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Preliminary investigation of the pathogenic effectors of *Paramyrothecium eichhorniae* by heat treatment and membrane separation

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Abstract *Paramyrothecium eichhorniae* causes leaf blight in water hyacinth. The nature of the pathogenic effectors of spore suspension was investigated by heat-treatment and diafiltration. The supernatant of the spore suspension heated at 85 ºC was not shown different levels of pathogenicity and xylanase and pectinase activities compared to the untreated supernatant. The pathogenicity of autoclaved supernatant was abolished. Diafiltration experiment with a 5 kDa membrane showed that the pathogenic effectors could permeate through the membrane, but the xylanase activity could not pass through. The results indicated that the major pathogenic effectors of *P. eichhorniae* are small molecules, and xylanase activity of spore suspension is insufficient to cause the leaf blight symptoms.

Keywords: Biological control, Leaf blight disease, Mycotoxin, Phytotoxin, Water hyacinth

Introduction

Paramyrothecium eichhorniae is previously reported as *Myrothecium roridum* which causes leaf blight of water hyacinth (*Eichhornia crassipes*) (Piyaboon *et al.*, 2016; Pinruan *et al.*, 2022). Water hyacinth is a prominent invasive aquatic weed (Villamagna and Murphy, 2010; Dagno *et al.*, 2012) that affects human populations globally (Kriticos and Brunel, 2016). Water hyacinth is eliminated by conventional methods such as conventional herbicides or mechanical removal, which are costly (Dagno *et al.*, 2012; Okunowo *et al.*, 2013). *Myrothecium roridum* Tode: Fries and other isolates of *M. roridum* have been reported to cause disease in water hyacinth, but never been developed into a bioherbicide in a large scale (Okunowo *et al.*, 2019). Therefore, the use of *P.*

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eichhorniae as a biocontrol agent is of great interest because it is specific to water hyacinth and easy to produce (Piyaboon *et al.*, 2016).

In order to effectively manufacture *P. eichhorniae* spore suspension as a biocontrol agent in a larger scale, the nature of the pathogenic effectors in the spore suspension needs to be investigated. *Myrothecium roridum* was reported to produce trichothecene A and D toxins, which were found to be toxic to *Plasmodium falciparum* and cultured human cancer cells, but the effects of the isolated toxin on water hyacinth had not been addressed (Lakornwong *et al.*, 2019). In a different report, roridin A, which is a type of trichothecene mycotoxins, was purified from *Myrothecium roridum* Tode: Fries (IMI 394934) and was shown to be toxic to water hyacinth as well as to lemongrass, peanut, and cowpea (Okunowo *et al.*, 2019). In contrast, Piyaboon *et al.* (2016) reported that the crude extract of *M. roridum* was not toxic to lemongrass, peanut, and cowpea. Thus, it remains unclear if roridin A and trichothecene A and D toxins are the main pathogenic effectors for *P. eichhorniae*.

Some pathogenic fungi secrete cell wall degrading enzymes or other small proteins while they attack their plant hosts (de Jonge *et al.*, 2011). Xylanase has been found in several pathogenic fungi, and their corresponding inhibitors has been detected in the attacked hosts, suggesting that the enzymes play a role as pathogenic effectors (Misas-Villamil and van der Hoorn, 2008). Low levels of β-1,4-endoglucanase, β-glucosidase, xylanase, and pectinase activity had been detected in the spore suspension of *M. roridum* (Piyaboon *et al.*, 2016). Whether or not the enzymes are sufficient to cause the leaf blight symptoms had not been demonstrated.

In this study, the nature of the pathogenic effectors in the spore suspensions of *Paramyrothecium eichhorniae* was investigated by heat treatment and diafiltration, followed by observing the pathogenic activity on water hyacinth leaves and measuring the cell wall degrading enzyme activities of the treated spore suspensions.

