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Inhibitory activity of *Athyrium sinense* extracts against *Clavibater michiganense* subsp. *sepedonicum*

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Summary

Clavibater michiganense subsp. *sepedonicum*, the causal agent of a serious disease called bacterial ring rot of potato, is widespread under both greenhouse and field cultivation. In order to find a promising method for the control of this disease, *Athyrium sinense* extracts were investigated in the study presented here. Results showed that the *A. sinense* extracts had significantly inhibitory activity against *C. michiganense* subsp. *sepedonicum*. Eight organic solvents were used to obtain *A. sinense* extracts, and ethanol was selected as an ideal extraction solvent. Based on single factor experiment results and $L_{27}3^{(13)}$ orthogonal experiment results, optimum extraction conditions were obtained. They were $A_2B_3C_3$, solid to liquid ratio 1:20 (g:mL), temperature 97 °C and extraction time 10 h. Stability assays revealed that Zn^{2+}, Fe^{3+}, acidic and neutral conditions enhanced inhibitory activity, and extracts were stable under ultraviolet (UV). Transmission electron microscopy (TEM) showed drastic changes caused by *A. sinense* extracts, including lack of cytoplasmic materials, formation of vacuoles and bacterial deformation. These results revealed *A. sinense* extracts have strong inhibitory activity against *C. michiganense* subsp. *sepedonicum*, and can be used as a potent phytochemical fungicide.

Introduction

Potato is an important vegetable crop grown throughout the world. The Gram-positive coryneform bacterium *Clavibater michiganense* subsp. *sepedonicum* (Spieckermann & Kotthoff) Davis et al. is a causal agent of a serious disease called bacterial ring rot of potato (BRR). The disease has occurred in major potato-growing area (Fousek and Mráz, 2003). Currently, BRR become most destructive in China and yield loss was up to 60% (Fu et al., 2005). Chemical bactericides are used for controlling BRR (Chen et al., 2010). However, these chemicals are highly toxic to environment (Cardoso et al., 2010). In an attempt to modify this condition, some alternative methods of control have been adopted. Recent efforts have focused on developing environmentally safe, long lasting and effective biological control methods for the management of plant diseases (Nguyen et al., 2009). Plants are exposed to various pathogenic fungi and bacteria (Asano et al., 2013). As a countermeasure, plants produce physiologically active substances to inhibit phytopathogens, which include antibacterial substances (Yazaki et al., 2008; Sabat and Gupta, 2009; Chen and Dai, 2012; Tabti et al., 2014; Elshafie et al., 2015). Use of plant products and biological control agents has been shown to be eco-friendly and effective against many plant pathogens (Sivakumar et al., 2008; Latha et al., 2009; Dan et al., 2010; Hashem et al., 2010; Matusinsky et al., 2015).

Athyrium sinense Rupr. is a fern, and commonly used as a traditional Chinese medicine for preventing influenza and epidemic encephalitis B (Li and Xu, 2009). However, no attempts have been made for using *A. sinense* extracts to biological control of *C. michiganense* subsp. *sepedonicum*. In the present study, the objectives were to determine inhibitory activity of *A. sinense* extracts against *C. michiganense* subsp. *sepedonicum*; to optimize antibacterial substance extraction of *A. sinense*, which can give maximal inhibitory activity; to evaluate the stability of *A. sinense* extracts under different environmental conditions; to investigate the interior damage by using transmission electron microscopy (TEM).

Materials and methods

Plant material and pathogen

Athyrium sinense Rupr. was collected from Pangquangou Nature Reserve, Shanxi Province, North China (37°45′-37°55′N, 111°22′-111°33′E), in August 2010. Taxonomic identification was performed by Prof. Shulian Xie from School of Life Science, Shanxi University, where the specimens (SAS2010208) are deposited. *Clavibater michiganense* subsp. *sepedonicum* (Spieckermann & Kotthoff) Davis et al. (ATCC 33113) was provided by Chinese Academy of Agricultural Science.

Solvent selection

A. sinense was washed, and dried for 20 days at room temperature (Abdel-monaim et al., 2011). Dry *A. sinense* was grounded using the blender. Extraction was performed as previously described (Khadri et al., 2010) using eight solvents with different polarities. The powdered *A. sinense* (10 g of each) were extracted respectively with 100 mL of methanol, ethanol, acetone, chloroform, ethyl acetate, butanol, benzene and petroleum ether 8 h at corresponding solvent boiling temperatures. After filtration, the solvents were evaporated to dryness by using rotary evaporation. Then dryness of each sample was dissolved in dimethyl sulphoxide (DMSO) to give a final concentration of 1 mg/mL. The crude extracts were evaluated for their inhibitory activities as described below.

Inhibitory activity determination was performed by agar diffusion method (ADM; Michielin et al., 2009). The agar surface was perforated with 10 mm holes and subsequently filled with 200 µL of each extract sample. Since DMSO does not inhibit microorganism growth (Smânia et al., 1999), it was used as control. Plate was incubated at 28 °C for 24 h. Inhibitory activity was determined by measuring the inhibition zone around each hole. Each treatment was replicated four times.

