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Cell wall-degrading enzymes of *Didymella bryoniae* in relation to fungal growth and virulence in cantaloupe fruit

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Abstract

Didymella bryoniae is an important pathogen of cucurbits worldwide. Virulence factors of *D. bryoniae* were investigated in regard to fungal growth and the production of cell wall-degrading enzymes, polygalacturonase (PG), pectate lyase (PL), pectin lyase (PNL), β -galactosidase (β -Gal) and cellulase (Cx). Virulence levels of five *D. bryoniae* isolates were determined by the severity of inoculated cantaloupe fruit decay. The highly virulent isolates had more mycelial growth than the moderately virulent isolates in different media. PG activities produced by the highly virulent isolates in shake cultures and in decayed fruit were greater than those of the moderately virulent isolates. PNL, but not PL, in decayed fruit was higher with the highly virulent isolates compared to the moderately virulent ones. The highly virulent isolates showed higher Cx activity than the moderately virulent ones in decayed fruit and in fruit tissue shake culture. β -Gal activities of the highly virulent isolates in pectin shake culture and in decayed fruit were greater than those of the two moderately virulent isolates although fruit also produced β -Gal. Protein analysis showed two fungal β -Gal isozymes in decayed fruit compared to those of healthy fruit. Correlation analysis indicated that the activities of PG, PNL, β -Gal and Cx in cultures and in decayed fruit positively correlated with fungal growth and fruit decay severity. The results of this study suggest that PG, PNL, β -Gal, and Cx appear to be virulence factors of *D. bryoniae* in cantaloupe decay with PG and β -Gal as the most predominant fruit decay enzymes.

Keywords

Cucumis melo; black rot; pectolytic enzymes; polygalacturonase; pectate lyase; pectin lyase; cellulase; β -galactosidase.

Introduction

Didymella bryoniae (Auersw.) Rehm is the causal agent of gummy stem blight, foliar leaf spot, and black rot of fruit, causing severe cucurbit crop losses worldwide (Keinath 2011; Sitterly and Keinath 1996; Zitter 1996). The pathogen is most common in the southern

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United States, and subtropical and tropical areas of the world (Sitterly and Keinath 1996). Black rot symptoms occur on the fruit of all cucurbits (Zitter 1996), and can be severe in both pre- and post-harvest developmental stages of cantaloupe (*Cucumis melo* var. *cantaloupensis* Naud.) fruit (Miller et al. 1997; Zhang et al. 1999). During the gummy stem blight and black rot epidemic of cantaloupe fruit in the lower Rio Grande Valley of Texas of USA in 1997, black rot was observed on all fruit developmental stages, and many fields suffered 100% loss (Zhang et al. 1999). The control of black rot of cantaloupe fruit is achieved by both pre-harvest gummy stem blight control and appropriate post-harvest handling and treatments (Zitter, 1996). However, new effective methods and products for controlling diseases caused by *D. bryoniae* on cucurbit plants and fruits are still needed.

The understanding of *D. bryoniae* pathogenesis and virulence factors may provide new information to develop effective control methods of black rot on cantaloupe fruit. Fungal virulence factors include any microbial characteristic that determines the capacity for virulence in the host. Steekelenburg (1982) reported that variation in virulence of *D. bryoniae* isolates on cucumber fruit was correlated with the extent of fruit rot and *in vitro* radial growth of the fungus. Keinath et al. (1995) tested pathogenicity of 19 *D. bryoniae* isolates collected from different locations in the United States and found that 17 of them were pathogenic to watermelon and cantaloupe plants. Chilosi and Magro (1998) investigated pectolytic enzymes of melon seedling hypocotyls inoculated with a *D. bryoniae* isolate. They found that pectin lyase was the principal pectolytic enzyme associated with fungal pathogenesis.

Plant cell wall-degrading enzymes, especially pectolytic enzymes, produced by pathogens have been implicated in playing an important role in pathogenesis (Bateman and Basham 1976; Collmer and Keen 1986). Polygalacturonase (PG, EC 3.2.1.15), pectin lyase (PNL, EC 4.2.2.10), and pectate lyase (PL, EC 4.2.2.2) have been implied as virulence factors for a number of plant pathogens (Akagi and Stotz 2007; Barras et al. 1994; Brown et al. 1992; Chilosi and Magro 1998; Cleveland and Cotty 1991; Crawford and Kolattukudy 1987; Hancock 1968; Huang and Allen 1997; Lei et al. 1985; Punja et al. 1985; Rodriguez-Palenzuela 1991; Zhang et al. 1999). Further degradation of plant cell wall components, hemicellulose and cellulose by pathogens is due to the actions of pathogen produced hemicellulases and cellulases. A number of plant pathogens are known to produce hemicellulases and cellulases (Bateman and Basham 1976; Misaghi 1982; Vu et al. 2012). Hemicellulases include enzymes such as β -xylosidase and β -galactosidase (β -Gal, EC 3.2.1.23) (Misaghi 1982). Cellulases are a group of enzymes including endoglucanase (Cx, EC 3.2.1.4, endo- β -1,4-glucanase), exoglucanase (C_1 , β -1,4-cellobiohydrolase) and β -glucosidase (Dori et al. 1995). The role of hemicellulases and cellulases in pathogenicity and virulence of plant pathogens are largely undetermined (Novo et al. 2006). However, cellulolytic activity of *Penicillium digitatum* and *P. italicum* was reported to correlate with fungal growth and pathogenesis in citrus fruit (Barkai-Golan and Karadavid 1991). Recently, Vu et al. (2012) determined that cellulases were virulence factors of *Magnaporthe oryzae*. When the major genes coding endoglucanases in *Xanthomonas campestris* pv *campestris* were disrupted, the pathogen virulence on turnip and radish decreased (Gough et al. 1988). *Mycocentrospora acerina* produced PG, PL, pectin methylesterase and β -1,4-glucanase in a liquid culture with carrot cell walls as the carbon source, and the activity of

these enzymes was positively correlated with the aggressiveness of the fungal isolates on carrot (Le-Cam et al.1994).