Materials and methods

Fungal preparation

The spore suspension of *Paramyrothecium eichhorniae* was prepared by following the protocol used by Piyaboon *et al.* (2016). The fungus was cultured on potato dextrose agar (PDA) plates at room temperature (27–30 ºC) for 30 days with a 12 h photoperiod and subsequently cultured into boiled paddy rice, then incubated at room temperature (27–30 ºC) for 21 days with a 12 h photoperiod. Afterward, the spore suspension was prepared by suspending the spores on the

boiled paddy rice in sterile distilled water. The solid media was filtered out with cheese cloth. The spore suspension was collected, and the spore count of the suspension was adjusted to $1x10^8$ spores per ml. The suspension was stored at 4 ºC until used.

Determination of the enzymatic activity

The supernatants of the spore suspensions were prepared by the following procedure. Fifty milliliter of each spore suspension sample was centrifuged at 200 RCF, 4 ºC for 5 min in 50 ml plastic conical tubes. The supernatant from each sample was collected and transferred into new microcentrifuge tubes, then centrifuged again in a refrigerated micro-centrifuge at 12,000 RPM for 10 min at 4 ºC. Afterward, the supernatants of the samples were transferred into new containers and kept at -20 ºC until used. For every enzymatic activity measurement, the amount of product released in the reaction between the clarified supernatant and the corresponding substrate was subtracted by the amount of product released in the blank substrate reaction, *i.e.* containing no supernatant, and by the amount of product released in the blank supernatant reaction, *i.e.* containing supernatant but not the substrate. The net amount of product release was subsequently used to calculate the rate of reaction. One unit per ml of enzyme releases 1 µM of sugar per min.

β-1,4-Endoglucanase activity

The level of β-1,4-endoglucanase activity was determined using the method described by Piyaboon *et al.* (2016), using carboxymethylcellulose (CMC) as the substrate. The released reducing sugar was measured by the reaction with dinitrosalicylic acid (DNS). One milliliter of the substrate solution containing 1% CMC in 50 mM sodium acetate (pH 5.0) was mixed with 1 ml of the clarified fungal supernatant. The reaction was incubated in a 50 ºC shaker for 30 min, then terminated by adding 1 ml of DNS. The mixture was then heated in a boiling water bath for 5 min. The absorbance was read at 540 nm by using a spectrophotometer. The standard curve for the amount of reducing sugar was determined by using glucose as the standard.

β-Glucosidase activity

The level of β-glucosidase activity was determined using the method described by Jaijoi *et al.* (2022), using p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate. Half a milliliter of the substrate solution containing 5.0 mM pNPG in 50 mM sodium acetate (pH 5.0) was mixed with 0.3 ml of the clarified fungal supernatant. The reaction was incubated in a 50 ºC shaker for 30

min, then terminated by adding 50 µl of saturated sodium tetraborate ($Na_2B_4O_7$). The absorbance was read at 410 nm by using a spectrophotometer. One unit of enzyme was defined by the amount of enzyme that releases 1.0 µmol p-nitrophenol·ml^{-1}·min^{-1}, using the molar extinction coefficient of pNPG: ε 410 = 200 l·mol^{-1}·cm^{-1}.

Xylanase activity

The level of xylanase activity was determined using the method described by Piyaboon *et al.* (2016), using birch wood xylan as the substrate. One milliliter of 1% birch wood xylan in 50 mM sodium acetate (pH 5.0) was mixed with 1 ml of the clarified fungal supernatant. The reaction was incubated in a 50 ºC shaker for 30 min, then terminated by adding 1 ml of DNS. The mixture was then heated in a boiling water bath for 5 min. The absorbance was read at 540 nm by using a spectrophotometer. The standard curve for the amount of reducing sugar was determined by using xylose as the standard.

Pectinase activity

The level of pectinase activity was determined using the method described by Piyaboon *et al.* (2016), using citrus pectin as the substrate. One milliliter of 1% citrus pectin in 50 mM sodium acetate (pH 5.0) was mixed with 1 ml of the clarified fungal supernatant. The reaction was incubated in a 50 ºC shaker for 30 min, then terminated by adding 1 ml of DNS. The mixture was then heated in a boiling water bath for 5 min. The absorbance was read at 540 nm by using a spectrophotometer. The standard curve for the amount of reducing sugar was determined by using galacturonic acid as the standard.

