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Growth inhibition of *Hydrilla verticillata* by freshwater fungi

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Abstract. *Triadiati T, Sukarno N, Rahmah IS. 2021. Growth inhibition of* Hydrilla verticillata *by freshwater fungi. Biodiversitas 22: 2876-2882.* The uncontrolled growth of hydrilla (*Hydrilla verticillata* (L.f.) Royle) in Mekarsari Fruit Garden, Bogor causes various losses. A Freshwater fungus is one of the alternatives to control hydrilla growth. Therefore, the study aimed to investigate the damage and growth inhibition of hydrilla using freshwater fungi. Freshwater fungi were isolated from Lake Mekarsari Fruit Garden. Hydrilla growth characteristics observed were stem length, stem nodus number, number of healthy leaves, leaf number, leaf damage, wet and dry weight. The results showed that a total of seven isolates of freshwater fungi were obtained from Lake Mekarsari Fruit Garden. Two species, i.e. *Myrothecium* sp. and *Stachybotrys* sp. were selected to control hydrilla growth. Fungal treatment reduced the stem length and leaf number of hydrilla. The combination of both fungal isolates showed less leaf damage than *Myrothecium* sp. The damage of hydrilla leaves by *Myrothecium* sp. and *Stachybotrys* sp. were 98.07% and 78.71%, respectively.

Keywords: Biocontrol agent, *Hydrilla verticillata*, *Myrothecium*, *Stachybotrys*

INTRODUCTION

Hydrilla (*Hydrilla verticillata* (L.f.) Royle, Family: Hydrocharitaceae) is an invasive plant that grows in various types of freshwater and has spread in the world. Hydrilla is considered one of the worst aquatic weeds in the world. The problem with hydrilla in Indonesia, for example, in the lake in the Mekarsari Fruit Garden, West Java, Indonesia is its fast growth, causing the lake to become shallow. The fast growth of hydrilla in freshwaters can cause severe losses. Economic severe losses and ecological damage occur when hydrilla impedes navigation, clogs drainage and irrigation canals, interferes with recreational activities, and disrupts wildlife habitats (Baniszewski et al. 2016). This plant overgrows, tolerates very low light intensities, and produces specialized hibernating organs (turions). It can survive in unfavorable conditions for growth and outcompete other species. The lake at Mekarsari Fruit Garden, West Java, Indonesia, is used for recreation, water sports, and fishing. The lake becomes shallow due to the rapid and uncontrolled growth of hydrilla, thereby affecting lake activity and reducing fish populations.

Hydrilla is a submerged plant with a fast growth rate. It can survive in adverse environmental conditions such as the availability of nutrients and low light (Baniszewski et al. 2016). Also, calm lake water flow can support its growth (Dewiyanti 2012). Hydrilla distribution is not controlled only by abiotic factors (temperature, length of vegetation period, hydrochemical features of the water bodies), and biotic factors; there is a competition with the more aggressive neophyte *Elodea canadensis* (Efremov et al. 2018). The efforts have been made mechanically and chemically to reduce the hydrilla population in the lake. This is affecting the fish population in the lake.

Hydrilla is controlled mainly through the use of chemical herbicides or mechanical removal. The high cost of these control measures and concern for the environment has increased interest in biological control of this noxious weed. Diseases of submerged weeds are poorly known, and very few plant pathogens have been found on hydrilla. One of the biological agents of biocontrol is freshwater fungi. The use of naturally occurring fungi on aquatic plants in the USA to develop a mycoherbicide is an example of inundative biological control (Hussner et al. 2017). Fungi are the biocontrol agents that can be used as an alternative to chemical herbicides (Ray and Hill 2013). Mycoherbicide or fungal phytotoxins are secondary metabolites that play an important role in the induction of disease symptoms in agriculture and forest plants and weeds (Evidente et al. 2013; Cimmino et al. 2015; Vurro et al. 2018). The application of phytopathogenic fungi positively impacts controlling aquatic plant *Eichhornia crassipes*, with a maximum deterioration of 88–94% (Moreira et al. 2018). Previous studies have successfully controlled the growth of hydrilla by using the fungi *Fusarium culmorum*, *Mycoleptodiscus terrestris* (Shearer et al. 2007), and *Macrophomina phaseolina* (Zilli et al. 2018). The fungus *M. terrestris* is an endemic fungal pathogen and a potential biological control agent for hydrilla as observed in laboratory, greenhouse, and field trials. *Mycoleptodiscus terrestris* can cause chlorosis, necrosis, and the decay of the hydrilla (Shearer et al. 2007). Freshwater fungus belonging to the genus Diaporthe have bioherbicides activity for rice weed (Souza et al. 2017).

