Plant Pathology Bulletin 13: 275-282, 2004

Effect of Water-soluble Extracts of Radish Seed Meal on Control of Lettuce Brown Leaf Spot (*Acremonium lactucae* Lin *et al*.)

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Accepted for publication: October 08, 2004.

ABSTRACT

Muto, M., Huang, J. W., and Takahashi, H. 2004. Effect of water-soluble extracts of radish seed meal on control of lettuce brown leaf spot (*Acremonium lactucae* Lin *et al*.). Plant Pathol. Bull. 13: 275-282.

Brown leaf spot caused by *Acremonium lactucae* Lin *et al*., has become a serious disease of lettuce grown in organic farming in Taiwan. Of the water-soluble extracts from 11 plant species showing strong inhibitory effects on germination of conidia of *A. lactucae*, extracts from *Raphanus sativus* (radish) and *Rheum palmatum* (Chinese rhubarb) were most effective in reducing disease severity of brown leaf spot of lettuce. The effectiveness of radish seed extracts was increased with concentration increment of the extract from 0 to 1% (w/v). Furthermore, radish seed extracts reduced disease severity most effectively when applied concurrently with inoculation of the pathogen, although radish seed extracts appeared to retain significant antifungal properties when applied one day prior to or after inoculation. Water-soluble compounds from radish seed extracts were separated by SPE and HPLC and tested for suppressive effects on *A. lactucae*. One compound was noted to have potent inhibitory effects on conidial germination of *A. lactucae*, and was identified as the isothiocyanate-containing glucoraphenin derivative, sulforaphene by UV, CI-Mass, ^{1}H and ^{13}C NMR, and IR spectometry. This study suggests that seed meal extracts of radish contain antifungal substance, sulforaphene, with potential for control of brown leaf spot of lettuce.

Key words : *Acremonium lactucae*, brown leaf spot, lettuce, *Lactuca sativa*, medicinal plant materials, plant extracts, radish seed meal, and sulforaphene

INTRODUCTION

Brown leaf spot caused by *Acremonium lactucae* Lin *et al*. is an important disease of lettuce (*Lactuca sativa* L.) in Taiwan [19, 20]. Recent outbreaks of this disease on lettuce crop grown by organic farming methods resulted in severe economic losses to the organic farming industry in Taiwan [18].

Presently, there are no effective and safe products for control of brown leaf spot of lettuce. Although the use of chemical fungicides may be an economical and effective means for control of fungal diseases in crops, many of these chemicals may potentially be harmful to growers and consumers as well as the environment [10]. Moreover, numerous plant pathogens have also been observed to develop tolerance to chemical fungicides [28]. Thus, the development of environmentally safe, natural products as alternatives to chemical pesticides has become an important focus in modern agriculture.

Numerous species of higher plants such as neem tree, pyrethrum, tobacco, derris roots, and herbs are known to contain natural fungicidal substances that are used as botanical pesticides [7, 26, 31]. Several reports indicated that some medicinal plants may be a potential source of materials for control of plant diseases [9, 25]. In spite of this, there are only a handful of plant materials being developed and commercialized as insecticides in North America and Europe, with few new products on the threshold of commercialization [12]. In addition, some pesticides made of plant materials such as the neem-based pesticide using for tea tree, cotton, and vegetable [9] have been embraced by organic food producers, but have only been applied on a limited scale in conventional crop production.

In a previous study, 103 medicinal plant extracts including Compositae, Labiatae, Leguminosae, Rosaceae, and Rutaceae were evaluated for their antifungal activities [(23)] and 11 plant species were identified for this study.

The objectives of this study were to: (1) screen and select effective medicinal plant materials for control of brown leaf spot of lettuce caused by *A. lactucae*; (2) determine the effectiveness of water-soluble extract of radish seed meal on controlling brown leaf spot of lettuce; (3) identify effective compounds in radish seed meal.

MATERIALS AND METHODS

Inoculum preparation of *Acremonium lactucae*

Two single-spore isolates AL0818 and AL1114 of *A. lactucae* obtained from infected lettuce leaves at Hsilo in Yun-lin County, Taiwan, were used in this study. Stock cultures were maintained on potato dextrose agar (PDA) (Difco Laboratory, Detroit, MI, USA) at room temperature (22-25℃) under 12 h diurnal illumination.