β -Gal is a hydrolase enzyme that catalyzes the hydrolysis of β -galactosides into monosaccharides. Both microbes and plants including fruits can produce β -Gal. Fruit synthesized β -Gal has been suggested to play a major role in cantaloupe fruit softening during the fruit ripening process by degrading the pectin polymers and hemicellulose fractions of the cell wall (Ranwala et al. 1992). However, degradative enzyme production and changes in fruit are regulated by plant genes and gene expression during fruit development. Pathogen-produced degradative enzymes can help the pathogen infect fruits by breaking down the major cell wall constituents of fruit tissues. Although *D. bryoniae* has been reported to produce pectin methylesterase, PG, PNL, cellulase and β -Gal (Bruton et al. 1996; Chilosi and Magro 1998; Curren 1969; Reshetnikova and Uspenskaya 1978; Zhang et al. 1999), there is little information regarding the relationships between the production of cell wall-degrading enzymes by *D. bryoniae* and the fungal virulence in cucurbit fruit. Information regarding the *D. bryoniae* cell wall-degrading enzymes of cantaloupe will lead to further understanding of the molecular interactions between hosts and pathogens, the pathogenesis and virulence factors, and help develop new effective control strategies for this pathogen.

The objectives of this study were to: (1) determine the virulence levels of five *D. bryoniae* isolates based on their decay capacities on cantaloupe fruit; (2) test the mycelial growth rates of the five fungal isolates in different media and substrates; (3) analyze the activities of PG, PL, PNL, β -Gal and Cx produced by *D. bryoniae* isolates or cantaloupe fruit; and (4) determine the relationship of fungal virulence in cantaloupe fruit to the *in vitro* and *in vivo* production of each of these enzymes and fungal growth *in vitro*.

Materials and methods

Fungal isolates

Didymella bryoniae isolates were collected from decayed cantaloupe fruit in the fields in Oklahoma and Texas. Based on virulence levels on inoculated cantaloupe fruit, five representative *D. bryoniae* isolates (OK963095, TX97046, TX97128, TX97059 and TX97025) were chosen for this study. The isolate OK963095 was obtained in Lane, Oklahoma, and the other four isolates were from the Rio Grande Valley of Texas. The isolates were grown on potato dextrose agar (PDA) at 25°C for 4 days, and hyphal tipped for isolate purification. The isolates were then grown on PDA and transferred to glass vials containing moist artificial soil-mix (Scotts-Sierra, Marysville, OH, USA) for long term storage (21-22°C). As needed during the experiments, the fungal isolates were recovered from storage vials and grown on PDA at 25°C.

Fungal virulence determination

The virulence of the five *D. bryoniae* isolates was determined based on lesion sizes on inoculated cantaloupe fruit. Cantaloupes (cv. Magnum 45) were grown at the USDA-ARS Research Station, Lane, Oklahoma using recommended cultural practices. To accurately

determine the fruit maturity stage, female flowers were tagged and marked at the day of flowering in the field. When fruit developmental stage reached 10 days (post-pollination) after female flowers were tagged, fruit were harvested and brought to the laboratory for inoculation with the fungus. Ten day-old-fruit were used for fungal inoculations. Previous research showed that 10 day fruit were more susceptible to *D. bryoniae* decay than more mature fruit (Zhang et al. 1999). Therefore, 10 day-old-fruit was considered most suitable and discriminating for determining virulence levels of *D. bryoniae* isolates. Fruit were inoculated by surface disinfecting the melon with 80% ethanol, and then removing tissue aseptically (1-cm deep) with a cork borer (0.7-cm diameter). The fungal isolates were grown on PDA for 4 days at 25°C prior to inoculation. A PDA plug (0.5-cm diameter) colonized by the fungus was placed into each inoculation site followed by a small autoclaved cotton ball and sealed with Kwik™ Seal Caulk (DAP Inc., Dayton, Ohio, USA). Two inoculation sites were established equatorially on each of six replicate fruit for each isolate. Fruit inoculated with PDA plugs without the fungus served as controls. After 4 days, each lesion was cut perpendicularly to the lesion center, and the profiles of lesions inside the fruit were traced on transparency sheets. The areas of decay profiles on the transparent sheets were calculated using an area meter (Li-Cor, Lincoln, NE, USA). Decayed and healthy tissues were collected separately, and stored at -20°C until further processing. The experiment was conducted twice.

Shake cultures for fungal enzyme and mycelial growth determination

A modified Richard's liquid medium (Martinson and Baker 1962) containing KNO₃ (10 g l⁻¹), KH₂PO₄ (5 g l⁻¹), MgSO₄·7H₂O (2.5 g l⁻¹), and FeCl₃·6H₂O (0.02 g l⁻¹) was used. Three carbon sources were used in the liquid medium: 1% (w/v) orange pectin (Sigma, St. Louis, MO), 1% (w/v) carboxymethyl cellulose (CMC, Sigma, St. Louis, MO), and 10% (w/v) homogenized fresh fruit mesocarp tissue (10-day-old fruit). The initial pH of autoclaved liquid medium with pectin, CMC and fresh fruit tissue was 4.7, 5.1 and 6.0, respectively. Each of the five isolates was grown on PDA for 4 days at 25°C prior to inoculation. Two 0.7-cm PDA plugs colonized by the fungus were introduced into a 250-ml flask containing 100 ml of the liquid medium. The flasks were placed on an orbital shaker (100 rpm) at 25°C with 4 replicates per isolate. After 7 days, the contents of each flask were filtered through Miracloth™ (Calbiochem-Behring, La Jolla, CA, USA). The filtrates were collected, and their pH values were also recorded. The filtrates were subsequently stored at -20°C until further processing. Mass of fungal mycelia collected on the Miracloth was dried in an oven at 70°C for seven days and weighed. Miracloth was pre-dried and weighed for fungal mass calculation. The experiment was repeated twice.

