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Changes in *Botrytis cinerea* Conidia Caused by *Berberis vulgaris* Extract

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Abstract

Testing plant extracts for controlling fungal diseases is a main biocontrol method. More interesting is to see what happens to the fungus treated with the plant extract. Therefore, the aim of the study was to evaluate the antifungal activity of *Berberis vulgaris* extract on *Botrytis cinerea* and to examine the ultrastructural changes in *B. cinerea* conidia caused by the minimum inhibitory concentration (MIC), using SEM and TEM. The antifungal activity of *B. vulgaris* bark extract was investigated using agar dilution method, and compared to that of berberine. Fluconazole was used as the positive antimycotic control. It was found that (1) *B. vulgaris* bark extract had significant antifungal activity against *B. cinerea*, and its effect was stronger than that of pure berberine. It was also noted that (2) *B. vulgaris* MIC caused severe structural changes of the conidia, comparable with berberine MIC effect; therefore (3) *B. vulgaris* bark extract might be recommended to be tested as a biocontrol agent against *B. cinerea*.

Keywords: berberine, fungi, plant extract, SEM, TEM

Introduction

Plant diseases constitute a threat to global food security (Strange and Scott, 2005). *Botrytis cinerea* is a necrotrophic opportunistic plant pathogenic fungus, also known as "gray mould fungus", which causes serious pre- and postharvest diseases in more than 200 plant species, including agriculturally important crops and harvested commodities, such as grapes, tomatoes, strawberries, cucumbers, bulb flowers, cut flowers, and ornamental plants (Kars *et al.*, 2005). The broad host range of *B. cinerea* results in great economic losses, not only during growth but also during the storage and transportation of products (Elad, 2003). Necrotrophs kill their host cells by secreting toxic compounds or lytic enzymes and also produce an array of pathogenicity factors that can subvert host defences (Makovitzki, 2007).

Plant fungicides based on synthetic chemicals cause severe and long-term environmental pollution, are highly and acutely toxic, and are sometimes even carcinogenic to humans and wild animals. Pathogens can also become resistant to many of these chemicals. *Botrytis cinerea* strains are highly genetically and physiologically variable, and several strains have developed resistance to most of the fungicides used to control them (van Baarlen *et al.*, 2004; Silva *et al.*, 2006). Consequently, the aim of new antifungal strategies is to develop drugs that combine sustainability, high efficacy, restricted toxicity, safety for humans, animals, host plants, and ecosystems, and low cost of production. Because fungicides of biological origin have been demonstrated to be specifically effective on target organisms and are also biodegradable, biological control has become popular worldwide (Barker and Rogers, 2006; Carrillo-Munoz *et al.*, 2006; Fatehi *et al.*, 2005; Ienascu *et al.*, 2008).

Berberis vulgaris (barberry) is a common garden bush, native to Europe and the British Isles, and naturalized in North America. It has played a prominent role in herbal healing for more than 2,500 years. Twenty-two alkaloids of medicinal importance have been reported so far from the roots, stems, leaves, and fruit of this plant (Arayne *et al.*, 2007). The alkaloid content differs in *Berberis* from different areas, different species, and different organs (Di *et al.*, 2003). The main alkaloid that has been isolated from the roots and bark of *B. vulgaris* is berberine, an isoquinoline alkaloid with antibacterial and antifungal properties (Singh *et al.*, 2001; Soffar *et al.*, 2001).

The aim of this study was to evaluate the antifungal activity of a hydroalcoholic extract of *B. vulgaris* bark on *B. cinerea* and to examine the ultrastructural changes in

B. cinerea conidia caused by the minimum inhibitory concentration (MIC) of *B. vulgaris*.

Materials and methods

Chemicals

Fluconazole (2 mg ml^{-1}) (Krka, Novo Mesto, Slovenia), berberine (Merck KGaA, Darmstadt, Germany), Czapek agar (BD Difco, Budapest, Hungary), ethanol (70% EtOH), ammonium acetate (Merck KGaA, Darmstadt, Germany), acetonitrile (Merck KGaA, Darmstadt, Germany) and all other used chemicals and reagents were of the highest grade commercially available.

