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Berberine as a natural compound inhibits the development of brown rot fungus *Monilinia fructicola*

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ABSTRACT

Peach brown rot is a severe disease caused by ascomycete fungus *Monilinia fructicola* and is primarily controlled by chemical fungicides. However, the utilization of fungicides has caused many problems such as microbial resistance, environmental pollution, and fruit contamination. The growing concern for food safety requires alternative management methods that are safe to humans and benign to the environment. Here we report that the extract from *Coptis chinensis* (a Chinese herb named "Huang Lian") demonstrates a strong inhibition to *M. fructicola*. The 50% effective concentration (EC_{50}) of *C. chinensis* extract to *M. fructicola* was only 0.91 mg/mL, while the EC_{50}s to other fungi such as *Botrytis cinerea* and *Alternaria solani* were 14.09 mg/mL and 27.35 mg/mL, respectively. These results indicate that the extract of *C. chinensis* has a specific inhibition to *M. fructicola*. Subsequent partitioning of the ingredients in *C. chinensis* extract revealed that berberine, the conventional anti-bacterial alkaloid, was the predominant ingredient that exerted robust inhibition against *M. fructicola*. The EC_{50} and minimum inhibitory concentration (MIC) against *M. fructicola* were as low as 4.5 μg/mL and 46.9 μg/mL, respectively. Compared to berberine's analogs in *C. chinensis* such as palmatine, berberine tannate and jatrorrhizine, berberine showed the strongest inhibition against *M. fructicola*. This finding provided insight into the structure–activity relationship between berberine and its analogs. Notably, not only can berberine prevent spore germination and hyphal growth, it also inhibits the activity of cutinase secreted by *M. fructicola*, implying the potential function of berberine in reducing the pathogenesis of *M. fructicola*. In addition, the strong *in vivo* inhibition of berberine against *M. fructicola* was observed with no visual cytotoxicity noted to peach fruits, even at berberine concentration of 400 μg/mL. Note that this was much higher than its MIC value (46.90 μg/mL). Overall berberine, as a natural compound, may be a promising candidate in control of brown rot.

1. Introduction

Brown rot, caused by ascomycete fungus *Monilinia fructicola* (G Wint) Honey, is a severe disease that considerably reduces the yield of peaches (*Prunus persica* (L.) Batsch) and many other stone fruits (Forster and Adaskaveg, 2000; Luo et al., 2005; Giobbe et al., 2007). *M. fructicola* may invade twigs and blossoms, and may eventually destroy the fruits (Luo and Michailides, 2003). In past decades, the management of this disease in the commercial orchards of the Beijing region relied primarily on pre-harvest applications of benzimidazole fungicides such as benomyl, thiophanate-methyl and sterol demethylation inhibitor fungicides (DMIs). These fungicides are currently the most effective, and almost exclusively recruited treatment options to control peach brown rot. As a measure to ensure food safety, fungicide spray is generally prohibited at least one week prior to harvest. Unlike the pre-harvest strategy, postharvest management of this disease depends on one or a combination of the following measures including low temperature storage, gas adjustment (Ahmadi et al., 1999; Lazar et al., 2008), fumigation (Liu et al., 2002; Holb and Schnabel, 2008a), irradiation treatment (e.g., ultraviolet light) (Stevens et al., 1998; Marcaki, 1998), or biocontrol (Yao and Tian, 2005; Giobbe et al., 2007; Larena et al., 2007; Xu et al., 2008; Zhou et al., 2008). Despite the diversity of measures, fungicidal application is presently the key to controlling this disease.

Owing to extensive application of benzimidazoles and DMIs, new strains of *M. fructicola* resistant to fungicide have emerged in many countries (Jones and Ehret, 1976; Sonoda et al., 1983; Penrose, 1990; Zehr et al., 1991; Ma et al., 2003; Schnabel et al. 2004; Holb

