a negative control, and 0.06% Strepto in water as a positive control were applied in separate plastic boxes. Application was performed to each individual flower of a series by pumping two times 150 μL spray solution with a hand sprayer from above. The plastic boxes with the different spray applications were supplemented with 32.6% aqueous glycerol solution at their bottoms to provide a constant air humidity of 70% after closure. After 2 days of incubation at 22 °C, 50 mL of water with fungicide (5 g Cantus/L) was sprayed uniformly in each opened box with a manual pump sprayer, simulating a moisture event that would transport the bacterial cells to the hypanthium of the flowers. After a total of 8−10 days of incubation at 22 °C, flowers were evaluated for infection symptoms (bottom of flowers with brown to black color, wet looking; excluding browning of styles or petals as infection symptoms), and the number of infected flower per series was determined. The whole assay was considered to be successfully performed only if at least a 50% infection rate was seen with the water/negative control. An efficacy percentage was calculated as $\eta = (1 - (\text{rate of infections with test compound})/(\text{rate of infections with water control treatment})) \times 100$.

Field Testing with Trees Inoculated during Bloom. Field tests with artificially inoculated trees and juglone were performed in the years 2010 and 2011 at two isolated test fields in Vogt and Mühlingen, which are organized by the Kompetenzzentrum Obstbau-Bodensee (Bavendorf, Germany). Test plants were in each series 10 3-year-old potted apple trees of the cv. Gala grafted on M9 rootstocks and additional potted apple trees of other cultivars placed closely as pollinators (all tree sizes = 1.4 m, diameters = 0.75 m). Each experimental variant was performed in four parallels and was randomized. Inoculation was performed indirectly by placing one additional inoculated tree (sprayed with 2.6×10^8 CFU/mL of a mixture of three virulent *E. amylovora* strains (797, 839, 894) per tract) and by positioning a bee hive in the experimental orchard field to provide an efficient measure for spreading the infection. Application of the test compound and controls was performed 2 h before inoculation and two or three times within the next days, either 2 h after fire blight infection conditions were reached or when regional warning status was reached.

Streptomycin as a positive control was applied as a 0.06% solution Strepto (containing 21.2% streptomycin sulfate) (0.3 kg/500 L; 500 L/ha and m tree height); juglone was applied in 2010 three times as 0.1 mM solution (predissolved as a 10 mM solution in ethanol; diluted 1:100 in water directly before application; equals 8.7 g/500 L; 500 L/ha and m tree height). In 2011 juglone was applied four times at 0.5 mM in 0.1% citric acid (5% ethanol from predissolving) adjusted to pH 4.5 with sodium hydroxide. For application a motor backpack spraying device was used. The rating was performed by discriminating and counting infected against uninfected inflorescences. The whole tests were performed according to EPPO PP1/166(3).

Test for Juglone Stability with UV and Visible Light. The stability of juglone with UV light and with visible light was tested by exposing 1 mM juglone in ethanol to UV plus visible light (bright sunlight) and to only visible light (bright sunlight behind UV nontransparent glass screen) for 1 h. Together with juglone incubated in the dark as a control, these solutions were applied with a nominal concentration of 10 μM, which corresponds to the MIC and should completely inhibit growth of *E. amylovora* cells in the suspension culture.

Histochemical Staining for Tyrosinase Activity. The presence and distribution of tyrosinase activity at flowers of *M. domestica* cv. Gala were analyzed with the reagent 3-methyl-2-benzthiazolinonhydrazone (MBTH).[17] Spraying was performed with the test solution of 6 mM MBTH and 15 mM of the substrate tyrosine in 1 M NaP_i, pH 7.0, with 3% ethanol and 0.03% Tween 20. The flowers were incubated for 15 min with the applied test solution and documented afterward using a binocular microscope.

