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In vivo Testing of Plant Extracts in Controlling Rice Brown Spot Disease Through Folia Application

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ABSTRACT

Background: Rice brown spot disease is caused by the fungus *Bipolaris oryzae* (Breda de Haan), which is one of the most significantly devastating diseases in rice. Nowadays, biological control agents and plant extracts as botanical fungicides are used to develop an alternative method to control this disease and reduce the use of synthetic fungicides. Therefore, the efficacy levels of *Hydnocarpus anthelminthicus* Pierre ex Laness., *Crateva magna* (Lour.) DC., *Caesalpinia sappan* L., *Xanthophyllum lanceatum* J. J. Sm. and *Carallia brachiata* (Lour.) Merr. crude extracts were tested *in vitro* against *B. oryzae* and their control of rice brown spot disease under greenhouse conditions.

Methods: Five plants namely; *H. anthelminthicus, C. magna*, *C. sappan*, *X. lanceatum* and *C. brachiata* were cleaned with tap water and air dried at 28±2°C then cut into small pieces and ground into fine powder and stored at 4°C until used. Plant crude extracts was prepared using ethanol as solvent.

Result: The results showed that the *H. anthelminthicus* crude extract showed the best antifungal activity against *B. oryzae* at the highest dose tested, causing 93% mycelial growth inhibition. Under greenhouse testing, the application of the *H. anthelminthicus*, *X. lanceatum*, *C. brachiata*, *C. magna* and *C. sappan* crude extracts at a concentration of 50,000 ppm effectively suppressed and reduced rice brown spot incidence caused by *B. oryzae* when applied once 30 days after transplanting (DAT). Interestingly, the *H. anthelminthicus* crude extract at a concentration of 10,000 ppm displayed the greatest suppression of the development of rice brown spot disease in terms of disease incidence when applied twice 30 DAT and 45 DAT compared with unprotected control. The results of this study indicated that *H. anthelminthicus* could provide botanical fungicide protection against rice brown spot disease to reduce the use of synthetic fungicides.

Key words: *Bipolaris oryzae*, *Hydnocarpus anthelminthicus*, Rice brown spot disease.

INTRODUCTION

Rice is one of the most important cereal crops in Thailand, planted for both local consumption and exports. In 2014, Thailand was the world's number one exporter of milled rice (Charoenrak and Chamswarng, 2015). Unfortunately, rice production confronts many diseases and infections, including fungi, which constrain both quantitative and qualitative yield losses of up to 45% (Nalley *et al.*, 2016; Law *et al.*, 2017; Raveloson *et al.*, 2018). Rice brown spot disease caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker [telemorph: *Cochliobolus miyabeanus* (Ito and Kuribayashi) Dreches. ex Dastur] was recorded as one of the most significant diseases in rice and results in yield losses of about 10% whenever the disease occurs and up to 90% in grain yield losses (Ghose 2000; Savary *et al.*, 2000; Sunder *et al.*, 2014). The disease is widespread worldwide in rice-growing areas and it manifests through blemishes that are visible on several parts of the rice plant, including the leaves, sheath, panicles and grain (Mohd Anuar *et al.*, 2020). The typical brown spot symptom appears as a brown oval or cylindrical spot with a gray or whitish center surrounded by a yellow halo on rice leaves in the growing stage until the maturity stage (Sunder *et al.*, 2014; Kumari *et al.*, 2015). However, grain discoloration occurs after *B. oryzae* infects the rice glumes (Marchetti and Petersen, 1984).

