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Radicinin from *Cochliobolus* sp. inhibits *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevine

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ABSTRACT

The fastidious phytopathogenic bacterium, *Xylella fastidiosa*, poses a substantial threat to many economically important crops, causing devastating diseases including Pierce's Disease of grapevine. Grapevines (*Vitis vinifera* L.) planted in an area under Pierce's Disease pressure often display differences in disease severity and symptom expression, with apparently healthy vines growing alongside the dying ones, despite the fact that all the vines are genetic clones of one another. Under the hypothesis that endophytic microbes might be responsible for this non-genetic resistance to *X. fastidiosa*, endophytic fungi were isolated from vineyard cvs. 'Chardonnay' and 'Cabernet Sauvignon' grown under high Pierce's Disease pressure. A *Cochliobolus* sp. isolated from a Cabernet Sauvignon grapevine inhibited the growth of *X. fastidiosa in vitro*. Bioassay-guided isolation of an organic extract of *Cochliobolus* sp. yielded the natural product radicinin as the major active compound. Radicinin also inhibited proteases isolated from the culture supernatant of *X. fastidiosa*. In order to assess structure–activity relationships, three semi-synthetic derivatives of radicinin were prepared and tested for activity against *X. fastidiosa in vitro*. Assay results of these derivatives are consistent with enzyme inactivation by conjugate addition to carbon-10 of radicinin, as proposed previously.

1. Introduction

Xylella fastidiosa (*Xf*) is a Gram-negative bacterial phytopathogen that causes many devastating plant diseases, including Pierce's Disease (PD) of grapevine, phony peach disease, alfalfa dwarf disease, plum leaf scald, citrus variegated chlorosis, and leaf scorch of almond, coffee, elm, oak, oleander, pear, and sycamore (Hopkins and Purcell, 2002). The bacterium is transmitted by xylem-feeding insects belonging to the Cercopidae and Cicadellidae families, primarily sharpshooters. Once inside the xylem, the bacterium systemically colonizes this tissue and the symptoms manifest in a manner similar to, but not exactly like, water stress (Choi et al., 2013; Thorne et al., 2006). The symptoms include a characteristic marginal leaf necrosis, irregular leaf abscission, irregular periderm development, raisining of berries, general vine stunting and eventual death of the plant (Hopkins, 1989; Hopkins and Purcell, 2002; Varela et al., 2001). Presently, there is no cure for *Xf* infection aside from severe pruning of vines before they become chronically infected. Current control of PD relies largely on decreasing the insect vector population with insecticides (Purcell et al., 2013).

Natural products from endophytic microorganisms may provide an alternate strategy for controlling PD. In vineyards under high PD pressure, irregular patterns of disease incidence have been observed (Jones, 2004). Often a single healthy vine, which appears to have escaped the disease, can be found among many heavily-infected vines. Because grapevine plantings are genetically clonal, these field observations suggest a non-genetic avenue for host resistance to *Xf*. One hypothesis consistent with these observations is that endophytic microorganisms may impart resistance to PD by inhibiting the growth of *Xf* in the xylem. Specifically, this study focuses on endophytic fungi (*i.e.*, the fungi inhabiting plant tissues without causing symptoms of disease). Endophytic fungi are poised to have evolved chemical mechanisms for competing with phytopathogenic bacteria such as *Xf*, and previous studies have

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shown that endophytic fungi can increase the tolerance of their plant hosts to stress and pathogens. For example, fungi have been used as biocontrol agents against avocado white root rot (Rosa and Lopez Herrera, 2009), powdery mildew of strawberries (De Cal et al., 2008), and downy mildew and trunk diseases of grapevines (John et al., 2008; Perazzolli et al., 2008). Endophytic fungi are also well-documented prolific producers of bioactive natural products (Aly et al., 2010; Gunatilaka, 2006; Kusari et al., 2012; Strobel et al., 2004), many with the potential for commercial applications. Indeed, the number of US patents awarded for bioactive natural products from endophytic fungi has dramatically increased in the past 20 years (Priti et al., 2009). These examples suggest that an investigation of the fungal endophytes associated with healthy grapevines, or those exhibiting mild PD symptoms, may yield antibiotic natural products which could be inhibitory to *Xf*.

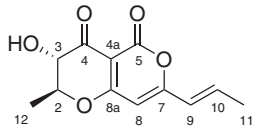
Radicinin (**1**) is a fungal natural product first isolated from *Stemphylium radicinum* in 1953 (Clarke and Nord, 1953) and later observed from a variety of other plant-associated fungi, including *Cochliobolus lunatus* (Nukina and Marumo, 1977), *Phoma andina* (Noordeloos et al., 1993), *Curvularia* sp. (Kadam et al., 1994) and several members of the genus *Alternaria* (Pryor and Gilbertson, 2002; Robeson and Strobel, 1982; Tal et al., 1985). The structure of radicinin (**1**) was first reported in 1964 (Grove, 1964), with the absolute stereochemistry proposed in 1977 (Nukina and Marumo) and confirmed by X-ray crystallography in 1982 (Fig. 1, Robeson et al., 1982). While the antibacterial (Li et al., 2014; Suzuki et al., 2012) and phytotoxic (Canning et al., 1992; Hansen, 1954; Li et al., 2014; Nakajima et al., 1997; Solfrizzo et al., 2004) activities of radicinin (**1**) are well-documented, little is known about a possible mechanism of action. A target-directed microbial screen identified it as an inhibitor of the human rhinovirus 3C protease (Kadam et al., 1994), and theoretical mechanistic studies have predicted that the mechanism of enzyme inactivation is a nucleophilic attack of cysteine-147 in a conjugate addition, resulting in covalent modification of the enzyme (Scheme 1) (Steindl et al., 2005). However, to our knowledge, no one has looked into the protein target against an ecologically relevant organism (such as *Xf*). Moreover, the proposed conjugate addition to radicinin (**1**) has not been demonstrated chemically.