Test for Juglone Stability with Tyrosinase Activity. Due to the unavailability of suitable amounts of tyrosinase from apple flowers, the effect of mushroom tyrosinase on juglone was tested at pH 7.2 and 4.5. For this, from a growing suspension culture of *E. amylovora* 295/93 a volume equivalent was centrifuged resulting in an OD_{600} of 0.2 after resuspending the cells in each 1 mL of the different test solutions. All combinations of 25 μM juglone (diluted from 10 mM stock in ethanol) present or absent and 0.5 mg mushroom tyrosinase present or absent were tested at pH 7.2 and pH 4.5 in King's B medium, including 0.25% ethanol for every preincubation, also in the controls without juglone addition. The preincubation lasted for 1 h, then each 10 μL of the bacterial suspensions were serially diluted 1:100, 1:1000, 1:10000, and each 10 μL of the respective dilutions of each variant were plated on King's B agar plates and incubated overnight for colony growth and counting.

In Vitro Pollen Germination Test and in Vivo Fertilization Test. The germination rate of apple pollen (cv. Golden Delicious) was tested at variable pH values. Pollen had been collected 2 weeks before, air-dried, and kept at −20 °C. Pollen germination media were prepared according to the method of Flachowsky et al.[18] and consisted of 10% sucrose, 25 mg/L H_3BO_3, and 250 mg/mL $Ca(NO_3)_2 \cdot 4H_2O$ and were adjusted to variable pH values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and

7.5) before dissolving in 1% agar by boiling. Each 2 mL of the warm solutions was spread on glass slides. In parallel, the dried pollen had been rehydrated in sterile deionized water. After solidification of the agar media on the glass slides, three times 10 μL pollen suspensions were spread on the media of each pH and were incubated for 20 min at room temperature in a dark and moist box. Pollen germination rates were determined by counting pollen tubes under the binocular microscope.

The fertilization rate in vivo at pH 4.5 was tested by spraying of 0.1% citric acid and 0.1% Tween 20 (adjusted with sodium hydroxide to pH 4.5) or water with 0.1% Tween 20 to potted trees during bloom, on which flowers had been protected from uncontrolled pollination before by paper bags. On each plant labeled flowers were pollinated by hand after drying of the applied solutions, using pollen from a compatible cultivar as a donor.

Phytotoxicity Test. In 2011 phytotoxicity of juglone solutions was tested on a large scale with the apple cv. Golden Delicious clone B, which is especially sensitive to fruit russeting upon flower treatments with agrochemicals. Testing was performed at the Kompetenzzentrum Obstbau-Bodensee (Bavendorf). The application was performed 3-fold during bloom (BBCH 63, BBCH 65, and BBCH 67) with a Holder Querströmer applying 200 L/ha and m tree height. Rating of fruit russeting was performed with 1000 harvested fruits, differentiating the following classes of fruit russeting: 1, no fruit russeting; 2, up to 10%; 3, 10−30%; 4, >30% fruit russeting. An index was calculated: fruit russeting index = (fruits in class 1 × 1 + fruits in class 2 × 2 + fruits in class 3 × 3 + fruits in class 4 × 4)/total number of fruits.

RESULTS AND DISCUSSION

Screening of Plant Quinones for Bactericidal Activity on *E. amylovora*. Much variation in the MICs of the screened quinones (Figure 1) was found. Figure 2 shows the effect of some quinones at a concentration of 100 μM (A) and of juglone at concentrations from 5 to 25 μM (B) in suspension cultures. Juglone, **3**, and naphthazarin, **6**, showed MICs of only 10 μM, 1,4-benzoquinone, **1**, plumbagin, **4**, and shikonin, **7** of approximately 100 μM, menadione, **5** of 250 μM, and lawsone, **8**, of 2.5 mM. Duroquinone, **2** and bilawsone, **9**, showed no inhibitory effect. Streptomycin showed a MIC of about 10 μM under these conditions (data not shown).