Protein content determination

The protein concentration was measured using the Bradford reagent, which contains 0.01% Coomassie Brilliant Blue (G-250), 4.7% ethanol, and 8.5% phosphoric acid (Bradford, 1976). Chicken egg white albumin (Sigma-Aldrich) was used to construct the standard curve. Four hundred microliters of the clarified supernatant were added to 1.6 ml of the Bradford reagent. The absorbent at 595 nm was read using a spectrophotometer within 2 to 3 min of mixing.

Plant inoculation

The experiment was conducted using a randomized complete block design (RCBD) by 14 to 24 healthy leaves per treatment. Healthy water hyacinth

plants with leaf size 25-50 cm^2 were prepared for inoculation. Each of the spore suspension was inoculated on the entire surface of a leaf using a cotton tip applicator. The disease symptoms were recorded at 14 days after inoculation. The level of disease severity was rated by visual examination as the following: 0 = no sign of disease observed; 1 = some leaf spotting or slight chlorosis, but without necrosis, the affected area was less than 30% of the leaf; 2 = prominent discoloration or chlorosis, but without necrosis, affecting 30%-50% of the leaf; 3 = prominent discoloration with necrosis affecting 30%-50% of the leaf; 4= prominent discoloration with necrosis in more than 50% of the leaf. For each experiment, a group of leaves was applied with deionized water to observe possible contaminations.

Heat treatment of spore suspension

Fifty milliliter of the spore suspension was centrifuged at 200 RCF, 4 ºC for 5 min in 50 ml plastic conical tubes. The supernatants were decanted into new conical tubes, and the spore pellets were stored at 4 ºC for the duration of the treatment. For the 85 ºC treatment, the 50 ml plastic conical tubes containing the supernatant were submerged in a 85 ºC water bath for 30 min. Afterward, the tubes were cooled in an iced water bath for 15 min. Subsequently, the treated supernatants were added back to the collected spore pellets. For the 95 ºC treatment, the collected supernatants were added into 250 ml glass bottles and heated in a pot of boiling water for 30 min. The temperature of the liquid in the bottles was at 95 ºC. Afterward, the bottles were cooled in an iced water bath until the temperature reached 10 ºC. Subsequently, the treated supernatants were added back to the collected spore pellets. For the autoclave treatment, the collected supernatants were added into 250 ml glass bottles, then autoclaved with a 15 min liquid cycle. Afterward, the bottles were cooled in an iced water bath until the temperature reached 10 ºC. Subsequently, the treated supernatants were added back to the collected spore pellets. For the positive control, the untreated supernatant was added back to the collected spore pellet until the volume reached 50 ml. For the negative control, deionized water was added back to the collected spore pellet until the volume reached 50 ml.

Variable volume diafiltration of spore suspension

Diafiltration of the supernatant fractions were performed using the Millipore Labscale TFF System, which is a tangential flow filtration system consists of a 500 ml acrylic reservoir with a magnetic stirrer, a diaphragm pump, two pressure gauges, and a retentate valve. The flow paths were completed using

