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In vitro evaluation of antifungal activity of endophytic fungal extracts from selected rice plant pathogens

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Abstract Fungal endophytes are considered as natural bioactive reservoir that could be used as agents for antifungal activity. *Echinochloa colonum* (L.) Link an invasive rice weed compete with plant resources but houses several essential endophytes. However, a notable gap exists since few to none were conducted regarding the use of fungal extracts isolated from the weed *E. colonum*. Thus, evaluation of fungal endophytes from weeds needs to be established that may lead to development of biocontrol agent against fungal pathogens. Therefore, extracts from endophytic fungi isolated from the leaves of *E. colonum* were evaluated its antifungal activity against rice plant pathogens such as *Aspergillus flavus, Fusarium oxysporum*, *Rhizoctonia solani*, and *Aspergillus oryzae*, *Curvularia umbiliciformis*, *Curvularia chiangmaiensis*, and *Bipolaris panici-miliacei* were successfully isolated from the leaves of *E. colonum* and were identified based on cultural, morphological and molecular data. Additionally, using morphological and cultural techniques, *Aspergillus* sp. was also identified as another endophyte. Identified fungi were fermented using solid state fermentation to obtain fungal extracts. The 50µL of endophytic fungal extracts were used and exhibited varying antifungal activities in the agar well diffusion method. Extracts from *Aspergillus* sp., and *B. panici-miliacei* showed absence to weak antifungal activity against different fungal rice pathogens. While, *C. chiangmaiensis* extracts showed weak (11.83 ± 0.06 mm) to comparable (30.83 ± 0.40 mm) antifungal activity against *A. flavus* and *F. oxysporum* after 48 hours of incubation. Among the four fungal extracts, *C. umbiliciformis* extracts showed antifungal activity against *A. flavus* (18.53±0.40 mm), *F. oxysporum* (11.23±0.15 mm), and *R. solani* (31.73 ± 1.00 mm), and *A. oryzae* (36.77 ± 4.89 mm) after 48 hours of observation. Furthermore, statistics revealed that *C. umbiliciformis* extract showed comparable antifungal activity against *R. solani*. Therefore, fungal endophytic extracts can be expressed as a good source of antifungal agent against selected rice pathogen.

Keywords: Biocontrol, *Echinochloa colonum*, Endophytic fungi, Plant pathogen, rice, *Rhizoctonia solani*

Introduction

Agriculture stands as a crucial asset of a country. It serves as a primary source of income and fulfils the daily food requirements. Rice crop, is greatly

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cultivated in the Philippines and serves as a staple food. With the escalating population, the demand for food security arises in various Asian countries, including the Philippines (Sureshkumar *et al.,* 2016).

Rice crops in the Philippines and other parts of the world experience threats from various factors like crop diseases and weed management (Mohanty *et al.,* 2013). Weeds deemed economically insignificant, are removed from farmlands. They are known to compete with nutrients, space, and light needed by rice crops for growth (Chaudhary *et al*., 2019). The presence of weeds on farmlands are recorded to significantly reduce yield during organic farming (Toru *et al.,* 2020). Thus, abundance of weed species in ricefields of Southeast Asia could contribute to lower yields (Moody, 1989).

Echinochloa colonum (L.) Link is a weed found on rice fields in different Asian countries, including the Philippines. The weed is also known as Dakayang or barnyard grass from the family Poaceae. The weed mimics rice during its vegetative stages and is a great resource competitor. *E. colonum* is one of the troublesome weeds in rice and is prone to developing herbicide resistance (Zabala *et al.,* 2019).

In the Philippines, plant diseases caused by various pathogenic fungi contributes to about 50% to 85% yield loss (Leung, 2018). Fungal pathogens could affect the preharvest and postharvest of rice and is also associated with global harvest loss (Savary *et al.,* 2019). Some of the leading rice plant pathogens, including bacterial leaf streak, blight disease, brown leaf spots, bacterial palea browning, black kernel grain, and seedling blight, are some of the problems encountered by farmers. Plant-related pathogens including *Aspergillus flavus*, *Fusarium oxysporum* and *Rhizoctonia solani* are some of the fungal species that could be harmful on rice.

