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Decolorization of Synthetic Textile Dyes by Fungal Endophytes Isolated from the Leaves of Philippine Mangrove *(Avicennia marina)*

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Abstract

Textile dyes in wastewater can be harmful pollutants when released into the environment without treatment. Biodegradation of textile dye effluents by different microbes, including fungi, has become popular as an alternative to physicochemical methods. The mangrove *Avicennia marina* is known to harbor endophytic fungi which have the potential to carry out dye degradation. Therefore, this study assessed the ability to decolorize synthetic dyes of endophytic fungi isolated from the leaves of *A. marina.* Of the nine fungal endophytes, *Aspergillus niger, Syncephalastrum racemosum* and *Penicillium citrinum* exhibited the highest mycelial growths in solid media, while all endophytes adsorbed Congo red. Through liquid decolorization assay, four isolates decolorized Congo red at greater than 89% decolorization rates. *P. citrinum* (55.45%), Mycelia sterilia (85.19%), *A. flavus* (44.91%) showed the highest decolorization rates of Methylene blue, Malachite green and Rhodamine B, respectively. The ligninolytic enzymes produced by the endophytic fungi, laccase exhibited the highest activity with values higher than the positive control.

Keywords: Fungal endophyte; Mangrove; Decolorization; *Avicennia marina*

1. Introduction

Synthetic dyes are aromatic molecular compounds used in the food, cosmetic, papers, plastic, and textile industries. About 100,000 commercial dyes are produced annually including acidic, basic, reactive, diazo, anthraquinone dyes. About 5-10% of the dyestuff becomes part of the effluent (Campos *et al.,* 2001; Sathiya *et al.,* 2007). Industries release approximately $8x10^6$ tons of dye wastewater annually (Wang *et al.,* 2017), with the textile and dyestuff industries contributing significantly (Yaseen and Scholz, 2018). Textile dye effluents are particularly difficult to treat due to various complicated molecular structures, increased biochemical oxygen demand, suspended solids, and the presence of heavy metals. These dyes are toxic and carcinogenic (Srivastava *et al.,* 2004; Lellis *et al.,* 2019), which necessitates their treatment before release into the environment.

Various physicochemical and biological processes are employed to treat textile dye wastewater. Physicochemical treatments include adsorption, flocculation, photooxidation, membrane filtration, and electrokinetic coagulation (Ghaly *et al.,* 2014; Yaseen and Scholz, 2018). Biological treatments employ microbes such as bacteria and fungi (Khan *et al.,* 2013; Imran, 2015) and have been proposed as a cheaper and less environmentally intrusive alternative for dye wastewater treatment. Fungi are known to decolorize textile dyes particularly white rot fungi (Suwannawong *et al.,* 2010; Kumari *et al.,* 2019; Singh *et al.,* 2020; Bhuvaneswari *et al.,* 2020)

Fungal endophytes are microorganisms that grow among cells within the living tissues of host plants. Unlike pathogenic fungi, they do not cause disease upon colonization of host plant tissues; instead, they interact symbiotically with the host plant (Petrini, 1991; Sun *et al.,* 2012). As mutualistic associates of plants, fungal endophytes produce secondary metabolites with biological activities (Schulz *et al.,* 2002) that allow them to adapt to unfavorable environments (Kumaresan and Suryanarayanan, 2002) and may also produce enzymes that may be involved in tissue decomposition when the host plant dies (Pointing *et al.,* 1998; Jayasinghe *et al.,* 2008). Numerous plant species are hosts to endophytic fungi. Plant-associated endophytic fungal mycobiota are highly diverse, with one host plant harboring fungal endophyte assemblages consisting of more than 30 fungal species (Pimentel, 2011; Krishnamurthy and Naik, 2017).

Endophytic fungi have the potential to remove pollutants from soil, water, and substrates by close interactions with their host plants (Krishnamurthy and Naik, 2017). However, few studies have been carried out to determine the ability of endophytic fungi to decolorize synthetic dyes, especially mangrove-associated ones.

This study aimed to evaluate the potential of fungal endophytes associated with the leaves of *Avicennia marina* to decolorize the synthetic textile dyes: Congo red (azo), Malachite green (triphenylmethane), Methylene blue (heterocyclic), and Rhodamine B (fluorine).