silicone tubing. The retentate was configured to flow back into the reservoir. The separation was performed with a Pellicon® XL50 cassette with Biomax® polyethersulfone (PES) membrane, which has the molecular weight cutoff at 5 kDa and has 50 cm^2 of membrane area (PXB005A50). The membrane was equilibrated with distilled water. For each sample, 100 ml of the spore suspension was centrifuged at 200 RCF, 4 ºC for 5 min in two 50 ml plastic conical tubes. Subsequently, 80 ml of the supernatant was collected and added to the reservoir, and the spore pellets were stored at 4 ºC for the duration of the experiment. The diafiltration process was performed in batches at 25 ºC, starting by adding one diavolume (80 ml) of deionized water to the supernatant in the reservoir and mixing with the magnetic stirrer. Afterward, the pump was turned on and the feed flow rate was adjusted to approximately 32 ml/min, the average transmembrane pressure was adjusted to approximately 36 psi. The flow rate and the transmembrane pressure were kept constant until 80 ml of the permeate was collected. This first permeate fraction (permeate 1) was stored for further experiments. Next, one diavolume (80 ml) of deionized water was added to the reservoir, and the process was repeated three more times, until a total of 4 diavolumes were added. Finally, the retentate fraction was collected from the reservoir once the volume of the reservoir was reduced to 80 ml. The entire process took about 2 hours for each sample. The membrane was cleaned with 0.5M NaOH according to the manufacturer protocol after each sample. Four different spore suspension samples were diafiltrated. For the plant inoculation experiments, each of the treated supernatant fractions was added back to the corresponding spore pellet until the volume reached 50 ml. For the positive control, the untreated supernatant was added back to the collected spore pellet until the volume reached 50 ml. The resulting spore suspensions were stored at 4 ºC. Three sets of diafiltrated samples were used to inoculate plants. For the enzyme activity assays, each of the treated supernatant fractions was further clarified by centrifugation in a refrigerated micro-centrifuge at 12000 RPM for 10 min at 4 ºC. The supernatants were collected and stored at -20 ºC for further analysis. Four sets of diafiltrated samples were used in the enzymatic assays.

Statistical analysis

For disease severity, a Kruskal-Wallis test was performed for each set of the plant inoculation experiment at $\alpha = 0.05$. Once a significant difference was detected, pairwise two-tailed Mann-Whitney U tests were performed, using the Bonferroni corrected threshold to produce the overall $\alpha = 0.05$. For the protein concentrations of the diafiltrated samples, one-way ANOVA was performed, using Tukey's HSD as the post-hoc test at $\alpha = 0.05$. For comparing the enzyme

activity between the supernatant samples heated to 85 ºC and the control samples, paired T-test was used, at α = 0.05. The raw data of the levels of enzyme activity between the diafiltrated samples and the control samples were not normal (Shapiro-Wilk test p value = 0.02). Consequently, they were transformed with log(x+1), which produced a normally distributed data set (Shapiro-Wilk test p value = 0.07). Afterward, the transformed data set was tested with one-way ANOVA, using Tukey's HSD as the post-hoc test at α = 0.05. The statistical tests were performed using SPSS for Windows statistical software, v. 16.0, Chicago, IL, USA.

Results

Effects of heat treatment on the pathogenicity and on the levels of enzymatic activities

The effects of heat treatment on the pathogenicity of the spore suspension and on the levels of selected cell wall degrading enzyme activities were investigated. The reconstituted spore suspension, made with the 85 ºC heat-treated supernatant and untreated spores, caused leaf blight symptoms at a similar level to the untreated control (Figure 1). The levels of leaf blight symptoms in both groups did not significantly differ from one another. The negative control, made with deionized water and untreated spores, produced a significantly different level of disease severity from the other groups (Figure 2).

Figure 1. Leaf blight symptoms on water hyacinth leaves inoculated with the 85 ºC heat-treated spore suspension (A), the positive control (B), and the negative control (C). The positive control was the untreated spore suspension. The negative control was the spore suspension that had the supernatant replaced with deionized water. The photos were taken at 14 days post inoculation. Bar = 1 cm