Extraction of enzymes from fruit

The extraction for polygalacturonase (PG), cellulase (Cx) and β-galactosidase (β-Gal) from decayed or healthy fruit tissue was conducted following a procedure described previously (Zhang *et al.* 1999). In brief, 10 g of decayed tissue taken from inoculated fruit were placed in 10 ml of 0.1 M Na-acetate buffer (pH 5.0) containing 1 M NaCl and 1 mM EDTA in a centrifuge tube (2.6 × 10.5 cm). The contents were homogenized using a polytron (Kinematica, Littau, Switzerland). The homogenates were centrifuged at 32,000 ×g for 15 min, and supernatants were collected.

The extraction of pectate lyase (PL) and pectin lyase (PNL) from decayed or healthy fruit tissue followed the same procedure as described above except that the extracting buffer was 0.1 M Tris-HCl (pH 8.5) containing 1 M NaCl. Healthy tissues were processed by the same procedure and served as controls. All extracts obtained were stored at -20°C until needed for enzyme assays.

PG activity assay

PG activity was assayed by measuring reducing groups using the 2-cyanoacetamide method (Gross 1982). The substrate was 0.2% polygalacturonic acid in 50 mM Na-acetate buffer (pH 5.0). The reaction mixture consisted of 50 μl of sample and 150 μl of substrate, and incubated for 30 min at 40°C . The reaction was terminated by adding 1.2 ml of 0.1 M borate buffer (pH 9) containing 0.2% 2-cyanoacetamide, and boiling for 10 min. The absorbance of the samples at 276 nm was recorded using a spectrophotometer (Shimadzu Inc., Kyoto, Japan) after the reaction mixtures were equilibrated to room temperature. Controls (zero time), to which the terminating buffer was added prior to the substrate solution, were run simultaneously with each sample. Galacturonic acid at various concentrations was used to establish a standard curve. One unit of enzyme activity was defined as one μmole of reducing groups released by the enzyme per min at 40°C .

PL and PNL activity assays

PL activity was measured spectrophotometrically by following the procedure described by Collmer *et al.* (1988). An enzyme sample (0.5 ml) was mixed with 2.5 ml of substrate solution of 60 mM Tris-HCl, pH 8.5, 0.6 mM CaCl_2 and 0.24% (w/v) polygalacturonic acid (P-3889, Sigma-Aldrich Co., St Louis, MO, USA) in a 3-ml cuvette with a 1-cm light path. The subsequent increase of absorbance at 232 nm was monitored over time with a spectrophotometer (Shimadzu Inc., Kyoto, Japan). One unit of enzyme activity was defined as one μmole of 4,5-unsaturated products released by the enzyme in one min at 30°C . The enzyme activity was calculated based on the molar extinction coefficient of $4,600 \text{ M}^{-1} \text{ cm}^{-1}$ for the unsaturated products at 232 nm (Collmer *et al.* 1988). Activity of PNL was measured using the same procedure as that used for assaying PL activity, except that polygalacturonic acid was replaced with orange pectin (P-9135, Sigma-Aldrich Co., St Louis, MO, USA). One unit of enzyme activity was defined as the formation of one μmole of 4,5-unsaturated products in 1 min at 30°C .

Cx activity assay

Cx activity was assayed using the same procedure as the PG activity assay described previously, except that enzyme substrate was 0.5% CMC, and incubation time was 4 hrs (Bonghi *et al.* 1992). One unit of Cx activity was defined as one nmole of product released by the enzyme per min at 40°C .

β -Gal activity assay

β -Gal activity was assayed according to a procedure described by Biles *et al.* (1993). The substrate solution was 50 mM Na-acetate buffer (pH 5.0) containing 0.1% p-nitrophenyl β -D-galactopyranoside, 0.09% NaCl and 0.06% bovine serum albumin (BSA). The reaction

mixture containing 100 μl of enzyme sample and 900 μl of substrate solution was incubated at 25°C for 1 h. The enzyme reaction was stopped by adding 1 ml of 0.2 M Na_2CO_3 . The concentration of released p-nitrophenyl groups was measured with a spectrophotometer at 400 nm. Controls (zero time), to which the terminating buffer (0.2 M Na_2CO_3) was added prior to the substrate solution, were run simultaneously with each sample. A linear series of p-nitrophenol (p-nitrophenol standard solution, 10 $\mu\text{mol}/\text{ml}$, Sigma Diagnostics, Inc. St Louis, MO, USA) concentrations was used to establish a standard curve. One unit of the enzyme activity was defined as one nmole of products released by the enzyme in one min at 25°C.

β -Gal isozyme analysis

β -Gal isozymes from *D. bryoniae* (isolate OK963095) infected fruit, healthy cantaloupe fruit (10 days old) and fungal pectin shake culture were assayed using cation exchange chromatography. Samples prepared from healthy and decay fruit tissue as well as from fungal pectin shake culture, were applied to a Bio-Scale S₂ cation-exchange column (7 $\times$ 52 mm) (Bio-Rad, Hercules, CA, USA) attached to a HPLC Biologic chromatography system (Bio-Rad, Hercules, CA, USA). The column was equilibrated with 25 mM Na-acetate buffer, pH 4.0, prior to loading samples. After the samples were loaded onto the column, a 0 to 1.0 M NaCl concentration gradient was initiated over a 30 min period. Flow rate was 1 ml per min, and fractions were collected every min. β -Gal activity was assayed for each of the fractions. Protein profiles were detected by recording absorbance at 280 nm during chromatography.