Aspergillus is a common fungal genus related to food spoilage. Several species of *Aspergillus* are known to have pathogen activity against grains like maize and rice (Phan *et al.,* 2021). *Aspergillus* species are known to contaminate crops and strains the economic production process (Szalewski *et al.,* 2018). On the other hand, fusarium wilt and root rot (Husna *et al.,* 2020; Behera *et al.,* 2022) and seedling blight (Li *et al.,* 2019) are caused by *Fusarium* species which greatly affect rice. Rice sheath blight is regarded as one of the most severe diseases on rice and ranks second to rice blast (Pan *et al.,* 1999; Senapati *et al.,* 2022). *R. solani* is another major fungal pathogen and was successfully identified as the causative agent for rice sheath blight (Dayong *et al*., 2021) which is known to be one of the serious diseases in rice second only to rice blast (Wang *et al*., 2015). In the Philippines, *R. solani* was reported to reduce 5-80% of rice grain produce affecting the yield and its economic impact (Cumagun *et al*., 2020).

Control of fungal pathogens to prevent crop loss is done through fungicide administration. However, continuous usage of strong chemicals could contribute to resistant microorganisms. Therefore, the need for a natural product that possesses antimicrobial properties is needed to address the problem of the growing number of resistant microorganisms. Thus, antimicrobial studies are utilized to develop antimicrobial drugs to help address crop diseases (Mohanty *et al*., 2013).

Weeds do not possess any relevant economic value and are considered a threat in rice farming, together with various plant diseases. Weeds and plant diseases could contribute to economic and yield loss. Though, they are considered threat and shade out desirable plants, they house various endophytes. Fungal endophytes are known to have plant protection, imparting tolerance against several pathogens. Their isolation could provide new insights into clinical utility and a vital role in solving the growing cases of rice problems. However, few studies were conducted about the effects of endophytic fungi isolated from *E. colonum.* Moreover, endophytic fungi from the weed *E. colonum* were not yet evaluated against *A. flavus, F. oxysporum, R. solani* and *Aspergillus oryzae.*

In this study, the weed *E. colonum* was used as a specimen for fungal isolation of endophytes and determining the antifungal efficacy against selected rice plant pathogens. This research aimed to determine the different endophytic fungi found in the leaves of *E. colonum* and determined their antifungal activity against *A. flavus, F. oxysporum*, *R. solani*, and *A. oryzae*.

Materials and Methods

Collection and preparation of samples

Fresh leaves of *Echinochloa colonum* (L.) Link were collected from ricefields in Nueva Ecija, Philippines. Within 24 hours after the collection, the samples were surface sterilized (Shankar Naik *et al.,* 2009) using 70% ethanol for 5 minutes, 2% sodium hypochlorite for 5 minutes, and another 30 seconds in 70% ethanol. Then, surface sterilized samples were rinsed in sterile distilled water twice (Araujo *et al.,* 2002).

Identification of the isolated endophytic fungi

Potato Dextrose Agar (PDA) was sterilized and poured to sterile petri plates which was used to isolate fungal endophytes. The leaves of *E. colonum* was cut to 6 cm segments and was aseptically placed on top of PDA plates supplemented with streptomycin (250 mg/ml). Each plate contains five segments

and were done in triplicates. The samples were incubated for 7 days at room temperature.

The isolated fungal endophytes were purified and was identified morphologically, culturally, and molecularly. Three-point inoculation technique was done to observe the cultural charateristics on the obverse and reverse side after 7 days of incubation (Waing *et al.,* 2015). Slide culture technique was used to observe the spores and hyphae of the isolated endophytes under the microscope. Pure cultures were sent to Macrogen, Korea through Kinovett Scientific Co, Quezon City for molecular identification.