Inoculum from *A. lactucae* isolates AL0818 and AL1114 was prepared from 14-day-old PDA slant cultures by adding 10 ml sterile distilled water and rubbing the surface of the colony with a scalpel to make a conidial suspension in each test tube. The conidial suspensions were filtered through two layers of cheesecloth to remove hyphae and agar fragments. Conidia in the filtered solution were determined using microsyringe method [(15)], and the conidial suspension was adjusted by adding sterile water to required concentrations according to the following experiments.

Testing lettuce for brown leaf spot disease

Seeds of lettuce, cv. Round leaf lettuce, were put on a No. 1 filter paper (9 cm in diameter; Toyo Roshi Co., Japan) moistened in water and kept in a Petri dish at room temperature (22-25℃) for 1 day. The germinated seeds were sown in peat moss in trays, 128 cells/tray and 1 seed/cell. After one week, individual seedlings were transplanted to plastic pots (8 cm in diameter) filled with sandy soil, 1 plant/pot, and kept in a greenhouse for four weeks with daily watering.

Conidial suspensions of *A. lactucae* were inoculated on each plant at 8 ml/plant and 10^5 conidia/ml, using a compressed air-sprayer (SIL-AIR, Werther International, Italy). There were three replicates (pots) for each isolate of *A. lactucae*. Plants sprayed with sterile distilled water were used as controls. All pots were placed in moist plastic bags, and kept in a moist chamber at 28℃ under 12 h diurnal illumination. The plastic bags were removed after one-day incubation and the plants were examined for symptoms and disease severity of brown leaf spot at one week after inoculation.

The disease severity of each plant was assessed by the formula of James [(13)]:

Disease severity (%) = $[(0xn_0 + 1xn_1 + 2xn_2 + 3xn_3 + 4xn_4)/Nx4]$ x 100, where n_0 = number of healthy leaves per plant, n_1 = number of diseased leaves (1-10% total area infected), n_2 = number of diseased leaves (11-25% total area infected), n_3 = number of diseased leaves (26-50% total area infected), n_4 = number of diseased leaves (>50% area infected), and N = total number of leaves of a plant. The experiment was repeated twice.

Table 1. Concentration dependent inhibition of conidial germination of *Acremonium lactucae* isolates AL0818 and AL1114 using water-soluble medicinal plant extracts

Medicinal plant material	Conidial germination (%)[2]					
	AL0818			AL1114		
	0.25 %	0.5%	1.0%	0.25%	0.5%	1.0%
Aster tataricus	0.0 c[3]	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Astragalus mongholicus	30.5 b	0.0 b	0.0 b	84.7 b	0.0 c	0.0 b
Citrus sinensis	0.7 c	0.0 b	0.0 b	5.9 d	0.0 c	0.0 b
Cornus officinalis	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Crataegus pinnatifida	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Paeonia veitchii	90.8 a	0.0 b	0.0 b	59.5 c	36.8 b	0.0 b
Polygala tenuifolia	95.1 a	84.6 a	0.0 b	86.8 b	0.0 c	0.0 b
Raphanus sativus	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Rheum palmatum	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Sanguisorba officinalis	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
Terminalia chebula	0.0 c	0.0 b	0.0 b	0.0 d	0.0 c	0.0 b
None (CK)[1]	92.4 a	85.0 a	94.4 a	98.1 a	97.8 a	96.7 a

[1] Sterilized distilled water was used as the control (CK).
[2] Conidial germination was calculated from one hundred of conidia per 20 μl drop. Data shown was derived from the average of four replicates per isolate of *A. lactucae*.
[3] Means (n = 4) indicated by identical letters are not significantly different ($P > 0.05$) according to Duncan's multiple range test.

Effect of water-soluble extracts of medicinal plants on *Acremonium lactucae*

Extracts were prepared by immersing ground plant material of plants (Table 1) in sterilized distilled water at room temperature for 24 h, and filtered through No. 1 filter paper to obtain the extract at required concentrations according to the following experiments.

The conidial germination bioassay was conducted to evaluate the effect of water-soluble extracts of medicinal plants on *A. lactucae*. This was done by placing four drops (10 μl/droplet) of conidial suspension (10^4 conidia/ml) of *A. lactucae* isolate AL0818 or AL1114 on a glass slide. Ten microliter of 2% w/v medicinal plant extracts were added to each drop of the conidial suspension. For controls, 10 μl of sterile distill water was added to each drop of the conidial suspension. The glass slides were placed on a moist filter paper in a Petri dish and incubated at 28℃ for 12 h. Conidial germination was terminated by adding approximately 20 μl of cotton blue to each drop. One-hundred conidia were scored for germination under a light microscope (model CH-2, Olympus, Optical Co., LTD. Japan).