Single factor experiments

Solid to liquid ratio (g:mL), extraction temperature (°C) and extraction time (h), are three factors, which can affect extraction efficiency. Appropriate levels of orthogonal experimental design would be decided by single factor experiments. For each single factor, five different levels were designed, with other factors keeping constant. For each assay, 10 g sample was added to corresponding volume of ethanol and extracted as Tab. 1. Then, inhibitory activity was analyzed by ADM. Each treatment was replicated four times.

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Tab. 1: Single factor experiment design

Factors	Conditions	Levels				
		1	2	3	4	5
Solid to liquid ratio (g:mL)	Extraction time 8 h Temperature 85 °C	1:10	1:15	1:20	1:25	1:30
Temperature (°C)	Extraction time 8 h Solid to liquid ratio 1:10	40	55	70	85	97
Extraction time (h)	Temperature 85 °C Solid to liquid ratio 1:10	4	6	8	10	12

Orthogonal experimental design

On the basis of results of single factor experiments, three levels of three factors were selected as described in Tab. 2. Then $L_{27}(3^{13})$ orthogonal assay was used to determine the optimum extraction conditions of antibacterial substances in *A. sinense* (Jia et al., 2010). For each experiment, 10 g *A. sinense* sample was added to corresponding volume of ethanol and extracted as Tab. 3. Then, inhibitory activity was analyzed by ADM. Each treatment was replicated four times.

Tab. 2: Factors and levels of orthogonal experiment

Levels	Factors		
	Solid to liquid ratio (A)	Extraction temperature (B)	Extraction time (C)
1	1:15 (g:mL)	70 °C	6 h
2	1:20 (g:mL)	85 °C	8 h
3	1:25 (g:mL)	97 °C	10 h

Stability assays

The effect of pH on inhibitory activity in *A. sinense* extracts were examined by pH stability assays (Wu et al., 2008; Cheikhyoussef et al., 2009). Tests were conducted in two sets: test sets of *A. sinense* extracts were adjusted with 5M NaOH or 5M HCl to different pH values ranging from 2 to 12. The control sets were prepared using the same method with DMSO except that no *A. sinense* extract was added. To test effect of metal ions, tests were conducted in four sets: solutions of Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, and Fe^{3+} were separately added to *A. sinense* extracts (1 mg/mL) to a final concentration of 1 mol/L. One negative control set was prepared using the same method with DMSO except that no *A. sinense* extract was added. Another negative control set was prepared only with DMSO. The positive control set was prepared with *A. sinense* extract, but no metal ion added. To test the impact of UV, *A. sinense* extracts were incubated under UV light (256 rim, 6 W, 5 cm) for a period ranging from 1 h to 5 h. For all stability assays, *C. michiganense* subsp. *sepedonicum* was inoculated into separate mixtures at a concentration of 10^7 CFU/mL. Then, inhibitory activity was analyzed by agar diffusion method. Each treatment was replicated four times.

Transmission electron microscopy (TEM)

Logarithmic phase cells of *C. michiganense* subsp. *sepedonicum* (each approximately 10^9 CFU/mL) were treated with *A. sinense* extracts at 0.06 g/L for 8 h. No treatment with *A. sinense* extracts was as control. TEM was performed as previously described (Cai et al., 2013). The samples were transferred to fresh 0.5% glutaraldehyde, and kept for 30 min at 4 °C, centrifuged, and fixed in 3% glutaraldehyde. The cells were further fixed in 4% osmium tetroxide, dehydrated in graded solutions of acetone, and embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a transmission electron microscope (Model JEM-1011, JEOL, Japan) at 80 kV.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test were carried out to determine significant ($P<0.05$) differences between the means. The analyses were carried out using SPSS package software (Version 17.0).

Results

Inhibitory activity and solvent selection

Extracts obtained by methanol, ethanol, acetone, chloroform, ethyl acetate and butanol showed obviously inhibitory activities against *C. michiganense* subsp. *sepedonicum*. However, benzene and petroleum extracts showed no inhibitory activity. The ethanol extracts had the highest inhibition zone value and were significantly ($P<0.05$) different from other solvents (Fig. 1).

Single factor experiments

Solid to liquid ratio (g:mL), extraction temperature (°C), and extraction time (h) were assessed individually (Fig. 2). As the ratio of solid to liquid increasing, inhibitory activity of *A. sinense* extracts was increased. Maximum antibacterial substances ($P<0.05$) were obtained at 1:20 ratio. Then, inhibitory activity decreased, when solid to liquid ratio was greater than 1:20 (Fig. 2A). ANOVA showed that best solid to liquid ratios with significant ($P<0.05$) difference were Level 2 (1:15), Level 3 (1:20), Level 4 (1:25), and selected them for orthogonal experimental design in Tab. 2. Temperature increasing led to greater inhibitory activity (Fig. 2B), and the maximum inhibitory activity ($P<0.05$) was observed at 85 °C. However, inhibitory activity was not improved, when temperature was at 97 °C. ANOVA showed that best extraction temperatures with significant ($P<0.05$) difference were Level 3 (70 °C), Level 4 (85 °C), Level 5 (97 °C), and selected them for orthogonal experimental design in Tab. 2. Fig. 2C showed the effect of extraction time on inhibitory activity in *A. sinense* extracts. The inhibitory activity increased with extraction time extended. The highest inhibition zone value, with significantly ($P<0.05$) difference, was observed at 8 h. Thereafter, inhibitory activity decreased. ANOVA shows that best extraction times with significant ($P<0.05$) difference were Level 2 (6 h), Level 3 (8 h), Level 4 (10 h), and selected them for orthogonal experimental design in Tab. 2.

