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Reaction of Pea Cultivars to Metabolites of *Mycosphaerella pinodes* Detected Using Thin-layer Chromatography

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ABSTRACT

Metabolites were extracted from culture filtrates of two weakly aggressive (A1 & A2) and two strongly aggressive (B1 & B2) isolates of *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr., which causes mycosphaerella blight on pea. Using thin-layer chromatography, metabolites were detected using four of the five solvent systems assessed. The best separation of spots (representing one or more metabolites) was obtained with a mixture of ethyl acetate: water: acetone (5: 2: 5, v: v) viewed under 365 nm UV light. Fewer compounds were observed at 254 nm (short-wave UV). Five spots were visible from each isolate using the best solvent and light system. Four of these spots had similar retention factor (R_f) values in the highly aggressive and weakly aggressive isolates. One metabolite was recovered only from the weakly aggressive isolates and another only from the highly aggressive isolates. Four pea lines, representing a range of reaction to mycosphaerella blight, were assessed in a detached-leaf assay. Application of crude extracts from the highly aggressive isolates produced necrotic lesions that were significantly larger than those from the weakly aggressive isolates. The smallest lesions developed in cv. Radley (least susceptible). Line JI 181 (highly susceptible) developed the largest lesions in two of the three repetitions of the assay. Following separation of the crude extracts using thin-layer chromatography, two compounds from A2 (weakly aggressive) and three from B2 (highly aggressive) produced necrotic symptom in the leaf bioassays. These metabolites may eventually be useful in evaluating cultivar resistance to mycosphaerella blight.

Key Words: Toxic metabolite; *Mycosphaerella pinodes;* Resistance; Thin-layer chromatography (TLC); Detached-leaf

INTRODUCTION

Mycosphaerella blight is a disease complex caused by three similar fungal pathogens: *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr., *Ascochyta pisi* Lib. and *Phoma medicaginis* var. *pinodella* (L.K. Jones) Boerema (Xue, 2003). The disease is widely distributed on pea and is especially prevalent in regions, where cool wet weather provides favourable conditions for disease development e.g., central Alberta. *Mycosphaerella pinodes* is the most prevalent pathogen and severe yield losses have been reported (Wallen, 1965; Wang *et al*., 1999; Su *et al*., 2002). Application of foliar fungicide can reduce blight severity and yield loss, but is frequently not economical when yield and pea prices are low (Hnatowich, 2000; Xue, 2003). No pea cultivars are immune to mycosphaerella blight but some cultivars carry partial resistance (Kraft *et al*., 1998; Xue & Warkentin, 2001; Zhang *et al*., 2006). Like many other plant pathogens, isolates of *M. pinodes* differ in virulence and aggressiveness (Nasir & Hoppe, 1991; Zhang *et al*., 2003). Su *et al*. (2006) analyzed the virulence of 83 isolates of *M. pinodes* collected in central Alberta in 2001 and identified six pathotypes using 10 pea cultivars/lines.

Plant pathogen-produced toxins and toxic metabolites have been actively studied for many decades, because researchers believe that disease development, host selectivity and disease resistance have a chemical basis (Scheffer, 1983). These pathogen-produced toxins have been classified as either pathogenicity factors that are essential for the pathogen to cause disease, or as a virulence factors that can increase the extent of disease (Yoder, 1980; Mitchell, 1984). These toxic metabolites may be a useful experimental tool for selecting disease resistance *in vitro* (Yoder, 1980; Weiergang *et al*., 2002).

It would be highly desirable if a convenient lab-scale screening system could be developed to assess cultivar resistance to *M. pinodes*. Hwang *et al*. (2004) extracted and characterized a toxic metabolite from *M. pinodes* that produced discoloration on detached pea leaves. The main component of this compound was $C_{16}H_{12}N_3O_6$. Also, the toxin ascochitine, which has a similar chemical structure to the toxin citrinin that is produced by some *Penicillium* and *Aspergillus* spp. (Iwai & Mishima, 1965; Betina, 1984), is produced by *A. pisi* and is toxic to pea plants (Kaur & Deshpande, 1980; Lepoivre, 1982; Abouzeid & El-Tarabily, 2003).