While the application of synthetic fungicides was mainly used for rice disease control, the repeated and continued use of synthetic fungicides has resulted in the development of pathogen resistance and harm to the environment, the local ecology, non-target microorganisms and human health (Thind, 2012; Nicolopoulou-Stamati *et al.*, 2016; Van de Wouw *et al.*, 2017). Hence, there is an urgent need to solve

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these problems and find an effective way to manage these rice diseases, replace the use of synthetic fungicides and promote organic crop production and sustainable agriculture. Nowadays, several studies seek to discover new potential antifungal activity from biological control agents and plant extracts as botanical fungicides to develop an alternative bio-fungicide to control rice disease with effectiveness similar to that of synthetic fungicides (Zhang *et al*., 2014). Some plant species may contain rich amounts of bioactive compounds by producing diverse groups of secondary metabolites against plant pathogens (Naveenkumar *et al*., 2017; Dethoup *et al*., 2019; Kokkrua *et al*., 2020). Jantasorn *et al*., (2016) reported antifungal activity from the extracts of *Hydnocarpus anthelminthicus* and *Xanthophyllum lanceatum*, best effective against *Pyricularia oryzae and Rhizoctonia solani*, which cause rice diseases *in vitro*. However, the *H. anthelminthicus* leaves extract showed a strong inhibitory effect on the appressorium formation and conidial germination of *Colletotrichum higginsianum*, causing anthracnose disease in Chinese cabbage (Hsieh, 2018). The crude ethanol extract of *Acorus calamus* at a concentration of 5 g/L displayed the ability to significantly reduce brown spot incidence by 47% (Dethoup *et al*., 2019). Indeed, the use of natural products to develop botanical fungicides for fungal management is considered one of the better alternatives that can decrease the current use of synthetic fungicides, which contaminates and poisons other organisms present in the environment (Gujar *et al*., 2012; Plodpai *et al*., 2013; Shetty and Shruthi, 2015). Therefore, the objective of this study was to investigate the antifungal effects of the plant extracts of *H. anthelminthicus*, *C. magna*, *C. sappan*, *X. lanceatum* and *C. brachiata* on *B. oryzae* in vitro as well as their fungal activity to control the brown spot disease of rice seedlings, as assessed under greenhouse conditions.

MATERIALS AND METHODS

Plant materials and extractions

Five plant samples-namely, *H. anthelminthicus* (fruit), *C. magna* (fruit), *C. sappan* (bark), *X. lanceatum* (fruit) and *C. brachiata* (bark)-were collected from the riparian forest at Bodhivijjalaya College, Srinakharinwirot University, Sakaeo campus. Rice cv. KDML 105 seeds were obtained from the Surin Rice Research Center in Surin Province, Thailand. Each plant material was washed with tap water, air-dried at room temperature, cut into small pieces, ground into a fine powder and stored at 4°C until used. The extraction method was then performed (Jantasorn *et al*., 2016; Jantasorn *et al*., 2017). Briefly, 200 g of each plant powder sample was extracted twice, macerated with 95% ethanol and incubated at room temperature for 1 week. The aqueous ethanol extracts were filtered through three-layer sterile cheesecloth and then the solutions were concentrated under reduced pressure to produce the crude ethanol extracts.

In vitro mycelial growth inhibition test

The mycelial growth inhibition activity of the plant crude extracts was evaluated on *B. oryzae in vitro via* the dilution plate method, as described by Dethoup *et al.* (2019). Briefly, each plant crude extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in sterile water using the serial dilution method. Then 1 mL of each crude extract solution was mixed with 9 mL of warm potato dextrose agar (PDA) to obtain the final concentrations of 1, 10, 100, 1,000 and 10,000 ppm in separate petri dishes. A mycelial plug (5 mm in diameter) of *B. oryzae* obtained from an active growing colony of a seven-day-old culture was placed at the center of each petri dish. A mycelial plug control for tested fungus was placed on the petri dishes containing 1% DMSO. Each tested treatment plate was incubated at room temperature (28±3°C) for 14 days and the experiment was repeated twice with five replicates. Mycelial growth inhibition (MGI) was calculated using the following formula:

$$\%MGI = \frac{G1\text{-}G2}{G1} \times 100$$

Where

G1 represents the diameter of the colony radius of the fungi in the negative control and G2 is the diameter of the colony radius of *B. oryzae* treated with a plant crude extract at different concentrations.