Solid state fermentation of fungal endophytes

The isolated fungal endophytes were subjected for extraction and further screenings for antifungal activity. The isolated fungal endophytes were fermented following the protocol of Alade *et al.* (2018) and Ibrahim *et al.* (2021). A total of 200 g of commercially available white rice was soaked in distilled water (200 mL) for 10 minutes in a clean container. The flask containing the soaked rice was sterilized and was allowed to cool (Alade *et al.,* 2018). Four small parts of mycelium from the isolated endophytic fungi were inoculated on the rice medium under sterile conditions. The fungal strain was incubated at room temperature to allow growth on the rice medium for 5 weeks. The flask containing autoclaved rice medium without the inoculum served as control (Ibrahim *et al.,* 2021).

After incubation, 250 mL of ethyl-acetate was added. The flask with the culture and ethyl-acetate was laid overnight at room temperature. The fungal mixture was the filtered using a Buchner funnel and the collected supernatant was subjected to the rotary evaporator to obtain the extract (Ibrahim *et al.,* 2021).

Antifungal activity of the endophytic fungal extracts

Fungal rice plant pathogens were used as the test organisms. Pure cultures of *A. flavus, F. oxysporum* and *A. oryzae*, were obtained from Philippine Center of Postharvest and Development Mechanization (PhilMech) and *R. solani* from the College of Veterinary Science and Medicine, CLSU.

Following the methods of Moron *et al.* (2018) with modifications, 50 µL of the endophytic fungal extracts were used in the study. Commercially available fungicide (Folicur) was used as the positive control. Pure extracts from the endophytic fungi were tested for its antifungal activity.

Fungal strains were grown on PDA for 7 days at 35°C. The inoculum was adjusted to 1 x 10^6 spores/mL to 2.7 x 10^6 spores/mL using the hemocytometer (Petrikkou *et al*., 2001; Chavez-Quintal *et al*., 2011; Serrano *et al*., 2018).

For the inhibition of fungal growth, agar well method was used (Garcia, 2014). Wherein, a layer of molten PDA was allowed to solidify on sterile petri dishes. Seven (7) sterile improvised tubes were aseptically placed on top of the solidified agar before pouring the second layer of PDA containing the fungal pathogen (Sharma *et al.,* 2012; Serrano *et al*., 2018). The improvised tubes served as an improvised cork borer to form the agar wells and 50 µL of each treatment was added to the hole (de Rodriguez *et al*., 2005; Shakhatreh *et al.,* 2016; Serrano *et al*., 2018). The zone of inhibition (mm) was measured using a vernier caliper after 48 hours of incubation (Chavez-Quintal *et al.,* 2011).

Statistical analysis

The zone of inhibition (mm) was measured using a digital vernier caliper. The zone of inhibition for the fungal endophytic extract was measured to test the antifungal efficacy of the isolated endophytes. The zone of inhibition was recorded as mean of the three replicates. One-way Analysis of Variance (ANOVA) with Tukey HSD was used to test the difference between the commercially available control and the fungal endophytic extracts. A significance level of 5% was used in the study.

Results

Identity of the isolated fungal endophytes

Morphological, cultural, and molecular techniques were utilized to identify the isolated fungal endophytes. A total of 4 fungal endophytes were successfully isolated and purified. Among the isolates, 3 were identified using molecular techniques and 1 was identified using morphological and cultural techniques.

Curvularia umbiliciformis, illustrated velvety surface, with white center and dark colony, creamy black reverse tints, with flat elevation and undulate margin. Moreover, concentric rings were observed on the reverse side of the sample. The colony diameter ranges from 42.3 mm to 48.5 mm on the 7th day of incubation (Figure 1). Using a light microscope, the isolate exhibited multiple conidia found at the apical region (Figure 2a) and septated hypha (Figure 2b).