The current studies were initiated to investigate the possibility of using toxic metabolites from *M. pinodes* to evaluate pea cultivar susceptibility under controlled conditions. Metabolites were extracted from two strongly aggressive and two weakly aggressive isolates of *M. pinodes*, separated using thin-layer chromatography and evaluated for toxicity *in vitro* on four pea lines that differed in susceptibility to *M. pinodes*.

MATERIALS AND METHODS

Isolates and plant materials. Two weakly aggressive isolates of *M. pinodes* (A1 & A2) and two strongly aggressive (B1 & B2) isolates (Su *et al*., 2006) were grown on 1% oat meal agar. These isolates were originally isolated from pea plants with symptoms of mycosphaerella blight collected from central Alberta in 2001 (Su *et al*., 2002).

Four pea lines (JI181, JI96, Espace & Radley) were selected to represent a range of disease reaction to mycosphaerella blight. JI181 is highly susceptible, Radley is the least susceptible and other two lines have an intermediate disease reaction (Su *et al*., 2006). Seeds of each line were sown in 15-cm-diameter fibre pots in pasteurized soil mix. They were maintained in a greenhouse at 20/16°C day/night temperature and 16-h photoperiod with light intensity of 250 – 300 μE m^{-2} s^{-1} provided by a mixture of cool-white fluorescent and incandescent bulbs. The relative humidity ranged from 30 to 40%. Seedlings were watered daily. Leaves of 4- to 5-week-old plants were excised and used in the bioassay.

Production of metabolites. Cultures of *M. pinodes* were grown in a liquid medium containing 20 g glucose, 2.5 g peptone, 2 g K_2HPO_4, 1 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 l distilled water (Oku & Nakanishi, 1963). The pH of the medium was adjusted to 5.6 using 1 N HCl and the media was autoclaved at 120°C for 15 min. Vitamins, consisting of 6 mg thiamine mononitrate, 2 mg pyridone·HCl, 2 mg riboflavin and 3 mg calcium pantothenate, were filter-sterilized and added to the medium after autoclaving. For production of metabolites, the medium was distributed into 1-l flasks (500 mL in each) and inoculated with agar plugs from actively growing colonies of *M. pinodes*. The inoculated liquid medium was cultured on a shaker at 110 rpm in the dark at 20 – 25°C for 30 days.

Extraction, detection and separation of metabolites. After harvesting the liquid culture, metabolites of *M. pinodes* were extracted using a procedure modified slightly from that described by Hwang *et al*. (2004). To collect the culture filtrates, the liquid culture was centrifuged for 10 min at 4,470 x *g* and the supernatant was run through a 0.22 μm cellulose acetate filter (Corning Incorporated, Corning, NY). The filtrate from each culture flask was adjusted to pH 3.0 with HCl and extracted with 200 mL chloroform. The chloroform phase was purified by two extractions with 100 mL aqueous solution of 0.4% Na_2CO_3. The aqueous phase was acidified to pH 3.0 and extracted again with 100 mL chloroform. The chloroform was evaporated under reduced pressure at 45°C with a Buchi rotavapor (Labortechnik AG, Flawil, Switzerland). The dried residue was dissolved in 2 mL of hot methanol and used for analysis by thin-layer chromatograph (TLC) and in the initial detached-leaf bioassays.