As part of an ongoing project examining the role of endophytes in relation to host resistance to *Xf*, several endophytic fungal strains were isolated from grapevines grown under high PD pressure but which were apparently healthy or exhibiting mild PD symptoms. *Xf*-inhibitory activity in one of these fungal strains, belonging to the genus *Cochliobolus* (anamorph *Bipolaris*, *Curvularia*), was traced to the natural product radicinin (**1**). Additionally, it was demonstrated that radicinin inhibits proteases secreted by *Xf*. To shed light on the structure–activity relationships of radicinin (**1**), three semi-synthetic derivatives were prepared and tested for activity against *Xf*, with the ultimate goal of developing bioactive fungal natural products as an alternative strategy for managing PD.

2. Results and discussion

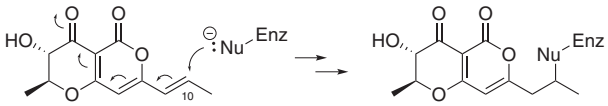
2.1. Isolation and identification of Xf-inhibitory fungi

Several endophytic fungi were recovered from grapevine shoots belonging to the genera *Aureobasidium*, *Cladosporium*, *Alternaria*, *Chaetomium*, *Cryptococcus*, and *Cochliobolus*, with the two former taxa being the most abundant. Both *Aureobasidium* and *Cladosporium* were re-isolated most frequently from grapevine shoots (7 out of the 10 vines) in comparison to *Chaeotomium* (4 vines), *Alternaria* (3 vines), *Cryptococcus* and *Cochliobolus* (2 vines). All of these taxa were identified by percent homology to ITS rDNA nucleotide sequences from specimens posted in the NCBI database.



radicinin (**1**)

Fig. 1. Structure of radicinin from *Cochliobolus* sp.



Scheme 1. Proposed mechanism of enzyme inactivation by radicinin (**1**). An enzyme nucleophile (such as cysteine or serine) could attack the electrophilic carbon at C-10 in a conjugate addition, leading to covalent modification and inactivation of the enzyme.

The genera *Aureobasidium*, *Cladosporium* and *Alternaria* have previously been associated with grapevines, as either epiphytes on pruning wounds (Munkvold and Marois, 1993) or as endophytes in shoots (Pancher et al., 2012).

All fungi were screened for their ability to inhibit *Xf* in our *in vitro* agar-diffusion inhibition assay. *Cochliobolus* sp. strain COC1 and *Cryptococcus* sp. strain CRY1 were able to curtail *Xf* growth, as indicated by the clearing zone (*i.e.*, no *Xf* growth) surrounding the margin of the fungal colony (Fig. 2). These data suggest that these fungi produce and secrete natural products that inhibit growth of *Xf*. The *Cochliobolus* sp. strain COC1 was further investigated to identify these potential bioactive natural product(s).[1]

Several species of *Cochliobolus* and their secondary metabolites have been described (Manamgoda et al., 2011). This genus is cosmopolitan and is commonly found in association with grasses but several *Cochliobolus* species can be pathogenic and cause disease in food crops (Manamgoda et al., 2011). To our knowledge, this study is the first to report *Cochliobolus* associated with grapevine. This strain shared 100% ITS nucleotide sequence homology (520 nucleotides in length) with *Cochliobolus* (anamorph *Curvularia*) strains posted in NCBI; accession numbers HE861842 (da Cunha et al., 2013) and DQ836798 (Desnos-Ollivier et al., 2006).

2.2. Purification and identification of the bioactive agent from Cochliobolus sp.

Cochliobolus sp. strain COC1 was propagated in Potato Dextrose Broth (PDB) and the organic-soluble metabolites extracted with EtOAc. The *Xf* inhibition assay was used to guide isolation of the active compound from the crude extract through two rounds of flash column chromatography (EtOAc: hexanes eluent), and one round of high-performance liquid chromatography (HPLC, CH_3CN: H_2O) to yield a single active compound, **1**. A time-course study established that levels of **1** in the culture broth peaked around 14 days, ultimately yielding ~61 mg of pure radicinin (**1**) per liter of culture broth.

High-resolution mass spectrometry (ESI-TOFMS) of **1** gave a molecular ion $[M+H]^+$ of 237.0758, consistent with a molecular formula of $C_{12}H_{12}O_5$ (calcd 237.0757). Its ^{13}C-NMR spectrum showed twelve signals including two methyl carbons (δ 17.8 and 18.6), two carbons adjacent to oxygen (δ 72.1 and 80.0), four sp^2-hybridized

[1] Extracts of *Cryptococcus* sp. strain CRY1 contained a complex suite of trace metabolites that complicated isolation efforts. The other fungal isolates displayed only minor inhibition of *Xf*.