2. Materials and Methods

2.1 Collection of mangrove leaves and Isolation of Endophytic Fungi

Avicennia marina plants were identified from a mangrove forest in Unisan, Quezon Province, Philippines (13° 50' 23.6508'' N, 121° 58' 26.796'' E). Healthy leaves were randomly collected from forty individual plants along a 10 x 10 m transect. Leaf samples were washed with distilled water and placed individually in sterile plastic bags. Collected leaf samples were stored at 40^0C and processed within 48 hours in the laboratory.

Four 6-mm discs were randomly cut from each leaf sample with a sterile cork borer and were surface sterilized using a standard triple sterilization technique described by Kumaresan and Suryanarayanan (2002). Each disc was plated on potato carrot agar (PCA) amended with seawater and 0.5 mL Streptomycin and incubated at room temperature for 2 - 4 weeks. Fungi growing from the leaf discs were isolated by hyphal tipping.

2.2 Identification of Endophytic Fungi

Microscopic examination of fungal isolates was conducted following the procedure by Riddell (Webber and Pitt, 2000), and this included the spore morphology, color, shape, wall ornamentation or texture, sizes, conidial formation, and other relevant characteristics such as phialide and conidiation pattern in addition to descriptions of colony morphology (colony surface, hyphal pigments, growth rates and exudates, among others. Similar isolates were grouped into morphotypes. Identification of fungi, at least up to the genus level, was based on Frölich and Hyde (2000), Domsch *et al.* (2007), Pitt and Hocking (2009), Watanabe (2010), and Campbell *et al.* (2013). Other online keys and databases were also consulted.

2.3 Solid Phase Decolorization Assay

The ability of mangrove-associated fungal endophytes to decolorize dyes was tested on four aromatic dyes: Congo Red CR, Malachite Green MG, Methylene Blue MB, and Rhodamine RB in solid media. A 5-mm agar disc containing mycelia from each fungal isolate was placed on the center of potato dextrose agar (PDA) plates added with 500 mg/L of individual dyes. A fungus known as an effective dye decolorizer, the white rot fungus *Trametes versicolor* (Amaral *et al.,* 2004; Singh *et al.,* 2020), was used as the positive control. Uninoculated PDA plates containing each of the dyes were used as the negative control. Plates were incubated at 25^0C for 10 days, after which fungal growth was determined by measuring colony diameter using a Vernier caliper. Each test was replicated three times. Dye decolorization activity was assessed visually.

2.4 Liquid Phase Decolorization Assay

Liquid phase decolorization assay was carried out following the method of Jayasinghe *et al.* (2008). Briefly, five 5-mm agar plugs of mycelia (acquired from the edge of actively growing mycelia on agar plates) were inoculated into 100 ml potato dextrose broth (PDB) medium containing 100 mg/L of individual dye followed by incubation at 25^0C for 10 days. Negative controls consisted of uninoculated PDB with dye. Each day a 2-ml aliquot was taken and centrifuged for 2 min at 4000 rpm before spectrophotometric measurement of absorbance with APEL PD-303 UV Spectrophotometer (APEL Co., Ltd., Saitama, Japan). Dye decolorization was calculated using the formula:

$$\textbf{Decolorization rate (\%)} = (A_0 - A) \times 100/A_0$$

where A_0 is the initial absorbance and A is maximum absorbance at the current time. Triplicates were made per experimental set up. Student's t-test was used to compare the decolorization rates of isolated endophytic fungi with the positive control.

2.5 Ligninolytic Enzyme Assay

Each fungal endophyte was grown in 20% PDB supplemented with 1% glucose and 1% naphthalene for 10 days at room temperature. *Trametes versicolor* cultured in the same medium was used as the positive control. After 10 days, the liquid culture was filtered through Whatman No. 2 filter paper to remove the fungal mycelia from the supernatant (Jayasinghe *et al.,* 2008). Laccase activity in the supernatant was assayed based on the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). A 100-µl of the culture supernatant was added to 5 mM. ABTS substrate in 0.1 M sodium acetate buffer (pH 5.0). The mixture was incubated at 30^0C for 2 min, followed by absorbance measurement at 420 nm (Bourbonnais *et al.,* 1995). As the negative control, ABTS solution minus the supernatant was used.