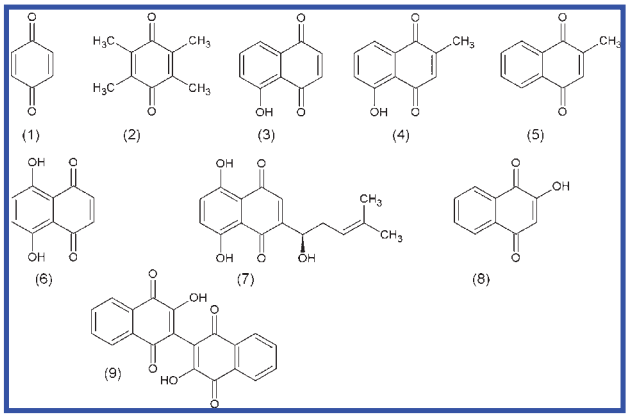

Figure 1. Chemical structures of plant-derived quinones (or basic quinone structures) used for screening of bactericidal effects on *Erwinia amylovora*: 1,4-benzoquinone, **1**; duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone), **2**; juglone (5-hydroxy-1,4-naphthoquinone), **3**; plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), **4**; menadione (2-methyl-1,4-naphthaquinone), **5**; naphthazarin (5,8-dihydroxy-1,4-naphthoquinone), **6**; shikonin (5,8-dihydroxy-2-(1-hydroxy-5-methylpent-3-enyl)-1,4-naphthoquinone), **7**; lawsone (2-hydroxy-1,4-naphthoquinone), **8**; bilawsone 2,2′-bis(3-hydroxy-1,4-naphthoquinone), **9**.

Arbutin, the 1,4-hydroquinone glucoside of pear, has early been considered as a defense compound of pear against fire blight.[19] Despite an in vitro activity of arbutin or its oxidation products against the bacteria,[20] pear is generally more susceptible to infection in comparison to apple, which contains large amounts of dihydrochalcones instead of arbutin as its major secondray metabolite. Jin and Sato[21] clearly demonstrated the formation of 1,4-hydroquinone and 1,4-benzoquinone, **1**, from pear leaf extracts and the bactericidal activity of 1,4-benzoquinone against *E. amylovora*. A level of 1−2 ppm 1,4-benzoquinone (equals approximately 10−20 μM) was found as MIC with 10 min of exposure to it compared to a MIC of 100 μM in our study with another strain of *E. amylovora*. Despite this significant effect of a pear metabolite, pear susceptibility seems to be due to the absence of β-glucosidase in nectaries,[19] which are a main site of infection. However, this potential of a quinone as bactericidal compound triggered interest in quinones of other structural classes to be considered as exogenously applicable bactericides, even taking into account their known instability.

Structural Features of Active Quinones. 5-Hydroxylation of 1,4-naphthoquinones is a structural feature leading to high bactericidal activity against *E. amylovora*, as can be seen by the low MICs of juglone, **3**, naphthazarin, **6**, plumbagin, **4**, and shikonin, **7**, or the comparison of plumbagin, **4**, and menadione, **5**, with MICs of 100 and 250 μM, respectively. An additional 8-hydroxylation is not detrimental to such an activity, as can be seen with the respective pairs juglone, **3**/naphthazarin, **6**, each with a MIC of 10 μM, and plumbagin, **4**/shikonin, **7**, with 100 μM MIC. Juglone, **3**, versus plumbagin, **4**, and naphthazarin, **6**, versus shikonin, **7**, illustrate the negative effect of aliphatic substitutions in position 2, possibly influencing the distribution of the active compounds in the cytosol or membranes, by increasing their lipophily. The 2-hydroxylated 1,4-naphthoquinone lawsone, **8**, as an isomer of juglone, **3**, in contrast, shows only little activity. Additionally, several anthraquinones were tested besides the 1,4-naphthoquinones and 1,4-benzoquinones but exhibited no bactericidal effect (data not shown).