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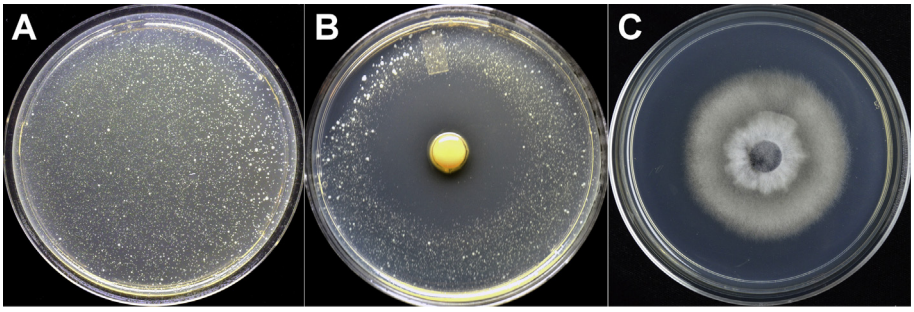


Fig. 2. *Xf* inhibition assay. Images of assay plates from the *in vitro* assay, showing: (A) uninhibited *Xf* growth on a control plate, (B) a halo of *Xf* growth inhibition around an agar plug of an established culture of *Cryptococcus* sp. strain CRY1, (C) complete inhibition of *Xf* growth around an agar plug of an established culture of *Cochliobolus* sp. strain COC1.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for radicinin (**1**) in $CDCl_3$. Observed values are provided along with published values (Nakajima et al., 1997).

Position	δ^{13}C (ppm) *observed*	δ^{13}C (ppm) *lit.*	δ^{1}H (ppm) *observed*	*J* (Hz) *observed*	δ^{1}H (ppm) *lit.*	*J* (Hz) *lit.*
2	80.0	80.0	4.36	*dq* (12.3, 6.2)	4.36	*dq* (12.4, 6.3)
3	72.1	72.0	3.98	*d* (12.3)	3.98	*d* (12.4)
4	189.1	188.6				
4a	97.8	97.9				
5	157.6	156.7				
7	164.1	164.3				
8	98.2	98.0	5.84	*s*	5.84	*s*
8a	176.3	176.3				
9	122.5	122.6	6.03	*dq* (15.5, 1.6)	6.03	*dq* (15.5, 1.8)
10	140.8	141.0	6.94	*dq* (15.5, 7.0)	6.95	*dq* (15.5, 7.0)
11	18.6	18.8	1.95	*dd* (7.0, 1.6)	1.95	*dd* (7.0, 1.8)
12	17.8	18.1	1.64	*d* (6.2)	1.64	*d* (6.3)

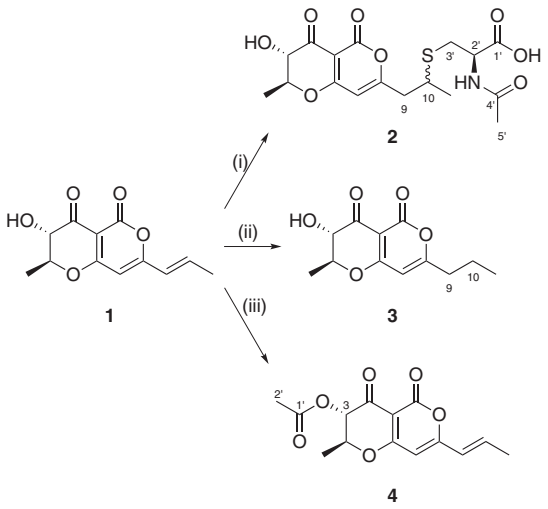
carbons (δ 97.8, 98.2, 122.5, and 140.8), and four sp^2-hybridized carbons that were especially deshielded (δ 157.6, 164.1, 176.3, and 189.1). The ^{1}H-NMR spectrum displayed only eight signals, including one methyl doublet (δ 1.64, *J* = 6.2 Hz), a methyl doublet of doublets (δ 1.95, *J* = 7.0, 1.6 Hz) and three vinylic hydrogens (δ 5.84, 6.03 and 6.94). A search of the AntiMarin natural products database (Blunt et al., 2012) yielded radicinin (**1**) as a likely candidate. Identity of **1** was confirmed by comparing literature ^{1}H and ^{13}C-NMR chemical shifts (Table 1; Nakajima et al., 1997).

2.3. Semisynthesis of radicinin derivatives

In order to explore structure–activity relationships of **1**, several derivatives **2**–**4** were prepared for testing in the *Xf* inhibition assay (Scheme 2). *N*-acetylcysteine adduct **2** was prepared by reacting **1** with *N*-acetyl-L-cysteine in the presence of triethylamine at room temperature. After purification by HPLC, the structure of **2** was confirmed by 1D- and 2D-NMR and HRMS. In addition to acting as a proof-of-concept of the proposed mechanism of enzyme inactivation via conjugate addition, **2** no longer possesses the accessible electrophile at C-10, and would be expected to be inactive in the *Xf* inhibition assay.