Plant extracts which showed significant inhibitory effects on conidial germination were selected for further testing at the lower concentrations of 0.25 and 0.5% (w/v). The experiment was done by the same procedure described previously. Each experiment on conidial germination was repeated three times with four replicates per treatment.

To test the effect of water-soluble extracts from medicinal plants on severity of brown leaf spot symptoms of lettuce, conidial suspension (4 ml at 10^5 conidia/ml) of *A. lactucae* isolate AL0818 or isolate AL1114 was mixed with an equal volume of plant extract at a concentration of 2% (w/v). The resulting mixtures were sprayed on 4-week-old lettuce using the same procedure described previously. Conidial suspensions mixed with sterile distilled water were used as controls. After incubation for one-week, plants were examined and assessed for severity of brown leaf spot using the method described previously. The experiment was repeated three times with three replicates per treatment.

Effect of water-soluble extracts of radish seeds on *Acremonium lactucae*

Seeds of radish (*Raphanus sativus*), cv. Wan Sheng Ta Mei Hua (WS-T) were used to collect extract for this experiment. The extract was prepared by the same procedure described previously. To determine time of application of radish seed extracts on control of brown leaf spot, radish seed extracts (0.5%; w/v) were sprayed on 3-week-old lettuce plants at daily intervals for upto 7 days prior to the inoculation of *A. lactucae*, or on 4-week-old lettuce plants at 1 and 4 days after inoculation of the pathogen. The amount of seed extracts was 8 ml per plant and the amount of conidial suspension (5×10^4 conidia/ml) of *A. lactucae* was 8 ml per plant. Plants sprayed with radish seed extracts alone, *A. lactucae* alone was used as control. Inoculated and control plants were kept in a moist chamber at 28℃ for 1-week and assessed for disease severity by the same method described previously. The experiment was repeated twice with three replicates per treatment.

Identification of antifungal compounds in water-soluble extracts of radish seed meal

Water-soluble extracts of radish seed meal (cv. WT-S) were obtained by incubating 20 g of the radish seed meal in 100 ml deionized water with agitation at 50 to 55℃ for 1 h, and filtering the slurry through No. 1 filter paper and a 0.45 μm poly (vinylidene difluoride) (PVDF) membrane filter (Millipore Co., Bedlford, MA, USA). Radish seed extracts were then separated by solid phase extraction (SPE) using a C18 SPE cartridge (500 mg, 3 ml; Lida, WI, USA), and assayed for their inhibitory effects on *A. lactucae* conidial germination (Fig. 1). Eluted fractions (fractions 2 to 6) showing inhibitory effects on *A. lactucae* were pooled and separated by reversed-phase HPLC. Five peaks were obtained

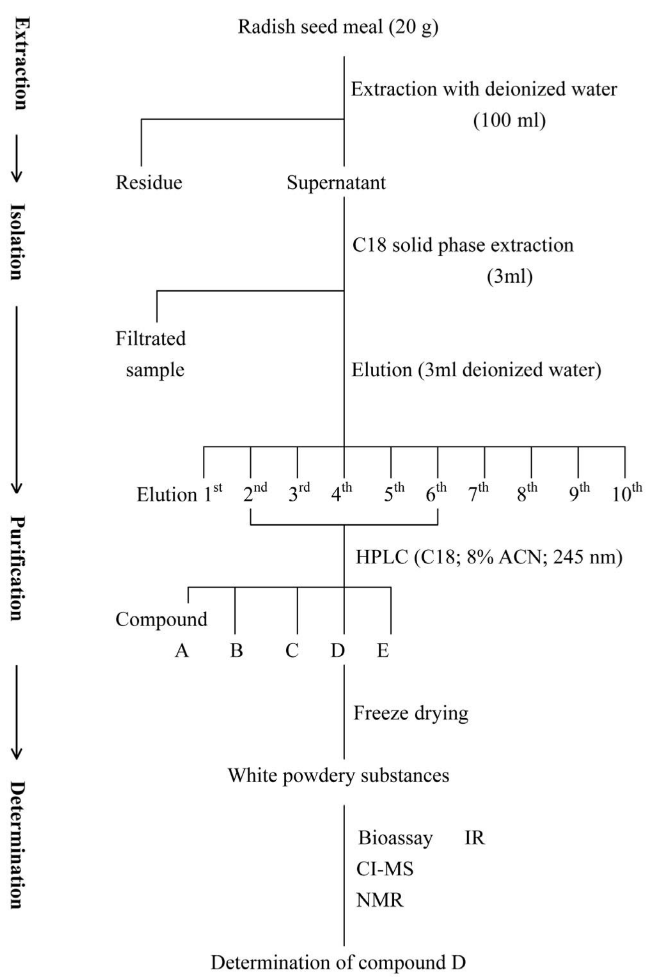

Fig. 1. Separation and analysis of water-soluble extracts of radish seed meal (cv. WS-T).