Protein assay

Protein concentrations of the samples were determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Bovine serum albumin was used to establish a standard protein curve.

Radial growth of *D. bryoniae* on PDA

The *D. bryoniae* isolates OK963095, TX97046, TX97128, TX97059 and TX97025 were grown on PDA for 4 days at 25°C. PDA plugs (5-mm diameter), taken from the colony margins of each of the five *D. bryoniae* isolates, were placed at the centres —of PDA plates (9-cm diameter). Five plates were used for each of the five fungal isolates. The plates were incubated at 25°C for 3 days, and diameters of fungal colonies were recorded. The experiment was conducted twice.

Data analysis

Analysis of variance of data was performed using a SAS statistical package (SAS Institute, Cary, NC). Treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple range test ($P = 0.05$). Correlation and regression analyses were conducted using Microsoft Excel data analysis procedures.

RESULTS

Fruit decay

All five *Didymella bryoniae* isolates caused decay by fruit inoculations, and varied in decay severity (lesion area) (Fig. 1). The average areas of lesions in cantaloupe fruit (10-days post-pollination) 4 days after inoculation with *D. bryoniae* isolates OK963095, TX97128 and TX97046, were greater ($P < 0.05$) than those caused by isolates TX97059 and TX97025 (Fig. 1). The lesion area produced by the isolates OK963095, TX97128 or TX97046, was similar ($P > 0.05$). In addition, the lesion area produced by TX97025 and TX97059 was also similar. Based on these results, the isolates OK963095, TX97128 and TX97046, hereafter, were referred to as highly virulent isolates, and TX97059 and TX97025 as moderately virulent isolates.

Production of PG, PL, PNL, Cx and β -Gal by *D. bryoniae* in shake cultures

When the five *D. bryoniae* isolates were grown in a liquid culture using pectin or cantaloupe fruit tissue as the sole carbon source, the PG activity (units/g dry fungal mass) of the three highly virulent isolates OK963095, TX97128 and TX97046 was greater ($P < 0.05$) than that of the two moderately virulent isolates TX97059 and TX97025 (Table 1). Among the three highly virulent isolates, the PG activity of the isolate TX 97046 was greater than that of the isolate OK963095, but was similar to that of the isolate TX97128 in pectin shake culture. All three highly virulent isolates produced a similar amount of PG activity in the shake culture using cantaloupe fruit mesocarp tissue as the sole carbon source. No difference in PG activity between the two moderately virulent isolates was observed in shake culture using either pectin or fruit tissue as the sole carbon source. Significant correlations were shown between fungal virulence and PG activity detected in pectin shake culture ($r = 0.908$, $p = 0.033$) and cantaloupe fruit tissue shake culture ($r = 0.973$, $p = 0.005$) (Table 1). The activity of PL or PNL was not detected with any of the five *D. bryoniae* isolates grown in pectin or cantaloupe fruit tissue shake culture under the test conditions (Table 1).

Average β -Gal activity (units/g dry fungal mass) produced by the three highly virulent isolates was 3.3 and 16.9 times greater ($P < 0.05$) in pectin shake culture and cantaloupe fruit tissue shake culture, respectively, when compared to the two moderately virulent isolates (Table 1). Among the three highly virulent isolates, β -Gal activity of the isolate TX97046 was greater than that of the isolate TX97128, but was similar to that of the isolate OK963095 in pectin shake culture. In fruit tissue shake culture β -Gal activity of the isolate TX97046 was higher than that of the isolate OK963095, but was similar to that of the isolate TX97128. The two moderately virulent isolates TX97059 and TX97025 grown in shake culture with either pectin or fruit tissue as the sole carbon source produced a similar ($P < 0.05$) amount of β -Gal activity. A significant correlation ($r = 0.953$, $p = 0.012$) was observed between fungal virulence and β -Gal activity of the five fungal isolates grown in pectin shake culture (Table 1).

When the five *D. bryoniae* isolates were grown in cantaloupe fruit tissue shake culture, the highly virulent isolates OK963095, TX97046 and TX97128 exhibited a greater Cx activity compared with the two moderately virulent isolates TX97059 and TX97025 (Table 1). No

difference in Cx activity was found among the three virulent isolates or between the two moderately virulent isolates in fruit tissue shake culture. A significant correlation ($r = 0.950$, $p = 0.014$) was shown between fungal virulence and Cx activity detected in cantaloupe fruit tissue shake culture (Table 1). When CMC was used as the sole carbon source in shake culture to grow the five *D. bryoniae* isolates, all five isolates produced a similar ($P < 0.05$) amount of Cx activity. No Cx activity was detected with any *D. bryoniae* isolates in pectin shake culture (Table 1).

PG, PL, PNL, β -Gal and Cx activities detected in healthy and *D. bryoniae* decayed cantaloupe fruit

PG activity (units/mg protein) in extracts obtained from decayed fruit tissues produced by each of the three highly virulent *D. bryoniae* isolates OK963095, TX97046 and TX97128, was greater ($P < 0.05$) than that of each of the moderately virulent isolates TX97059 and TX97025 (Table 2). However, significant differences in PG activity in decayed fruit tissue were detected among the three highly virulent isolates, but no difference was found between the two moderately virulent isolates. PG activity was not detected in healthy fruit tissue (Table 2). The PG activity detected in decayed fruit tissue was significantly correlated with disease severity ($r = 0.890$, $p = 0.043$) (Table 2).