Evaluation of plant crude extracts against brown spot disease on rice seedlings under greenhouse conditions

Rice cv. KDML 105 was used in this study, which is susceptible to rice brown spot disease caused by *B. oryzae.* The rice seeds were surface disinfected with a 1% (v/v) sodium hypochlorite solution (Dethoup *et al.,* 2019). Then the rice seeds were planted in a plastic tray and kept in nurseries for 7 days. Five seedlings of the same height and vigor were transplanted to a plastic pot (18 cm in diameter, 12 cm in height) containing sterile clay soil, placed in a greenhouse at 28±2°C for 4 weeks and watered as needed to maintain a water level of 1 cm above the soil surface throughout the experiment. The experiments were repeated twice and the pot used in a completely randomized design with five replicates. Five plant crude extracts were dissolved separately in DMSO and diluted in sterile water to give final concentrations of 10,000 and 50,000 ppm. The spore suspensions containing 10^6 spores mL^{-1} of *B. oryzae* were prepared as described by Komhorm *et al.* (2021). The effects of the five plant crude extracts were evaluated to suppress rice brown spot disease under greenhouse conditions in rice aged 30 days and 45 days. The rice seedlings were sprayed once 30 days after transplanting (DAT) and again at 45 DAT with 30 mL of each concentration of crude extract containing 0.01% (v/v) Tween-20 per pot on both sides of the leaves 24 hours before *B. oryzae* inoculation. Then 30 mL spore suspensions (10^6 spores mL^{-1}) of *B. oryzae* containing 0.01% (v/v) Tween-20 were inoculated on the treated rice seedling in each of the five pots (replications) per treatment; the treated pots were incubated at 28±2°C and 90%-100% humidity for 1 day and placed in the greenhouse. The five pots of rice seedlings were treated with 1% DMSO containing 0.01% (v/v) Tween-20 as a negative control. Disease incidence was evaluated 7 days after pathogen inoculation. Three leaves were collected from the top part of each plant and inspected for disease incidence (Komhorm *et al*., 2021). Difenoconazole (25% W/V EC, Syngenta Crop Science Co. Ltd.) was applied as a positive treatment.

Statistical analysis

The data was analyzed using the statistical program Statistix8 (Analytical Software, SXW, Tallahassee, FL, USA). The data in the experiments was evaluated using analysis of variance (ANOVA); the means were compared using least significance difference (LSD) with 95% statistical significance (p<0.05). Therefore, the data was obtained from the repeated experiments, pooled and submitted to analysis.

RESULTS AND DISCUSSION

In vitro antifungal activity of plant crude extracts against *B. oryzae*

The antifungal activity levels of the *H. anthelminthicus*, *C. magna*, *C. sappan*, *X. lanceatum* and *C. brachiata* crude extracts were tested against the mycelial growth of *B. oryzae* at different concentrations (1, 10, 100, 1,000 and 10,000 ppm) in *in vitro* conditions. The growth of *B. oryzae* was found to have an inverse relationship with the concentration of the crude extracts. The *H. anthelminthicus* crude extract showed the best antifungal activity against *B. oryzae* at the highest dose tested, causing 93% mycelial growth inhibition. Additionally, the *X. lanceatum* and *C. brachiata* extracts at a concentration of 10,000 ppm exhibited significant mycelial growth inhibition of *B. oryzae*, causing 82% and 67% inhibition compared with the control treatment (Fig 1). Meanwhile, the *C. magna* and *C. sappan* extracts inhibited the mycelial growth of *B. oryzae* via the dilution plate method on PDA by more than 50% (data not showed) and at low concentrations (1, 10 and 100 ppm), each crude extract failed to inhibit the mycelial growth of the brown spot disease pathogen.