(compounds A through E), and assayed for antifungal activity. Compound D gave the largest peak area, and was further characterized by UV, IR, ^{1}H and ^{13}C NMR spectrometry, and mass spectrometry. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of an isolated compound were measured in deuterium oxide (D_2O) at 400 and 300 MHz with a Varian Unity Inova-400 and VXR-300/51 spectrometer, respectively. The optimum UV spectrum of the extract was identified through a U-3000 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at 245 nm. Infrared (IR) spectra were obtained from KBr discs and in $CHCl_3$ solution with Bruker Equinox 55 FT-IR.

Statistical analysis

Data from the above experiments were analyzed by analysis of variance (ANOVA) with the SAS system for personal computers (SAS Institute, Cary, NC). Separation of means for the treatments of each experiment was accomplished by using Duncan's multiple range test.

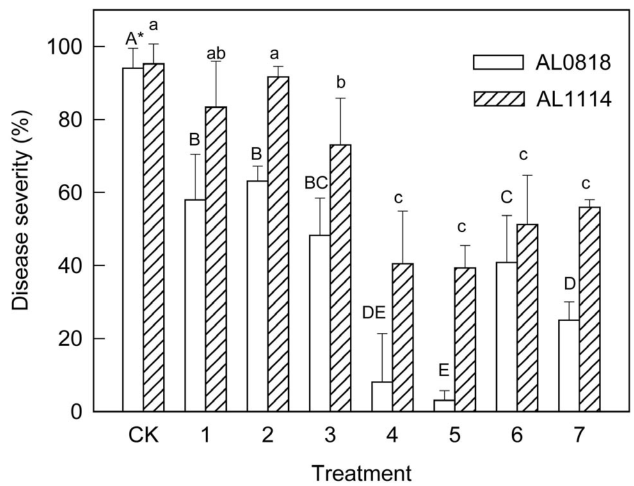

Fig. 2. Effect of medicinal plant extracts at 1% (w/v) on disease severity of lettuce brown leaf spot caused by *Acremonium lactucae* (isolates AL0818 and AL1114). (CK: sterilized distilled water (control); 1: *Aster tataricus*; 2: *Cornus officinalis*; 3: *Crataegus pinnatifida*; 4: *Raphanus sativus*; 5: *Rheum palmatum*; 6: *Sanguisorba officinalis*; 7: *Terminalia chebula*). Vertical bars denote standard deviation of the mean of three replicates.
* Means (n = 3) among eight columns per isolate followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple range test.

RESULTS

Effect of medicinal plant extracts on germination of conidia of *Acremonium lactucae*

Eleven medicinal plant extracts, including *Aster tataricus*, *Astragalus mongholicus*, *Citrus sinensis*, *Cornus officinalis*, *Crataegus pinnatifida*, *Paeonia veitchii*, *Polygala tenuifolia*, *Raphanus sativus*, *Rheum palmatum*, *Sanguisorba officinalis*, and *Terminalia chebula* completely inhibited conidial germination of both isolates of *A. lactucae* at the concentration of 1% (w/v). They were further assayed at 0.25 and 0.5% (w/v) to determine their dose-dependent effects (Table 1). At 0.5% (w/v), all medicinal plant materials, except for *Paeonia veitchii* and *Polygala tenuifolia*, had complete inhibitory effects on conidial germination of both isolates of *A. lactucae* (Table 1). At 0.25% (w/v) seven extracts, including *Aster tataricus*, *Cornus officinalis*, *Crataegus pinnatifida*, *Raphanus sativus*, *Rheum palmatum*, *Sanguisorba officinalis*, and *Terminalia chebula* completely inhibited conidial germination of both isolates. These results showed that at a concentration of 0.5% (w/v), most of the tested extracts completely inhibited germination of conidia of both isolates of *A. lactucae*, except for the extract from *Polygala tenuifolia* which resulted in 84.6% of germinated conidia of isolate AL0818 and the extract from *Paeonia veitchii* which resulted in 36.8% of germinated conidia of isolate AL1114. Germination of conidia of *A. lactucae* in the treatments of controls was higher than 85.0% for isolate AL0818 and higher than 97.8% for isolate AL1114 (Table 1).