TLC was used to characterize the different metabolite extracts. Analytical TLC plates of Uniplate™ coated with silica gel G at 250 microns (Analtech, Inc., Newark, DE) were activated at 110°C for 2 h, then 30 μL samples of extract were spotted on each TLC plate using a micropipet (borosilicate glass capillaries) and developed for a distance of 9 – 10 cm in different solvent mixtures. Five solvent systems were selected for assessment based on studies on ascochitine produced by *A. pisi* on pea (Lepoivre, 1982), citrinin produced by *Penicillium* and *Aspergillus* spp. on cereal grain (Hald & Krogh, 1973; Betina, 1984) and on a toxic metabolite produced by *M. pinodes* on pea (Hwang *et al*., 2004). The five solvent systems were benzene: methanol: acetic acid (24: 2: 1, BMA), chloroform: methanol: acetic acid (19: 10: 2, CMA), diethylether: methanol: water: formic acid (95: 4: 1: 1, DMWF), ethyl acetate: water: acetone (5: 2: 5, EWA) and toluene: ethyl acetate: formic acid (6: 3: 1, TEF). The developed plates were air-dried and metabolites produced by the pathogen were visualized under 254 and 365 nm ultraviolet (UV) light. The retention factor (R_f) was calculated by dividing the distance the solvent moved by the distance the individual spot moved for each metabolite detected in each solvent system. Metabolite strength in TLC plates under UV illumination was visually expressed as * (weakest) to **** (strongest).

Metabolites of *M. pinodes* isolates A2 and B2 were further separated on the TLC plates in the solvent system EWA by scratching off the silica gel in the target area and dissolving the material in 2 mL of hot methanol. The solution was filtered through a 0.2 μm filter to eliminate the silica gel. The separated metabolites were then assessed in detached-leaf assays.

Evaluation of metabolites on detached leaves. Detached-leaf assays were conducted on all four pea lines using the crude extracts of metabolites as described previously (Hwang *et al*., 2004). Stipules were excised when plants reached the 6 – 8 node stage and placed on a plastic screening laid inside petri dishes. Humidity within each petri dish was maintained by two layers of water-soaked filter paper beneath the screen. The treatments were crude extracts from each isolate; 30 μL of each crude extract was applied to a sterilized 5-mm filter paper disk. The discs were air-dried to evaporate the methanol. Pure methanol and sterile water were applied independently to discs as controls. To ensure direct contact with the metabolite (s), each leaflet was punctured with a sterile needle. A treated disc was placed over the puncture wounds in the detached leaves and 20 μL of sterilized water was applied to each disc. The petri dishes were sealed with paraffin film and maintained in an

incubator with 20 – 25°C. There were 10 replications for each treatment. After three days of incubation, the treated leaves were visually examined for symptoms. The necrotic lesions that developed underneath the disk were assessed on a scale of 0 to 3, where 0 = no visible lesion, 1 = visible lesions with the radius of less than 2 mm, 2 = lesion radius of 2 – 4 mm and 3 = necrotic lesions with radius of more than 4 mm on the leaf. Each assay was conducted three times.

The bioassays were also conducted on the pea lines JI181 and Radley using the separated metabolites from *M. pinodes* isolates A2 and B2. The method was as described above, except that the amount of extracts per disk was increased to 100 µL and there were six replications per treatment. Each assay was conducted two times.

Statistical analysis. Analyses were conducted using SAS software (version 9.1.3, SAS Institute Inc., Cary, NC). Data were assessed using the General Linear Model procedure for analysis of variance and Duncan's Multiple Range Test for means separation. Differences are significant at $P < 0.05$ unless specified. The reaction of pea lines to the metabolite extracts and effect of extracts in the three bioassays were compared using correlation analysis of the means (Steele *et al*., 1997). No lesions developed in the methanol or water controls, so these results were dropped from the data set prior to analysis.