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and Schnabel, 2007). The emergence of the new strain of *M. fructicola* and the increasing awareness of food safety have initiated the search for alternative fungicides that work effectively yet are safe for human consumption. Although biocontrol is being considered as a treatment option for brown rot (Yao and Tian, 2005; Giobbe et al., 2007; Larena et al., 2007; Xu et al., 2008; Zhou et al., 2008), in recent years increased efforts have been made to examine naturally occurring compounds from botanical resources, especially from medicinal herbs (Lam and Ng, 2002; Feng et al., 2008). Although numerous studies have confirmed the inhibitory activities of herb extracts against plant pathogenic fungi, it is rare that the specific bioactive compound(s) have been isolated. Although some antifungal compounds have been identified, they have unfortunately failed to play a major role in controlling brown rot disease due to their weak or moderate antifungal activities. In order to identify natural compounds that have strong inhibition to *M. fructicola*, arduous efforts have been invested into screening active antifungal compounds from various Chinese herbs. The goals of this study were to (i) identify natural compounds that display strong inhibition against *M. fructicola*, (ii) examine the inhibitory effects of these natural compounds on spore germination, mycelial growth, and cutinase activity, and (iii) determine whether these active natural compounds are toxic to peach fruits.

2. Materials and methods

2.1. Selection of Chinese herbs and preparation of extracts

Four Chinese herbs including *Coptis chinensis* Franch, *Tripterygium wilfkrdii* Hook, *Artemisia apiacea* Hance and *Melia toosendan* Sieb.et Zucc were chosen for extracting antifungal compounds based on their reported biological activities. All of these herbs were purchased from Anguo Chinese traditional medicine market in Hebei province of China. These herbs were ground into fine powder (less than 40 mesh) using a stainless-steel grinder and stored in pill vials at room temperature. An aliquot (200 g) of each powder was mixed with 2000 mL of 70% ethanol and extracted for 7 min by microwave method. After centrifugation at 4000 rpm for 30 min, the supernatants were harvested and evaporated to dryness in a rotary evaporator. A final concentration of 1 g/mL was attained by adding 200 mL of sterile water.

2.2. Screening of antifungal compounds from the extracts of Chinese herbs

Single spore isolate of *M. fructicola* was collected from a peach orchard in the Pinggu district in Beijing and was stored on grade 40 silica gel at 4 °C. Two filamentous fungi including *Botrytis cenerea* and *Alternaria solani* were preserved in the lab of Beijing University of Chemical and Technology. These fungi were cultivated at 27 °C and incubated on potato dextrose agar (PDA) slant at 4 °C.

To determine the EC_{50}, a 5 mm (diameter) mycelial plug was taken from the edge of a 3-day-old colony of *M. fructicola* and placed in the center of a PDA plate containing one of the serially diluted herb extracts. After incubation at 27 °C for 72 h, the radial growth (colony diameter) of each plug was measured, and the diameter of original mycelial plug was subtracted from each measurement. The PDA plate without herb extract was used as a control. The average of the colony diameters measured in two perpendicular directions were used for calculating the EC_{50}. All experiments were completed in triplicate.

Minimum inhibitory concentrations (MICs) of herb extracts were determined by broth dilution method (Schmourlo et al., 2005). Briefly, the PDA plate containing one of the serially diluted herb extracts was inoculated with 100 μL of spore suspension, which was evenly scattered over the PDA plate. The plate was then cultured at 27 °C for 72 h to determine the MIC required to fully inhibit growth of fungal cells. The PDA plate containing sterilized H_2O instead of herb extract was used as the control. Each treatment was completed in triplicate. To identify the compounds from *C. chinensis* that exhibit strong inhibition against *M. fructicola*, four previously reported alkaloids known to occur in *C. chinensis* extract were chosen to be investigated for their antifungal activities.

2.3. Microscopic observation of spore germination and fungal growth

To investigate the influence of herb extracts on spore germination and fungal growth, *M. fructicola* spores were harvested by flooding the colony with sterilized water, centrifuged, and suspended in PDA liquid media. 0.9 mL of spore suspension was mixed with 0.1 mL of each serially diluted herb extract on a clean microscope glass slide and covered with a thin glass plate, incubated at 27 °C for 72 h, then spore germination rates were counted. In the control, 0.9 mL of spore suspension was mixed with 0.1 mL of sterile water. Each treatment was done in triplicate with the average growth used for calculating the inhibitory rate according to the following formula: $B_i(\%) = (B_c - B_t)/B_c$, where B_i is the inhibitory rate on spore germination, B_c and B_t represent the spore germination of the control and treatment specimens, respectively. Morphology of fungal cells was observed under a binocular light microscope equipped with a digital camera.