Tab. 3: Orthogonal experiment $L_{27}(3^{13})$ and intuitive analysis

Experiment NO.	Factors			Inhibition zone (mm)
	Solid to liquid ratio (A)	Extraction temperature (B)	Extraction time (C)	*C. michiganense* subsp. *sepedonicum* [a]
1	1 (1:15; g:mL)	1 (70 °C)	1 (6 h)	13.77±0.92
2	1 (1:15; g:mL)	1 (70 °C)	2 (8 h)	13.85±1.02
3	1 (1:15; g:mL)	1 (70 °C)	3 (10 h)	13.62±0.88
4	1 (1:15; g:mL)	2 (85 °C)	1 (6 h)	13.38±1.67
5	1 (1:15; g:mL)	2 (85 °C)	2 (8 h)	13.58±1.18
6	1 (1:15; g:mL)	2 (85 °C)	3 (10 h)	14.71±0.90
7	1 (1:15; g:mL)	3 (97 °C)	1 (6 h)	16.72±0.60
8	1 (1:15; g:mL)	3 (97 °C)	2 (8 h)	15.13±0.84
9	1 (1:15; g:mL)	3 (97 °C)	3 (10 h)	16.37±0.36
10	2 (1:20; g:mL)	1 (70 °C)	1 (6 h)	14.49±0.46
11	2 (1:20; g:mL)	1 (70 °C)	2 (8 h)	16.96±1.40
12	2 (1:20; g:mL)	1 (70 °C)	3 (10 h)	15.48±1.41
13	2 (1:20; g:mL)	2 (85 °C)	1 (6 h)	15.72±0.23
14	2 (1:20; g:mL)	2 (85 °C)	2 (8 h)	13.69±0.57
15	2 (1:20; g:mLl)	2 (85 °C)	3 (10 h)	13.27±0.90
16	2 (1:20; g:mL)	3 (97 °C)	1 (6 h)	13.94±0.75
17	2 (1:20; g:mL)	3 (97 °C)	2 (8 h)	15.34±0.40
18	2 (1:20; g:mL)	3 (97 °C)	3 (10 h)	12.96±0.08
19	3 (1:25; g:mL)	1 (70 °C)	1 (6 h)	12.04±0.82
20	3 (1:25; g:mL)	1 (70 °C)	2 (8 h)	14.00±0.82
21	3 (1:25; g:mL)	1 (70 °C)	3 (10 h)	14.71±1.63
22	3 (1:25; g:mL)	2 (85 °C)	1 (6 h)	13.44±1.20
23	3 (1:25; g:mL)	2 (85 °C)	2 (8 h)	15.16±1.82
24	3 (1:25; g:mL)	2 (85 °C)	3 (10 h)	14.23±1.66
25	3 (1:25; g:mL)	3 (97 °C)	1 (6 h)	16.01±1.63
26	3 (1:25; g:mL)	3 (97 °C)	2 (8 h)	13.55±1.58
27	3 (1:25; g:mL)	3 (97 °C)	3 (10 h)	16.58±1.29
K_{1j} [b]	14.57	14.32	14.39	∑392.70
K_{2j}	14.65	14.13	14.58	
K_{3j}	14.41	15.17	14.66	
R[c]	0.24	1.04	0.27	

[a] DMSO was used as control since it does not inhibit microorganism growth. A negative result was defined as an inhibition zone of 10mm. Greater than 10mm indicated positive result of the presence of antibacterial substance. Each value was mean and standard deviation of four replications.
[b] K_{ij} = (1/9) ∑ mean inhibition zone of *A. sinense* at factor *j* (*j* = A, B, C).
[c] R = max $\{K_{ij}\}$- min $\{K_{ij}\}$, *j* and *i* mean factor and setting level here, respectively.

Optimization of extraction condition

Orthogonal experimental design was a main method for fractional factorial design. This design can screen out key variables effectively (Antony, 2006; Kilickap, 2010). From results, it was inferred that inhibitory activity of *A. sinense* was influenced by different factors and their interactions. The $L_{27}(3^{13})$ orthogonal array implied 27 groups of experiments (Tab. 3). Factors that influence inhibitory activity of *A. sinense* were listed in a decreasing order: B>C>A (Tab. 3). The levels within three factors were ranked as: A: 2>1>3; B: 3>1>2; C: 3>2>1 (Fig. 3).