PL and PNL activity (units/mg protein) was detected in *D. bryoniae* decayed fruit tissue, but not from healthy fruit (Table 2). The three highly virulent isolates produced higher PNL activity compared to the two moderately virulent isolates, although there was a difference ($P < 0.05$) in PNL activity among the three highly virulent isolates. No difference in PNL activity was found between the two moderately virulent isolates. Among the five isolates, the moderately virulent isolate TX97025 produced the highest amount of PL activity in decayed fruit tissue followed by the highly virulent isolate TX97046 (Table 2). The highly virulent isolates OK963095 and TX97128, and the moderately virulent isolate TX97059 produced a similar ($P < 0.05$) amount of PL activity. No significant correlation ($r = 0.408$, $p = 0.495$) was found between PL activity detected in decayed fruit tissue and fungal virulence (Table 2).

β -Gal activity (units/mg protein) was detected in both fungal decayed and healthy cantaloupe fruit tissue (Table 2). However, β -Gal activity in the healthy tissue was 2.5 to 7.8 times greater than in decayed tissue. β -Gal activity was higher ($P < 0.05$) in decayed tissue caused by the three highly virulent isolates when compared to fruit tissue decayed by the two moderately virulent isolates. A significant correlation between fungal virulence and β -Gal activity was detected in cantaloupe fruit tissue decayed by *D. bryoniae* isolates ($r = 0.880$, $p = 0.049$) (Table 2).

Cx activity (units/mg protein) was detected in cantaloupe fruit tissue infected by each of the five isolates, but no Cx activity was detected from healthy fruit tissue (Table 2). The Cx activity produced by the three highly virulent isolates was 2 – 3 fold greater ($P < 0.05$) than the moderately virulent isolates. Cx activity of OK963095 in decayed fruit was higher than that of isolates TX97046 and TX97128. Cx activity was not different ($P < 0.05$) between TX97046 and TX97128. The two moderately virulent isolates TX97025 and TX97059, exhibited a similar ($P < 0.05$) amount of Cx activity in decayed fruit. However, a significant

correlation ($r = 0.958$, $p = 0.010$) was shown between Cx activity of decayed fruit tissue and fungal decay severity among the five isolates (Table 2).

β -Gal isozyme detection

Cation exchange chromatography of the proteins extracted from healthy cantaloupe fruit tissue (10-days post-pollination) showed two β -Gal activity peaks, which were eluted with a salt concentration of 0.3 and 0.9 M, respectively (Fig. 2A). Four β -Gal activity peaks corresponding to a salt concentration of 0, 0.2, 0.3 and 0.9 M, respectively, were detected in the extract of *D. bryoniae* (OK963095) infected fruit tissue (Fig. 2B). A comparison of β -Gal activity peaks between the healthy and infected fruit tissue revealed that the two β -Gal isozymes eluted at a salt concentration of 0.0 and 0.2 M appeared to be of fungal origin, and the two β -Gal isozymes eluted at 0.3 and 0.9 M of salt to be of fruit origin. Orange pectin shake culture of *D. bryoniae* isolate OK963095, showed only one prominent β -Gal peak, which was eluted at a salt concentration of 0.1 M (Fig. 2C). This β -Gal isozyme had a different charge when compared to the β -Gal isozymes observed in decayed fruit and healthy fruit.

Mycelial growth of

***D. bryoniae* isolates *in vitro* and pH changes of fungal shake cultures**

The average radial mycelial growth rate of the highly virulent *D. bryoniae* isolates OK963095, TX97046 and TX97128 on PDA was 16.4% greater ($P < 0.05$) compared to that of the moderately virulent isolates TX97059 and TX97025 (Table 3). Mycelial growth rate was not different ($P < 0.05$) among the three highly virulent isolates or between the two moderately virulent isolates. There was a significant correlation ($r = 0.970$, $p = 0.006$) between fungal decay severity and mycelial growth on PDA (Table 3).

When the five *D. bryoniae* isolates were grown in a liquid medium using pectin or cantaloupe fruit tissue as the sole carbon source, mycelial growth rate of each of the highly virulent isolates OK96095, TX97046 and TX97128 was greater ($P < 0.05$) than that of each of the moderately virulent isolates (Table 3). In addition, mycelial growth rate was not significantly different ($P < 0.05$) among the three highly virulent isolates or between the two moderately virulent isolates. A significant correlation was observed between fungal virulence and fungal mycelial growth in pectin shake culture ($r = 0.931$, $p = 0.022$), and also between fungal virulence and fungal mycelial growth in cantaloupe fruit tissue shake culture ($r = 0.966$, $p = 0.007$) (Table 3). Mycelial growth rates of all five *D. bryoniae* isolates were similar ($P < 0.05$) when grown with CMC as the sole carbon source (Table 3).

The initial pH value of the modified Richard's liquid medium before fungal inoculations was 4.7, 6.0 and 5.1 for pectin, fruit tissue, and CMC as sole carbon sources, respectively. After 7 days of shake culture incubation at 25°C, the pH of pectin cultures of the highly virulent isolates OK96095, TX97046 and TX97128 was 6.60 to 6.73 compared to a range of 5.33 to 6.02 for the cultures of moderately virulent isolates TX97059 and TX97025. However, the pH of fruit tissue shake cultures of all five isolates after 7 days was 6.22 to 6.44. The pH values of CMC shake cultures of all five isolates were 5.40 to 5.42 after 7 days.