2.4. Inhibition of herb extracts on cutinase activity

Cutinases are inducible extracellular enzymes capable of degrading plant cell walls by catalyzing the cleavage of ester bonds of cutin, the major component of plant cuticle. Considering the capability of *M. fructicola* to generate and secrete cutinase (Wang et al., 2002), cutinase assay was thus conducted to determine whether the herb extracts affect cutinase activity. The cutin was prepared with tomato fruit peels. The mature tomato fruits were cooked for 5 min, cooled, and the adhering pulp was removed by gentle scraping. The peels were washed with de-ionized water and cooked in an oxalic acid buffer (containing oxalic acid and ammonium oxalate at concentrations of 4 g/L and 16 g/L, respectively) for 1 h, washed with water, dried at 105 °C and finally ground to powder. Strain culture was performed in the media containing 1.0% yeast extract and 0.1% cutin powder, using glucose as the principal carbon source. *M. fructicola* spore suspension was added to this medium, cultured at 27 °C for 21 days. Cutinase activity was measured by using the *p*-nitrophenyl butyrate (*p*PNB) method (Fett et al., 1992). Each treatment was completed in triplicate.

2.5. In vivo inhibition assay and cytotoxicity measurement

To examine *in vivo* antifungal activity of berberine, healthy fruits without physical injuries or infections were surface-disinfected with 2% sodium hypochlorite for 2 min then washed with tap water. After air-drying, the fruits were stabbed (4 mm deep and 3 mm wide) with a sterile nail. Then, 20 uL of 50 μg/mL berberine solution or 20 μL sterile distilled water (control) was put into each wound. After 24 h, 15 μL of *M. fructicola* suspension at 10^4 spores/mL was put into each of the wounds. The treated fruits were put in trays covered with plastic bags to maintain a relative humidity of approximately 95%, then stored at 22 °C. Disease incidence and lesion diameter were observed every 24 h. The experiment was performed in triplicate.

Based on determined MICs, serially diluted berberine solutions were prepared to determine the concentration at which the fruits

would undergo cytotoxicity. Briefly, healthy peach fruits were washed with sterilized water, immersed into berberine solutions for 5 min, stored at 4 °C, and subsequently observed for chemical damage 2 days later under a light microscope equipped with a digital camera.

2.6. Statistical analysis

All experiments were performed in triplicate. The data were treated with SPSS10.0 statistical software and presented as mean ± standard error of mean (S.E.M.). *T*-tests were used for statistical analysis; *P*-values equal or less than 0.05 were considered to be significant.

3. Results

3.1. Effect of C. chinensis extract on M. fructicola

The ethanol extracts of *C. chinensis*, *T. wilfkrdii*, *A. apiacea*, and *M. toosendan* were chosen to investigate their inhibitory activities on the plant pathogenic fungi *M. fructicola*, *B. cinerea* and *A. solani*. Among them, *C. chinensis* extract showed 100%, 74.4% and 80.7% inhibition rates against *M. fructicola*, *B. cinerea* and *A. solani*, respectively (Table 1). To study the dosage effect of *C. chinensis* extract on inhibition rates of *M. fructicola*, *A. solani* and *B. cinerea*, further experiments were conducted with extract concentrations ranging from 0 mg/mL to 100 mg/mL. The results suggested that extract of *C. chinensis* exhibited a dose-dependent anti-proliferative effect, i.e. higher concentration of *C. chinensis* extract exhibits a higher inhibition rate. In addition, the EC_{50}s of *C. chinensis* extract against *M. fructicola*, *B. cinerea*, and *A. solani* were 0.91, 14.09 and 27.35 mg/mL, respectively (Table 1).

3.2. Identification of compounds inhibiting M. fructicola

Compared to the other three analogs (berberine tannate, jateorrhizine and palmatine), berberine exerted the strongest inhibition against *M. fructicola* (Fig. 1, $P < 0.01$). As such, we eliminated the other three analogs as potential natural compounds providing significant inhibitory activity. This finding also illuminated the structure–activity relationship between berberine and its analogs. Interestingly, they share almost identical structure except for the differences in C-2 and C-3 (Fig. 1). Not surprisingly, it is the subtle differences that lead to divergent inhibitory activities towards *M. fructicola*.