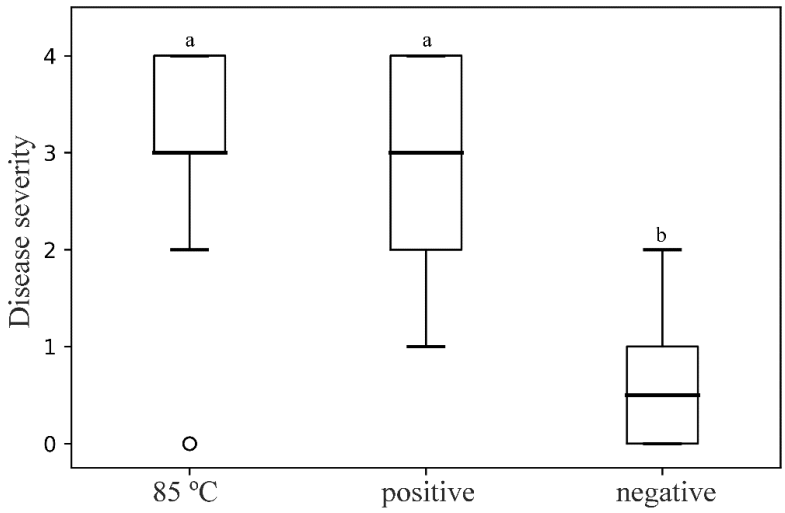

Figure 2. Disease severity levels caused by the 85 ºC heat-treated spore suspension. The positive control was the untreated spore suspension: The negative control was the spore suspension that had the supernatant replaced with deionized water. Samples with the same letter are not significantly different by Kruskal-Wallis test followed by pairwise Mann-Whitney U test at overall $\alpha = 0.05$, n = 24 per group

The levels of enzyme activities from the 85 ºC heat-treated supernatant samples and from the untreated control samples were measured. The control samples had a detectable xylanase and pectinase activity of 0.22 ± 0.09 U/ml and 0.44 ± 0.09 U/ml respectively, but had no detectable β-glucosidase and endo-glucanase activity (Table 1). Notably, the heat-treated samples did not have a significantly different level of enzymatic activity compared to the control samples. Paired t-tests between the heat-treated samples and the control samples yielded p-values of 0.45 and 0.65 for the xylanase and pectinase activity respectively (n=3).

The pathogenicity of the spore suspension reconstituted from the 95 ºC heat-treated supernatant and the autoclaved supernatant was investigated (Figure 3). The 95 ºC heat-treated samples produced a wide range of disease severity level with a slightly reduced median level from that of the positive control, but the difference was not statistically significant from either the positive control or the negative control group (Figure 3A). However, the autoclaved samples produced a reduced level of disease severity, which was statistically significant from the positive control group (Figure 3B and Figure 4).

Table 1. Enzyme activity levels of the 85 ºC treated fungal supernatant

Sample	Enzyme Activity (U/ml)[1/]			
	Xylanase	β-glucosidase	Endo-glucanase	Pectinase
Control	0.22 ± 0.09[a]	ND[2/]	ND	0.44 ± 0.09[a]
85 ºC	0.11 ± 0.18[a]	ND	ND	0.41 ± 0.18[a]

[1]/Samples with the same letter in the same column are not significantly different by paired t-test, at the significance level 0.05, n= 3 per group. Samples without the letter were not tested. [2]/ND = Not detected

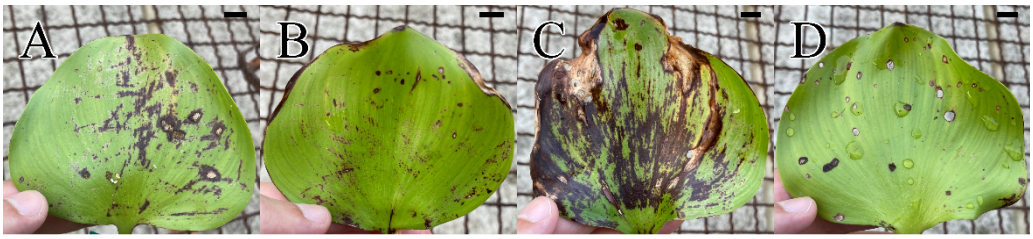

Figure 3. Leaf blight symptoms on water hyacinth leaves inoculated with the 95 ºC heat-treated spore suspension (A), the autoclaved spore suspension (B), the positive control (C), and the negative control (D). The positive control was the untreated spore suspension. The negative control was the spore suspension that had the supernatant replaced with deionized water. The photos were taken at 14 days post inoculation. Bar = 1 cm