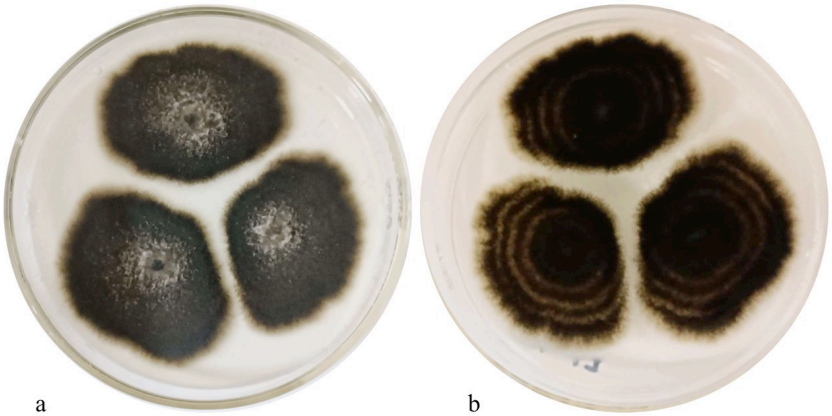

Figure 1. Cultural characteristics of the isolate EF1 (*C. umbiliciformis*), colonies on PDA in the obverse (a) and reverse (b) sides after 7 days of incubation

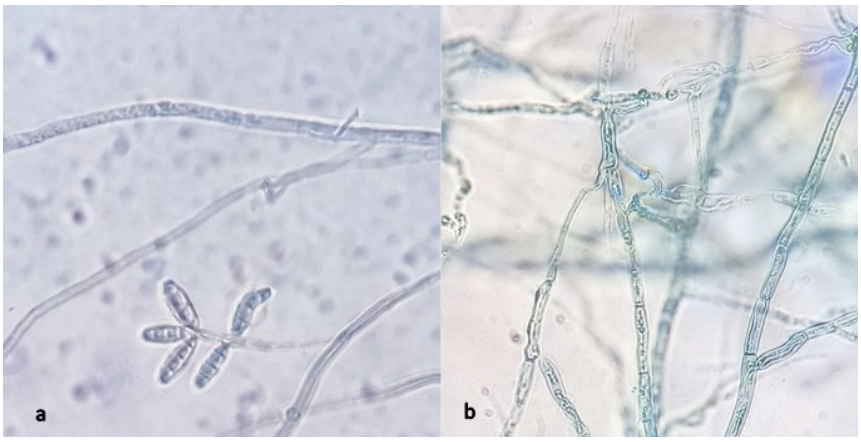

Figure 2. Morphological characteristics of EF1 (*C. umbiliciformis*) showing the conidia (a) and septated hyphae (b) under 400x magnification

Colonies in PDA of *Curvularia chiangmaiensis* are grayish at the center, white on its periphery, and has soft-shiny cotton texture (Figure 3a). The sample exhibits tri-color on the reverse plate; light brown on the periphery, dark brown at the center, and creamy-white edge (Figure 3b). The colony is flat on the surface and had an entire margin. Colony diameter ranges from 28 mm to 39.7 mm at the 7th day of incubation. Numerous conidia are also observed for *C. chiangmaiensis* under the microscope (Figure 4).

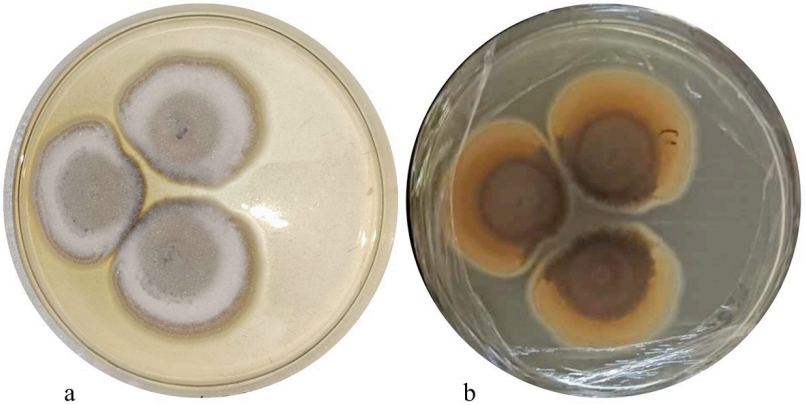

Figure 3. Cultural characteristics of the isolate EF2 (*C. chiangmaiensis*), colonies on PDA in the obverse (a) and reverse (b) sides after 7 days of incubation