Tab. 4 summarized the analysis of variance of factors and interactions that affect antibacterial substance extraction. In Tab. 4 "interaction" indicated by "×" symbol was used to describe this condition in which the effect of one factor's influence upon the results was dependent on condition of the other factors. *F*-ratio was defined as *F* = MSF/MSE. The MSF and MSE represented mean square of factors or interactions, and mean square of errors. SS, *df* and MS represented sum of squares, degree of freedom, and mean square. If the calculated value *F* was greater than critical value F_α [e.g. $F_{0.05}(4, 20)=2.87$], then that factor or interaction was statistically significant. In Tab. 4, if significant level α=0.05, interaction A (Solid to liquid ratio) × B (Temperature) was statistically significant interaction. Therefore, A×B interaction was regarded as dependent interaction in antibacterial substance extraction. A (Solid to liquid ratio), B

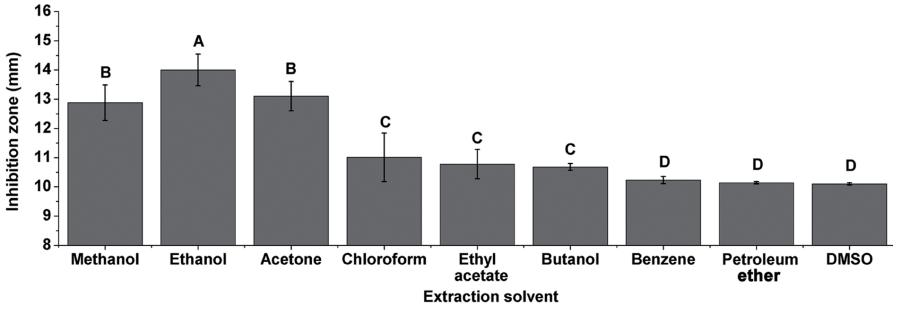

Fig. 1: Inhibitory activity of eight solvent extracts from *Athyrium sinense* against *Clavibater michiganense* subsp. *sepedonicum*. A negative result was defined as an inhibition zone of 10 mm. Greater than 10 mm indicated positive result of the presence of antibacterial substance. Different letters indicated significant differences ($P<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated four times.

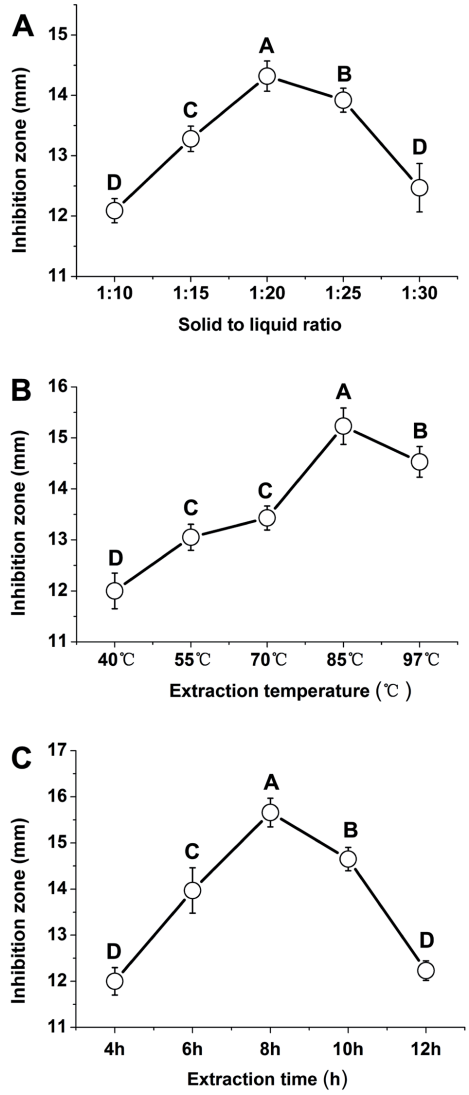

Fig. 2: Effect of solid to liquid ratio (A), extraction temperature (B), and extraction time (C) on inhibitory activity. DMSO was used as control. A negative result was defined as an inhibition zone of 10 mm. Greater than 10 mm indicated positive result of the presence of antibacterial substance. Different letters indicated significant differences ($P<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated four times.

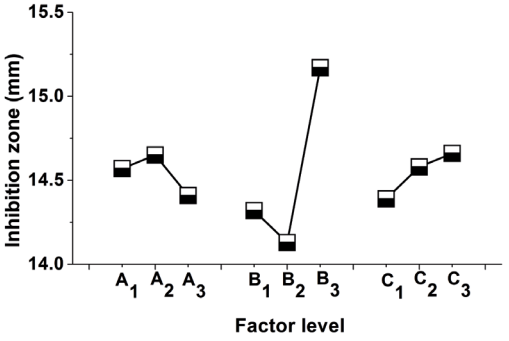

Fig. 3: Effect of parameters on inhibitory activity of *Athyrium sinense*: A, solid to liquid ratio: A_1: 1:15, A_2: 1:20, A_3: 1:25; B, temperature: B_1: 70 °C, B_2: 85 °C, B_3: 97 °C; C, extraction time: C_1: 6 h, C_2: 8 h, C_3: 10 h.