The study on the growth control of hydrilla living in West Java lakes, Indonesia using local freshwater fungi has not been conducted. Therefore, research is needed to obtain freshwater fungi from the lake where hydrilla develops in abundance and observed their effect on the growth of

hydrilla. This study aims to analyze the damage and growth inhibition of hydrilla in the lake using freshwater fungi.

MATERIALS AND METHODS

Water sampling strategy

Water and hydrilla were sampled every two months for one year from 10 sampling points in the lake (lake area of 25 ha) at the Mekasari Fruit Garden. Water samples were collected in submerged sterile bottles ±20 cm below the water surface. The bottle cap was opened and filled with water until all the water was filled, then the bottle was closed.

Isolation and identification of the freshwater fungi

The freshwater fungi were isolated on Potato Dextrose Agar (PDA) with 0.5 g chloramphenicol as antibiotic and 0.03 g rose bengal in 1 L solution. The suspension of freshwater samples was spread on media in a Petri dish and incubated at room temperature for 30 days. The isolated fungus was further purified by transferring them to PDA medium.

Spore suspension of freshwater fungi for inoculum

Spores of freshwater fungi were used as test fungi to test their potential as biocontrol agents through screening. Freshwater fungi were grown on PDA media for 14 days at room temperature. Spores were harvested from the surface of the media by inserting 10 mL of sterile aquadest into Petri dish. Spores were collected by rubbing the tip of sterile object-glass slide on the surface of the media to knock out the spores. Spore count was measured using a hemocytometer. The spore concentration used was $1x10^7 mL^{-1}$.

Screening of freshwater fungi against hydrilla in a test tube

Freshwater fungi were investigated to control hydrilla growth using a modification of Shabana et al. (2003) method. Seven centimeters from the top of hydrilla shoot (from Mekarsari Fruit Garden Lake) was cleaned with tap water and rinsed several times using sterile aquadest. Hydrilla was kept in a sterile test tube containing sterilized freshwater from Lake Mekarsari Fruit Garden. The test tubes were closed using plastic and sterile cotton. Test tubes were placed in a shaker at 10 rpm under a lamp (12 hours on, 12 hours off), at ± 25°C for one week to acclimatize. After acclimatization, one mL of spore suspension was added to each test tubes. The treatment was carried out for four weeks and then the level of damage to hydrilla leaves was observed four weeks after treatment (WAT).

Assessment of leaf damage level

Leaf damage was measured by observing and calculating the number of damaged (decay) leaves due to fungal treatments and the total number of hydrilla leaves in one plant. The level of leaf damage can be categorized on a scale of 0 to 4, namely 0: healthy, 1: 1-25% damaged, 2: 26-50% damaged, 3: 51-75% damaged, and 4: 76-100% damaged (100 % = dead) (Shabana et al. 2003). The damaged level of a leaf was calculated using the formula: Percentage of leaves damage (%) = (Number of damaged leaves/leaves total) x 100%.

Morphological identification of selected freshwater fungi

Freshwater fungi were identified using the slide culture method and morphological identification was confirmed by the key of Barnett and Hunter (1998).

Growth inhibition of hydrilla by freshwater fungi in the field

Growth inhibition of the hydrilla test in the field was carried out using a five-liter volume container equipped with an aerator. The lake water was used for this experiment. Each of the three stems of hydrilla measuring 7-12 cm from the Mekarsari Fruit Garden Lake was put into a container equipped with an aerator. Before being treated, hydrilla was acclimatized for one week. A total of 100 mL of spore suspension was added for each container. The spore concentration of freshwater fungi used was $1x10^7$ mL^{-1}. Freshwater fungi used for field testing were selected from screening tests that had damaged hydrilla at damage level 4 (76-100% damaged, 100% = dead).