3.3. MIC and EC_{50} of berberine for M. fructicola

To determine the MIC of berberine against *M. fructicola*, PDA plates containing serially diluted berberine were prepared as previously described and inoculated with *M. fructicola*. The results showed that the EC_{50} and MIC of berberine against *M. fructicola* were merely 4.55 μg/mL and 46.90 μg/mL, respectively.

Table 1
Toxicity of *Coptis chinensis* extract to three fungi *Botrytis cinerea*, *Monilinia fructicola* and *Alternaria solani*.

Pathogenic fungi	Inhibitory rate	Linear regression equation	Correlation coefficient (r)	EC_{50} (mg/ml)
Monilinia fructicola	100%	$Y = 5.0581 + 1.4747X$	0.9374	0.91**
Botrytis cinerea	74.4%	$Y = 3.3602 + 1.4272X$	0.9819	14.09*
Alternaria solani	80.7%	$Y = 3.1894 + 1.2600X$	0.8848	27.35*

Coptis chinensis extract vs control $^{*}P<0.05$, $^{**}P<0.01$, $n = 10$.

3.4. Effect of berberine on spore germination and hyphal growth

At the berberine concentration of 0.125 μg/mL, the inhibitory rate on spore germination was 80.2%, however, the inhibitory rate increased to ~100% when berberine concentration of 4 μg/mL was used, indicating the dosage effect. Other concentrations of berberine solution also led to high inhibitory rates on spore germination. In the control, the fungal spores germinated normally and grew aggressively. By contrast, when treated with berberine at the concentration of 0.125 μg/mL, although some of the berberine-treated spores could germinate, mycelial tube malformation manifested, subsequently resulting in slowed growth and finally stagnation. These results indicate the inhibition effect of berberine on spore germination and hyphal growth.

3.5. Effect of berberine on cutinase activity

The influence of berberine on cutinase activity was studied. During 21 days of strain culture and cutinase induction, the enzyme activities were assayed every 3 days. As demonstrated in Fig. 2, the cutinase activities of berberine-treated *M. fructicola* were at lower levels during the 21 days compared to the control, especially from 9th to 15th day. This finding implied that the cutinase expression was regulated by berberine via signal transduction pathway, or through its direct binding to cutinase. The underlying mechanisms require further study.

3.6. In vivo inhibition against M. fructicola and phytotoxic effect on peach fruits

To determine whether berberine exerted *in vivo* inhibition against *M. fructicola*, an inoculation experiment was performed. As shown in Fig. 3, berberine-treated fruits were healthy and uninfected, by contrast, the fruits in the control group (without injecting berberine) were severely infected by *M. fructicola* and manifested aggressive lesions ($P < 0.05$). This result clearly demonstrated the *in vivo* inhibition of berberine against *M. fructicola*.

To determine whether berberine can impart toxicity to peach fruits, peach fruits were immersed in berberine solution for 5 min, removed and stored at 4 °C for 2 days. Even when treated with berberine at concentrations up to 400 μg/mL (much higher than its MIC and EC_{50} value), few fruits showed visual damage suggesting the safety of berberine to peaches and also providing an indication that berberine can be applied as a field spray or used in storage management.

4. Discussion

M. fructicola can infect most of stone fruits, including peaches (*P. persica* L.), apricots (*Prunus armeniaca* L.), plums (*Prunus salicina* L.), and cherries (*Prunus pseudocerasus*) (Forster and Adaskaveg, 2000; Luo and Michailides, 2003; Luo et al., 2005; Giobbe et al., 2007). In particular, it causes brown rot that remarkably reduces the yield and quality of peaches around the world. Although tremendous efforts have been paid to the screening of safe and efficient natural fungicides, almost no such fungicides have come to dominate the market due to their relatively weak antifungal activities. In this study, *C. chinensis* extract was found for the first time to show strong inhibition against *M. fructicola* (Table 1; Figs. 1 and 3). Its EC_{50} value was much lower than that of *B. cinerea* and *A. solani*, clearly suggesting its inhibition preference to *M. fructicola* (Table 1). In addition, we also investigated the inhibitory activities of other