(Temperature), C (Extraction time) and interactions A×C, B×C were regarded as independent factors and interactions. Optimum conditions for extraction were $A_2B_3C_3$, solid to liquid ratio 1:20 (g:ml), temperature 97 °C and extraction time 10 h (Tab. 3). Through optimization test, the inhibition zone was up to 17.04 mm (Fig. 4).

Effect of pH, metal ions, and UV on the stability of extracts

As shown in Fig. 5, there were no statistically significant ($P>0.05$) differences between test sets and control sets in the range from pH10 to pH12. The result implied that, inhibitory activity (pH 10 to pH 12) might be the role of alkali, rather than *A. sinense* extracts. Based on *t*-test, we got the result that there were statistically significant ($P<0.05$) differences between test sets and control sets in the range from pH 2 to pH 9. In other words, inhibitory activity of *A. sinense* extracts was not impacted by the control in the range from pH 2 to pH 9. In order to exclude the effect of control, we only compared the inhibitory activity of test sets when pH values were between 2 and 9, based on ANOVA. The data showed that maximum efficiency of inhibitory activity was observed when pH was 2 ($P<0.05$), but it decreased rapidly when pH values were between 3 and 9.

The effect of metal ions on inhibitory activity in extracts was presented in Fig. 6. From these results, it is clear that Zn^{2+}, Fe^{3+} significantly ($P<0.05$) increased inhibitory activity in *A. sinense* extracts, and there was significantly ($P<0.05$) difference between *A. sinense* extracts with these metal ions, *A. sinense* extracts, DMSO with these metal ions and DMSO. On the other hand, Na^+, K^+, Mg^{2+}, and Ca^{2+} did not ($P>0.05$) changed the inhibitory activities of *A. sinense* extracts.

Tab. 4: Results of variance (ANOVA) analysis

Source	SS	*df*	MS	*F* [a]	Significance [b]
B	5.58	2	2.79	2.47	
A×B	14.02	4	3.50	3.10	*
Error	22.59	20	1.13		
Total	43.19	26			

[a] Significant parameter, $F_{0.05}(2, 20) = 3.49$, $F_{0.05}(4, 20) = 2.87$.
[b] * and Blank indicate significant different and no significant different, respectively.

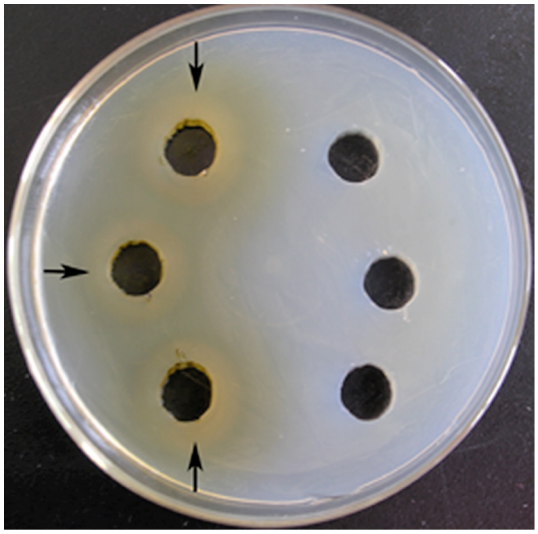

Fig. 4: Inhibitory activity of *Athyrium sinense* extracts against *Clavibater michiganense* subsp. *sepedonicum*, using optimum combination of extraction conditions (solid to liquid ratio 1:20, temperature 97 °C, extraction time 10 h; left). DMSO was used as control (right).

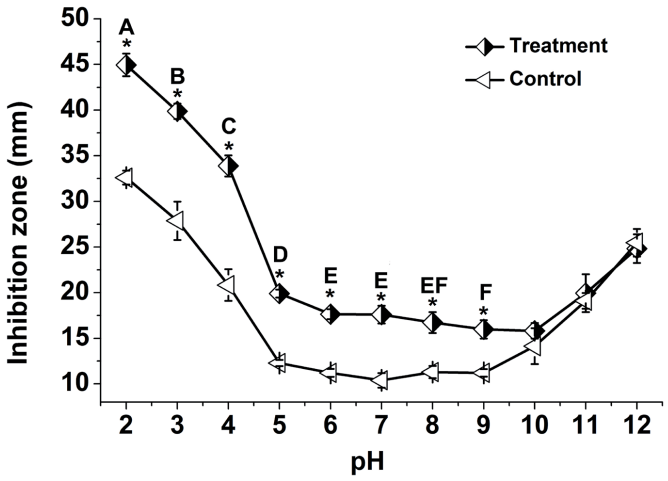

Fig. 5: Effect of pH on inhibitory activity of *Athyrium sinense* extracts against *Clavibater michiganense* subsp. *sepedonicum*. For pH effect, *t*-test was carried out to determine significant ($P<0.05$) differences between test sets and control sets. * indicated significant differences. ANOVA was carried out to determine significant ($P<0.05$) differences between test sets at different pH values ranging from 2 to 9. Different letters indicated significant differences ($P<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated four times.