The observation was carried out every week for five weeks. The experiment was performed in a Completely Randomized Design (CRD) with three treatments: control (sterile aquadest) and two selected freshwater fungi. For each treatment, there were five replications.

Growth characters of hydrilla in field test

The growth parameters observed included stem length, number of stem segments, number of healthy leaves, fresh plant weight, and plant dry weight. Stem length was measured from the base of the main stem to the end of the main stem. The number of plant stem segments was calculated from the bottom of the main stem to the end of the main stem. The number of leaves counted was healthy leaves in each plant sample. The fresh weight of plants was observed before treatment (week 0) and four weeks after treatment (WAT). Dry weight was observed at four WAT. The hydrilla from each treatment was weighed using an 80°C oven to obtain the dry weight. In addition, the level of leaf damage was observed at four WAT.

Leaf damage of hydrilla

Hydrilla leaves were observed every week after the treatment of selected freshwater fungal spores. Hydrilla leaf was placed on the object-glass slide with water, then cover using a cover glass. Observations were made using a microscope of 400x magnification.

Data analysis

Data were analyzed by analysis of variance (ANOVA) at a 95% confidence level using SPSS software version 25.0. Further testing was carried out using the Duncan Multiple Range Test (DMRT) at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Isolates of freshwater fungi

A total of seven freshwater fungal isolates 5MII, 5M, BKII-1, BKIII-2, BKI-2, 5MIII, and BKI-1 were isolated from the Lake of Mekarsari Fruit Garden. Inhibition of hydrilla growth by seven freshwater fungal isolates is presented in Figure 1 (A-H). The 5MIII and BKI-1 isolates cause decay in the leaves and stems of the hydrilla (Figure 1.G-H). The decay occurs in young leaves, then in the older leaves, and continued until the stem. The other freshwater fungi treatments (Figure 1.A-F) did not show severe damage during four weeks of treatment.

Damage in hydrilla leaves due to the treatment of freshwater fungi (tube test)

Damage in hydrilla leaves at 2 WAT by freshwater fungi can be seen in Figure 2 (A-C). The treatment of freshwater fungi caused damage in leaves. The leaf of hydrilla treated by 5MIII isolate showed that leaves forming tissue was not intact (Figure 2.B). Leaves were damaged in the BKI-1 isolate treatment, and many fungal spores were observed on the surface of the leaves (Figure 2.C). The 5MIII isolate caused severe damage in hydrilla leaves compared to the BKI-1 isolate. The percentage and level of hydrilla leaf damage due to freshwater fungal isolates are presented in Table 1. Two of the seven isolates, namely 5MIII and BKI-1 isolates, showed 100% damage in leaves, and eventually, the plant died at four WAT (Table 1). The damage of hydrilla leaves by *Myrothecium* sp. and *Stachybotrys* sp. were 98.07% and 78.71%, respectively at four WAT. The other treatments showed damage level 1 and the percentage damage of leaves ranged between 0.89-6.37 %.

Table 1. Damage percentage of hydrilla leaves by the treatment of seven freshwater fungi isolates at 4 WAT

Freshwater fungal isolates	Damage percentage of leaves (%)	Damage level (scale)
Control	0.89[a]	1
5MII	2.45[a]	1
5M	3.82[a]	1
BKII-1	1.92[a]	1
BKIII-2	4.30[a]	1
BKI-2	6.37[a]	1
5MIII	100[b]	4
BKI-1	100[b]	4

Note: Data followed by the same letter in the same column shows no significant difference (Duncan test, $p<0.05$); Damage level in scale 0-4, that is 0: healthy, 1: 1-25% damaged, 2: 26-50% damaged, 3: 51-75% damaged, and 4: 76-100% damaged (100% = die) (Shabana et al. 2003).