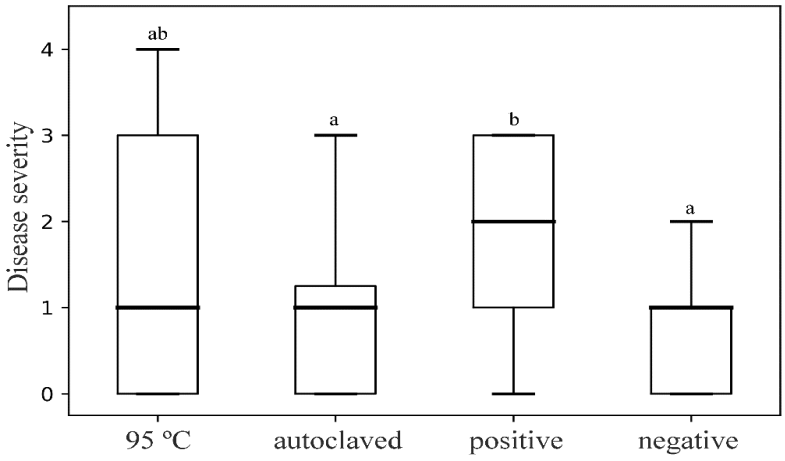

Figure 4. Disease severity levels caused by the 95 ºC heat-treated spore suspension and the autoclaved spore suspension: The positive control was the untreated spore suspension. The negative control was the spore suspension that had the supernatant replaced with deionized water. Samples with the same letter are not significantly different by Kruskal-Wallis test followed by pairwise Mann-Whitney U test at overall $\alpha = 0.05$, n = 14 per group

Effects of membrane separation on the pathogenicity and on the levels of enzymatic activities

The effects of diafiltration with a 5 kDa membrane on the pathogenicity of the spore suspension and on selected cell wall degrading enzyme activities had been investigated. In the diafiltration process, molecules in the diafiltrated fraction have molecular sizes larger than the size of the membrane pores and are retained by the membrane, while molecules in the permeate fraction have passed through the membrane. The untreated supernatant, the diafiltrated fraction, and the first permeate fraction of the process (the permeate 1 fraction) were tested on water hyacinth leaves to determine the levels of pathogenicity. The permeate 1 fraction produced a slightly lower median level of disease severity (Figure 5C), but the difference was not significantly different from the untreated supernatant (Figure 5A), while the diafiltrated sample caused a significantly reduced level of disease severity (Figure 5B and Figure 6).

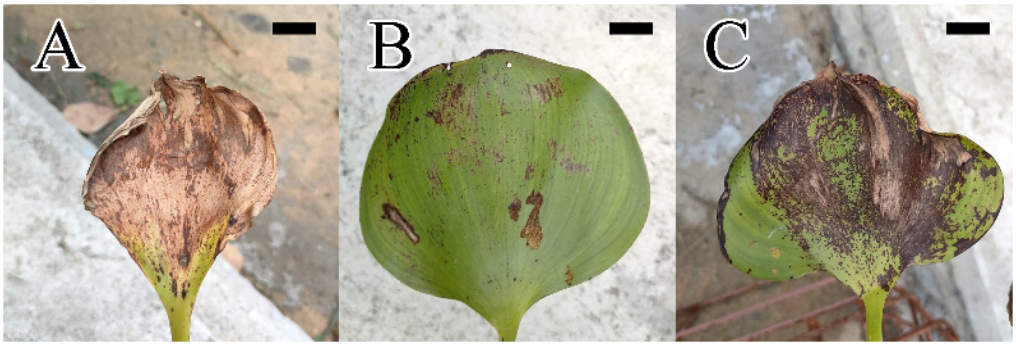

Figure 5. Leaf blight symptoms on water hyacinth leaves inoculated with the positive control (A), the diafiltrated spore suspension sample (B), and the permeate 1 spore suspension sample (C). The photos were taken at 14 days post inoculation. Bar = 1 cm

The protein content of the permeate 1 fraction, the diafiltrated fraction, and the untreated supernatant control was measured. The permeate 1 fraction had an undetectable level of protein, which was significantly different from the other two groups. The protein levels of the control and the diafiltrated fractions were not significantly different from one another (Table 2).

The level of xylanase activity from each of the membrane-separated samples was measured. The permeate 1 fraction had a reduced level of xylanase

activity compared to the untreated supernatant. The difference is statistically significant at α = 0.05. The xylanase activity of the diafiltrated fraction was not significantly different from that of either the untreated supernatant or the permeate 1 fraction (Table 3).