Selectively hydrogenated derivative **3** was prepared by stirring a solution of **1** under hydrogen gas, in the presence of palladium-on-charcoal. After filtering through celite and purification by HPLC, structure **3** was confirmed by 1D and 2D NMR and HRMS analyses. Like the *N*-acetylcysteine adduct **2**, the reduced product **3** lacks the double bond connecting C-9 and C-10. Without this easily accessible electrophilic site for conjugate addition, **3** would also be expected to be inactive in the *Xf* inhibition assay.

The acetylated derivative **4** was prepared by reacting **1** with acetic anhydride in the presence of dimethylaminopyridine and triethylamine under nitrogen. After purification by HPLC, structure **4** was confirmed by 1D and 2D NMR and HRMS analyses. Unlike **2** and **3**, acetate **4** was modified at a site remote from the proposed site of attack by an enzyme nucleophile. If **4** retained activity against *Xf*, the hydroxyl group of radicinin (**1**) may be a useful 'handle' for attaching a fluorescent or affinity tag to isolate and/or identify the protein target(s) of **1**.



Scheme 2. Preparation of derivatives (**2**–**4**) from radicinin (**1**). Reagents: (i) *N*-acetyl-L-cysteine, Et_3N, MeOH; (ii) H_2/Pd, MeOH; (iii) Ac_2O, DMAP, Et_3N, CH_2Cl_2.

2.4. Evaluation of inhibitory effects of radicinin (*1*) and derivatives (*2*–*4*) against *Xf*

Purified radicinin (**1**) from *Cochliobolus* sp. strain COC1 showed dose-dependent inhibition of *Xf* in an *in vitro* disk diffusion assay

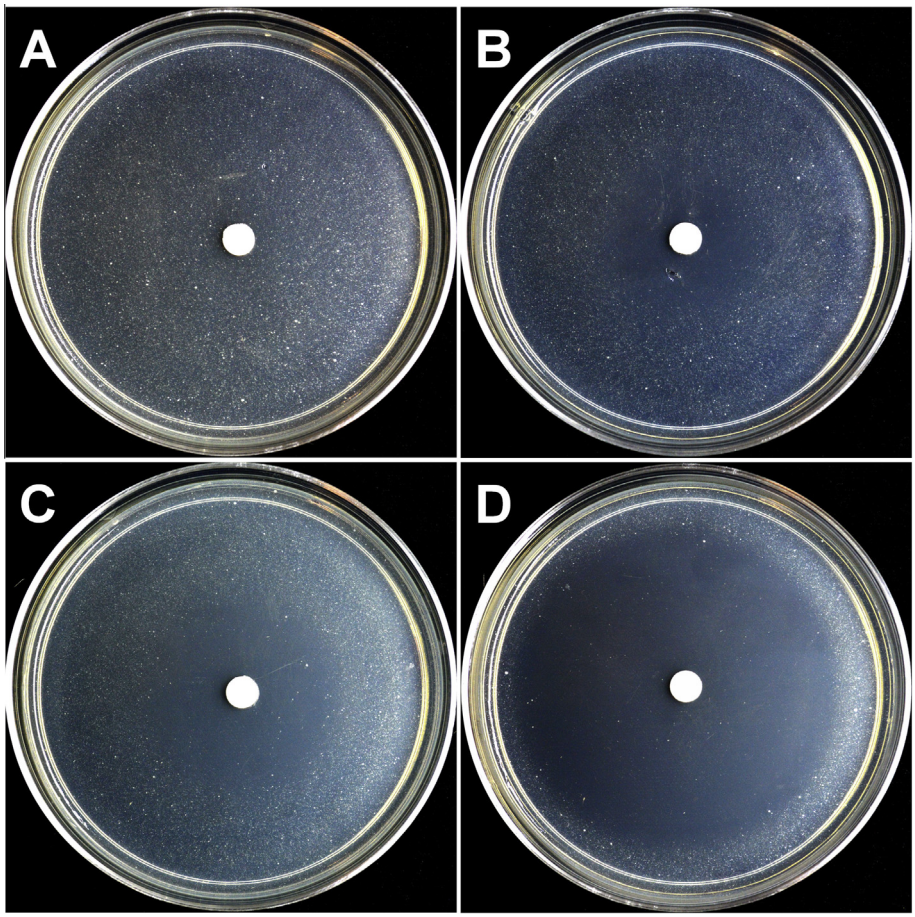


Fig. 3. Dose-dependent inhibition of *Xf* by radicinin. Images of assay plates from the *in vitro* assay, showing a halo of *Xf* growth inhibition around filter disks containing radicinin (**1**) at the following doses: (A) 10 μg, (B) 50 μg, (C) 100 μg, (D) 250 μg.

(Fig. 3). The assay results obtained with the semisynthetic radicinin derivatives **2**–**4** support the proposed mechanism of enzyme inactivation by radicinin (**1**). As shown in Scheme 1, enzyme inactivation is believed to occur by a conjugate addition of an enzyme nucleophile (such as a cysteine or serine) to the electrophilic carbon-10 of radicinin (**1**), leading to covalent modification and loss of activity.