RESULTS

Crude extracts of metabolites. Following extraction and separation of the crude extracts using TLC, different numbers of metabolites were observed from the five solvent systems under the two ultraviolet light sources. More compounds were visualized at 365 nm UV light than at 254 nm (data not shown). Characterization of compounds based on R_f values and relative strength under 365 nm UV light is presented in Table I. Five compounds were visualized in the EWA solvent system, while no product was found in the CMA solvent system. The metabolites that were visualized at 365 nm had the same R_f values in solvent systems of DMWF, TEF and BMA for each of the four *M. pinodes* isolates. The only exception was that one weak product (R_f = 0.53) was not visible from isolates B1 and B2 (highly aggressive) in the solvent BMA. In the solvent system EWA, four products with same R_f values as from the other solvent systems (R_f = 0.93, 0.70, 0.53 & 0.31) were observed from all four isolates, but an additional metabolite was observed from isolates A1 and A2 (R_f = 0.78) and another from B1 and B2 (R_f = 0.18) (Table I).

There was no interaction between isolate extract and pea line in the detached-leaf assays, so data from the four pea lines were combined within each repetition of the experiment to illustrate the effect of the isolates on lesion size (Fig. 1A). Crude extracts from isolates B1 and B2 (highly aggressive) induced larger lesions than from isolates A1 and A2 (weakly aggressive). In the first repetition of this experiment, the lesions developed slowly. Lesion size was about 0.4 on a 0-3 scale for extracts B1 and B2, while the weak aggressive isolates induced fewer, smaller lesions (mean = 0.03). In the second and third repetitions, extracts from all four isolates were toxic to pea leaves. Lesion reached 0.9 - 1.1 for crude extracts from isolates B1 and B2 and 0.3 - 0.7 from isolates A1 and A2. No lesions were observed from the methanol or water controls.

Different reactions to the crude extracts were observed among four pea lines (Fig. 1B). Lesions developed slowly in cv. Radley (least susceptible) in all three experiments, while line JI181 (highly susceptible) had the largest lesions in two of three repetitions. There was a correlation for the relative impact of isolate extracts among repetitions (r = 0.83 – 0.89,

Table I. R_f values and relative strength of the crude extracts of metabolites produced by four *Mycosphaerella pinodes* isolates (A1, A2, B1 & B2) in thin-layer chromatography (TLC) detected under 365 nm UV light

Solvent system	R_f[a]	Relative strength under 365 nm UV light			
		Isolate A1	Isolate A2	Isolate B1	Isolate B2
BMA (benzene: methanol: acetic acid, 24:2:1)	0.53	*[b]	*	nd[c]	nd
	0.41	****	****	****	****
	0.20	***	***	***	***
	0.11	***	***	***	***
CMA (chloroform: methanol: acetic acid, 19:10:2)	-	nd	nd	nd	nd
DMWF (diethylether: methnol: water: formic acid, 95:4:1:1)	0.95	***	***	***	***
	0.42	*	*	*	*
	0.93	****	****	****	****
	0.78	*	*	nd	nd
EWA (ethyl acetate: water: acetone, 5:2:5)	0.70	*	*	*	*
	0.53	**	**	**	**
	0.31	***	***	***	***
	0.18	nd	nd	**	**
TEF (toluene: ethyl acetate: formic acid, 6:3:1)	0.67	**	*	*	*
	0.61	***	***	**	**
	0.52	****	****	****	****

[a] R_f values are means of 10 replications in each of three repetitions
[b] Metabolite strength in TLC plates were visually expressed with * (weakest) to **** (strongest)
[c] nd = not detected

Table II. R_f values of the separated extracts of metabolites produced by two isolates of *Mycosphaerella pinodes* (A2 & B2) in thin-layer chromatography with solvent system of ethyl acetate: water: acetone (5: 2: 5 v: v) detected under 365 nm UV light

A2 (weakly aggressive)			B2 (strongly aggressive)		
Metabolite (s)	R_f (mean ± SD)[a]	Toxicity	Metabolite (s)	R_f (mean ± SD)	Toxicity
No matching product			B2 - 1	0.18 ± 0.02	No
A2 - 1	0.29 ± 0.02	No	B2 - 2	0.29 ± 0.02	Yes
A2 - 2	0.51 ± 0.02	Yes	B2 - 3	0.54 ± 0.02	Yes
A2 - 3	0.68 ± 0.03	No	B2 - 4	0.71 ± 0.02	No
A2 - 4	0.76 ± 0.02	No	No matching product		
A2 - 5	0.90 ± 0.01	Yes	B2 - 5	0.88 ± 0.02	Yes