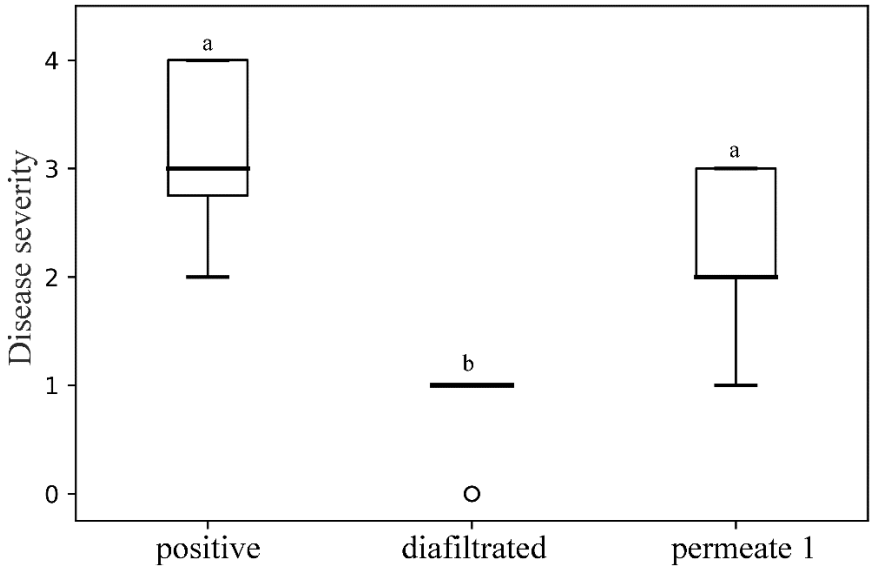

Figure 6. Disease severity levels caused by membrane separated spore suspension samples: Positive = the positive control was the untreated supernatant, diafiltrated = diafiltrated sample, permeate 1 = the first permeate fraction of the process. Samples with the same letter are not significantly different by Kruskal-Wallis test followed by pairwise Mann-Whitney U test at overall α = 0.05, n of fungal samples = 3, n of water hyacinth = 12,12,15

Table 2. Protein concentration of membrane separated samples (Bradford method)

Sample	**Protein concentration (mg/ml) [1/]**
Supernatant	0.12 ± 0.05^{a}
Diafiltrated	0.08 ± 0.02^{a}
Permeate 1	0.00 ± 0.00^{b}

[1]/Samples with the same letter in the same column are not significantly different by ANOVA followed by Tukey HSD, at the overall significance level 0.05, n= 4 per group.

Table 3. Xylanase activity levels of membrane-separated samples

Sample	Xylanase Activity (U/ml)[1/]
Supernatant	1.20 ± 0.61^{a}
Diafiltrated	0.74 ± 0.89^{ab}
Permeate 1	0.02 ± 0.03^{b}

[1]/Samples with the same letter in the same column are not significantly different by ANOVA followed by Tukey HSD, at the overall significance level 0.05, n= 4 per group. The test was performed on data transformed with log(x+1).

Discussion

Plant inoculation and enzymatic activity

Leaf blight symptoms similar to previous studies were observed from water hyacinth that had been applied with the spore suspension of *Paramyrothecium eichhorniae.* The median disease severity in most experiments were level 2 to 3, in which 30–50% of the area of the leaves was affected. This is similar to the report from Piyaboon *et al.* (2016), that on average, 30–75% of hyacinth leaf area was affected by the spore suspension.