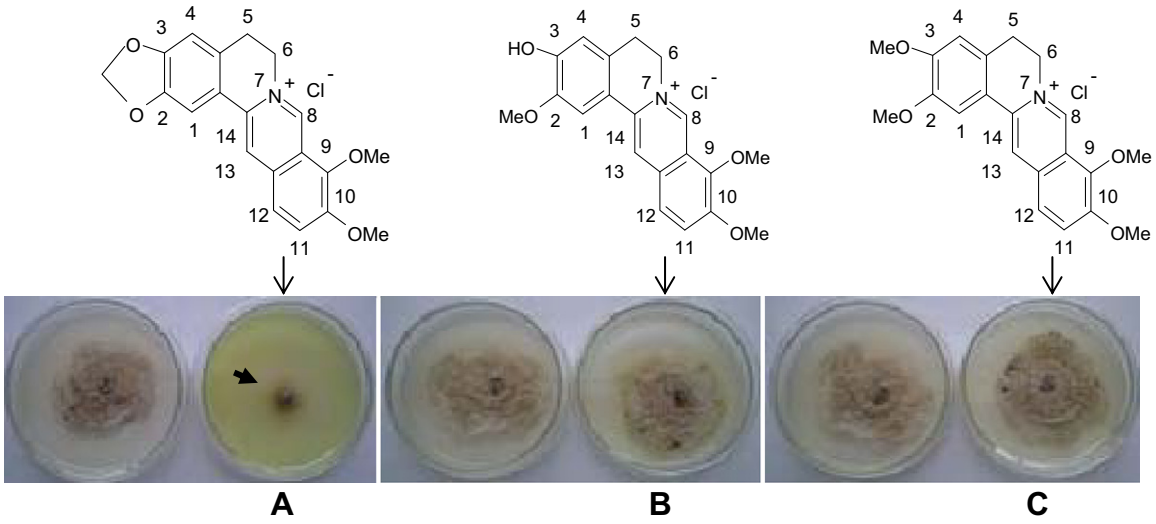

Fig. 1. Different inhibitory activities of berberine and its analogs against *Monilinia fructicola*. For each treatment, the left petri dish was the control, the right petri dish contains berberine (A), jateorrhizine (B), palmatine (C) at a concentration of 234.375 μg/mL. The striking inhibition of berberine on *M. fructicola* was highlighted with arrowhead.

herb extracts. Compared to extract of *C. chinensis*, extracts of *T. wilfkrdii*, *A. apiacea* and *M. toosendan* showed relatively weak inhibition against *M. fructicola*. Based on these findings, we conclude the presence of antifungal component(s) in *C. chinensis* extract. Subsequent partitioning of this extract revealed the robust inhibition of berberine against *M. fructicola* (Fig. 1). Although the EC_{50} and MIC values of berberine are somewhat higher than that of DMIs (EC_{50} <1 μg/mL), it can yet be regarded as a competitive natural fungicide due to its low cost and availability. As we know, it has been produced industrially on a large scale.

As a natural alkaloid, berberine is conventionally employed to kill both gram-positive and gram-negative bacteria (Iwasa et al., 1998; Stermitz et al., 2000). Here we uncovered its striking inhibition against *M. fructicola*. Its EC_{50} value is merely 4.55 μg/mL, clearly indicates its potential commercial value. The berberine's inhibitory activities include reduced spore germination rate, retarded mycelium growth, and diminished cutinase activity (Fig. 2). Regarding the effects of possible residue on peach fruits, because berberine is an antidiarrheal medicine traditionally administered in China and other Asian countries for centuries, possible negative health effects from ingesting this residue are not anticipated. In terms of aesthetics, this preliminary study showed