Radicinin (**1**) and derivatives **2**–**4** were tested in the *in vitro* disk diffusion assay at an equimolar dose of 1.89 μmol. At this dose, radicinin (**1**) exhibited complete inhibition and, as predicted, **2** and **3** showed no observable growth inhibition. Both compounds **2** and **3** lack an electron-poor alkene at C-10, and conjugate addition to these compounds could only occur at the more congested and less reactive C-7 or C-8a. By contrast, compound **4**, in which the electrophilic character at C-10 is maintained, exhibits similar activity to that of radicinin (**1**). Upon further testing, **4** displayed dose-dependent inhibition of *Xf* comparable to that of pure radicinin (**1**) (Fig. 4).

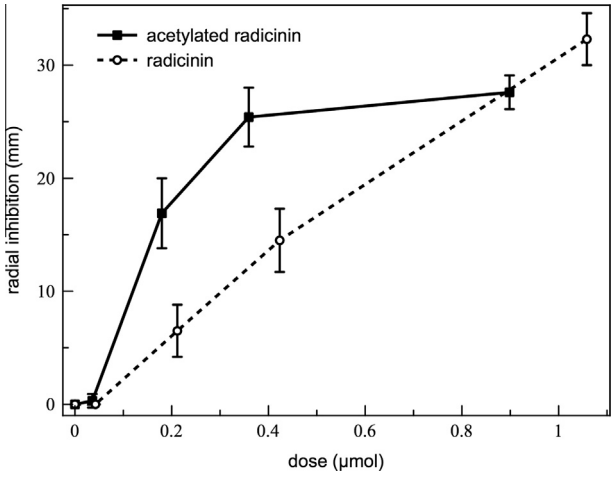


Fig. 4. *Xf*-inhibitory activity of **1** and **4**. Acetylated radicinin (**4**) displays similar dose-dependent activity in the *Xf* inhibition assay to that of radicinin (**1**). Neither the *N*-acetylcysteine adduct (**2**) nor dihydroradicinin (**3**) showed observable activity when tested at 1.89 μmol.

2.5. Evaluation of Xf protease inhibition by radicinin

Phytopathogenic organisms are known to release extracellular enzymes, including proteases, that can be intimately involved in pathogen–host interactions, and in certain cases, crucial for pathogen virulence (Dangl and Jones, 2001; Roper et al., 2007; Xia, 2004). Analysis of the *Xf* genome indicates 36 genes annotated as proteases, including three identified as putative serine proteases and two as putative cysteine proteases (Dehal et al., 2010; Van Sluys et al., 2003; Varani et al., 2012). Protease activity has been demonstrated in *Xf* (Fedatto et al., 2006; Fry et al., 1994; Wells et al., 1987) and a cysteine protease has been detected by immunoblot assays in cultures of *Xf* (a citrus and coffee isolate). Interestingly, this cysteine protease is differentially expressed in a non-pathogenic strain of *Xf* suggesting this protease may be a

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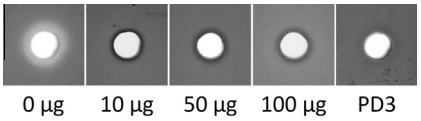

Fig. 5. Inhibition of protease activity in supernatants of *Xf* cultures. Protease activity was assessed by a radial diffusion assay using casein as a substrate. A halo of protease degradation was observed when *Xf* culture supernatants were incubated alone with no radicinin. Complete inhibition of protease activity was observed when *Xf* culture supernatants were incubated with either 10 µg, 50 µg or 100 µg of radicinin (**1**). No halo was observed in these wells and they were indistinguishable from the negative PD3-medium-only control.

promising target for combating *Xf* virulence (Leite et al., 2013; Nogaroto et al., 2006). To test the hypothesis that radicinin (**1**) inhibits *Xf* by a mechanism involving protease inactivation, proteases from the culture supernatant of *Xf* were isolated and used in a radial diffusion assay with casein as a substrate. Indeed, radicinin (**1**) abolished activity of proteases secreted by *Xf* at all concentrations tested (10 µg, 50 µg and 100 µg) (Fig. 5).

3. Conclusions

In this study, an endophytic fungus (*Cochliobolus* sp.) isolated from grapevines was found to inhibit the growth of the bacterial phytopathogen *Xf in vitro*. The natural product radicinin (**1**) was isolated from the culture broth of *Cochliobolus* sp. and its antibacterial activity demonstrated against *Xf* along with its strong inhibitory activity against proteases produced by *Xf*. These results add to the expanding body of evidence that proteases may be important virulence factors employed by *Xf* during host plant colonization. To begin to assess structure–activity relationships for radicinin (**1**), three semi-synthetic derivatives (**2**, **3**, and **4**) were prepared and assayed against *Xf* and these results were consistent with the proposed method of protease inactivation via conjugate addition at carbon-10 of radicinin (**1**). The acetylated derivative **4**, which was modified at the hydroxyl position on carbon-3 of radicinin yet retained *Xf*-inhibitory activity, was especially noteworthy. In future studies, it may be possible to capitalize on the 'handle' that this hydroxyl group represents. For example, modification with a charged group at this site should provide a water-soluble radicinin derivative which, if active against *Xf*, might be suitable for *in planta* studies. Alternatively, attachment of a fluorescent or affinity tag at this site might permit identification of the specific protein target of radicinin. If inhibition of *Xf* by radicinin can be demonstrated *in planta*, radicinin (**1**) will join a growing list of plant-protective natural products isolated from endophytes.