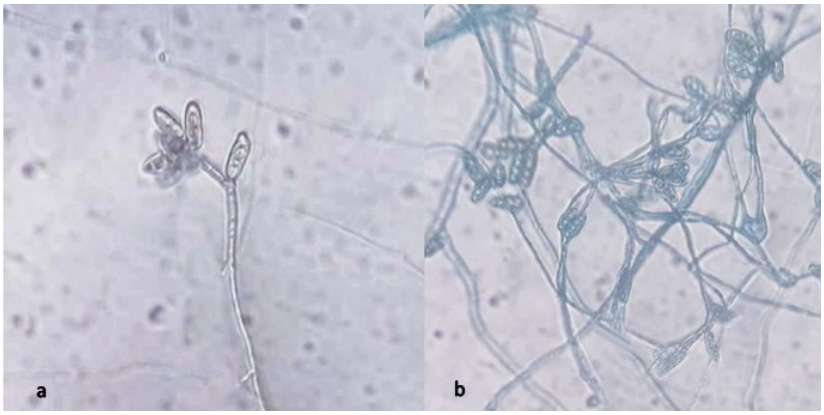

Figure 4. Morphological characteristics of the isolate EF2 (*C. chiangmaiensis*) showing conidiophores (a) and singly or branching conidiophores (b) under 400x magnification

Colonies of *Aspergillus* sp. in PDA are matte beige-yellowish to orange-brown, irregular margin, and have a diameter around 31.4 mm to 41.3 mm after 7 days of incubation (Figure 5a). The reverse side is yellowish-white, with no observable zonation. *Aspergillus* sp. produced columnar conidia (Figure 6a), smooth walled conidiophores, and measures around 20μm in diameter (Figure 6b).

Figure 5. Cultural characteristics of the isolate EF3 (*Aspergillus* sp.), colonies on PDA in the obverse (a) and reverse (b) sides after 7 days of incubation

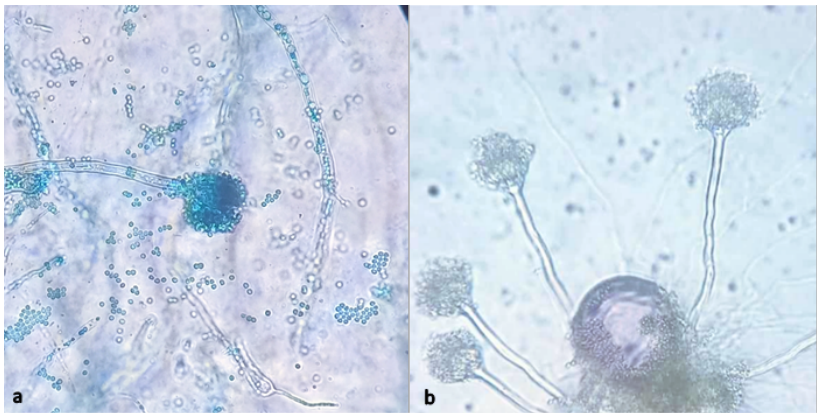

Figure 6. Morphological characteristics of the isolate EF3 (*Aspergillus* sp.) showing columnar conidia (a) and smooth-walled conidiophores (b) under 400x magnification

Bipolaris panici-miliacei colonies in PDA appears cottony, grayish black, with a grayish center, irregular margin, and the diameter ranges from 42.1 mm to 50.1 mm on the 7th day of incubation at room temperature (Figure 7a). On its reverse, is darker with grayish alternate with no zonation present (Figure 7b). *B. panici-miliacei* have septated conidia (Figure 8a) with hyaline conidiophore.