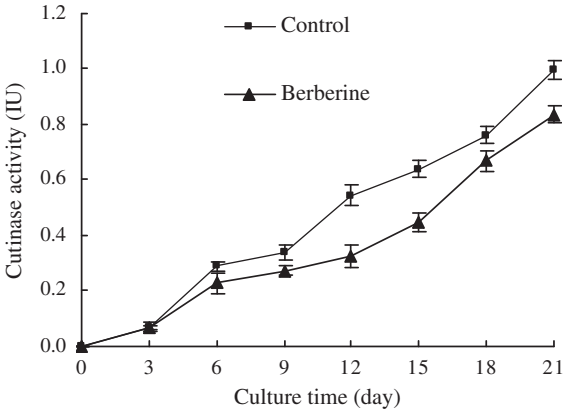

Fig. 2. Inhibition assay of berberine on cutinase activity of *Monilinia fructicola*. The control: without berberine. Culture time: the days after inoculation of *Monilinia fructicola*. Cutinase activity was assayed by calculating absorbance value under 405 nm.

that berberine does not visually damage peach fruit surfaces, even at a high berberine concentration. Presently, berberine can be produced industrially on a large scale. Owing to its availability, low cost (~12 USD/kg in China, purity of 99%), safety to humans, as well as compatibility with environment, it deserves putting into practice and might be of particular interest to scientists in this field.

As an alkaloid purified from *Berberis* species, berberine has been extensively studied for many years. It has shown significant antimicrobial activity towards a variety of organisms including bacteria, fungi, protozoans, viruses, chlamydia and helminthes (Basha et al., 2002). Berberine has been verified to be a valuable leading compound. For instance, by introducing aromatic groups in 13-C of berberine, a series of 13-(substituted benzyl) berberine derivatives were synthesized. The synthesized compounds exhibited stronger antifungal activities than berberine (Park et al., 2006). Hence, it is warranted that a more effective compound may emerge upon structural modification. In principle, structural modification makes the compounds more lipophilic, which increases the permeability of cell membranes. Forthcoming research may focus on structurally related compounds that might have antifungal activity superior to berberine, and thus establish structure–activity relationships among berberine analogs. In this study, berberine was found to impart considerable inhibitory activity towards *M. fructicola* (EC_{50} = 4.55 μg/mL, MIC = 46.90 μg/mL). Although its EC_{50} and MIC values were somewhat higher than that of DMI fungicides prevalent in the antifungal market, owing to its availability and low cost, berberine may be used alone or in combination with DMI fungicides, instead of being developed into novel compounds via structural modification. If so, the environmental pressure of DMI fungicides may be alleviated ascribing to the biodegradable property of berberine. Based on this preliminary study, berberine does not cause visual chemical damage to the peach fruit surface, meaning berberine will not produce cytotoxicity. In addition, berberine has been demonstrated to be safe for human consumption. Overall berberine may be a promising natural fungicide to control brown rot.

Compared to the growing body of literature on berberine's inhibition against bacteria, little is known about its antifungal mechanism. In this study, berberine was found to not only block spore germination, but also inhibit cutinase activity (Fig. 2). The mechanisms underlying berberine being uptaken by *M. fructicola* and the intracellular navigation remain unknown and require

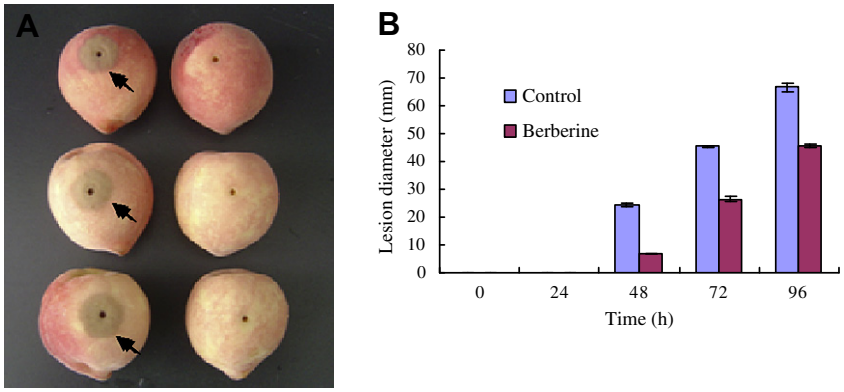

Fig. 3. *In vivo* inhibition analysis of berberine against *Monilinia fructicola*. (A) Prior to incubation, berberine at concentration of 234.375 μg/mL was put into the wounds of the treatment group (right), whereas sterilized water was put into the control (left). After 24 h, all fruits were inoculated with *M. fructicola*. The resulting lesions were highlighted with arrowheads. (B) Comparison of lesion diameters in control and berberine-treated peach fruits.