Extract preparation

The extract was prepared according to Squibb's re-percolation method (Anonymous, 1993; Ionescu-Stoian and Savopol, 1977): shade air-dried *Berberis vulgaris* L. stem bark was powdered in a cross beater mill equipped with a 3 mm sieve, and an aliquot (1 g) was extracted with one ml EtOH, and than filtered through a disc of filter paper.

Phytochemical screening

The total berberine content of the *B. vulgaris* bark extract was quantitatively determined by LC/MS/MS analysis, performed on an Agilent 1100 Series HPLC system (Agilent Technology Co., Ltd.). LC separation was performed on a Zorbax SB-C18 column (100 mm × 3.0 mm, i.d. 3.5 µm; Agilent) preceded by a 0.5 µm online filter. The mobile phase consisted of acetonitrile and 4 mM (v/v) ammonium acetate in water, in a ratio of 30:70 (v/v), delivered at a flow rate of 1 ml min^{-1}. The autosampler injection volume was set at 5 µl. The mass spectrometer operated using an ESI source in positive mode and was set for isolation and fragmentation of the berberine molecular ion with m/z = 336 (Fig. 1a). Quantification of berberine was based on the sum of ions with m/z = 291.9 and 321.0 from the MS spectrum of the parent ion (Fig. 1b). The calibration curve was linear in the range of 9.82-196.42 ng ml^{-1}, with a correlation coefficient of 0.9978. Due to enhanced sensitivity and selectivity of MS/MS (Fig. 2a) over the UV detection (Fig. 2b), we chose the former to use for quantification of berberine in samples (Vlase *et al.*, 2007; Vlase *et al.*, 2008; Wu *et al.*, 2005). The retention time for berberine was 3.6 min. (Fig. 2a, 2b).

Determination of antifungal activity

Antifungal activity was investigated with a radial growth inhibition assay (Bhandari *et al.*, 2000). The MICs were determined for *B. vulgaris* bark extract, berberine, and fluconazole against five strains of *B. cinerea* isolated from roses, at five days after inoculation. Fluconazole was used as the positive antimycotic control. A drug-free-growth negative control and an ethanol control (C) were

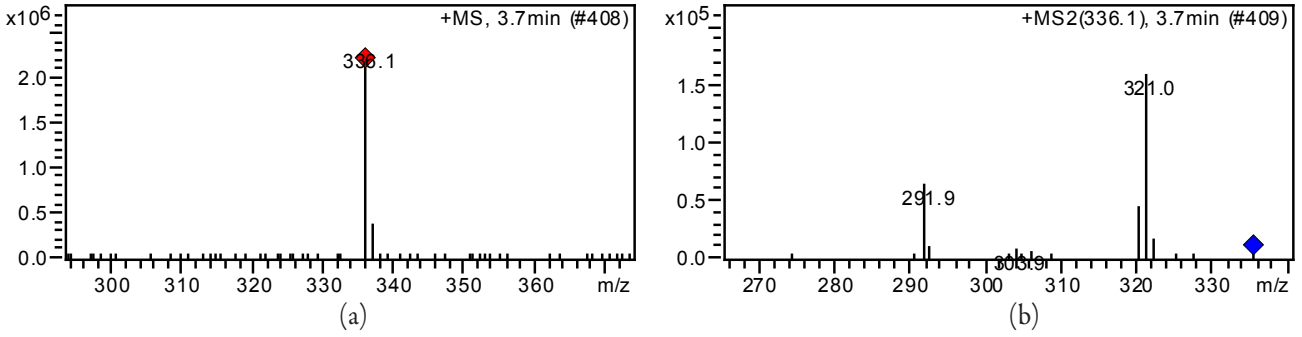

Fig. 1. (a) Full-scan MS spectrum of berberine in the mobile phase; (b) MS/MS spectrum of berberine in the mobile phase