Lignin peroxidase (LiP) activity was determined in a reaction mixture consisting of 40 µl of the culture supernatant and 2.2 ml of sodium tartrate buffer (50mM, pH 4 at 25^0C) into which 20 µl of H^2O^2 (0.2mM) was added. The absorbance at 310 nm was measured immediately (Tien and Kirk, 1983).

To determine the manganese peroxidase (MnP) activity, 200 µl of culture supernatant was added to 2.5 ml substrate. The reaction was initiated with the addition of 0.1 mM H_2O_2 to the mixture. After an incubation period of 2 min at 30^0C, the reaction was stopped by the addition of 5M NaOH, followed by the measurement of absorbance at 610 nm (Gold *et al.,* 1988).

One enzymatic unit of ligninolytic enzyme activity was defined as the quantity of enzyme that produced 1µmol of oxidized product (Jayasinghe *et al.,* 2008).

3. Results and Discussion

3.1 Identification of Endophytic Fungi

Fifty-five fungal isolates were obtained from the leaves of *A. marina* and were determined to belong to five genera, including *Aspergillus* and *Penicillium*, along with mycelia sterilia (Table 1, Figure 1).

They are all anamorphic stages. Of these, *Colletotrichum lindemuthianum* showed the highest frequency of occurrence.

Ananda and Sridhar (2002) reported 13 endophytes isolated from the roots of *A. marina* mangrove having the second-highest species richness next to *Rhizophora mucronata*. *Aspergillus* species has the highest frequency of 53.3%. Wacira *et al.* (2020) isolated and identified 76 fungal isolates in five mangrove species along the Kenyan coastal areas, with most species isolated from *Avicennia marina.* In the Red Sea mangrove forest, 21 endophytic fungi were isolated belonging to 7 genera (Shebany, 2012), while 13 taxa were isolated from *Avicennia shaueriana* leaves from Northeastern Brazil (Wanderley Costa *et al.,* 2012).

Table 1. Characteristics of the 9 fungal endophytes isolated from leaves of *Avicennia marina*

	Macroscopic characteristics	**Microscopic characteristics**
Aspergillus flavus	Colony color: yellowish-green, coarsely roughen, fast-growing	conidiophores- rough, yellowish; vesicle- globose, biseriate; conidia- globose, rough, average size 3.45 µm diameter
Aspergillus niger	Colony: obverse – black; reverse - colorless; coarsely roughen	Vesicle - globose, dark brown; metulae and phialides present (biseriate); Conidiophores - light brown, smooth, long; Conidia - rough, spinulose, brown, average size 4.21µm diameter
Aspergillus* sp., Sect. *Clavati	Colony color: yellowish green (young) to pale brown (old) culture; rough	Conidiophores - smooth, pale brown, erect; Conidia - rough, globose, pale green, average size 2.87 µm diam. Conidial head - clavate to subglobose, uniseriate
***Basitospora rubra* Cole & Kendrick**	Colony color: pale brown with black spots with aerial hyphae	Conidiophores - pale brown not well differentiated with hyphae; Conidia - in a chain (aleuriosporous) which developed basipetally, smooth, globose to subglobose, average size 9.25 µm diameter
***Colletotrichum lindemuthianum* (Sacchardo et Magnus)**	Colony: obverse - dirty white with scattered hints of brown (pale brown); reverse - colorless	Conidiophore - hyaline, smooth; Conidia - cylindrical, hyaline, 1-celled, rough, average size 15.7 x 4.8 µm diameter, attached to the conidiophore
Colletotrichum sp.	Colony color: light brown, somewhat radiating	Conidiophore - hyaline to pale brown; Conidia - pale brown, 1-celled, phialosporous, attached to phialide
Mycelia sterilia	Colony color: white mycelia colony, cottony	Hyphae- hyaline; no conidia present
Penicillium citrinum	Colony color: grey to green with narrow white margin, finely roughen, restricted growth	Conidiophore - smooth, light green, biverticilliate; Conidia -small, smooth, average size 2.61 µm
Syncephalastrum racemosum	Colony color: dark grey, fast growing, finely roughen	Sporangiophores - hyaline, erect, with rhizoids irregularly branched; Vesicle - globose with rod-like merosporangia; merosporangia - globose, smooth, brownish, average size 3.26 µm diameter