The levels of xylanase, cellulase (β-glucosidase, endo-glucanase), and pectinase activity in the clarified spore suspension were similar to the previous reports (Okunowo *et al.*, 2010; Piyaboon *et al.*, 2016). When comparing the amount of xylanase and pectinase to other cell wall-degrading fungi, the levels of activities found in this study are in the lower end of the range. For example, the levels of xylanase and pectinase produced by other groups of fungi such as *Aspergillus*, *Penicillium,* or *Trichoderma* were in the range of 0–24 unit/ml and 2.3–23 unit/ml respectively (Kvesitadze *et al.*, 1999; Kutateladze *et al.*, 2009). In this study, *P. eichhorniae* spore suspension had 0.22–1.20 unit/ml of xylanase activity and 0.44 unit/ml of pectinase activity. The level of β-glucosidase and endo-glucanase activity was below detection in this study. Similarly, Piyaboon *et al.* (2016) reported that the levels of activity of these enzymes were less than 0.2 unit/ml, which is also close to the detection limit in this study. The difference between the enzymatic activity of the control groups reported in Table 1 and Table 3 likely reflects the variability between different production batches of the spore suspension. Nonetheless, every batch of spore suspension was tested for their virulence on water hyacinth and was confirmed to be effective at producing the leaf blight symptoms.

Heat treatment experiments

The effects of heat treatment of the spore suspension on the pathogenicity in water hyacinth and on the levels of cell wall degrading enzyme activities were investigated to observe any association between the two factors. After the heat treatment at 85 ºC, no significant difference in both the levels of pathogenicity and the levels of enzymatic activities of the spore suspension was detected compared to the untreated control. Xylanase, pectinase, as well as other pathogenic factors, appeared to be moderately heat resistant; however, the autoclaved supernatant lost its potency. The pathogenic activity of the spore suspension made with the live spores suspended in the autoclaved supernatant was not significantly different from the suspension made with live spores and distilled water. While some trichothecenes were reported to resist high heat treatments, (He *et al.*, 2010; Sobrova *et al.*, 2010), the fungal effectors in *P. eichhorniae* were deactivated after being autoclaved. These results cannot conclusively determine whether the pathogenic effectors were enzymes or other non-enzyme toxins such as roridin A or trichothecenes.

Diafiltration experiment

In the diafiltration experiment, small molecules were separated from cell wall degrading enzymes based on their molecular sizes. From previous reports, the molecular weights of xylanases and pectinases from various sources are 13–95 kDa and 36–63 kDa respectively (Sunna and Antranikian, 1997; Samanta, 2019). In contrast, the trichothecene mycotoxins found in *M. roridum* fungal extract were about 0.5 kDa in size (Lakornwong *et al.*, 2019). The 5 kDa membrane was chosen for this study in order to retain most of the enzymatic activities while letting small molecular mycotoxins pass through the membrane. In this experiment, the permeate 1 fraction had about half the concentration of the small molecules from the spore suspension supernatant. When mixed with the spores, this fraction still caused leaf blight damage, indicating that the disease-causing activity permeated through the membrane. In the diafiltrated fraction, the concentration of small molecules capable of passing though the membrane was about 6.3% of the original concentration. When mixed with the spores, the pathogenicity of this fraction was significantly different from the untreated spore suspension. The effects of the spore suspension made with this diafiltrated fraction on water hyacinth leaves were similar to that of the spore suspension made with deionized water mixed with spores (data not shown). The

permeate 1 fraction had an undetectable level of protein and had a significantly different level of the xylanase activity compared to the untreated supernatant. In contrast, the level of protein and the level enzyme activities of the diafiltrated fraction were not significantly different from those of the untreated supernatant. These results indicate that the majority of the protein did not pass through the membrane during the diafiltration process, and that the membrane was not leaked or damaged.

The main pathogenic effectors in *P. eichhorniae* could permeate through the 5 kDa membrane, were not measured as protein by the Bradford assay, and did not have the xylanase activity. The molecules were also moderately heat-resistant. Results suggested that main pathogenic effectors which contribute to the leaf blight symptoms might be mycotoxins or other small molecules that can permeate through a 5 kDa membrane. Nonetheless, it is possible that some small proteins could act as pathogenic effectors at a low concentration. In addition, it could not be ruled out that xylanase might contribute to the severity of the leaf blight disease when working in conjunction with small molecular effectors, though xylanase alone is insufficient to cause the symptoms. Future *in planta* experiments with purified and quantified fungal toxins could give more information about this process.

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