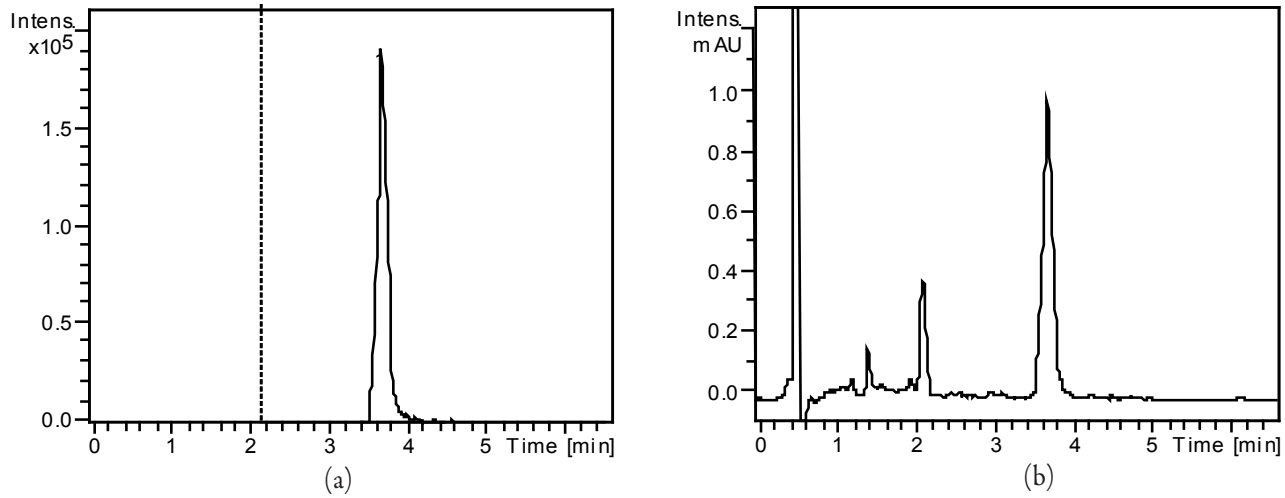

Fig. 2. Chromatograms of berberine from *Berberis vulgaris* extract (a) MS/MS signal; (b) UV signal at 343 nm

also used. The percentage of growth inhibition (P) was calculated by the formula: P = [C – T] × 100/C, where C is the diameter of the ethanol control colony and T is the diameter of the treated colony (Nidiry and Babu, 2005).

SEM and TEM examination of conidia

Samples containing *B. cinerea* conidia were incubated with *B. vulgaris* bark extract or berberine at its MIC for one h. The grids were examined by SEM with a JEOL JSM 5510 LV electron microscope (Vanky, 1994) and by TEM with a JEOL JEM 1010 electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) (Hayat, 2000).

Statistical analysis

Data are expressed as means ± SEM of six independent experiments. Comparisons of groups were performed by ANOVA test. Results expressed as percentages were analyzed with the Kruskal-Wallis test. Correlations were assessed by Pearson's and Spearman's correlation coefficients. For each test, the significance level was established at $P < 0.05$. Statistical calculations were performed with the Statistica for Windows software package (http://www.statsoft.com/textbook/stathome.html).

Results and discussion

The berberine concentration (expressed as berberine-HCl-2H_2O) in the *B. vulgaris* bark extract, determined by LC/MS/MS, was 0.6188 mg ml^{-1} of extract.

B. vulgaris bark extract, berberine, and fluconazole significantly inhibited the hyphal radial growth of *B. cinerea* ($P < 0.001$) (Tab. 1). The inhibitory effects correlated positively with the concentrations of *B. vulgaris* bark extract, berberine, and fluconazole ($r = 0.88$-0.99 and $r^2 = 0.78$-0.98). When the effects of *B. vulgaris* bark extract were compared to those of equivalent doses of pure berberine, the plant extract had a stronger inhibitory effect on *B. cinerea* growth ($P < 0.01$): the MIC of *B. vulgaris* bark plant extract against *B. cinerea* was 18.6 μg ml^{-1}, whereas the MIC of pure berberine was 27.8 μg ml^{-1}. The fluconazole MIC against *B. cinerea* was 120 μg ml^{-1}.