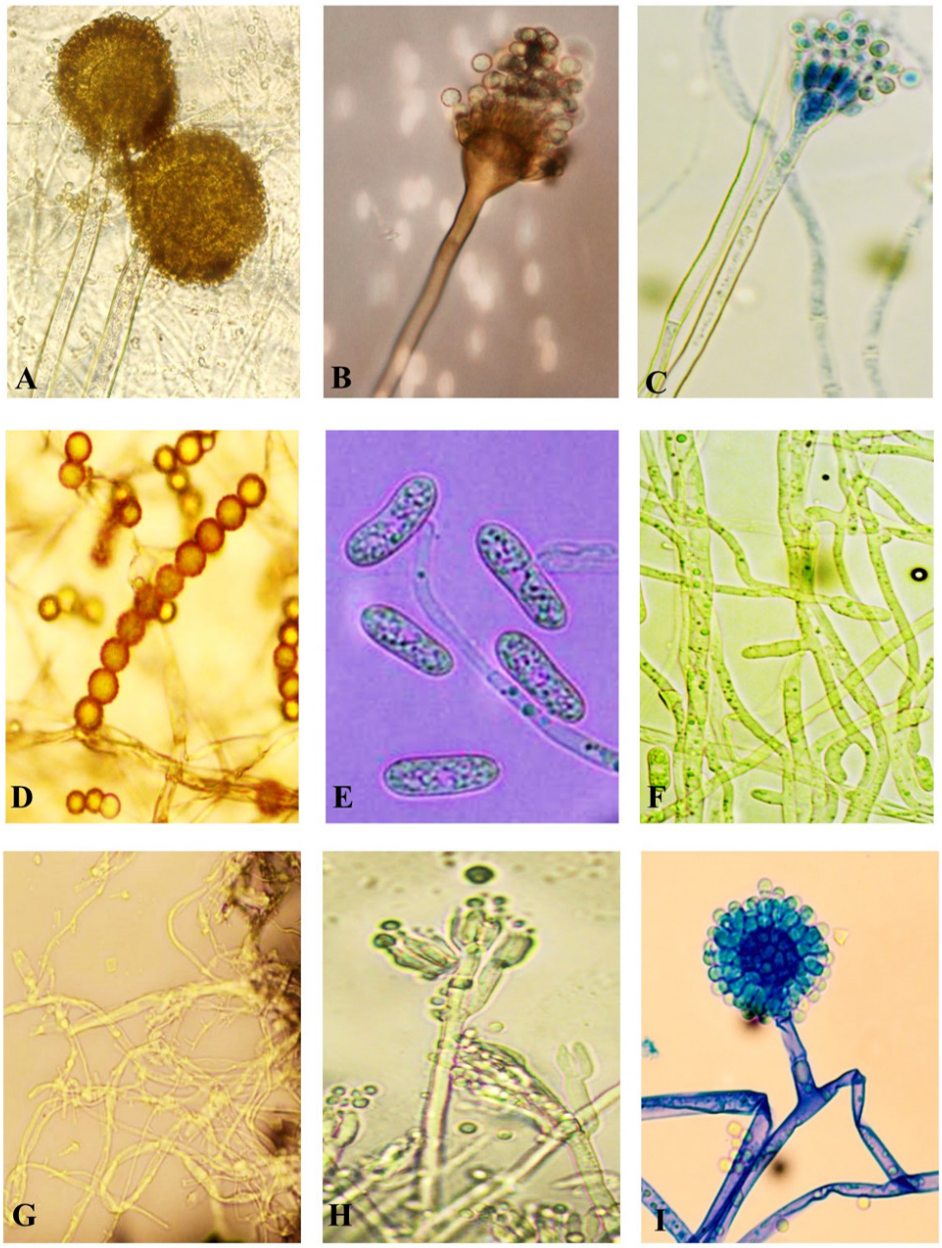

Figure 1. Photomicrographs of isolated fungal endophytes from *A. marina* leaves at 40x magnification (A- *Aspergillus flavus;* B- *Aspergillus niger;* C- *Aspergillus sp;* D- *Basitospora rubra;* E- *Colletotrichum lindemuthianum;* F- *Colletotrichum sp;* G- Mycelia sterilia; H- *Penicillium citrinum;* I- *Syncephalastrum racemosum)*