Effect of five plant crude extracts against *B. oryzae* affecting brown spot disease in rice cv. KDML 105 under greenhouse conditions

The antifungal activity test results of the five plant crude extracts at two concentrations in controlling *B. oryzae* causing rice brown spot disease compared with the control in greenhouse conditions are shown in Fig 2 and 3. *H. anthelminthicus*, *X. lanceatum*, *C. brachiata*, *C. magna* and *C. sappan* effectively suppressed the incidence of rice brown spot disease caused by *B. oryzae* when applied once at 50,000 ppm (30 DAT) compared with the water

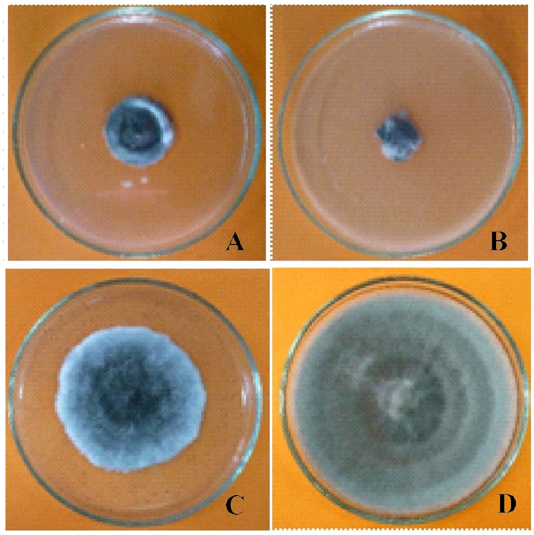

Fig 1: Antifungal effects of the tested plant crude extracts at a concentration of 10,000 ppm against *Bipolaris oryzae* in vitro using a dilution plate method: (A) *Xanthophyllum lanceatum*, (B) *Hydnocarpus anthelminthicus*, (C) *Carallia brachiata* and (D) control treatment.

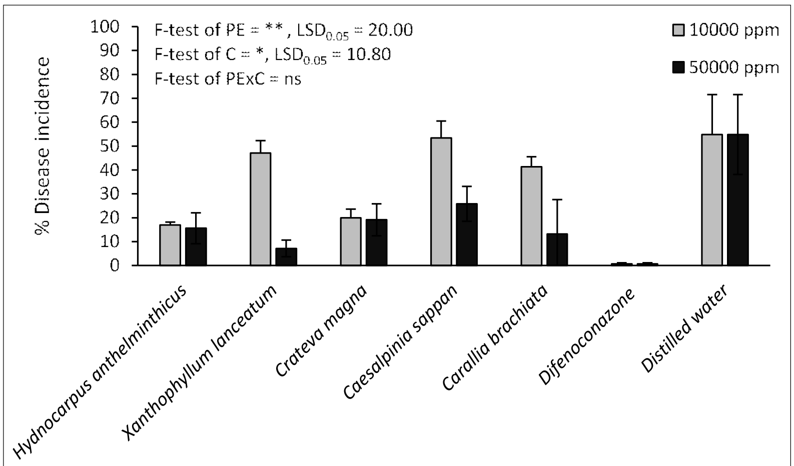

Fig 2: Efficacy of the five plant crude extracts against brown spot disease in rice cv. KDML 105 when applied once 30 DAT at concentrations of 10,000 ppm and 50,000 ppm. Error bars are the standard error of mean (n = 75). ns, not significant at p>0.05; * and ** indicate significant differences at p < 0.05 and p < 0.01, respectively. Comparisons were made for mean of plant extract (PE), concentration (C) and interaction between PE and C (PExC) at p < 0.05 by least significance difference test.

control (Fig 2). However, when the rice seedlings were sprayed twice, once 30 DAT and again 45 DAT, with the *H. anthelminthicus*, *X. lanceatum*, *C. brachiata*, *C. magna* and *C. sappan* crude extracts at a concentration of 10,000 ppm, they displayed the best levels of reduction and suppression of the development of brown spot incidence (Fig 3). Interestingly, the *H. anthelminthicus* crude extract at 10,000 ppm was found to induce the greatest level of suppression in the development of brown spot symptoms on rice seedlings when the plants were treated twice, 30 DAT and 45 DAT (Fig 4). However, the efficacy of the *H. anthelminthicus* crude extract being applied twice displayed the best reduction in the percentage of disease incidence of rice brown spot disease in greenhouse conditions, similar to the positive control with synthetic fungicides (difenoconazole). Furthermore, the rice seedlings in the water treatment showed typical brown spot symptoms.