Important antifungal activity of *Berberis* spp. have been demonstrated against some fungal strains with hydroalcoholic extracts, aqueous extract, methanolic or crude extracts, and alkaloidal fractions (Freile *et al.*, 2003; Iauk *et al.*, 2007; Li *et al.*, 2007; Parvu *et al.*, 2007; Parvu *et al.*, 2008; Singh *et al.*, 2007). Alcoholic extracts provide more complete extraction, and include fewer polar compounds (Webster *et al.*, 2008). The *in vitro* antifungal activity of berberine isolated from the same sources has also been investigated, and it was found that berberine alkaloids are cationic antimicrobials (Stermitz *et al.*, 2000).

The present results demonstrate important *B. vulgaris* bark extract activity against *B. cinerea* isolates, causing significant dose-dependent growth inhibition. Like previous studies of berberine (Fatehi *et al.*, 2005; Singh *et al.*, 2007), our results demonstrate that it possesses important antifungal activity in a concentration-dependent manner, and has lower antifungal activity against some fungi than *Berberis* spp. extracts (Iauk *et al.*, 2007). All these data suggest that other antimycotic compounds are present in the plant extract in addition to berberine.

Examination by SEM revealed that *B. vulgaris* bark extract, at its MIC, induced large-scale damage to the conidia of *B. cinerea*, because the surface protuberances from the control disappeared (Fig. 3a, 3b). On TEM micrographs *B. vulgaris* bark extract caused a disruption of the *B. cinerea* conidial cell wall, the external layer was more electron dense, the plasmalemma and the cytoplasm of the *B. cinerea* conidia had shrunk and detached altogether from the cell wall, the organelles and nucleus were also partly destroyed (Fig. 4a, 4b). Berberine treatment caused similar changes of the *B. cinerea* conidia as did *B. vulgaris* bark extract (Fig. 4c).

Other studies described *B. cinerea* conidial ultrastructure (Coley-Smith, 1980). The morpho-functional integrity of fungal cell components is required to maintain their viability and germination capacity (Segmüller *et al.*, 2008). Our electron microscopy data revealed that *B. vulgaris* bark extract, at its MIC, acts by causing irreversible ultrastructural changes to the *B. cinerea* conidia. Impor-

Tab. 1. *In vitro* radial growth inhibition of *Botrytis cinerea* by an ethanolic extract of *Berberis vulgaris*, berberine, and fluconazole

Berberis vulgaris (μg ml^{-1})	Colony diameter* (mm)	P* (%)	Berberine (μg ml^{-1})	Colony diameter† (mm)	P† (%)	Fluconazole (μg ml^{-1})	Colony diameter‡ (mm)	P‡ (%)
0	65	0	0	65	0	0	65	0
10	18.3	71.8±0.03	6.2	52.8	18.7±0.02	20	40.3	37.9±0.04
20	5.5	91.5±0.02	12.4	32.5	50±0.03	60	20.5	68.4±0.02
25	2.3	96.4±0.03	15.5	22	66.1±0.01	100	5	92.3±0.04
30	0	100	18.6	11.5	82.3±0.04	120	0	100
			24.8	2.15	96.7±0.01			
			27.8	0	100			

Legend: P = the percentage of growth inhibition; *The effect of *Berberis vulgaris* bark extract; †The effect of berberine; ‡The effect of fluconazole