3.2 Solid Phase Decolorization

Fungi are known to degrade aromatic compounds during secondary metabolism (Singh *et al.,* 2020; Bhuvaneswari *et al.,* 2020); hence the ability of mangrove-associated endophytic fungi to decolorize aromatic dyes in solid media was evaluated. Fading or disappearance of the dye from the solid media indicated biosorption by fungal mycelia. Of the fungal endophytes evaluated for ability to decolorize, species *Aspergillus niger* exhibited the highest adsorption of all dyes. All the fungal endophytes tested showed biosorptive capacities for Congo red. A similar result was observed by Bosco *et al.* (2016) in CR decolorization using *Phanerochaete chrysosporium* fungus. Singh and Singh (2010) reported that *Aspergillus flavus* decolorized CR as indicated by the change and disappearance of red color in the solid medium and the adsorption of dye.

A. niger, Aspergillus sp., S. racemosum and mycelia sterilia adsorbed Malachite green despite its cytotoxic and antifungal properties (Sudova *et al.,* 2007).

A. niger, S. racemosum P. citrinum and *A. flavus* were able to adsorb Methylene blue. These results are promising because MB has high stability, hence it is difficult to treat (Jian-xiao *et al.,* 2011).

Dye uptake of Rhodamine B was observed in *A. niger, S. racemosum, P. citrinum* and *A. flavus.* In the study of Itoh and Yatome (2004) RB was not decolorized by *Coriolus versicolor* fungus, which may be due to the dye's toxicity and rigid chemical structure.

Dye uptake and direct visual assessment of fungal strains were observed in the dye decolorization studies of Erum and Ahmed (2011) and Yesilada *et al.* (2002). Biosorption and bioaccumulation of industrial dyes are regarded as two methods for biodegradation of industrial dyes by fungi. (Bhuvaneswari *et al.,* 2020). Adsorption of dyes to microbial cell surface is the primary mechanism of decolorization followed by microbial metabolism (Knapp, 1995; Yesilada *et al.,* 2002). Although decolorization of dyes only demonstrates the transformation of the chromophoric group of a dye, it does not reveal the mode of degradation of the dye molecules (Hardin *et al.,* 2003).

A. niger, S. racemosum and *P. citrinum* exhibited higher mycelial growth compared with other endophytic fungi, including the positive control *T. versicolor* in all solid media. The smallest colony diameters were shown by *Colletotrichum spp.* and *A. flavus* in Congo red and Malachite green media, respectively, whereas *Basitospora rubra* showed the smallest colony diameter in Methylene blue and Rhodamine B media (Figure 2).

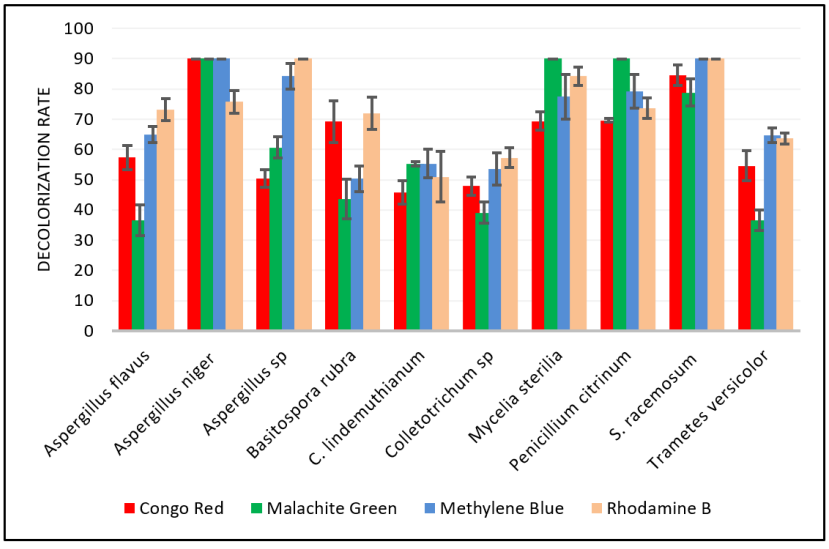

Figure 2. Colony diameters of the isolated fungal endophytes in different dyes.