4. Experimental

4.1. General experimental procedures

All chemicals were used as received from Sigma–Aldrich and reagent grade solvents were used as received from Spectrum Chemical, with the exception of anhydrous CH_2Cl_2, which was obtained from a SolvPure anhydrous solvent system using activated alumina. Fungal media were supplied by Beckton Dickinson and prepared according to manufacturer instructions. Reactions were monitored with TLC on glass plates coated with mesh 400 silica gel and visualized using UV and *p*-anisaldehyde stain. Flash column chromatography (CC) was performed on a CombiFlash Rf (Teledyne Isco, Inc., Lincoln, NE, USA), using a prepacked silica column (RediSep Rf, 12 g silica). HPLC separations were performed on a Beckman System Gold LC system (Beckman Coulter, Brea, CA, USA), equipped with a binary pump and diode array detector, using a semi-preparative reversed-phase column (Phenomenex, Luna C18 100A, 250 × 10.00 mm 5 µm). NMR experiments were performed on either a Varian INOVA 500 NMR spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) or a Bruker Avance 400 NMR spectrometer (400 and 100 MHz for ^{1}H and ^{13}C NMR, respectively) in $CDCl_3$ (Sigma–Aldrich), DMSO-d_6 (Cambridge Isotope Labs) or CD_3OD (Cambridge Isotope Labs) at ambient temperature. Chemical shifts are calibrated to the residual solvent peaks (δ^{13}C 77.2 for $CDCl_3$, 39.5 for DMSO, 49.0 for MeOH; δ^1H 7.26 for $CDCl_3$, 2.50 for DMSO, 3.31 for MeOH). Optical rotations were recorded using a Jasco P-1010 polarimeter. Fungal DNA extractions were performed with a DNeasy® Plant kit (Qiagen, Valencia, CA).

4.2. Plant material

Grapevines (*Vitis vinifera*) cvs. 'Chardonnay' and 'Cabernet Sauvignon' grown at the Agricultural Experiment Station at the University of California, Riverside were selected because this area is under constant and high PD pressure. Shoots from 10 one-year-old grapevines that were either healthy (exhibiting no PD symptoms) or ones exhibiting mild PD symptoms were sampled in August of 2009.

4.3. Fungal isolation and identification

Fungi inhabiting the sampled wood canes were isolated on culture medium as follows. Bark was removed from the shoots and wood samples were cut to 5-cm-long pieces, dipped in EtOH and surface-sterilized by flaming. Following this, shoot segments were longitudinally split open to expose the vascular system. Five wood chips (approximately 2 × 2 × 2 mm) were sampled from xylem of the shoot segments and then plated onto Potato Dextrose Agar (PDA) amended with tetracycline (100 mg l^{-1}) to inhibit bacterial growth. Two culture plates were used per shoot sample. Following ten days of incubation at room temperature, the fungi recovered were subsequently sub-cultured to new PDA plates. Following another ten days of incubation at room temperature, the fungi recovered were again sub-cultured to new culture medium in order to obtain a pure culture. Fungal isolates were identified by their ITS ribosomal DNA sequence using primer pairs ITS1 and ITS4 (White et al., 1990) as previously described (Rolshausen et al., 2014). PCR products were sequenced in both forward and reverse directions at the Genomic Core Sequencing Facility (University of California, Riverside). BLASTn searches identified matches to sequences with high homology to those deposited in GenBank.

4.4. Isolation and extraction of radicinin (*1*)

Agar plugs (1 cm^2) of *Cochliobolus* sp. strain COC1 were used to inoculate liquid cultures (8 × 250 ml PDB in 500 ml Erlenmeyer flasks). Cultures were incubated for 14 days at 20 °C with shaking at 200 rpm, extracted exhaustively with EtOAc (3 × 125 ml), and the resulting combined extracts were evaporated *in vacuo* to yield a bright pink solid residue (295.4 mg). The crude extract was fractionated by flash silica-gel CC at a flow rate of 30 ml min^{-1} with isocratic elution (EtOAc:hexanes, 6:1 v/v) to give radicinin (**1**) (121.0 mg) as a white powder. The NMR spectra were identical to those previously reported (Table 1, Nakajima et al., 1997).

4.4.1. Radicinin (*1*)

White powder; $[\alpha]_D^{23}$ – 11.0 ($CHCl_3$, c = 0.317); For ^{1}H and ^{13}C NMR ($CDCl_3$) spectroscopic data, see Table 1; HRMS (ESI-TOFMS): m/z 237.0758 $[M+H]^+$ (cald 237.0757).