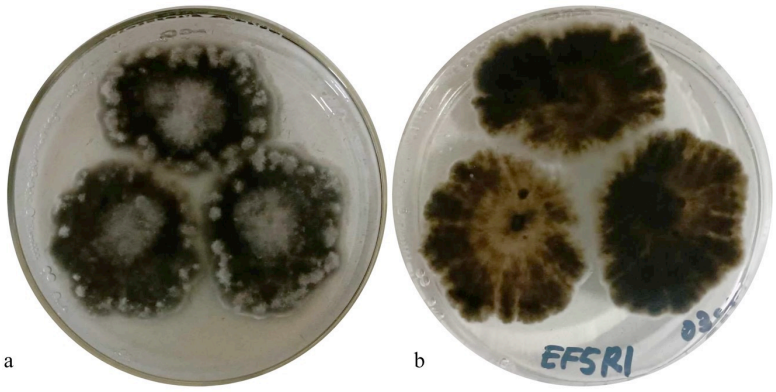

Figure 7. Cultural characteristics of the isolate EF5 (*Bipolaris panici-miliacei*), colonies on PDA in the obverse (a) and reverse (b) sides after 7 days of incubation

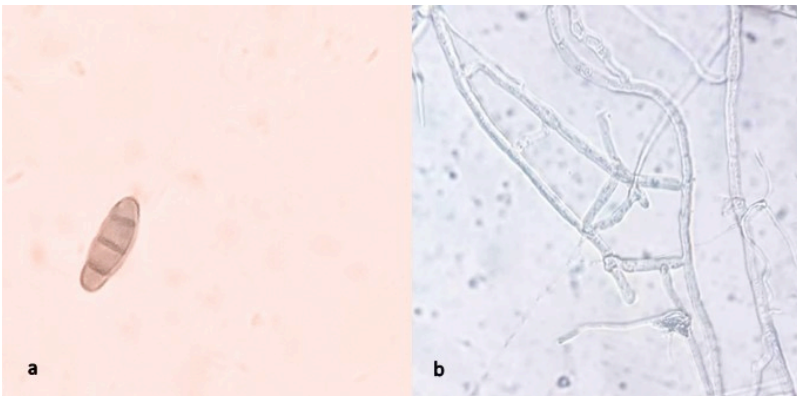

Figure 8. Morphological characteristics of the isolate EF5 (*B. panici-miliacei*) showing the conidia (a) and conidiophores (b) under 400x magnification

Result showed that, EF1 was identified as *Curvularia umbiliciformis* with 99% identity. The ITS sequence also revealed that EF2, was *Curvularia chiangmaiensis* with 99%, while EF5 was *Bipolaris panici-miliacei* with 100% identity (Table 1).

Table 1. Identity of the endophytic fungal isolates based on NCBI data

Sample code	Sample identity	Length (bp)	Percent identity	Accession number
EF1	*Curvularia umbiliciformis*	702	99	MN215713.1
EF2	*Curvularia chiangmaiensis*	704	99	MN215651.1
EF5	*Bipolaris panici-miliacei*	599	100	KJ909773.1

Antifungal activity of endophytic fungal extracts

The susceptibility of the rice plant pathogen, *A. flavus*, to different endophytic fungal extracts from *E. colonum* is presented in Table 2. Data shows that the *C. umbiliciformis* extract exhibited a mean value of 18.53 mm which is significantly different with *C. chiangmaiensis* extract with a mean of 11.83 mm, *Aspergillus* sp. (11.70 mm), *B. panici-miliacei* extract (12.00 mm) and rice extracts (12.10 mm).

Table 2. Zone of inhibition (mm) of endophytic fungal extracts against *A. flavus* after 48 hours of incubation

Treatments	Mean ± SD (mm) 48 hours
Curvularia umbiliciformis extract	18.53±0.40[b]
Curvularia chiangmaiensis extract	11.83±0.06[c]
Aspergillus sp. extract	11.70±0.10[c]
Bipolaris panici-miliacei extract	12.00±0.17[c]
Rice Extracts	12.10±0.20[c]
Folicur 25WP Systemic Fungicide, 1.875mg/mL	21.50±0.10[a]
Sterile Distilled Water	0.00±0.00[d]

Notes: Values are in the mean ± standard deviation format; Means with the same superscript letter are not significantly different at 5% level of significance.