Effect of medicinal plant extracts on disease severity

Extracts from *Aster tataricus*, *Cornus officinalis*, *Crataegus pinnatifida*, *Raphanus sativus*, *Rheum palmatum*, *Sanguisorba officinalis*, and *Terminalia chebula* which showed complete inhibition of conidial germination of *A. lactucae* at the concentrations of 0.5% or higher (Table 1) were individually assayed for their quantitative effects on disease severity. Disease severity of brown leaf spot of lettuce caused by *A. lactucae* isolate AL0818 was significantly ($P < 0.05$) reduced by the treatment of 1% (w/v) extracts from *Aster tataricus*, *Cornus sofficialis*, *Crataegus pinnatifida*, *Raphanus sativus*, *Rheum palmatum*, *Sanguisorba officinalis*, and *T. chebula*, compared to the control (Fig. 2). For isolate AL1114, the disease severity was significantly ($P < 0.05$) reduced only by the treatment of 1% (w/v) extracts from *Crataegus pinnatifida*, *Raphanus sativus*, *Rheum palmatum*, *Sanguisorba officinalis*, and *T. chebula*, compared to the control. Meanwhile, the extracts from *Rheum palmatum* and *Raphanus sativus* (radish) were most effective among the seven extracts tested and they suppressed disease severity below 50% in both isolates of *A. lactucae* (Fig. 2). Conidia of both isolates of *A. lactucae* treated with 1% (w/v) seed meal extract of radish, cv. Wan Sheng Ta Mei Hua (WS-T) resulted in the formation of swollen, deformed, or broken conidia (Figs. 3B and 3D).

Effects of concentrations of radish seed extracts on disease severity

Suppression of lettuce brown spot by radish seed extracts

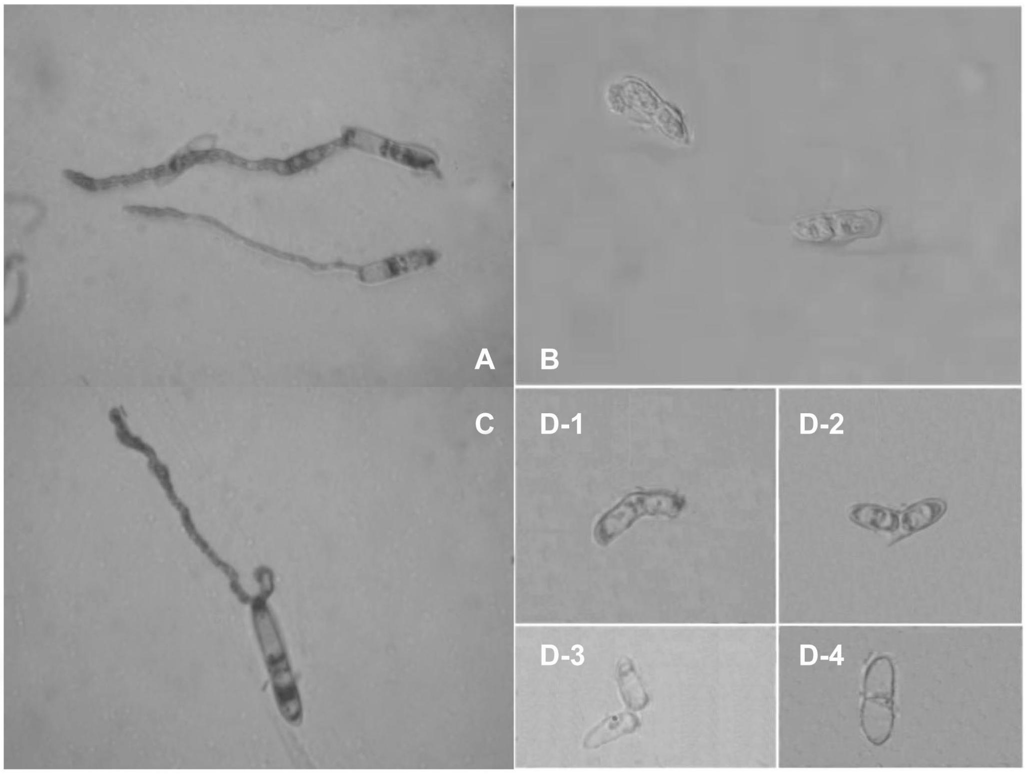

Fig. 3. Morphology of *Acremonium lactucae* isolates AL0818 and AL1114 conidia treated with water-soluble extract of radish seed meal cv. WS-T for 12h at 28℃.
Note: germination of conidia in the controls (A and C) and cell swelling or deformation in treated conidia (B and D).
A: Normal germinated conidia (AL0818); B: Swelling conidia (AL0818); C: Normal germinated conidium (AL1114); D-1: Deformed conidium (AL1114); D-2, D-3: Cell broken conidium; D-4: Swelling conidium.