pH Dependence of Juglone Activity. The most active compound, juglone, **3**, from this screening was studied for its specific characteristics. Taking into consideration the inhibiting activity of juglone and the known instability of quinones under alkaline conditions and to other nucleophilic agents,[22] the pH dependence of the juglone activity in the suspension culture assay was tested. For this, a concentration of 2.5 μM, less than half the MIC at pH 7.2, was used, and a pH range from 4.0 to 8.0 was tested. For most pH values tested, bacterial growth was still found with 2.5 μM juglone. However, the inhibitory effect of juglone proved to be strongly dependent on pH, with the highest activity of juglone at the lowest pH values (data not shown). The MIC for juglone for the pH value of 4.5 was determined and found to be at only 2.5−5 μM.

Minimal Inhibitory Concentration of Juglone in *hrp* Gene Inducing Medium. *E. amylovora* has a different physiology under conditions of infection. When this is simulated by special medium conditions, characteristic *hrp* gene expression does occur.[15] *hrp* genes of *E. amylovora* mediate bacterium−plant interactions and perform functions in the regulation, secretion, and code for secreted proteins of the bacterium[10] and hence are indicative for the perception of

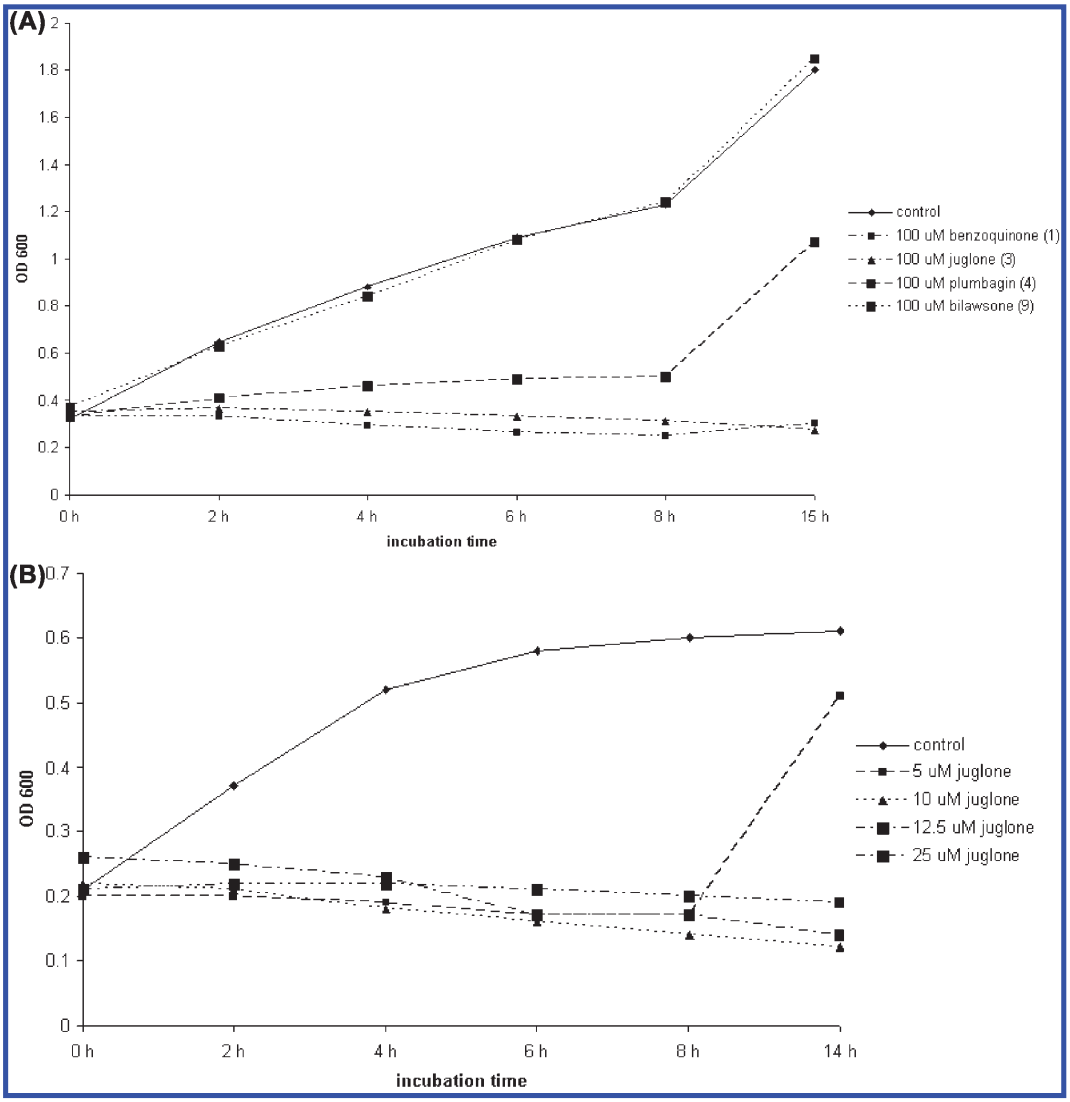

Figure 2. Exemplary screening results of the effects of 1,4-benzoquinone, **1**; juglone, **3**; plumbagin, **4**; and bilawsone, **9** on *Erwinia amylovora* at a concentration of 100 μM (A) and dependence of the bactericidal effect of juglone in a low concentration range of 5−25 μM (B). Last time point on abscissa is not to scale.

infection conditions by the bacterium. In our study, sufficient growth for the inhibition assay could be obtained only with 350 mM sorbitol (the major sugar alcohol of Rosaceae)[23,24] instead of 175 mM mannitol within this medium. The MIC of juglone was determined with this inducing medium (pH 7.0) to be only 5 μM. In comparison, a MIC of 10 μM under optimal growth conditions in King's B full medium (pH 7.2) was found. In conclusion, *E. amylovora* can also be expected to be sensitive to juglone under infection conditions at plant tissues.