further study. Based on existing literature concerning its antimicrobial mechanism (Ghosh et al., 1985; Letasiova et al., 2006; Jantova et al., 2007; Domadia et al., 2008), we may only envision the complexity of its antifungal strategy. As reported, berberine inhibits DNA and protein synthesis via its binding to the grove of DNA (Letasiova et al., 2006), and exhibits powerful anti-bacterial activity to both gram-positive and gram-negative bacteria (Iwasa et al., 1998; Stermitz et al., 2000) as well as some fungi (Basha et al., 2002). Judged by these available conclusions, we speculate that berberine likely inhibits *M. fructicola* through diverse modes including binding multiple targets, destroying cell membranes, hampering cell division, or other unknown strategies.

The fact that multiple mechanisms may exist for berberine's inhibition of *M. fructicola* provides some hope that *M. fructicola* may not develop resistance to berberine. As we know, DMI fungicides inhibit pathogens via binding one or limited targets, hence, pathogens can easily develop fungicide resistance just by over-expression of one or limited target genes or via gene mutation. For example, cytochrome P450 Lanosterol 14α-Demethylase gene (*MfCYP51*) is a demethylation inhibitor fungicide resistance determinant in *M. fructicola* field isolates from Georgia, USA. Just by the over-expression of *MfCYP51*, *M. fructicola* easily developed resistance to DMIs (Luo and Schnabel, 2008). Deduced from the available literature (Hirakawa et al., 2005; Letasiova et al., 2006; Domadia et al., 2008; Sinha and Kumar, 2009), unlike DMIs, berberine most likely binds multiple targets in *M. fructicola*. This is because berberine can target DNA (Hirakawa et al., 2005; Letasiova et al., 2006), protein (Domadia et al., 2008) or RNA (Sinha and Kumar, 2009). Thus, *M. fructicola* has vast difficulty in developing resistance to berberine barring simultaneous over-expression or mutation of a variety of genes. As mentioned above, *M. fructicola* has developed resistance to benzimidazole and DMI fungicides (Jones and Ehret, 1976; Sonoda et al., 1983; Penrose, 1990; Zehr et al., 1991; Ma et al., 2003; Schnabel et al. 2004; Holb and Schnabel, 2007). Different from DMIs, berberine inhibits the growth or cell division with diverse strategies, including destroying cell membranes, hampering cell division, or other unknown mechanisms. Moreover, its broad antimicrobial spectrum allows it to retain longer application time than DMI and other specific fungicides. To clarify the mechanisms, further studies are required.

To our knowledge, this is the first report of berberine exerting remarkable inhibition against *M. fructicola*. The underlying mechanisms warrant in-depth studies, including the mode of berberine passing through the cell membrane of *M. fructicola*, the interaction between berberine and DNA, RNA or protein, as well as the metabolic perturbation upon berberine stress. Among the unknown mechanisms, the targets of berberine are most fascinating. Forthcoming research is expected to decipher them, which may in turn provide implications for disease management. In view of existing studies, the strategy of integrating measures such as chemical spray and biocontrol may be a good approach for controlling brown rot (Emery et al., 2002; Yao and Tian, 2005; Holb and Schnabel, 2008a, 2008b; Xu et al., 2008). Hence, berberine in combination with the prevalent fungicides in market should contribute to the control of brown rot, and may be capable of attenuating the surging fungicide resistance. On the other hand, if mixed with other compounds, berberine will have wide applications. For example, a berberine–chitosan composite film and microsphere prepared in our lab also have been showed as robust inhibition against *M. fructicola* as berberine alone, but this self-made composite membrane and microsphere could slowly release berberine for more than one month, which would indicate a persistently retained antifungal activity during storage (unpublished data). With berberine as a principle component, fruit packing materials or composite fungicides will be developed. Taken together, these findings open the possibility for the broad use of berberine in controlling brown rot on stone fruits.

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