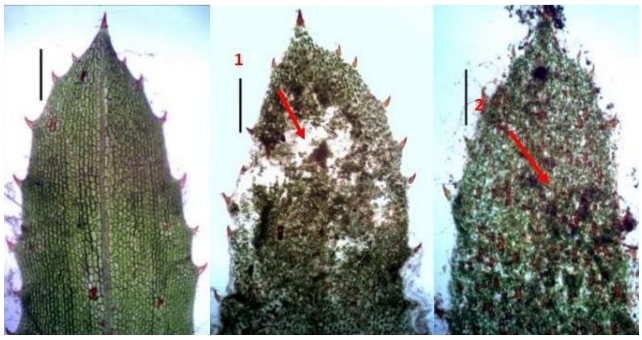

Figure 2. Hydrilla leaves in the first to third position from the tip at two WAT. (A) Control, (B) 5MIII, (C) BKI-1 isolates. Note: 1: damage leaf, 2: freshwater fungi spores' black dots). Scale bar = 500 µm.

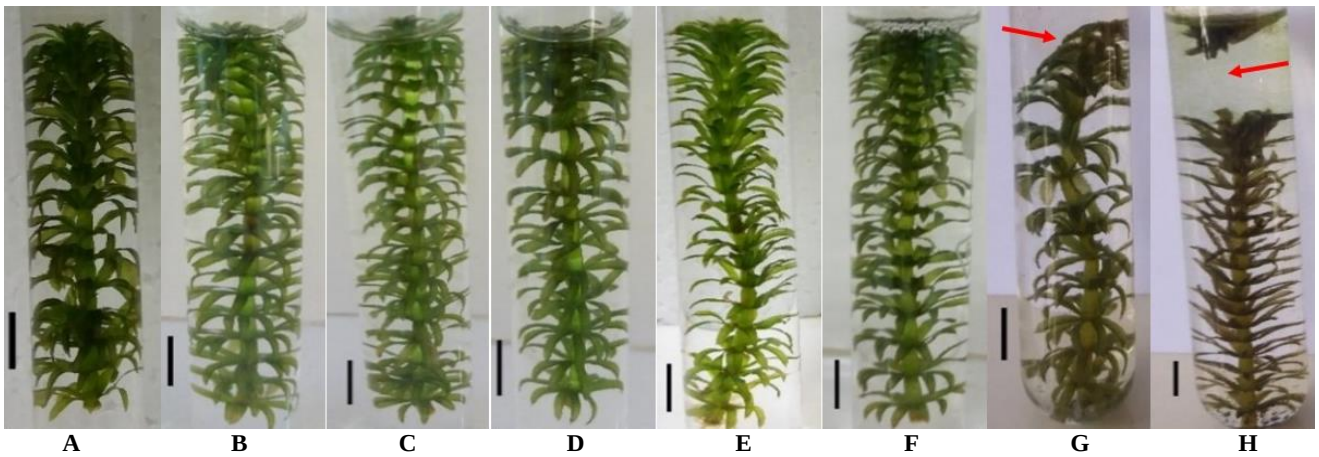

Figure 1. Decay in hydrilla leaves and stems (red arrow) at four WAT by seven isolates of freshwater fungi. A. Control, B. 5MII, C. 5M, D. BKII-1, E. BKIII-2, F. BKI-2, G. 5MIII, and H. BKI-1 isolates. Scale bar = 1 cm

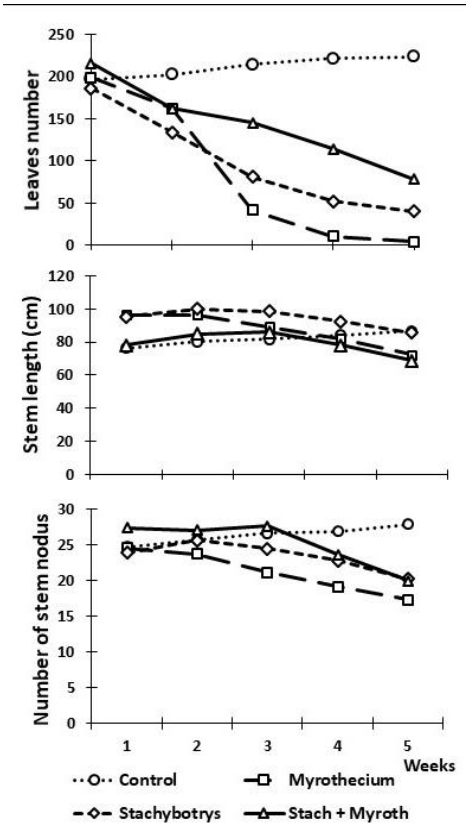

Figure 3. Leaves number, stem length, and number of stem nodus of hydrilla under treatment of *Stachybotrys* sp. and *Myrothecium* sp.