Specificity of the Bactericidal Effect of Juglone. Screening of quinones was performed with a representative isolate of *E. amylovora* (strain 295/93). Another 16 strains of *E. amylovora* were investigated with juglone in suspension culture for putative strain-specific effects on MIC. For this, strains of *E. amylovora* isolated in different geographic regions and from various host plants (all Maloideae) were chosen. MICs were all found to be between 10 and 20 μM juglone at pH 7.2 in suspension culture in King's B. Other species of *Erwinia* and other bacterial genera were also tested for their sensitivity for juglone, and a broad range of MICs was found. *E. pyrifoliae*, which is also pathogenic on pome fruit,[25] is comparably as sensitive as *E. amylovora* with a MIC of 20 μM. *E. tasmaniensis*, *E. billingiae*, and *P. agglomerans* (formerly *E. herbicola*) are all not plant pathogenic,[26,27] are commonly epiphytic on apple and pear, and are used for biological control of fire blight during bloom. These nonpathogenic species were found to be much less sensitive to juglone with MICs of 500, 1000, and 500 μM, respectively. The species *E. piriflorinigrans*, which is pathogenic on pear blossoms,[28] is also relatively insensitive, with a MIC of 500 μM. *P. carotovorum* (formerly *Erwinia carotovora*), a potato pathogen, has a relatively high MIC of 500 μM for juglone. In contrast, *B. subtilis*, an unrelated Gram-positive bacterium used for biological control of fire blight, is sensitive to juglone with a MIC of 50 μM. Another bacterium used for biological control of fire blight, *P. fluorescens* A506, is not sensitive and shows a MIC of >1000 μM. Also, *E. coli* proved to be almost insensitive up to 1000 μM juglone.