To test the UV stability of *A. sinense* extracts, we investigated the inhibitory activities of different treatments. There were no statistically significant ($P> 0.05$) differences between samples that were exposed to UV light for different times (Fig. 7), implying that *A. sinense* extracts were not impacted by exposure to UV light.

Observation of interior damage

Untreated cells showed no changes in cell morphology. Control cells showed a typical cell wall, cell membrane, and cytoplasmic content (Fig. 8A). In contrast, *C. michiganense* subsp. *sepedonicum* treated with *A. sinense* extracts (0.06 g/L), exhibited a wide range of abnormalities (Fig. 8 B-C). We could find big vacuoles inside the cells (Fig. 8 B 2-15; C 16-18). Some of them showed bacteria misshapen (Fig. 8 B 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14; C 16, 18).

Discussion

Use of plant products and biological control agents has been shown to be eco-friendly and effective against many plant pathogens (Harish et al., 2008). In this study, *A. sinense* extracts were showed inhibitory activity against *C. michiganense* subsp. *sepedonicum*. The result indicated that *A. sinense* has great potential to biological control of *C. michiganense* subsp. *sepedonicum*. This is the first report of *A. sinense* as a potential agent against bacterial ring rot of potato.

Solvent played the key role in inhibitory substance extraction from *A. sinense*. In this study, ethanol extracts of *A. sinense* were found to be highest effective against *C. michiganense* subsp. *sepedonicum* (Fig. 1). In addition, ethanol has several advantages such as low toxicity, economical, and lower boiling point (Xie and Lu, 2004). When solid to liquid ratio was too low, the contact between solvent and antibacterial substance was not sufficient enough. Therefore it was not conducive to extract maximal amount of antibacterial substance. When solid to liquid ratio was too high, concentration time might be longer, and antibacterial components would be decomposed (Fig. 2A; Yang et al., 2010). Higher temperature improved diffusivity, and thus the yield of inhibitory activity was increased (Liu et al., 2003). However, when temperature was too high, volatilization was accelerated, and solid to liquid ratio became lower. Therefore, yield of inhibitory activity was decreased (Fig. 2B). Shorter extraction time might result in incomplete extraction, longer extraction time might result in waste of energy and time, and antibacterial components would be decomposed (Fig. 2C; Bernardo-Gil et al., 2009).

Orthogonal experimental assay was used to determine optimization of parameters for efficient extraction of antibacterial substances from *A. sinense*. Its advantage is characterizing the complicated process in fewer experiments. However, orthogonal experimental design required a specialized experimental design to set up test, and to analyze data (Díez et al., 2008). The results (Tab. 3 and Tab. 4) revealed that interaction A (solid to liquid ratio) × B (temperature)