The antifungal activity of different endophytic fungal extracts from *E. colonum* against *F. oxysporum* is presented in Table 3. Data shows that only *C. umbiliciformis* extract induced a zone of inhibition with a mean value of 11.23±0.15 mm which is significantly different with the positive control with a mean of 34.37±3.27 mm after 48 hours of incubation. Moreover, no zone was recorded for *C. chiangmaiensis* extract, *Aspergillus* sp. extracts, *B. panici-miliacei* extracts, plain rice extracts, and sterile distilled water.

Table 3. Zone of inhibition (mm) of endophytic fungal extracts against *F. oxysporum* after 48 hours of incubation

Treatments	Mean ± SD (mm) 48 hours
Curvularia umbiliciformis extract	11.23±0.15[b]
Curvularia chiangmaiensis extract	0.00±0.00[c]
Aspergillus sp. extract	0.00±0.00[c]
Bipolaris panici-miliacei extract	0.00±0.00[c]
Rice Extracts	0.00±0.00[c]
Folicur 25WP Systemic Fungicide, 1.875mg/mL	34.37±3.27[a]
Sterile Distilled Water	0.00±0.00[c]

Notes: Values are in the mean ± standard deviation format; Means with the same superscript letter are not significantly different at 5% level of significance.

The susceptibility response of the rice plant pathogen, *R. solani,* to different endophytic fungal extracts from *E. colonum* is also presented at Table 4. Based on the results, the treatment from *C. umbiliciformis* extracts and *C. chiangmaiensis* showed a mean inhibition of 31.73 ± 1.00 mm and 30.83 ± 0.40 mm, respectively, after 48 hours of incubation which is comparable with the positive control. Moreover, smaller inhibitions were observed for *Aspergillus* sp. extract, *B. panici-miliacei* extract, and rice extracts.

Table 4. Zone of inhibition (mm) of endophytic fungal extracts against *R. solani* after 48 hours of incubation

Treatments	Mean ± SD (mm) 48 hours
Curvularia umbiliciformis extract	31.73 ± 1.00^{ab}
Curvularia chiangmaiensis extract	30.83 ± 0.40^{ab}
Aspergillus sp. extract	12.30 ± 0.80^{c}
Bipolaris panici-miliacei extract	10.73 ± 0.57^{c}
Rice Extracts	12.27 ± 1.54^{c}
Folicur 25WP Systemic Fungicide, 1.875mg/mL	30.00 ± 0.56^{ab}
Sterile Distilled Water	0.00 ± 0.00^{d}

Notes: Values are in the mean ± standard deviation format.
Means with the same superscript letter are not significantly different at 5% level of significance.

The antifungal susceptibility of *A. oryzae* to different endophytic fungi extracts isolated from *E. colonum* is presented in Table 5. Among the treatments *C. umbiliciformis* extract showed 36.77 ± 4.89 mm and the positive control had 38.73 ± 2.55 mm zone of inhibition after 48 hours which are comparable with each other. On the other hand, *Aspergillus* sp. extract, *B. panici-miliacei* extract, and rice extracts did not exhibit any antifungal activity.

Table 5. Zone of inhibition (mm) of endophytic fungal extracts against *A. oryzae* after 48 hours of incubation

Treatments	Mean ± sd (mm) 48 hours
Curvularia umbiliciformis extract	36.77 ± 4.89^{a}
Curvularia chiangmaiensis extract	0.00 ± 0.00^{b}
Aspergillus sp. extract	0.00 ± 0.00^{b}
Bipolaris panici-miliacei extract	0.00 ± 0.00^{b}
Rice Extracts	0.00 ± 0.00^{b}
Folicur 25WP Systemic Fungicide, 1.875mg/mL	38.73 ± 2.55^{a}
Sterile Distilled Water	0.00 ± 0.00^{b}

Notes: Values are in the mean ± standard deviation format.
Means with the same superscript letter are not significantly different at 5% level of significance.