Bactericidal Effect. Photometric measurements of optical density in the growth inhibition assay used for screening and MIC determination do not allow discriminating between living but not-growing cells and dead but nonlyzed cells of *E. amylovora*. Differentiation between the quality of the inhibitory effect, either bacteriostatic or bactericidal, was performed by brief exposure of the minimal inhibitory concentration of juglone to cells of *E. amylovora* in suspension culture and subsequent plating on King's B medium. This showed that exposure of a starting culture (approximately 10^5 cells treated) with 10 μM juglone for 30 min led to a completely bactericidal effect. In agreement with this result, also in the growth inhibition assay in suspension culture with permanent exposure of approximately 2×10^8 cells to the test compound juglone, a late restarting of growth from surviving cells has only been observed with juglone concentrations lower than the MIC of 10 μM. The very low MIC and the bactericidal mode of action make juglone a promising candidate compound for fire blight control.

In Vitro Flower Inoculation Assay. An in vitro flower inoculation assay was used for first tests for bactericidal activity of juglone applied to the host plant organs of *E. amylovora* in apple (Figure 3). For this, flowers of the *M. domestica* cv. Gala

Figure 3. In vitro test with inoculated flowers. Evaluation after 8−10 days: left, healthy; right, infected.

were transferred to in vitro conditions, inoculated with 10^4 CFU of a mixture of two strains of *E. amylovora* (295/93 and 763), and subsequently treated with juglone. Parallel water and streptomycin treatments provided negative and positive controls. An efficacy of 67% was found with 0.01 mM juglone in comparison to 75% for streptomycin. On the basis of this result, field testing was projected.

Field Testing with Trees Inoculated during Bloom. Field testing of juglone as a bactericide against *E. amylovora* was performed under orchard conditions with apple trees artificially inoculated during bloom, at geographically isolated localities maintained for field testing. Streptomycin treatment (0.06%) served as a positive control and water treatment as a negative control and for determination of the infection rate without control measures. In the first year of testing in 2010, juglone was tested as a 0.1 mM solution in water without any formulation. Two localities (Mühlingen, Vogt) in the Lake Constance region were used for testing. Due to weather conditions in 2010, which were unsuitable for fire blight infection with low temperatures during early blossom (6.8 and 12.4 °C during inoculation), only low infection rates of 2.5% (Mühlingen) and 2.8% (Vogt) were reached in the control, not fulfilling EPPO standards with 5% infection rate for secure analysis. With this reservation, at Mühlingen an efficacy of 85.6% was observed for the streptomycin positive control and an efficacy of 75.6% was found for juglone. At Vogt, streptomycin treatment showed an efficacy of 86.1% and juglone an efficacy of 52.6% (statistical validation was not possible for both experiments with each four parallels due to lack of homogeneity of variance). In 2011, juglone was tested with a higher concentration of 0.5 mM in 0.1% citric acid at pH 4.5 to stabilize juglone and to prevent its enzymatic degradation and inactivation on the plant tissues. Weather conditions were more suitable for fire blight infection in 2011 at both test localities; a 9.8% general infection rate were reached at Mühlingen and 12.3% at Vogt, fulfilling EPPO test standards for fire blight control test applications. At Mühlingen, where applications were performed in the morning, streptomycin reached an efficacy of 78% and juglone, of 40%. At Vogt, where applications were performed in the afternoon, streptomycin showed an efficacy of 95% and juglone, of 82%.

Phytotoxicity. In 2009, phytotoxicity has been tested on a small scale with a low concentration of 0.05 mM juglone on flowers of the apple cv. Idared and the pear cv. Williams Christ. Browning reactions on the petals of opened flowers were found as the only visible reaction (Figure 4). An influence on flower

Figure 4. Phytotoxic reaction of *M. domestica* cv. Idared flowers 3 days after treatment with 0.05 mM juglone solution (unaffected petals had still been closed at the time of application).

attractiveness to bees, which could lower the pollination rate and fruit set, may occur. In early August, fruits derived from such flowers were evaluated, and no fruit russeting was observed. Hence, in 2011 a larger scale standard test for fruit russeting caused by application of agrochemicals to apple flowers was performed with Golden Delicious clone B, which is quite sensitive to such reactions. A concentration of 0.5 mM juglone at pH 4.5 was tested, in parallel to 0.06% streptomycin and an untreated control. Fruit russeting indices were 1.57 for juglone, 1.79 for streptomycin, and 1.75 for the untreated control, clearly indicating no influence of juglone on fruit russeting.