[*Raphanus sativus* (cv. WS-T)] was dependent on extract concentration, where disease severity was decreased by increasing concentrations of radish extract (Fig. 4). Specifically, the disease severity in treatments inoculated with *A. lactucae* isolate AL0818 was greatly reduced (< 50%) by application of radish seed extracts at concentrations of 0.5 to 1% (w/v).

Effect of time of application of radish seed extracts on disease severity

Disease severity of lettuce brown spot was most strongly suppressed when radish extracts (cv. WT-S) were applied concurrently with inoculation of *A. lactucae* (Fig. 5), but disease severity increased when application of radish extracts occurred 1 to 7 days prior to inoculation of the pathogen. Application of radish extracts 0 to 3 days prior to inoculation had little effect in reducing disease severity, compared to the control for both *A. lactucae* isolates. Similarly, application of radish extracts at 1 to 4 days after inoculation of *A. lactucae* abrogated the inhibitory effects on disease severity in both isolates of the pathogen, compared to concurrent application of radish extracts with *A. lactucae* (Fig. 6).

Identification of water-soluble antifungal compounds in radish seed extracts

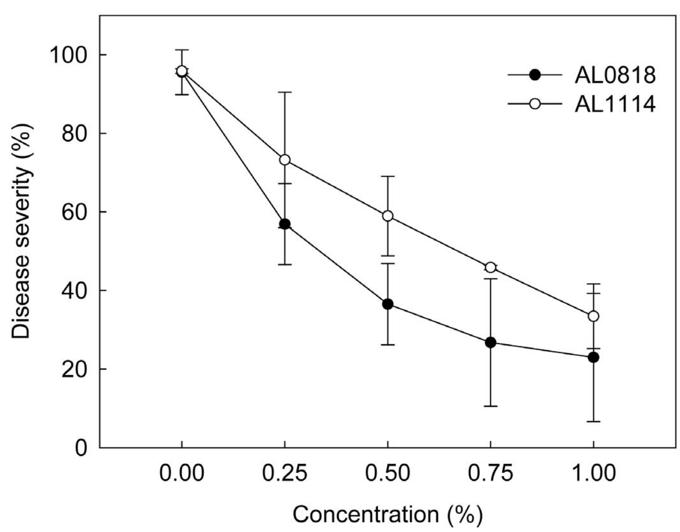

Fig. 4. Concentration-dependent effects of radish seed extracts (cv. WS-T) on disease severity of lettuce brown leaf spot caused by *Acremonium lactucae* (isolates AL0818 and AL1114). Vertical bars denote the standard deviation between means derived from three replicates.

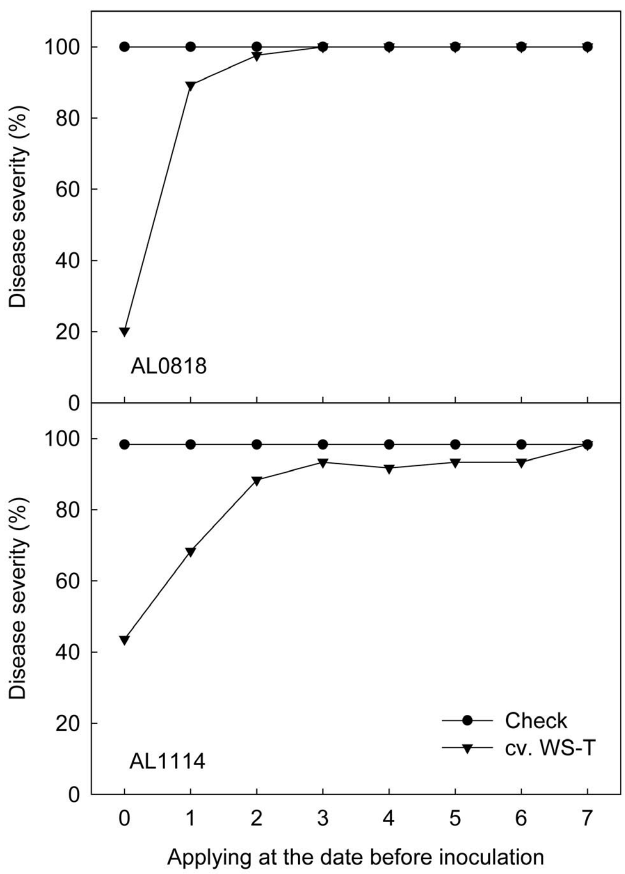

Fig. 5. Effect of applying radish seed extracts (cv. WS-T) prior to the pathogen inoculation on disease severity of lettuce brown leaf spot caused by *Acremonium lactucae* (isolates AL0818 and AL1114).