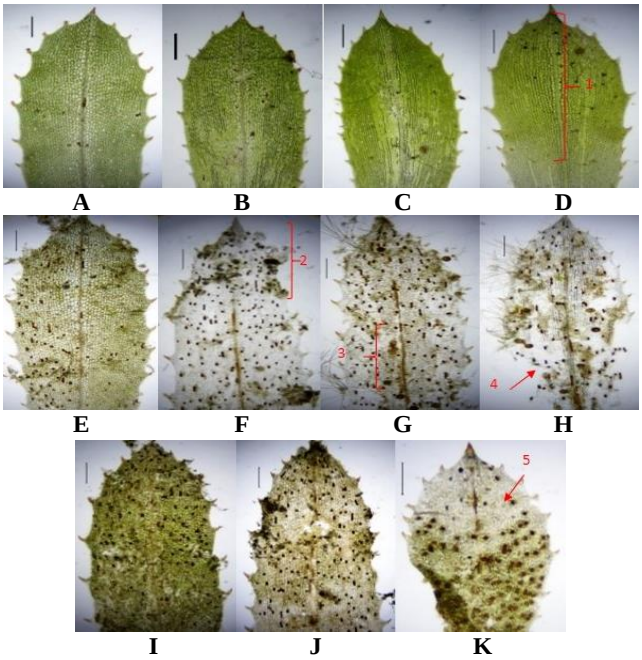

Figure 4. Morphology of hydrilla leaves in the first to third position from the tip at four WAT (magnification 400x). A-D. Control, E-H. *Myrothecium* sp., I-K. *Stachybotrys* sp. Note: 1: healthy leaf, 2: damage in the leaf tips, 3: damage to the middle leaf, 4: damage to the base of the leaf, 5: Reduction in chlorophyll content (chlorosis). Scale bar = 1 mm.

Table 2. Leaves number and damage percentage of hydrilla leaves by *Myrothecium* sp. and *Stachybotrys* sp. at four WAT

Treatments	Leaves number		Damage leaves (%)
	Healthy leaves	Damage leaves	
Control	224[a]	8[c]	3.83[c]
Myrothecium sp.	4[c]	195[a]	98.07[a]
Stachybotrys sp.	40[b]	146[b]	78.71[b]
Stachybotrys sp and *Myrothecium* sp.	86[ab]	130[b]	60.46[b]

Data followed by the same letter in the same column showed no significant difference (Duncan's Test, p <0.05)

Table 3. Fresh and dry weight of hydrilla by *Myrothecium* sp. and *Stachybotrys* sp. at four WAT

Treatments	Fresh weight (g)			Dry weight at 4 WAT (g)
	0 WAT	4 WAT	Difference	
Control	0.83[a]	0.92[a]	0.09[b]	0.14[a]
Myrothecium sp.	0.91[a]	0.41[b]	-0.50[a]	0.08[b]
Stachybotrys sp.	0.80[a]	0.58[b]	-0.22[ab]	0.07[b]

Note: Data followed by the same letter in the same column showed no significant difference (Duncan's Test, p <0.05)

Identification of freshwater fungi

The two freshwater fungal isolates, 5MIII and BKI-1 were able to inhibit the growth of hydrilla at the damage level of scale 4. These fungal isolates were *Myrothecium* sp. (5MIII isolate) and *Stachybotrys* sp. (BKI-1 isolate).

Myrothecium sp. On PDA fungal colony was white with black spots on the top and soft; reverse colony uniform; colony growth was slow and flat (non-aerial growth). Hyphae septate, hyaline to greenish-white; conidiophore hyaline to greenish-white, soft, branching, and spore produced at the terminal. Spores were oval, hyaline to light green, one cell, and 5.02-8.2 x 2.66-4.17 µm.