Stability of Juglone with Environmental Influences. Degradation of juglone exposed to environmental conditions at the plant was considered. Two seemingly important processes were experimentally tested. The stability of juglone with UV light and with visible light was tested by exposing it to UV plus visible light (bright sunlight) and to only visible light (bright

sunlight behind UV nontransparent glass screen) for 1 h, in comparison to juglone incubated in the dark. Applying these solutions at minimal inhibitory concentration of nominally 10 μM to growing suspension cultures, the inhibitory effect of juglone was completely suppressed by the UV plus visible light treatment, whereas with the visible light treatment the juglone kept part of its activity, and the untreated juglone control showed full bactericidal activity (Figure 5). The varying

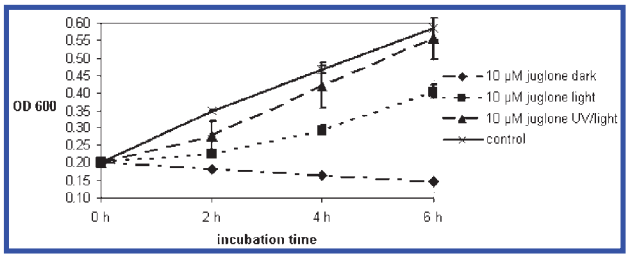

Figure 5. Bactericidal activity of juglone preincubated with UV−visible light and visible light in the suspension culture test. Standard deviations from 3-fold replication.

efficacies for juglone in the field testing in 2012 (40 vs 82%) may be interpreted as an effect of UV inactivation due to different application times (morning vs afternoon) and emphasizes the requirement of appropriate formulations for future field applications.

Juglone is also a suitable substrate for tyrosinases, as can be demonstrated by photometric assays with mushroom tyrosinase (data not shown). Tyrosinase activity in apple flowers was histochemically demonstrated with the MBTH reagent (Besthorn's hydrazone) for *o*-quinones formed from tyrosine. At flowers tyrosinase activity is detectable at the styles, opened stamina, nectaries, and local injuries; these are essentially all open tissues that are not protected by a cuticle. By incubation with mushroom tyrosinase at neutral pH, juglone loses its bactericidal activity as demonstrated by a preincubation/plating assay with *E. amylovora*, but this inactivation is prevented under low pH conditions (Table 1).

In contrast, air oxidation during spraying does not occur or lead to an inactivation, as tested by spraying of a juglone solution, re-collecting, and testing for remaining bactericidal activity (data not shown).

Pollen Germination and Fruit Set at Low pH. After application of juglone at a pH of 4.5 for stabilization, apple flowers could suffer from reduced pollen germination and fertilization; hence, both were tested at pH 4.5. Pollen germination was tested in vitro on pollen germination media with a pH range from 4.5 to 7.5 (Table 2). A reduction of the

Table 2. In Vitro Pollen Germination Rates of Apple (*Malus* × *domestica* cv. Golden Delicious) at Variable pH

pH	pollen germination rate in vitro, mean of triplicate (±standard deviation)
4.5	5.0 (±3.6)
5.0	10.3 (±4.7)
5.5	12.0 (±4.6)
6.0	12.0 (±5.3)
6.5	13.0 (±6.6)
7.0	11.7 (±1.5)
7.5	12.7 (±4.5)

pollen germination rate from about 12% in the neutral range to 5% at pH 4.5 was observed, but there was remaining pollen viability. A negative effect on pollen tube length was also observed but was not quantified. The fertilization rate in vivo was tested for these conditions by spraying 0.1% citric acid at pH 4.5. In a small-scale experiment with single trees with individually pollinated flowers, no negative influence of low pH on fruit set was observed (data not shown).