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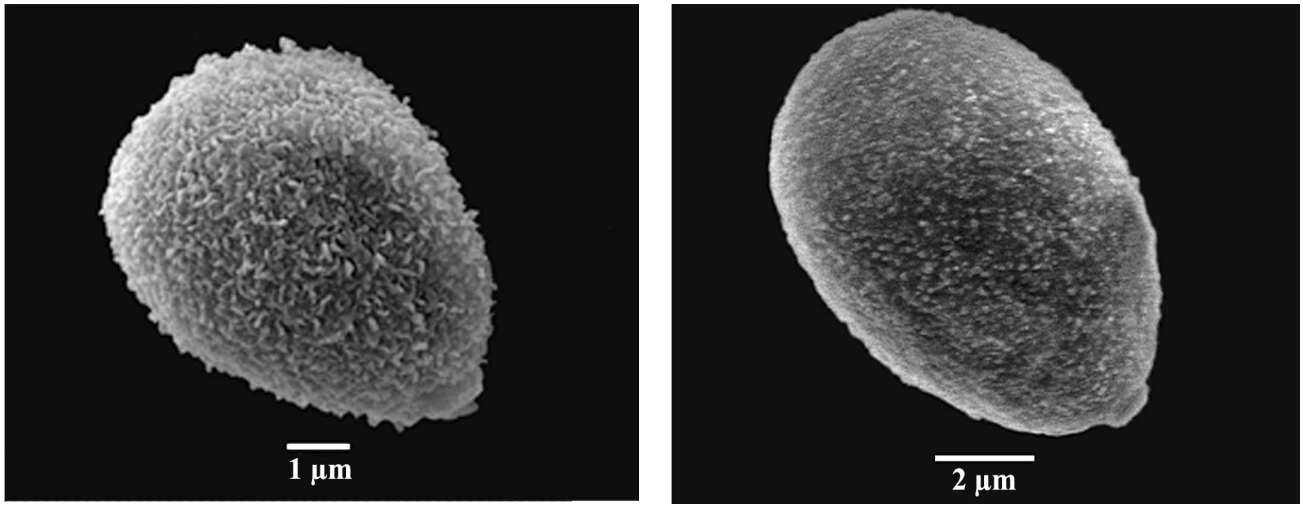
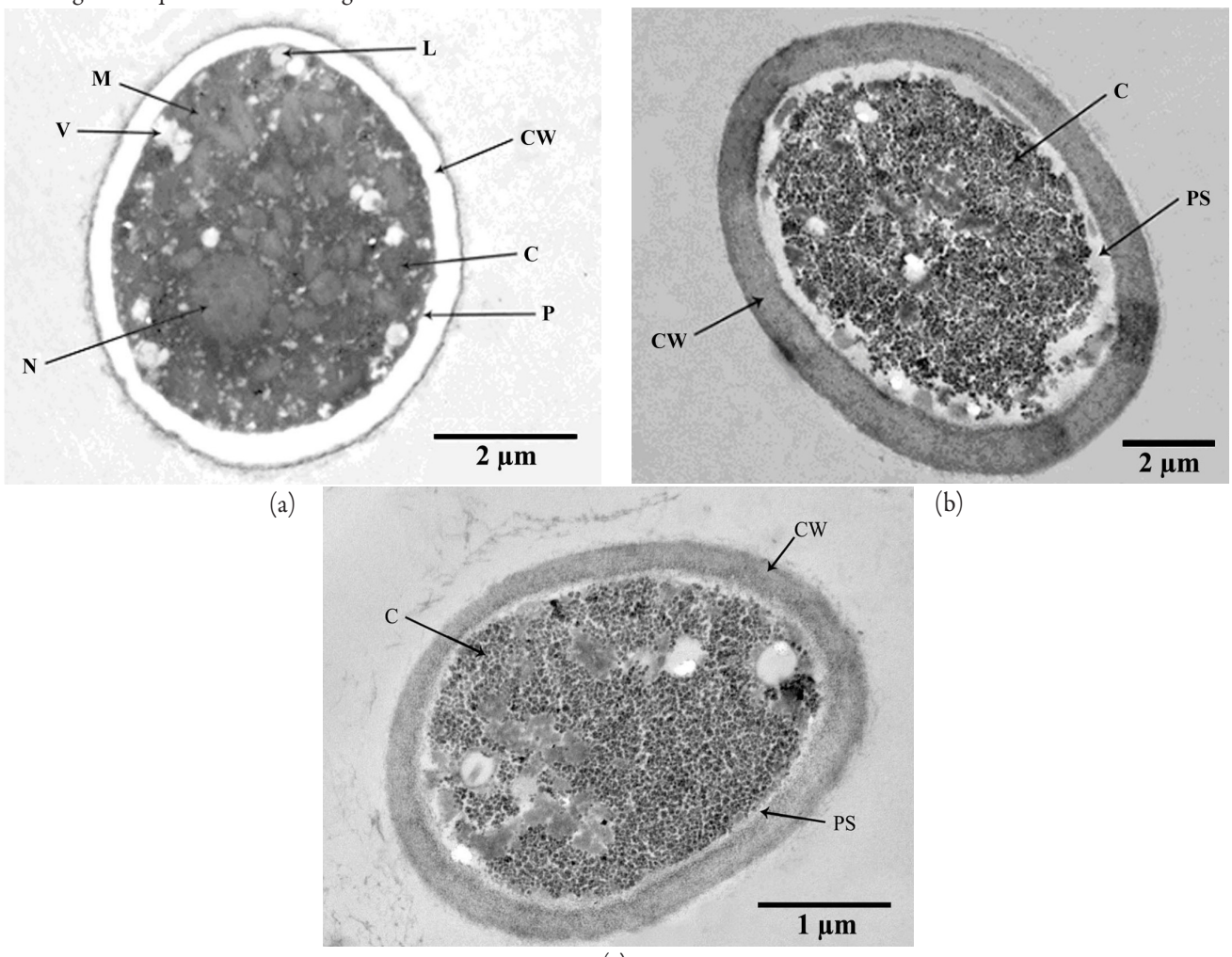