Nowadays, several researchers seek new approaches to decrease the use of synthetic fungicides in rice disease management, which causes pathogen resistance. However, rice diseases caused by fungi can constrain both quality and yield losses of more than 50% in rice production (Spence

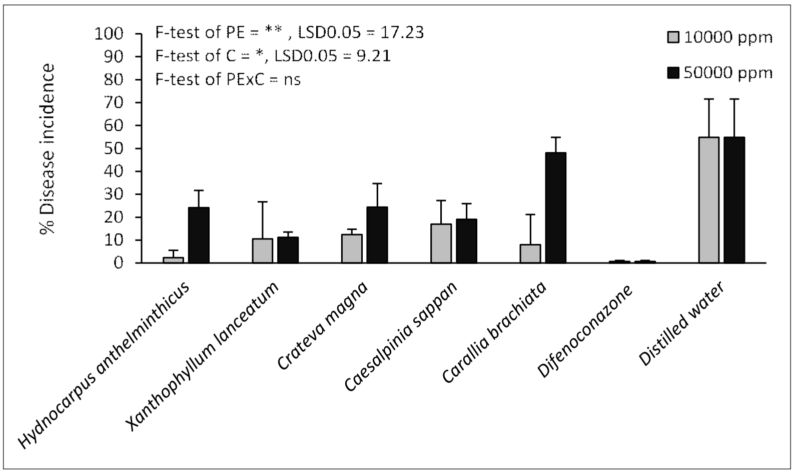

Fig 3: Efficacy of the five plant crude extracts against brown spot disease in rice cv. KDML 105 when applied once 30 DAT and again 45 DAT at concentrations of 10,000 ppm and 50,000 ppm. Error bars are the standard error of mean (n=75). ns, not significant at $p>0.05$; * and ** indicate significant differences at $p<0.05$ and $p<0.01$, respectively. Comparisons were made for mean of plant extract (PE), concentration (C) and interaction between PE and C (PExC) at $p<0.05$ by least significance difference test.

Fig 4: Effect of the *Hydnocarpus anthelminthicus* extract against brown spot disease in rice cv. KDML 105 when applied once 30 DAT and again 45 DAT at a concentration of 10,000 ppm: (A) *Hydnocarpus anthelminthicus*, (B) synthetic fungicide (difenoconazole) and (C) water control treatment.

et al., 2014; Awla *et al*., 2017). Biological control and botanical fungicides are promising approaches to rice disease management; however, the bioactive compounds from plant extracts to control rice diseases are still limited (Torres *et al*., 2017; Shao *et al*., 2018). In this study, the main antifungal activity of the *H. anthelminthicus* extract was observed both *in vitro* and *in vivo*. The *H. anthelminthicus* extract displayed potent *in vitro* antifungal activity against *B. oryzae* at high doses. Moreover, the antifungal activity of the *H. anthelminthicus* extract inhibited the mycelial growth of *Pyricularia oryzae*, *Rhizoctonia solani* and *Phytophthora palmivora* by 100% at a concentration of 10,000 ppm and the *X. lanceatum* extract at high doses showed a complete mycelial growth inhibition of *P. oryzae* (Jantasorn *et al*., 2016). Hsieh (2018) reported that the *H. anthelminthicus* crude ethanol extract from leaves at a concentration of 2.5% (v/v) displayed a strong inhibitory effect on the conidia germination and appressorium formation of *Colletotrichum higginsianum*, causing anthracnose in Chinese cabbage. However, several studies reported that the efficacy levels of plant extracts against *B. oryzae* have been tested (Bhuyan *et al*., 2010; Nguefack *et al*., 2013; Dethoup *et al*., 2019; Kokkrua *et al*., 2020).