DISCUSSION

Many studies have supported that pectin degrading enzymes of plant pathogens play an important role in pathogenesis (Akagi and Stotz, 2007; Bateman and Basham 1976; Collmer and Keen 1986). Curren (1969) studied pectolytic enzymes of *D. bryoniae* and their relation to black rot of squash fruit, and found that PG appeared to be an important enzyme involved in the breakdown of squash fruit. In contrast, Chilosi and Magro (1998) investigated pectolytic enzymes produced by *D. bryoniae* in hypocotyl tissue of melons, and reported that PNL was the predominant degradative enzyme related to the onset of disease and disease severity of melon seedlings. In our study, *D. bryoniae* produced PG, PL and PNL in decayed cantaloupe fruit tissue. The specific PG or PNL activity, but not PL activity, of *D. bryoniae* was higher in decayed cantaloupe fruit caused by the highly virulent isolates than that of the moderately virulent isolates. Significant correlations among fungal decay virulence, PG and PNL activities were observed. PG was also detected in pectin and cantaloupe fruit tissue shake cultures. However, PL and PNL were only detected in *D. bryoniae* decayed fruit tissue in our study. Therefore, PG seems to be the first pectolytic enzyme to degrade tissue by *D. bryoniae*, and PL and PNL production by the fungus appears to require some other factors such as elicitors which are available in cantaloupe fruit tissues *in vivo*, but not in *in vitro* autoclaved fruit tissue and pectin shake cultures. The alkalization of fungal culture and host fruit tissue may also regulate the production of different pectolytic enzymes by *D. bryoniae* isolates. The high pH (such as 7.5-8.5) is more favorable for PL and PNL activities while relatively low pH (such as 5-6) is more favorable for PG action. Chilosi and Magro (1998) reported that pH in Czapek's liquid culture containing pectin as the sole carbon source during *D. bryoniae* growth increased from 5.0 to 8.7, and PG and PNL were detected. In the current study, pH increased from an initial 4.7 to 6.7 for pectin cultures, and from an initial pH of 6.0 to 6.4 for fruit tissue cultures with *D. bryoniae*. In these cultures, PG was detected, but not PL and PNL. Chilosi and Magro (1998) reported that pH increased from 6.2-6.4 (controls) to over 7.3 in *D. bryoniae* infected melon seedling hypocotyl tissue. PNL was detected in the hypocotyl tissue experiments, but PG was not detected. Since PG, PL and PNL enzymes were detected in *D. bryoniae* infected cantaloupe fruit in the current study, it is hypothesized that initial fruit tissue pH is favourable to PG activity during disease onset, and then subsequent increase of pH during decay development resulted in pH conditions that were more favourable for PL and PNL activities. Our data also showed that the pectin liquid cultures of the three highly virulent *D. bryoniae* isolates had relatively higher pH values compared to those of the two moderately virulent isolates.

Previous work (Zhang et al. 1999) demonstrated that a *D. bryoniae* isolate produced at least 11 PG isozymes in a modified Richard's liquid medium using pectin as the sole carbon source, but Chilosi and Magro (1998) detected only one inducible PG isozyme with a *D. bryoniae* isolate grown in a modified Czapek's liquid medium. Apparently, many factors including different host tissues, media, and pH induce and possibly regulate the production of different pectolytic enzymes in distinct *D. bryoniae* isolates. Highly virulent isolates of other fungal pathogens have also been reported to produce greater amounts of pectolytic enzymes compared to the less virulent isolates (Barker and Walker 1962; Benard and Punja 1995; Carder et al. 1987; Chan and Sackston 1970; Le-Cam et al. 1994; Paquin and

Coulombe 1962; Punja et al. 1985). Zhang et al. (1999) also reported that black rot severity of cantaloupe fruit was positively associated with *D. bryoniae* PG activity in decayed fruit tissue. Multiple fungal PG isozymes were detected both *in vitro* and *in vivo*.

Although plant pathogens have been known to produce cellulases, the role of pathogen-produced cellulases in pathogenesis is still poorly understood (Bateman and Basham 1976; Misaghi 1982; Novo et al. 2006; Vu et al. 2012). A few reports have shown that Cx was related to virulence (Barkei-Golan and Karadavid 1991; Gough et al. 1988; Le-Cam et al. 1994). In this study, the Cx activity of *D. bryoniae* did not correlate with the virulence of the fungal isolates grown in the CMC shake culture, but the fungal Cx activity in fruit tissue shake culture and in decayed cantaloupe fruit was correlated with fungal virulence. Cx activity was not detected from healthy cantaloupe fruit tissue (10-days post-pollination) under the current test conditions, suggesting that the Cx activity detected in decayed fruit tissue was of fungal origin. Cx activity among the five *D. bryoniae* isolates in CMC shake culture *in vitro* was similar. The Cx activity of the highly virulent isolates was greater in fruit tissue when compared to the moderately virulent isolates. This suggests that Cx of the highly virulent isolates are stimulated to a greater extent by the fruit substrates and probable elicitors.

Pathogens produce cell wall-degrading enzymes in sequence with pectolytic enzymes forming first, and hemicellulose and cellulose degrading enzymes produced later (Bateman and Basham 1976; Cooper and Wood 1975). PG appeared to facilitate the ability of other cell wall-degrading enzymes to attack their substrates (Karr and Albersheim 1970). In the current study, the Cx activity of *D. bryoniae* was detected in decayed fruit, CMC shake cultures, and fruit shake cultures. However, -only Cx activity in decayed fruit and in fruit tissue shake culture was correlated with the fungal virulence. Since the highly virulent *D. bryoniae* isolates produced much higher PG and β -Gal than the moderately virulent isolates in fruit tissue shake culture and in infected cantaloupe fruit, more Cx substrates may be released and be available to the highly virulent isolates compared with those of the moderately virulent isolates. This may partially explain why the highly virulent *D. bryoniae* isolates produced higher Cx activity than the moderately virulent isolates in fruit tissue shake culture and in decayed fruit, but not in CMC shake culture. In CMC shake culture, all isolates had similar levels of Cx substrate present. In addition, we also observed that no Cx activity was detected with any of the five *D. bryoniae* isolates when they were grown in a shake culture using pectin as the sole carbon source and no Cx substrate available in the medium. All *D. bryoniae* isolates were similar in Cx production in CMC shake culture, indicating that they are genetically similar in their ability to produce Cx. These results suggest that *D. bryoniae* Cx production is a substrate-inducible enzyme. Curren (1968) reported that Cx activity was higher in the center than at the margin of the lesion caused by *D. bryoniae* on squash fruit. Cx was not significant in the early stages of squash black rot since the initial tissue maceration was effected primarily by the pectolytic enzymes with the cellulose of the cell walls being attacked only at a later stage of disease development. As implicated in our results and previous published work (Bateman and Basham 1976; Cooper and Wood 1975; Curren 1968), cellulase appears to be induced by pectolytic enzyme products and the greater availability of cellulose as a substrate.