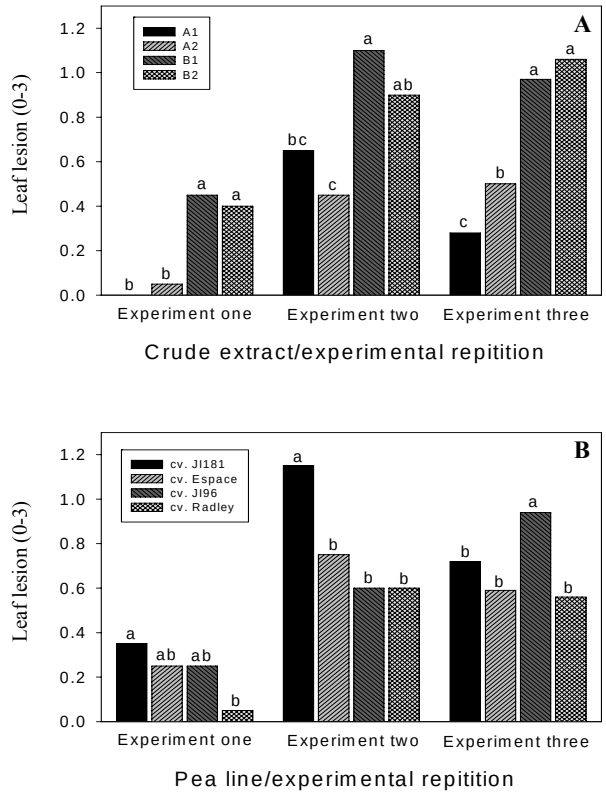
[a] R_f values are means of six replicates in each of two repetitions

$P \leq 0.02 - 0.04$) and for the reaction of pea lines between repetitions one and two ($r = 0.95$, $P \leq 0.05$), but no correlation with the third repetition.

Separated extracts of metabolites. Metabolites of isolates A2 and B2 were separated using the EWA solvent system in TLC analysis and assessed using 365 nm UV light. There were five products (based on different R_f values of visible spots) per isolate. Four of the spots had the same or similar R_f values for both isolates (Table II) and each pair of spots had similar strength (data not shown). However, a product with a unique R_f value was observed in each isolate; the product from isolate A had an R_f value of 0.76 and the product from isolate B had R_f value of 0.18.

Two of the five extracts from isolate A2 and three of five extracts from isolate B2 were toxic to pea leaves (Table II) and produced necrotic lesions around the inoculation site. No lesions were produced in treatments with methanol or water, or with five other products (A2-1, A2-3, A2-4, B2-1 & B2-4). Two of the toxic products were produced by both isolates: A2-2 and B2-3; A2-5 and B2-5. Extract A2-1 (R_f = 0.29) from isolate A was not toxic, but the product with a similar retention time from isolate B (B2-2, $R_f = 0.29$) was toxic (Fig. 2A). The most toxic of the five toxic extracts (A2-2 & B2-2) produced lesions with a mean rating of 0.8, while the least toxic extract B2-3 had lesion rating of 0.5. Collectively, lesions were smaller in cv. Radley than on line (Fig. 2B).

Fig. 1A. Effect of crude extracts from four isolates of *Mycosphaerella pinodes* on lesion development in detached-leaf assays (data combined across pea lines) and (B) reaction of four pea lines to crude extracts of metabolite from *Mycosphaerella pinodes* (data combined across isolates). Bars capped by the same letter in each experiment do not differ based on Duncan's Multiple Range Test at $P \leq 0.05$