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4.5. Preparation of radicinin derivatives

4.5.1. Preparation of N-acetylcysteine adduct (**2**)

Radicinin (**1**) (13.4 mg, 56.8 μmol) was added to a solution of Et_3N (10 mg, 100 μmol) and *N*-acetyl-L-cysteine (9.3 mg, 56.9 μmol) in MeOH (2.0 ml) and stirred for 2.5 h. The ^1H NMR of the crude mixture showed complete conversion. The product was purified via HPLC with a flow rate of 3 ml min^{-1} with CH_3CN (A) and H_2O acidified with 0.1% CF_3CO_2H (B) as mobile phase in the following gradient elution: 0–20 min 10–100% A; 20–25 min 100% A. The peak recorded by UV/Vis detector at R_t: 10.5 min was collected and dried to give **2** as a white solid mixture of diastereomers in an approximate 2:1 ratio (4.7 mg, 11.7 μmol, 21%).

Compound **2**; white solid: ^1H NMR of the major diastereomer (DMSO-d_6, 400 MHz) δ 8.26 (1H, *d*, *J* = 6.8 Hz, NH), 6.35 (1H, *s*, H-8), 4.57 (1H, *m*, H-2), 4.36 (1H, *m*, H-2′), 3.99 (1H, *d*, *J* = 10.8 Hz, H-3), 3.19 (1H, *m*, H-10), 2.92 (1H, *m*, H-3′a), 2.77 (1H, *m*, H-3′b), 2.73 (2H, *m*, H-9), 1.85 (3H, *s*, H-5′), 1.46 (3H, *d*, *J* = 6.3 Hz, H-12), 1.24 (3H, *d*, *J* = 6.1 Hz, H-11); ^{13}C NMR of the major diastereomer (DMSO-d_6, 100 MHz) δ 188.8 (C, C-4), 174.8 (C, C-8a), 172.1 (C, C-1′), 169.4 (C, C-4′), 168.9 (C, C-7), 157.0 (C, C-5), 100.7 (CH, C-8), 97.9 (C, C-4a), 79.6 (CH, C-2), 71.9 (CH, C-3), 52.2 (CH, C-2′), 40.4 (CH_2, C-9), 37.4 (CH, C-10), 30.8 (CH_2, C-3′), 22.4 (CH_3, C-5′), 20.9 (CH_3, C-11), 17.5 (CH_3, C-12); HRMS (ESI-TOFMS): *m*/*z* = 400.1063 $[M+H]^+$ (calcd 400.1061).

4.5.2. Preparation of dihydroradicinin (**3**)

Radicinin (**1**) (14.2 mg, 42.3 μmol) was dissolved in MeOH (3.0 ml), and Pd/C (5.1 mg) was added. The reaction mixture was stirred vigorously under H_2 (1 bar) at room temperature for 90 min. The crude product was filtered through celite and then concentrated *in vacuo*. The product was purified via HPLC with a flow rate of 3 ml min^{-1} with CH_3CN (A) and H_2O acidified with 0.1% CF_3CO_2H (B) as mobile phase in the following gradient elution: 0–2 min 10–30% A; 2–20 min 30% A; 20–22 min 30–100% A; 22–32 min 100% A. The peak recorded by UV/Vis detector at R_t: 16.0 min was collected and dried to give **3** as a white solid (1.4 mg, 5.9 μmol, 14%).

Compound **3**; white solid: ^1H NMR (CD_3OD, 400 MHz) δ 6.18 (1H, *s*, H-8), 4.52 (1H, *dq*, *J* = 11.5, 6.3 Hz, H-2), 4.07 (1H, *d*, *J* = 11.5 Hz, H-3), 2.54 (2H, *t*, *J* = 7.5 Hz, H-9), 1.71 (2H, *sextet*, *J* = 7.5 Hz, H-10) 1.60 (3H, *d*, *J* = 6.3 Hz, H-12), 1.00 (3H, *t*, *J* = 7.5 Hz, H-11); ^{13}C NMR (CD_3OD, 100 MHz) δ 191.3 (C, C-4), 177.8 (C, C-8a), 174.1 (C, C-7), 100.7 (CH, C-8), 99.0 (C, C-4a), 81.5 (CH, C-2), 73.6 (CH, C-3), 36.9 (CH_2, C-9), 21.0 (CH_2, C-10), 18.1 (CH_3, C-12), 13.7 (CH_3, C-11); HRMS (ESI-TOFMS): *m*/*z* = 239.0914 $[M+H]^+$ (calcd 239.0914).

4.5.3. Preparation of acetyl radicinin (**4**)

4-Dimethylaminopyridine (catalytic), Ac_2O (150 mg, 1.5 mmol), and Et_3N (120 mg, 1.2 mmol) were added to a solution of radicinin (**1**) (12.0 mg, 50.8 μmol) in anhydrous CH_2Cl_2 (3.0 ml) and stirred at ambient temperature under inert atmosphere for 20 min. The crude product was washed with 1 M HCl (2 × 3 ml), saturated Na_2CO_3 (2 × 3 ml), and brine (2 × 3 ml) and concentrated *in vacuo*. The product was purified via HPLC with a flow rate of 3 ml min^{-1} with CH_3CN (A) and H_2O acidified with 0.1% CF_3CO_2H (B) as the mobile phase in the following gradient elution: 0–2 min 10–30% A; 2–20 min 30% A; 20–22 min 30–100% A; 22–32 min 100% A. The peak recorded by UV/Vis detector at R_t: 16.5 min was collected and dried to give **4** as a white solid (5.2 mg, 18.7 μmol, 37%).