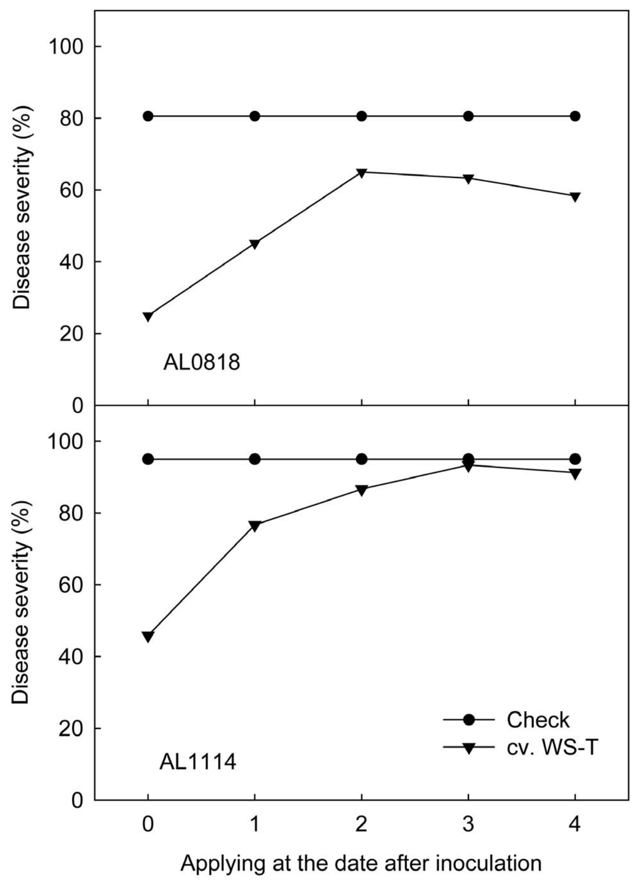

Fig. 6. Effect of applying radish seed extracts (cv. WS-T) following the pathogen inoculation on disease severity of lettuce brown leaf spot caused by *Acremonium lactucae* (isolates AL0818 and AL1114).

Reversed-phase HPLC analyses showed that five peaks (namely compound A to E) were identified in seed meal extracts of radish. Compound D was found to have potent antifungal activity, and was observed to completely inhibit both *A. lactucae* isolates. However, Compound D appeared to have little effect on conidial morphology, and failed to cause deformation of conidia of *A. lactucae*, when examined under a compound microscope. Through UV, IR, ^{1}H and ^{13}C NMR, and mass spectrometry (CI method), compound D was identified as the isothiocyanate-containing sulforaphene (4-methylsulfinl-e-butenyl isothiocyanate).

DISCUSSION

This study reveals that among the water soluble extracts from 11 plant species investigated, the extracts of radish seed meal and *R. palmatum* are most promising for control of brown leaf spot of lettuce, because of their strong inhibition to the germination of conidia of *A. lactucae* at low concentration (0.25%) and their effective control of the disease at the concentration of 1% (w/v). Radish seed extracts have been used medicinally for promoting digestion, relieving dyspepsia, eliminating phlegm, decreasing energy expulsion, and lowering blood pressure [(11)]. Present study suggests that in addition to the medicinal value, radish seed extract may also be a strong candidate for developing natural products for control of brown leaf spot of lettuce.

The effectiveness of control of brown leaf spot of lettuce depends on the concentration and time of application of the radish seed extracts. Application of 8ml radish seed extracts to each plant, 1 day prior to or after inoculation at a concentration of 1%, was found to be good for treating lettuce crops for the control of the disease under greenhouse conditions. Results of these greenhouse trials suggest that radish seed extracts may provide an inexpensive yet effective method for control of brown leaf spot in commercial production of lettuce.