β -Gal is involved in the degradation of galactose-containing pectic and hemicellulosic cell wall polysaccharides by attacking 1,4- β -galactosidic cross-linkages (Sawick and Kacperska 1995). There is little published information regarding the possible role of plant pathogen produced β -Gal in pathogenesis or virulence (Lehtinen 1993; Bruton et al. 1996). In our study, β -Gal activity of *D. bryoniae* was detected in pectin and fruit tissue shake cultures, and decayed and healthy cantaloupe fruit. β -Gal activity of *D. bryoniae in vitro* was correlated with fungal virulence. The total β -Gal activity detected from decayed fruit was also correlated to fungal virulence although healthy fruit had much higher β -Gal activity compared with that of decayed fruit. The β -Gal activity detected in decayed fruit tissue could be a mixture of fungus- and fruit-produced β -Gal. Cation exchange chromatography showed two distinct β -Gal activity peaks in *D. bryoniae* decayed fruit tissues when compared to healthy fruit tissue, suggesting that *D. bryoniae* produced at least two β -Gal isozymes which may play a role in tissue degradation. Bruton et al. (1996) also reported that cantaloupe vine decline fungal pathogen *D. bryoniae* produced β -Gal *in vitro*. High levels of β -Gal were detected from the shake cultures of *D. bryoniae* using pectin as the sole carbon source and *D. bryoniae* growth rate was positively correlated with the total β -Gal activity, which implies that β -Gal is involved in the degradation of pectic polysaccharides. Our results also showed that β -Gal activity was lower in decayed fruit when compared to healthy fruit, suggesting that cantaloupe fruit originated β -Gal might be inhibited or degraded during *D. bryoniae* infection and disease development. The presence of β -Gal has been found in cantaloupe fruit (Ranwala et al. 1992) and a number of other plant tissues (Bartley 1974; Biles et al. 1997; Carrington and Pressey 1996; DeVeau et al. 1993; Golden et al. 1993; Gross et al. 1986; Kitagawa et al. 1995; Konno et al., 1986). β -Gal was reported to degrade the hemicellulose fraction of the cell wall, and play an important role in the ripening of cantaloupe fruit (Ranwala et al. 1992) and other fruits (Golden et al. 1993; Biles, et al. 1997; Bartley 1974; Gross et al. 1986). *In vitro* β -Gal extracted from ripe cantaloupe fruit has been shown to be involved in the degradation of larger pectin polymers from pre-ripe fruit (Ranwala et al. 1992). The complex interactions among fungal infection, β -Gal of the fungus and β -Gal of the cantaloupe fruit are not known, and further study is needed.

Steekelenburg (1982) reported that there was a linear relationship between the growth of 11 *D. bryoniae* isolates on potato dextrose agar and virulence on cucumber fruit. In the present study, our data agreed with the report of Steekelenburg (1982). Among the five *D. bryoniae* isolates the correlation between fungal virulence and fungal growth *in vitro* were significant except for growth in CMC liquid culture. Similar relationships between fungal growth and virulence have been reported for other fungal pathogens (Barker and Walker 1962; Punja et al. 1985). Inferior mycelial growth of the moderately virulent isolates in comparison to the highly virulent isolates in liquid culture using pectin or cantaloupe fruit tissue as the sole carbon source appeared to be due to the limited ability of the moderately virulent isolates to produce PG and β -Gal. This would result in a corresponding reduction in sugars available for fungal respiration. When the five *D. bryoniae* isolates were grown in liquid culture using CMC as the sole carbon source, the growth of all isolates was similar, and there was no difference in Cx production, implicating that pectolytic enzymes and β -Gal are primary cell wall-degrading enzymes associated with the virulence of *D. bryoniae*.

In this study, the demonstration of cell wall-degrading enzymes as virulence factors of *D. bryoniae* to cantaloupe fruit decay may be helpful in developing new fungal decay control methods. For example, fungal cell wall-degrading enzyme inhibitors can be considered and pursued as new approaches for cantaloupe fruit decay. Genetic manipulation of plant and fruit for regulating or suppressing the production of fungal cell wall-degrading enzymes may lead to effective controls or reduction of fungal infections and disease development. The current study has focused on the interactions of fungal virulence, cell wall-degrading enzyme production, and fungal growth using five representative fungal isolates. In future studies more fungal isolates with different virulence levels will be tested for their cell wall-degrading enzymes. In addition, other approaches such as gene expression and gene manipulation of *D. bryoniae* isolates will be pursued to further elucidate the relationship between fungal cell wall-degrading enzyme production and fungal virulence in cantaloupe fruit.

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Abbreviations

HPLC	High Pressure Liquid Chromatography
Cx	cellulases

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Fig. 1.