DISCUSSION

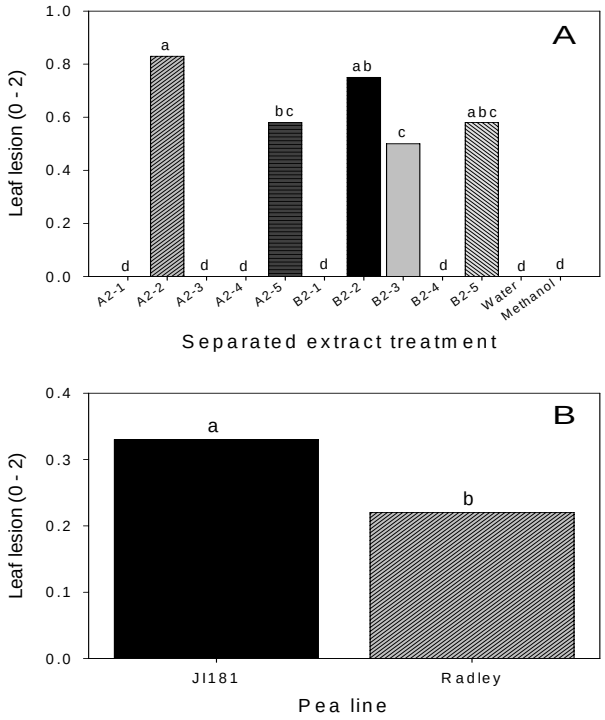
The current study demonstrated that several metabolites that are toxic to pea leaves occur in culture filtrates of *M. pinodes*. All of the crude culture extracts produced small necrotic lesions when applied to wounded pea leaves, regardless of the aggressiveness of the source isolate. However, only five out of ten separated products produced necrotic lesions in detached-leaf assays. Two of the products were from weakly aggressive isolate and three were from the strongly aggressive isolate of *M. pinodes*. Also, consistently smaller lesions developed on cv. Radley (least susceptible to *M. pinodes*) than on the other lines in the study. These findings indicate that it may be useful to evaluate the potential of these toxic metabolites for use in evaluations of disease resistance.

TCL is widely used in detection and quantification of mycotoxin (Scott *et al*., 1970; Gorst-Allman & Steyn, 1984; Coker *et al.,* 1993). However, only compounds that absorb UV can be detected and highly polar compounds will not be isolated with the solvent extraction procedures (Macko, 1983). Also, multiple forms of toxins are common in filtrates of toxin-producing pathogens (Yoder, 1980) and even products that have been separated using TLC can still contain a mixture of several metabolites. For example, only a single metabolite was obtained in a previous study (Hwang *et al.*, 2004) of metabolites of *M. pinodes*, but several products were separated in the current study. This variation could be caused by the different detection approaches we used compared to the previous study, where the TLC plates were sprayed with the reagents $FeCl_3$ and ρ-anisaldehyde to detect a characteristic compound. However, all visible compounds were analysed in our study without applying any spray reagent.

Several factors can affect the production and concentration of toxic metabolites in culture. One important factor is the growth media, because substrate affects toxin production (Lacey, 1985). The amount of toxic metabolites produced in the current study was quite low, but it was adequate to extract quantities for lab-scale bioassays.

Not all diseases are associated with toxins. Even for *M. pinodes*, a non-toxic diffusible factor is reported to be involved in tissue colonization and host selection (Oku *et al*., 1980). However, toxic metabolites from *M. pinodes* and *A. pisi* have been reported previously (Kaur & Deshpande, 1980; Lepoivre, 1982; Hwang *et al.,* 2004) and the results of the current study indicate that they may be associated with differences in disease reaction among cultivars. Further

Fig. 2. Effects of the separated extracts of metabolites from two *Mycosphaerella pinodes* isolates on lesion size in a detached-leaf assay. Data are combined (A) across the two pea lines and (B) across the two isolates. Bars capped by the same letter in each graph do not differ based on Duncan's Multiple Range Test at $P \leq 0.05$



study is required to determine how these toxins affect disease development and their mode of action. Such investigations could lead to application of toxic metabolites in resistance evaluation and other related fields.

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