(a) (b)

Fig. 3. Scanning electron micrographs of: (a) control *Botrytis cinerea* conidium, showing randomly positioned surface protuberances; (b) *Botrytis cinerea* conidium treated with *Berberis vulgaris* plant extract at the minimum inhibitory concentration (MIC), showing surface protuberance damage



(a) (b)

(c)

Fig. 4. Transmission electron micrograph of: (a) cross section of a control *Botrytis cinerea* conidium, showing the ultrastructural components; (b) cross section of a *Botrytis cinerea* conidium treated with *Berberis vulgaris* plant extract at the minimum inhibitory concentration (MIC), showing irreversible ultrastructural changes; (c) cross section of a *Botrytis cinerea* conidium treated with berberine at the minimum inhibitory concentration (MIC), showing irreversible ultrastructural changes. CW = cell wall; P = plasmalemma; PS = periplasmic space; C = cytoplasm; M = mitochondrion; N = nucleus; V = vacuole; L = lipids

tantly, the antifungal effect was rapid (one h incubation), which should make it difficult for the pathogen to develop resistance. Even when berberine MIC was bigger than that of *B. vulgaris* bark extract, the electron microscopy examination showed that it had similar effects on the *B. cinerea* conidia. Due to these results, it is likely that most of the morpho-functional changes induced by *B. vulgaris* bark extract are due to the berberine content.

B. vulgaris bark extract significantly inhibits the radial growth of *B. cinerea*, and its effect was stronger than that of berberine. *B. vulgaris* bark extract MIC caused the conidia to lose viability because of severe structural changes. The mechanism of the antifungal activity of *B. vulgaris* bark extract has yet to be fully clarified, but berberine seems to be one of the most important antifungal compounds because its MIC caused comparable structural effects.

Because of the increasing resistance of plant pathogens to currently available antimicrobial agents and the emerging need to eliminate toxic chemicals from agricultural use, *B. vulgaris* bark extract could serve as a viable biocontrol treatment alternative to conventional antifungal agents.

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