Discussion

Endophytic fungi are considered as a natural bioactive reservoir that could be used as favorable agents for antifungal activity. Moreover, compounds extracted from endophytes is vital to combat the increasing number of microbial resistance (Hashem *et al*., 2022). Studies are much needed to identify the different endophytic fungi present from the weeds that competes with resources needed by rice and has no recorded economic potential example of which is *E. colonum.*

Based on the results of the study, four species of fungi were successfully isolated from the leaves of *E. colonum*. The isolated endophytic fungi from the weeds of *E. colonum* are velvety, powdery, and cottony in texture, which are usually light and gray and eventually changed to dark black, light brown, grayish-black, and yellowish-white after 7 days of incubation on potato dextrose agar plates. These were *Aspergillus* sp., *B. panici-miliacei, C. chiangmaiensis* and *C. umbiliciformis.*

As noted by Lass-Florl *et al.* (2021), *Aspergillu*s sp. are able to become floccose and the columnar conidial heads that could reach 50µm in diameter. Meanwhile, in the study of Manamgoda *et al*. (2014) *B. panici-miliacei* have straight or curved conidia, obclavate, and hyaline when immature which then turn olive green upon maturation. Moreover, species of *Curvularia* exhibits ellipsoid conidial shape and with brown to black color (Rashid, 2001; Yanagihara *et al*., 2010). According to Marin-Felix *et al*. (2017), the conidiophores of *C. chiangmaiensis* are varied and could appear singly or in groups, septated, and could be branched.

Molecular sequencing is a way to confirm the identity of an unknown fungal isolate. ITS sequencing shows diverse sequences, application, and showcases high probability of accurate identification (Buehler *et al*., 2017; Jiang *et al*., 2022). Various studies, including those by Madrid *et al*. (2014), Ittuerietta-Gonzalez *et al*. (2020), and Connally *et al*. (2022), used ITS sequencing for the identification of *Curvularia* species. Despite numerous investigations on *Curvularia* species, limited information is available for *C. umbiliciformis* and *C. chiangmaiensis* from *E. colonum*. Notably, *C. chiangmaiensis* was isolated from *Zea mays* in Chiang Mai, Thailand in 2017. Additionally, other species of *Curvularia* were isolated from the weed *Eleusine indica* which supports the presence of the fungal endophytes (Marin-Felix *et al*., 2017).

Numerous species of endophytic microorganisms are known to produce secondary metabolites that are biologically active. Most studies employed the use of antagonistic approach using endophytes against several pathogens. In the study conducted by Catambacan and Camagun (2021), several endophytes from weeds were tested on *Fusarium* wilt and records a biocontrol activity using

antagonistic activity which could support the results of the study. Aside from endophytic microorganisms, various studies are explored and directed on testing various fungicides against *F. oxysporum.* In the study of Somu *et al.* (2014), several fungicides were used to combat *F. oxysporum* on banana. Varying concentrations were used and revealed that carbendazim, carboxin, propiconazole, and benomyl are able to inhibit the growth of *F. oxysporum.*

Meanwhile, several studies were conducted to determine various antifungal agents against *R. solani*. *Paecilomyces*, is an endophytic fungus used against *R. solani* and was reported to inhibit up to 70% of its growth (Hawar *et al*., 2023). Also, similar with the findings of Donayre and Dalisay (2015), endophytes from *Echinochloa* are found to be effective against *R. solani*. Similarly, in the study conducted by Motlagh *et al.* (2022), the volatile compounds present in *Curvularia lunata* showcased an antagonistic result against *R. solani* and disease reduction on rice infected with *R. solani*. Also, other fungal species are found to be effective in agricultural practice like *Trichoderma virens* which showed high antagonistic results against *R. solani*. Results presented that the endophytic fungal extracts, particularly *C. umbiliciformis* showed antifungal activity against *A. flavus*, *F. oxysporum*, *R. solani*, and *A. oryzae*.

The antifungal activity of tested endophytic fungal extracts isolated from *E. colonum* could be contributed to the possible secondary metabolites present in the extract. Several studies indicated that endophytic fungal extracts are rich in bioactive compounds (Wen *et al*., 2022; Gupta *et al*., 2023; Hashem *et al*., 2023). However, further studies are needed to evaluate the different bioactive compounds present in the endophytic fungal extracts from *E. colonum*.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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