3.3 Liquid Phase Decolorization

A total of 21 endophytic fungi that showed dye decolorization in solid media were further tested for the ability to decolorize in liquid media. Of the nine isolates that showed the ability to decolorize Congo red, *C. lindemuthianum* exhibited the highest decolorization rate (Table 2). The result showing that all endophytes tested achieved higher decolorization rates (> 89%) compared to the positive control *T. versicolor* (83.38%) suggests that massive potential of these *A. marina* associated fungal endophytes in the biodegradation of Congo red. Ado *et al.* (2018) reported 75% decolorization efficiency of CR by *Trametes sp.* after 72 hours.

Most studies on liquid decolorization of synthetic textile dyes were done using white rot fungi, which are known to completely decolorize azo dyes including Congo Red. Jayasinghe *et al.* (2008) reported that a 90% decolorization rate of Congo red in aqueous assays was observed in *Pycnoporus cinnabrius, Naematoloma fasciculare, Ganoderma lucidum* and *Pycnoporus coccineus*. Yang *et al.* (2009) reported that CR was nearly completely degraded by Trametes sp. after 6 days of incubation in liquid medium, while Ramsay and Nguyen (2002) observed that CR was completely decolorized by *Trametes versicolor* in about 22 hours. In other studies, CR was decolorized by *Irpex lacteus* by 58% (Novotny *et al.,* 2001) and *Aspergillus flavus* by 33.33% (Singh and Singh, 2010), which were lower compared to the isolated *A. marina* leaf endophytes.

Four isolates found to have decolorization activity in the solid assay were tested in liquid decolorization assay for Malachite green, Methylene Blue, and Rhodamine B (Table 2).

For Malachite green, all fungal endophytes used showed the ability to decolorize. Mycelia sterilia and *Aspergillus sp.* achieved higher than 50% decolorization rates while only mycelia sterilia showed a decolorization rate higher than that of *T. versicolor* ($p < 0.05$). Ali *et al.* (2009) observed higher MG degradation rates in *Aspergillus flavus* and *A. solani* having 97.43% and 96.91%, respectively. However, Jayasinghe *et al.* (2008) reported no MG decolorization among the white rot fungi they tested except for *F. fomentarius.* The low MG decolorization rate results may be due to dye toxicity (Yang *et al.,* 2009).

In the test for MB decolorization, all the fungal endophytes tested showed the ability to decolorize MB, with *P. citrinum* and *S. racemosum* exhibiting the highest decolorization rates. The decolorization rates for MB of *P. citrinum* and *S. racemosum* were higher than the positive control *T. versicolor* ($p < 0.05$). In an observation time of 20 days, Jayasinghe *et al.* (2008) observed 80% MB degradation by *P. cinnabarinus* and *G. lucidum* and only 20% degradation of *Pleurotus pulmonaris* and *Fomitopsis rosea.* No MB decolorization was observed in liquid media with *Pleurotus ostreatus* (Novotny *et al.,* 2001).

For RB dye, the endophytic fungi exhibited decolorization rates lower than 50%. *A. flavus* showed the highest decolorization rate higher than that of the positive control ($p < 0.05$). Suwannawong *et al.* (2010) reported 90% RB decolorization in less than three days by the laccase from *Lentinus polychrous* fungus. Decreased decolorization rates may be attributed to the reaction of the dyes with the enzymes secreted by the fungal endophytes (Jayasinghe *et al.,* 2008).

3.4 Assays for Ligninolytic Enzymes

Biodegradation of industrial dyes by fungi involved the utilization of ligninolytic enzymes. These enzymes cause various complex reactions, including alcohol oxidation, side chain cleavage, and demethylation to convert dyes to non-toxic forms (Singh *et al.,* 2020). Activities of the fungal oxidative enzymes laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) constitute the decolorization process of endophytes.

The deep green color in the assay using ABTS (Ruqayyah *et al.,* 2011) and absorbance values considered positive for laccase production indicated that all fungal endophytes (except for *A. niger*) isolated from the leaves of *A. marina* produce laccase. Only *S. racemosum, B. rubra, P. citrinum* and mycelia sterilia showed the ability to produce MnP, whereas only *C. lindemuthianum, A. niger, Aspergillus sp.,* and *Colletrotrichum sp.* could produce LiP.

Table 2. Decolorization rates of *A. marina* leaf endophytes in liquid dye media.