Our observations indicate that radish seed extracts contain sulforaphene which is highly inhibitory to the germination of conidia of *A. lactucae*. Previous studies have shown that radish seeds contain four antifungal proteins Rs-AFP1 through 4 [(6)]. Remarkably, a 15-mer peptide derived from Rs-AFP2 was found to be sufficient in producing anti-pathogenic activity against several fungal species. Despite our findings that sulforaphene alone is effective in inhibiting conidial germination of *A. lactucae*, whether the antifungal activity of radish seed extracts are due to the synergistic activity of several compounds, or primarily by sulforaphene alone warrants further investigations.

Sulforaphene (also called Raphanin) was previously described as a natural anti-microbial substance found in radishes [27]. It is an isothiocyanate derivative of glucoraphenin (a glucosinolate) 4-(methylsulfinyl)-3-butenylglucosinolate [1, 2, 4, 16, 17]. A total of 104 glucosinolates have been found in 450 higher plant species within 16 families [8, 14]. Interestingly, during the study of the 103 medicinal plant species for inhibitory effects on *A. lactucae* [23], two of the species, namely *R. sativus* and *Brassica alba*, contain glucosinolates and only one of these two species, *R. sativus*, is inhibitory to *A. lactucae*.

Glucosinolates are hydrolyzed by the plant enzyme myrosinase with injury to plant tissue, and are enzymatically modified into a wide range of breakdown products. Isothiocyanates represent the most common breakdown products of the glucosinolates, and are generally considered to have greater toxic effects in comparison to glucosinolates [3, 4, 5]. Thus far, the abundance of sulforaphene in radish seed extracts is unclear, although the quantity and quality of isothiocyanates have been previously noted to be dependent on growth conditions such as pH [21, 22, 24, 29, 30]. Furthermore our results suggest that a 1% (w/v) radish seed extract concentration is sufficient to inhibit *A. lactucae* conidial germination and disease severity, indicating that at these concentrations, a sufficient quantity of sulforaphene (perhaps together with other anti-pathogenic compounds) is extracted from radish seeds to produce its anti-pathogenic effects.

In summary, radish seed extracts may be a valuable natural substance for developing cost-effective products for control of brown leaf spot of lettuce. Although sulforaphene was identified as a potential antifungal substance in radish extracts, the mechanism and effective concentration by which this compound inhibits conidial germination remains unclear. Furthermore, it may be of interest to determine the efficacy of radish extracts in treating other similar fungal pathogens. Future study may address these questions, and further develop the use of glusinolates and glucosinolate-containing plant substances in the biological control of fungal pathogens.

ACKNOWLEDGEMENTS

The authors express their special appreciation to Drs. H. C. Huang and T. Y. Huang for their valuable suggestions and constructive criticism of the manuscript. Thanks are also extended to Dr. J. F. Jen for providing advice on chemical analysis and research staffs of Department of Chemistry at National Chung Hsing University for technical assistance.

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摘　要

武藤眞知子[1]、黃振文[2,3]、高橋久光[1]. 2004. 蘿蔔種子粉水溶性抽出物防治萵苣褐斑病的功效. 植病會刊 13：275-282. ([1.] 東京農業大學國際農業開發系；[2.] 國立中興大學植病系；[3.] 連絡作者，E-mail: jwhuang@dragon.nchu.edu.tw；傳眞：+886-4-22851676)

由 *Acremonium lactucae* Lin *et al*. 引起的萵苣褐斑病是台灣新發生的一種病害，目前已成爲有機栽培田的新問題。由十一種具有抑制 *A. lactucae* 分生孢子發芽功效的植物水溶性抽出物中，發現蘿蔔種子 (*Raphanus sativus*) 及大黃 (*Rheum palmatum*) 兩者最具有顯著 ($P = 0.05$) 降低萵苣褐斑病發生的效果；其中蘿蔔種子粉水溶性抽出物之抑菌效應，隨它的濃度由 0 提高至 1 % 而逐漸增強。在接種病原菌當天、前或後一天分別噴佈蘿蔔種子粉水溶性抽出液 (0.5, w/v) 於萵苣植株，可有效抑制萵苣褐斑病的發生。利用 SPE 及 HPLC 的方法分離蘿蔔種子粉抽出物中具有抑菌的有效成分；進而透過 UV、CI-MS、^{1}H NMR、^{13}C NMR 及 IR 等分析方法的鑑定，證實異硫氰化物 (isothiocyanate) 群中的萊菔素 (sulforaphene) 是蘿蔔種子粉具有抑制本菌的主要成分。

關鍵詞：萵苣褐斑病菌、植物源農藥、萵苣、萵苣褐斑病、藥用植物、蘿蔔種子、萊菔素