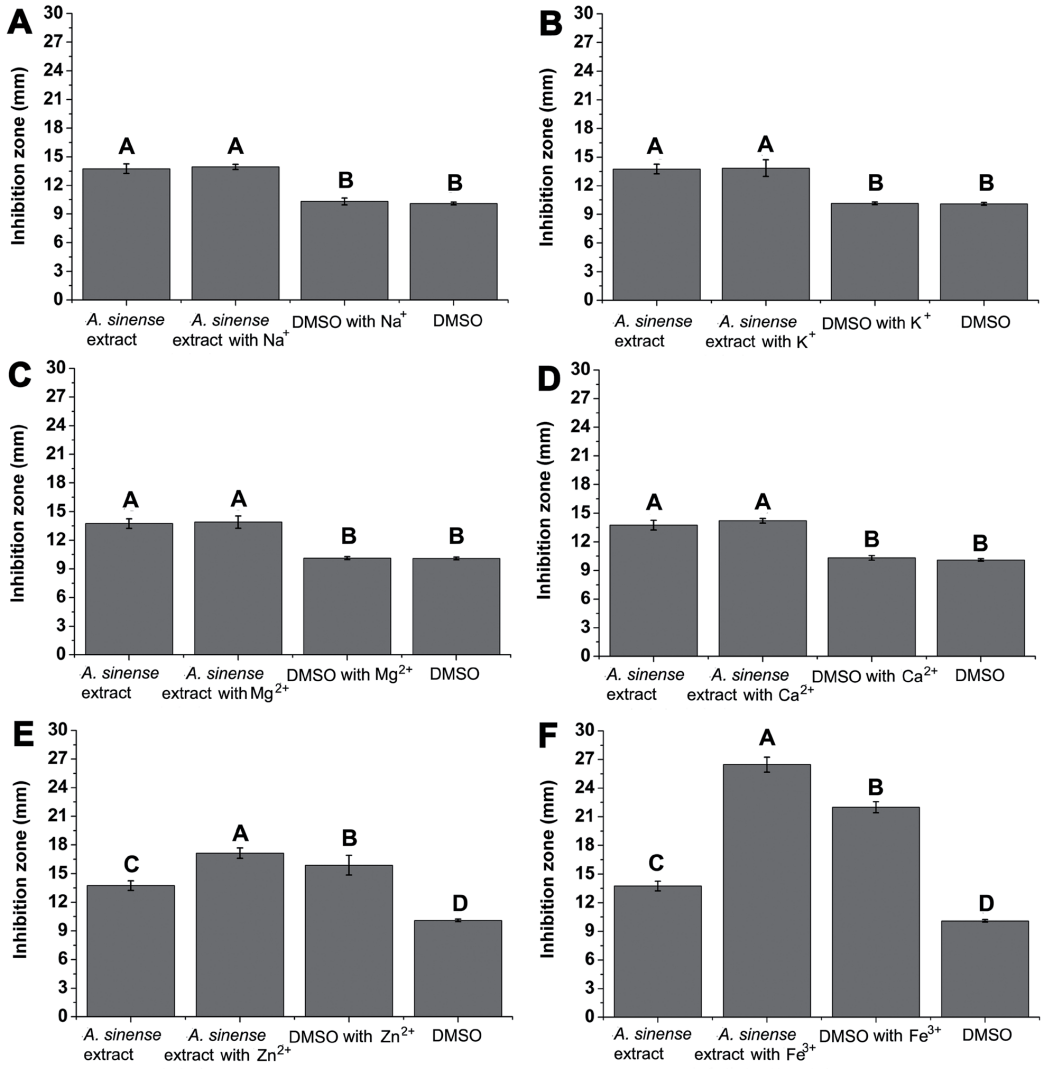

Fig. 6: Effect of metal ions on inhibitory activity of *Athyrium sinense* extracts against *Clavibater michiganense* subsp. *sepedonicum*. Different letters indicated significant differences ($P<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated four times.

had significant effect on the inhibitory activity of *A. sinense*, while other factors and interactions were not identified as significant factors and interactions. We concluded that interaction A (solid to liquid ratio) × B (temperature) was major factor affecting *A. sinense* extraction. Thus, we should pay more attention to interaction in *A. sinense* extraction. Thus, optimum extraction conditions of *A. sinense* were defined as: solid to liquid ratio 1:20 (g:mL), temperature 97 °C, and extraction time 10 h. Compared with conventional extraction, our optimum extraction in this study are efficient economic, and convenient (Li et al., 2010).

Environmental conditions can affect inhibitory activities in *A. sinense* extracts. Metal ions, pH, and UV are the major environmental factors. The pH value of extraction solution is a significant factor, which may affect the extraction procedure. Data (Fig. 5) showed that the highest ($P<0.05$) inhibitory activity was observed when pH was 2, excluding the effect of control. It was observed that the extracts had a wider pH stability range and the greater inhibitory activity in extract was obtained under neuter and acid conditions. This indicated that either organic acids or other pH-dependent antibacterial compounds were responsible for the inhibitory effect (Li et al., 2012). Metal ions exist in abundance in the natural environment. Some of them are vital components of living systems and known as essential metal ions (Raza et al., 2010).To avoid interference from a high ionic strength, tests were conducted in four sets: *A. sinense* extracts, *A. sinense* extracts with metal ions, DMSO with metal ions, and DMSO. As can be seen in Fig. 6, only Zn^{2+}, Fe^{3+} significantly ($P<0.05$) increased inhibitory activity in *A. sinense* extracts, and there was significantly ($P<0.05$) difference between *A. sinense* extracts with these metal ions, *A. sinense* extracts, DMSO with these metal ions and DMSO. On the other hand, Na^{+}, K^{+}, Mg^{2+}, and Ca^{2+} did not ($P>0.05$) changed the inhibitory activities of *A. sinense* extracts. Antibacterial compounds in *A. sinense* extracts were likely to be chelated with Zn^{2+}, Fe^{3+} and thus resulted in increased inhibitory ability. As exposure time changed, no statistically significant ($P>0.05$) differences were observed between different UV treatments. The results (Fig. 7) showed that extracts were stable following exposure to UV.

TEM was used to investigate possible changes in morphology of cells. *C. michiganense* subsp. *sepedonicum*, treated with *A. sinense* extracts, showed formation of vacuoles, lack of cytoplasmic materials, and bacteria misshapen (Fig. 8). The result indicated that *A. sinense* extracts had penetrated cell membrane and interacted with bacteria contents. It was most likely that inhibitory activity of *A. sinense* extracts was not attributable to one mechanism, since there were some targets in *C. michiganense* subsp. *sepedonicum*. It was speculated that *A. sinense* extracts disrupted cell membrane of *C.*

michiganense subsp. *sepedonicum*, causing leakage of cell content. Then, *C. michiganense* subsp. *sepedonicum* retained empty, and some vacuoles (Fig. 8 B 2-15; C 16-18), deformation cells (Fig. 8 B 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14; C 16, 18) would be observed. When antibacterial materials in *A. sinense* extracts made a break through into membrane, and entered inner cell, cell membrane was disrupted. The disruption and dysfunction of membrane, interference with energy generation system, and enzyme inhibition may lead to death of bacterial cells (IBRAHIM et al., 1996; HOLLEY and PATEL, 2005; KHAN and AHMAD, 2011). These phenomena suggested possible antibacterial mechanisms by *A. sinense* extracts inhibiting *C. michiganense* subsp. *sepedonicum* growth.