In greenhouse testing, the plant extracts had different levels of inhibitory activity to suppress the development of brown spot symptoms on rice seedlings and significantly reduced the incidence of the disease. In addition, the concentration and spraying quantity of the *H. anthelminthicus*, *X. lanceatum*, *C. brachiata*, *C. magna* and *C. sappan* crude extracts are critical for the effective control of rice brown spot disease. The *X. lanceatum* extract at a concentration of 50,000 ppm displayed the best effects in suppressing the development of brown spot symptoms caused by *B. oryzae* when applied once 30 DAT before pathogen inoculation. However, the *H. anthelminthicus* extract, when applied once at both concentrations (10,000 and 50,000 ppm) 30 DAT, showed a strong antifungal effect on *B. oryzae* growth. In addition, the efficacy of *H. anthelminthicus* against *B. oryzae* when applied once and twice at 10,000 ppm at both stages of growth (30 DAT and 45 DAT) was tested under greenhouse conditions. This phenomenon may be due to differences in the fungicidal activity of plant extracts. The percentage of brown spot disease incidence was lower than in the plants treated with the *X. lanceatum*, *C. brachiata*, *C. magna* and *C. sappan* extracts. This revealed that the *H. anthelminthicus* extract had higher fungicidal activity compared with the other plant extracts tested in this study. Thus, the *H. anthelminthicus* extract exerts the highest level of preventive activity to reduce the disease incidence of *B. oryzae* under greenhouse conditions. Also, the efficacy of the *H. anthelminthicus* ethanol crude extract at a concentration of 0.5% significantly reduced the incidence and severity of anthracnose in Chinese cabbage under greenhouse conditions (Hsieh, 2018).

Rice brown spot disease caused by *B. oryzae* can constrain both the yield and quality of rice production; many attempts have been made to find plant extracts to control this disease. Although many have reported on the antifungal activity of plant extracts against *B. oryzae in vitro* and *in vivo* (Nguefack *et al*., 2013; Dethoup *et al*., 2018; Dethoup *et al*., 2019), none have reported on the effect of the *H. anthelminthicus* crude extract against this disease *in vivo.* Our results in the greenhouse experiment indicated that the spraying of plant crude extracts once and twice on the leaves of the rice seedling plants resulted in a significant decrease in pathogen infection by *B. oryzae*, whereby all the treatments with the tested plant extracts reduced the disease incidence and suppressed the development of brown spot symptoms in rice seedlings compared with the unprotected control. Based on our results, the *H. anthelminthicus* crude extract at a concentration of 10,000 ppm was the most effective in suppressing the disease incidence by more than 80%, with two sprays (30 DAT and 45 DAT) and inoculation with *B. oryzae.* In this study, the rice seedling leaves did not show any phytotoxic symptoms when the *H. anthelminthicus* crude extract was applied at the highest dose of 50,000 ppm. So far, botanical fungicide products derived from plant extracts are still limited to development for commercialization. This study indicated that the *H. anthelminthicus* crude extract has higher antifungal activity against *B. oryzae*, which causes brown spot disease in rice seedlings, since it caused a reduction in disease incidence at 10,000 ppm both *in vitro* and *in vivo*. The *H. anthelminthicus* crude extract is safer for human beings and the results of this study demonstrate that this crude extract is a promising alternative botanical fungicide against *B. oryzae* to replace the use of synthetic fungicides.

CONCLUSION

Our results demonstrated that the *H. anthelminthicus* crude extract exhibited significant and potent botanical fungicide activity against *B. oryzae*, preventing it from causing the foliar disease of rice *in vitro* and *in vivo*. It was shown to strongly prevent *B. oryzae* infection and reduce disease development. Moreover, the *H. anthelminthicus* crude extract displayed the most effective antifungal activity against rice brown spot disease and reduced the incidence of this disease. Thus, the *H. anthelminthicus* crude extract can be applied to control rice brown spot disease caused by *B. oryzae* and can be developed as an alternative botanical fungicide to reduce the use of chemical fungicides. However, further studies are required to test the *H. anthelminthicus* crude extract against *B. oryzae* and to evaluate its control efficiency compared with that of synthetic fungicides in paddy fields.

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Conflict of interest

The author declare that they have no conflict of interest.

Human and animal rights

This article does not contain any studies involving human participants or animals by any of the authors.

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