Perspective. Juglone is a secondary metabolite of *Juglans* and *Carya* species within the family Juglandaceae (walnut family).[29] It is formed in *Juglans regia* L. (walnut tree) as hydrojuglone-4-*O*-βD-glucopyranoside, set free by a β-glucosidase[30] especially after cell damage, and is readily oxidized at the air, forming juglone. Leaves of walnut trees could be used for its isolation. Juglone as a chemical has the status of being toxic, but has traditionally been used for staining of hair or wool, and its effect in staining fingers when walnuts exocarps are peeled off is quite familiar. Physiologically, its allelopathic effect on plants, especially the phytotoxicity on seedlings growing below walnut trees, is well-known.[31] Also, lethal effects of walnut trees on apple trees have been described.[32] Hence, it may seem unsuitable to be considered as a potential plant protectant for apple trees. However, unlike the permanent exposure from competing walnut trees, which emit larger amounts of the precursor glucoside, juglone application in fire blight control would present a different situation. Primary infection with fire blight in pome fruit is restricted to the period of bloom and even less to a few days during bloom with suitable temperature and moisture conditions.[2] Hence, necessary exposure time for control is some days during bloom. Furthermore, juglone

Table 1. Incubation−Plating Assay for the Effect of Mushroom Tyrosinase on the Bactericidal Effect of Juglone against *Erwinia amylovora* in the pH Range of 4.5−7.5

E. amylovora 295/93	colonies after plating on King's B medium (pH 7.2)		
preincubation in King's B medium, pH 7.2	10 μL 1:100 dilution	10 μL 1:1000 dilution	10 μL 1:10000 dilution
only King's B medium (control)	confluent	1947	220
25 μM juglone	0	0	0
0.5 mg/mL tyrosinase	confluent	1986	241
25 μM juglone + 0.5 mg/mL tyrosinase	confluent	1776	280
preincubation in King's B medium, pH 4.5			
only King's B medium (control)	confluent	1200	182
25 μM juglone	0	0	0
0.5 mg/mL tyrosinase	confluent	2076	254
25 μM juglone + 0.5 mg/mL tyrosinase	0	0	0

shows a MIC of only 2.5−5 μM at pH 4.5, a pH that was found to be still compatible with pollen germination and fruit set in apple. It can be expected that practical application be maintained at low concentrations and, together with the given instability of the compound, long-term phytotoxic effects or unfavorable environmental effects would not be expected. Fruit russeting after application during bloom would have been an obstacle, but was not observed with apple and pear. Preliminary tests also did not indicate bee toxicity. The relative insensitivity of other *Erwinia* species, which are applied during bloom for biological fire blight control, is a favorable coincidence. Problems with quinone instability due to enzymatic degradation can mostly be overcome by the use of low pH, but UV protection must also be taken into account and would have to be counteracted for practical application via protective formulation and optimal application time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Conditions for field testing with trees inoculated during bloom. Histochemical staining of *Malus domestica* cv. Gala flowers for tyrosinase activity (Figure S1). Anthroquinones used for screening for bactericidal effects on *Erwinia amylovora* (Table S1). Strains of *E. amylovora* isolates from different geographic regions and from various host plants used for the determination of the MICs of juglone (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

The study was supported by The Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (DaFNE Project 100404) and the Austria Wirtschaftsservice (aws, PRIZE 2010, Z100355). M.G. acknowledges the support from the Verein zur Förderung der Pflanzenwissenschaften.

Notes

The authors declare the following competing financial interest(s): A patent application has been filed on this matter (Fischer, T. C.; Gosch, C.; Stich, K. Pflanzenschutzmittel. Austrian Patent AT 509 501 B1 2012-06-15; International Patent Appl. PCT/AT2011/000092, filed by Vienna University of Technology) to allow technology transfer and further development.

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