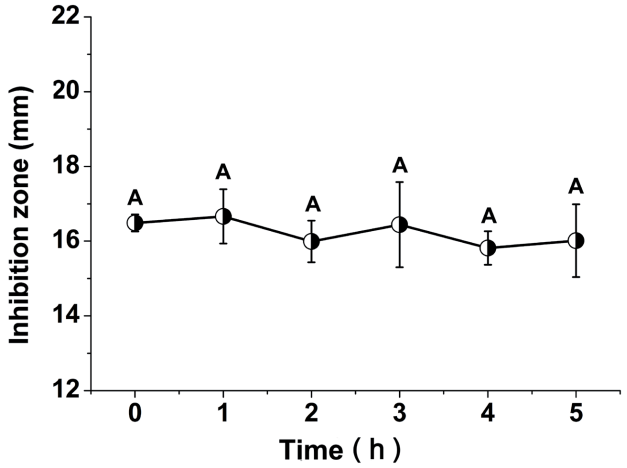

Fig. 7: Effect of UV on inhibitory activity of *Athyrium sinense* extracts against *Clavibater michiganense* subsp. *sepedonicum*. Different letters indicated significant differences ($P<0.05$; one-way analysis of variance (ANOVA) and Duncan's multiple range test). Bars represent the means ± standard deviation. Each was replicated four times.

Conclusion

A. sinense extracts consistently showed significant ($P<0.05$) inhibitory activity against *C. michiganense* subsp. *sepedonicum*. Optimum extraction condition was investigated using single factor and $L_{27}3^{(13)}$ orthogonal experimental design. Stability assays revealed that Zn^{2+}, Fe^{3+}, acidic and neutral conditions enhanced inhibitory activity, and extracts were stable under UV. The TEM investigated possible mechanism of *A. sinense* extracts against *C. michiganense* subsp. *sepedonicum*. These findings indicated that *A. sinense* extracts had a great potential for inhibiting *C. michiganense* subsp. *sepedonicum*.

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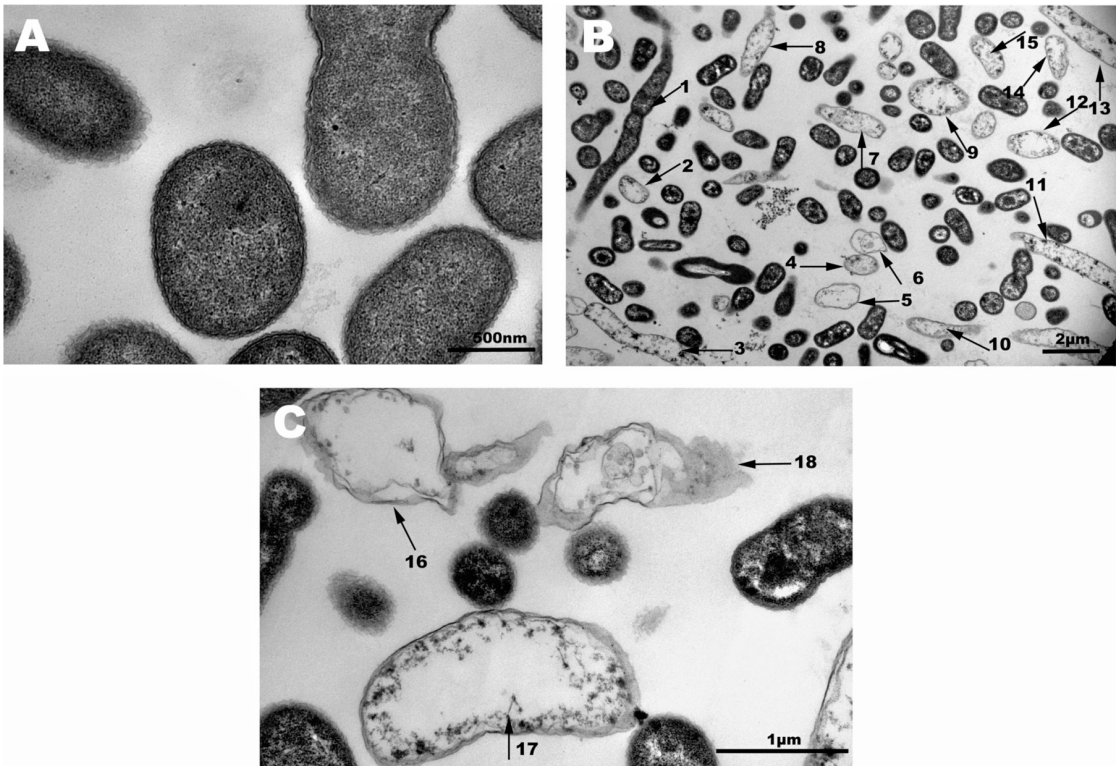

Fig. 8: TEM observation of *Clavibater michiganense* subsp. *sepedonicum* cells. Untreated cells (A 60,000x); Treated with *Athyrium sinense* extracts (0.06 g/L; B 10,000x and C 40,000x). Formation of vacuoles and loss of contents inside the cells (B 2-15; C 16-18). Malformation of cells (B 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14; C 16, 18).

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