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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; Perazzoli 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.

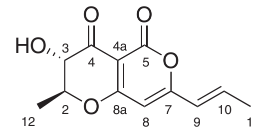
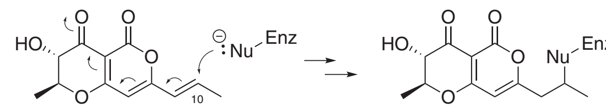


Fig. 1. Structure of radicinin from *Cochliobolus* sp.



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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