Compound **4**; white solid: ^1H NMR ($CDCl_3$, 400 MHz) δ 6.95 (1H, *dq*, *J* = 15.5, 7.0 Hz, H-10), 6.04 (1H, *dq*, *J* = 15.5, 1.7 Hz, H-9), 5.86 (1H, *s*, H-8), 5.24 (1H, *d*, *J* = 11.0 Hz, H-3), 4.71 (1H, *dq*, *J* = 11.0, 6.4 Hz, H-2), 2.20 (3H, *s*, H-2′), 1.96 (3H, *dd*, *J* = 7.0, 1.7 Hz, H-11), 1.54 (3H, *d*, *J* = 6.4 Hz, H-12); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 182.7 (C, C-4), 175.5 (C, C-8a), 169.6 (C, C-1′), 164.3 (C, C-7), 157.1 (C, C-5), 141.2 (CH, C-10), 122.7 (CH, C-9), 99.0 (C, C-4a), 98.0 (CH, C-8), 77.7 (CH, C-2), 72.5 (CH, C-3), 20.6 (CH_3, C-2′), 19.0 (CH_3, C-11), 17.7 (CH_3, C-12); HRMS (ESI-TOFMS): *m*/*z* = 279.0861 $[M+H]^+$ (calcd 279.0863).

4.6. Xf inhibition assay

Fungi recovered from grapevines, fungal crude extracts, radicinin (**1**) and radicinin derivatives (**2**–**4**) were evaluated in an *in vitro* assay for their ability to inhibit *Xf* growth. *Xf* was streaked onto solid PD3 medium (Davis et al., 1978) and incubated for seven days at 28 °C. Following this, the bacteria were subcultured in liquid PD3 medium for four days at 28 °C while shaking at 180 rpm. The culture was vortexed and adjusted to OD_{600} = 0.1 (approx. 10^7 CFU ml^{-1}) and 300 μl of the *Xf* cell suspension was added to 3 ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture was overlayed onto a Petri plate containing solid PD3 medium. For the fungi, a sterile circle of agar was drawn from the margin of an actively growing pure culture and was placed onto the plates previously inoculated with *Xf*. Crude extracts, radicinin (**1**) and radicinin derivatives (**2**–**4**) were each dissolved in MeOH and applied to sterile filter disks (Difco) to achieve a dose of 1.0 mg per disk in the case of the crude extract, and 1.89 μmol per disk in the case of the pure compounds (450 μg for **1** and **3**, 750 μg for **2**, and 530 μg for **4**). After the MeOH had been allowed to evaporate, the filter disk was placed onto the plates previously inoculated with *Xf*. In each case, plates were incubated at 28 °C for seven days and then observed for an inhibition zone around the fungal colony or paper disk. Pure compounds that showed activity at 1.89 μmol (namely **1** and **4**) were tested at lower doses (ranging from 0.04 to 1 μmol) to observe the dose response (Fig. 4).

4.7. Protease inhibition assay

Xf (Temecula1) wild type was grown for 7 days at 28 °C on solid PD3 medium. Following incubation, agar squares containing colonies were cut from plates, and one square was used to inoculate individual 50 mL Falcon tubes containing 20 mL of liquid PD3 medium. After 4 days of growth at 28 °C in a rotary shaker at 180 rpm, liquid cultures were adjusted to OD_{600} = 0.1 and harvested by centrifugation at 7000 rpm for 25 min at 4 °C. The supernatant was assayed for protease activity using a radial diffusion assay. In brief, the *Xf* cell supernatant was put into dialysis tubing (MWCO ⩽ 1000 Da). The supernatant was concentrated by placing it in polyethylene glycol (MW 15,000–20,000 Da) (Sigma Aldrich #P2263) until the volume decreased 4-fold. The concentrated supernatant (~30 μL) was added to 5 mm wells punched into agar plates containing 0.01% casein. To determine the inhibitory effects of radicinin (**1**) on *Xf* protease activity, supernatant (15 μL) was incubated with radicinin (15 μL) at final concentrations of 10 μg, 50 μg, or 100 μg per well (dissolved in DMSO). PD3 medium mixed with DMSO was used as a negative control for the assay. After incubation at 28 °C for 3 days, plates were fixed and stained as described previously (Gallagher et al., 1986), with slight modification. All steps were performed with gentle shaking. Briefly, gels were immersed in TCA (Cl_3CO_2H):H_2O (400 mL, 90:10 v/v) for 10 min, after which TCA was replaced with EtOH:H_2O (95:5 v/v) for 10 min to dehydrate the agarose. The fixed and dehydrated gels were stained in 100 mL of an aqueous solution containing 0.025% Coomassie blue R-250, 40% MeOH, and 7% AcOH until significant background was evident. Gels were de-stained in 100 mL MeOH:H_2O containing 7% AcOH (40:60 v/v,) until diffusion spots were visible. PD3 medium only served as the negative control for the assays and each assay was performed in triplicate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.03.015.

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