Cantaloupe fruit (10-days post-pollination) black rot caused by the inoculation with five *Didymella bryoniae* isolates. Lesion areas were calculated from profiles of perpendicularly cut fruit lesions four days post-inoculation. The data from two separate tests were combined for analysis since the results were similar. Standard errors (*vertical bars*) were calculated from 15 replicates. Means with the same letter on the top of each bar are not significantly different ($P < 0.05$) based on the Ryan-Einot-Gabriel-Welsch multiple range test.

Fig. 2.

Cation-exchange chromatography of β -galactosidase (β -Gal) activity of healthy cantaloupe tissue (A), *Didymella bryoniae* infected cantaloupe tissue (B), and extracellular β -galactosidase of *D. bryoniae* grown in 1% pectin shake culture (C). Two ml of each sample were loaded onto a Bio-scale S_2 cation-exchange column (Bio-Rad, Richmond, CA), and 1-ml fractions collected every min. The activity of β -Gal was assayed for each fraction.

Enzyme activity (polygalacturonase [PG], pectate lyase [PL], pectin lyase [PNL], β -galactosidase [β -Gal] and cellulase [Cx]) of five *Didymella bryoniae* isolates grown *in vitro* shake cultures of modified Richard's medium supplemented with different carbon sources, and correlation of fungal enzyme activity to fungal virulence in cantaloupe fruit decay.

Table 1

Carbon sources in Richard's medium	<i>D. bryoniae</i> isolates Enzyme activity (units/g dry fungal mass)				Correction with fungal virulence ^x
	OK963095	TX97046	TX97128	TX97059	TX97025
PG					
Pectin	287.4 B ^y	361.2 A	317.5 AB	185.2 C	137.8 C
					$r=0.908, p=0.033$
Fruit tissue	25.2 A	21.3 A	22.6 A	15.8 B	13.3 B
β -Gal					$r=0.973, p=0.005$
Pectin	54.4 A	55.5 A	42.7 B	16.4 C	14.6 C
					$r=0.953, p=0.012$
Fruit tissue	10.3 B	20.1 A	15.3 AB	1.0 C	0.8 C
					$r=0.860, p=0.061$
Cx					
Pectin	ND	ND	ND	ND	ND
Fruit tissue	0.7 A	0.7 A	0.5 A	0.1 B	0.1 B
					$r=0.950, p=0.014$
CMC ^Z	4.8 A	4.5 A	3.9 A	5.8 A	4.5 A
					$r=0.551, p=0.335$
PNL					
Pectin	ND	ND	ND	ND	ND
Fruit tissue	ND	ND	ND	ND	ND
PL					
Pectin	ND	ND	ND	ND	ND
Fruit tissue	ND	ND	ND	ND	ND

ND = enzyme activity was not detected under the test conditions.

^xCorrection was calculated based on fruit decay area and enzyme activity.

^yMeans with the same letter within the same row are not significantly different ($P \leq 0.05$) based on the Ryan-Einot-Gabriel-Welsch multiple range test.

^ZCMC = carboxymethyl cellulose.

Table 2

Enzyme activity (polygalacturonase [PG], pectate lyase [PL], pectin lyase [PNL], β -galactosidase [β -Gal], and cellulase [Cx]) of five *Didymella bryoniae* isolates inoculated to cantaloupe fruit, and correlation of enzyme activity to fungal virulence in cantaloupe fruit decay.

Enzymes	<i>D. bryoniae</i> isolates Enzyme activity (Units/mg protein)					Control ^y	Correction with fungal virulence ^x
	OK 963095	TX 97046	TX 97128	TX 97059	TX 97025		
PG	0.349 A ^z	0.235 B	0.176 C	0.059 D	0.054 D	ND	$r=0.890, p=0.043$
PNL	0.173 A	0.062 C	0.117 B	0.009 D	0.010 D	ND	$r=0.891, p=0.042$
PL	0.074 C	0.151 B	0.019 C	0.034 CD	0.243 A	ND	$r=0.408, p=0.495$
β-Gal	2.322 C	2.040 C	3.182 B	1.353 D	1.021 D	8.01 A	$r=0.880, p=0.049$
Cx	18.692 A	13.386 B	14.672 B	7.420 C	6.380 C	ND	$r=0.958, p=0.010$

ND = no enzyme activity was detected under the test conditions.

^xCorrection was calculated based on fruit decay area and fungal enzyme activity in respective medium.

^yHealthy fruit tissue without fungal inoculation.

^zMeans with the same letter on each row are not significantly different ($P \leq 0.05$) based on the Ryan-Einot-Gabriel-Welsch multiple range test.

Table 3

Mycelial growth of five *Didymella bryoniae* isolates in different media *in vitro*, and its correlation to fungal virulence in cantaloupe fruit decay.

Medium	<i>D. bryoniae</i> isolates Mycelial growth ^y (mm radial diameter /day or mg dry mass/day)					Correction with fungal virulence ^x
	OK 963095	TX 97046	TX 97128	TX 97059	TX 97025	
Potato dextrose agar	17.74 A ^z	17.26 A	16.85 A	15.14 B	14.53 B	$r=0.970, p=0.006$
Pectin liquid culture	43.74 A	51.03 A	47.13 A	28.71 B	20.12 B	$r=0.931, p=0.022$
Fruit tissue liquid culture	48.98 A	48.32 A	48.22 A	44.01B	41.37 B	$r=0.966, p=0.007$
Cellulose liquid culture	6.81 A	8.75 A	8.67 A	6.75 A	7.54 A	$r=0.436, p=0.463$

^xCorrection was calculated based on fruit decay area and fungal mycelial growth in respective medium.

^yFungal growth rate was calculated by the total dry mycelial mass or radial diameter divided by number of days after incubation.

^zMeans with the same letter within the row are not significantly different ($P \leq 0.05$) based on the Ryan-Einot-Gabriel-Welsch multiple range test.