Stachybotrys sp. The fungal colony was black, soft, reverse colony was irregular; colony growth slow and non-aerial. Hyphae septate, conidiophore single; phialide short, smooth, hyaline to light brown. Conidia was oval, light to dark brown, and 7.59 -10.85 x 2.89-4.68 µm.

Growth characteristics of hydrilla in greenhouse

Inoculation of selected freshwater fungi reduced the number of hydrilla healthy leaves (Figure 3A). The number of healthy leaves has decreased at one WAT. A significant ($p < 0.05$) reduction in the number of hydrilla leaves was observed with the treatment of *Myrothecium* sp. (Table 2).

Inoculation of selected freshwater fungal isolates caused a reduction in stem growth (Figure 3.B). The stem of the hydrilla exhibited rot in the apical part which reduced stem length. Hydrilla stem growth was inhibited by the treatment of *Myrothecium* sp. than by *Stachybotrys* sp.

The number of hydrilla stem nodus was found to decrease in the treatment of freshwater fungal isolates (Figure 3C). *Myrothecium* sp. decreased in the number of stem nodus at 1 WAT, whereas *Stachybotrys* sp. reduced

the number of stem nodus on two WAT. The combined treatment of two freshwater fungal isolates had the same effect as the treatment of *Stachybotrys* sp. Treatment of *Myrothecium* sp. resulted in the most significant reduction in the number of stem nodus segments at four WAT among the other treatments.

Fresh and dry weight of hydrilla

The fresh weight and dry weight of hydrilla are presented in Table 3. Treatment of freshwater fungal isolates affected ($p < 0.05$) the fresh weight of hydrilla at four WAT. A dry weight of hydrilla was influenced by freshwater fungal isolates at four WAT. The dry weight of control was significant ($p < 0.05$) higher than the freshwater fungal isolates treatments.

Discussion

A total of seven fungal isolates, namely 5MII, 5M, BKII, BKIII-2, BKI-2, 5MIII, and BKI-1, were isolated from the water lake of Taman Buah Makersari. The isolated water fungi were candidates as biocontrol agents for hydrilla growth in the screening test. Screening results showed that two fungal isolates namely, 5MIII and BKI-1, were able to inhibit the growth of hydrilla at four WAT. Leaf damage first appeared on young leaves after several days of inoculation. Young leaves are more susceptible to infection because they are still in the development stage, so the tissues that make up the leaf organs are not fully formed (Hoagland et al. 2012).

A field experiment showed that inoculating fungus rapidly inhibited the growth of hydrilla stem length at four WAT. The inhibition was indicated by the reduction in stem length due to decay. The hydrilla stem segment was reduced to four WAT. If the stem segment is damaged, it will affect the formation of leaves. Four to seven days after inoculation with the freshwater fungus, symptoms of the disease appear, cause chlorosis on the leaves of the hydrilla. Leaf tissue damage due to water fungal infection is caused by enzymatic reactions that damage leaf tissue, allowing pathogenic water fungi to enter into the lower epidermal cells (Shearer et al. 2011).

After the fungi enter, they will colonize the cells that make up the leaf, resulting in cell wall damage and cell death followed (Abdallah et al. 2018). In the field experiment, hydrilla leaves exhibited chlorosis from the top to the base of the leaf due to damage to the cells. Besides, more severe damage causes the leaf structure to be damaged and incomplete. Cimmino et al. (2015) stated that pathogenic fungi cause chlorosis in plants that are characterized by color changes and damage in plant cells. Hydrilla leaf damage in four WAT reached the worst condition with *Myrothecium* sp. and *Stachybotrys* sp. 98.07% and 78.71%, respectively. Okunowo et al. (2013) reported that *Myrothecium roridum* was capable of inducing disease symptoms (necrosis on leaves) on water hyacinth leaves three days post-inoculation. *Myrothecium* sp. also caused leaf spot symptoms in Begonia (Fujinawa et al. 2020).

The selected fungi i.e. *Myrothecium* sp. (5MIII isolate) and *Stachybotrys* sp. (BKI-1 isolate) were effective as a hydrilla biocontrol agent. *Myrothecium roridum* is a pathogenic fungus that can be used as a biocontrol of aquatic plants, *E. crassipes* (Okunowo et al. 2010; Piyaboon et al. 2016; Okunowo et al. 2019) and water lettuce (Okunowo et al. 2011). Kongjornrak et al. (2019) reported that *Myrothecium inundatum* could be used for controlling water lettuce. The other species of *Myrothecium*, *M. verrucaria*, also has phytotoxin implications for *Pueraria montana* var. lobata (Hoagland et al. 2012). *Stachybotrys* also produce plant pathogenic mycotoxins (Abdallah et al. 2018). Besides, Li et al. (2002) reported that *Stachybotrys chartarum* is a plant pathogenic fungus found in soybean.

The tissue structure of hydrilla leaves consists of two layers of cells, with the epidermal cells at the top more extensive than the bottom and each cell having a thin cuticle on the outer wall (Baniszewski et al. 2016). The simple type of hydrilla leaf organ allows the fungal infection process to occur more quickly. Fungal infection on hydrilla leaves causes leaf damage. The number of healthy leaves has decreased, starting to appear at one WAT. The decrease in the number of leaves increased to four WAT. The infection causes cells to lose their cytoplasm, chloroplasts swell and burst (Zilli et al. 2018). Shearer et al. (2007) reported that *M. terrestris* (Gerd.) damaged the hydrilla after fourteen days' post-inoculation. When the present study is compared to Shearer et al. (2007), it was found that selected fungal isolates inhibit the growth of hydrilla at a slow rate. It is suspected that there are differences in the growth-inhibiting compounds produced by these fungi. Nevertheless, the fungus has the potential as an inundative biological control agent for the management of hydrilla (Shearer et al. 2007).

Hydrilla leaves treated with freshwater fungal isolates in screening test showed structural damage in two WAT. The results indicated that *Myrothecium* sp. and *Stachybotrys* sp. play an essential role in inhibiting hydrilla growth. Treatment using a combination of both fungi has less effect than *Myrothechium* sp. treatment. It can be assumed that the two fungi have a mutually suppressive effect on hydrilla. If only *Myrothecium* sp. was treated, it can grow well on hydrilla leaves and act as a biocontrol agent.

The mechanism of fungal infection in hydrilla is believed to be the use of enzymes to degrade hydrilla cell walls. *Myrothecium gramineum* secretes cellulase enzymes that may play a role in the degradation of plant cell walls (Das et al. 2016). Saritha et al. (2015) also stated that *Myrothecium roridum* is capable of producing high amounts of lignocellulolytic enzymes to be used as environmentally friendly biocontrol applications. *Stachybotrys* sp. is also able to damage cell walls. *Stachybotrys atra* can form cellulase enzymes that play a role in the degradation of one of the cell wall compilers, namely cellulose (Picart et al. 2008). Furthermore, *Myrothecium* sp. and *Stachybotrys* sp. produce mycotoxin, which inhibits protein synthesis in plants (Chen et al. 2016).

The fresh hydrilla weight at weeks 0 and four WAT were not significantly different ($p > 0.05$). The most

significant reduction in fresh weight was observed in the treatment of *Myrothecium* sp. The lowest dry weight of hydrilla was found with the *Myrothecium* sp. treatment. Fungal infections that cause damage to plants can reduce plant biomass. Shearer et al. (2011) reported a decrease in hydrilla biomass after four weeks of inoculation due to *M. terrestris*. The reduction in hydrilla biomass due to fungi can affect the primary metabolism of hydrilla. The pathogenic organisms in plants cause an increase in nutritional requirements as they manipulate carbohydrates for their needs. This has an impact on reducing the availability of carbohydrates for host plants, resulting in inhibited host plant growth (Chanclud and Morel 2016). The present study revealed that *Myrothecium* sp. and *Stachybotrys* sp. can act as bioherbicides to control hydrilla growth. The use of bioherbicides is an important step towards sustainability in agriculture (Cordeau et al. 2016).

This study concluded that two freshwater fungal isolates namely, *Myrothecium* sp. and *Stachybotrys* sp. selected from Mekarsari Fruit Garden Lake can act as a biocontrol agent in controlling hydrilla. These two fungal isolates can be able to inhibit hydrilla growth by *Myrothecium* sp. and *Stachybotrys* sp. was 98.07% dan 78.71%, respectively at four WAT.

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