Fungal endophyte	Decolorization rates (%)			
	Congo Red	Malachite Green	Methylene Blue	Rhodamine B
Aspergillus flavus	95.02 ± 1.35	-	36.76 ± 3.49	44.92 ± 1.46
Aspergillus niger	90.46 ± 2.58	37.28 ± 4.92	42.17 ± 2.99	15.90 ± 0.00
Aspergillus sp.	94.40 ± 2.11	59.05 ± 1.98	-	-
Basitospora rubra	89.56 ± 4.58	-	-	-
Colletotrichum lindemuthianum	99.51 ± 0.28	-	-	-
Colletotrichum sp.	92.82 ± 0.90	-	-	-
Mycelia sterilia	92.93 ± 1.23	85.19 ± 0.43	-	-
Penicillium citrinum	96.08 ± 2.61	-	55.45 ± 5.84	13.95 ± 3.39
Syncephalastrum racemosum	93.05 ± 1.11	33.07 ± 3.24	50.32 ± 2.55	15.90 ± 0.00
Trametes versicolor	83.38 ± 3.22	75.00 ± 0.72	48.08 ± 3.47	10.03 ± 0.00

Table 3. Enzyme Activity by *A. marina* leaf endophytes.

Fungal Endophyte	Enzyme Activity (Uml^{-1})		
	Laccase	Lignin Peroxidase	Manganese Peroxidase
Aspergillus niger	0.270	0.360	0.136
Aspergillus sp.	0.430	0.360	0.130
Basitospora rubra	0.810	0.190	0.149
Colletotrichum lindemuthianum	0.520	0.360	0.131
Colletotrichum sp.	0.520	0.360	0.133
Mycelia sterilia	0.460	0.354	0.204
Penicillium citrinum	0.780	0.196	0.151
Syncephalastrum racemosum	0.950	0.237	0.150
Trametes versicolor	0.420	0.360	0.324

Of the eight fungal endophytes that produced laccase, *S. racemosum* exhibited the highest laccase activity. Most of the endophytes exceeded laccase activity of *T. versicolor.* The assay for LiP activity showed that *C. lindemuthianum, Aspergillus spp*. and *Colletotrichum sp*. have LiP activity levels comparable to that of *T. versicolor.* Mycelia sterilia gave the highest level of MnP activity of all the endophytes tested while showing lower MnP activity compared to the positive control *T. versicolor* (Table 3).

Production and the utilization of the laccase, LiP and MnP enzymes in dye decolorization were different for every fungal species (Wang *et al.,* 2017). LiP and MnP were generated more than laccase enzyme by endophyte *Bjerkandera adusta* for triphenylmethane dye decolorization with the high activity found in Malachite green decolorization (Gao *et al.,* 2020). Manganese peroxidases were also found to be more common among white rot fungi than lignin peroxidases. The lower enzymatic activity of LiPs may be due to their ability to catalyze the oxidation of non-phenolic aromatic compounds (Hofrichter, 2002).

Ligninolytic enzymes by various fungi, especially white rot fungi, were associated with decolorization activities.

Laccases, LiP, and MnP from *Peyronellaea prosopidis* degraded scarlet RR dye (Bankole *et al.,* 2018), while laccases and MnP produced by *Trametes hispida* and *Pleurotus ostreatus* were correlated with industrial dye decolorization (Rodriguez *et al.,* 1999). Laccases from *Trametes versicolor, Aspergillus oryzae,* and *Paraconiothyrium variabile* have decolorized Congo red, Remazol brilliant blue, Bromophenol blue, Methylene blue and Malachite green (Foroontanfar *et al.,* 2012; Ado *et al.,* 2018). *T. hirsuta* and *Sclerotium rolfsii* laccases degraded indigo textile dye (Campos *et al.,* 2001) while *Irplex lacteus* manganese peroxide was able to degrade Malachite green dye (Duan *et al.,* 2018).

4. Conclusion

This study showed that endophytic fungi associated with the leaves of the Philippine mangrove *A. marina* have the ability to decolorize synthetic dyes, which could be attributed to their production of ligninolytic enzymes. These enzymes, laccase exhibited the highest activity. These results suggest that besides white rot fungi, fungal endophytes from *A. marina* and most likely from other plants are capable of decolorizing synthetic dyes. Thus, isolation and evaluation of other potential dye decolorizing fungi from other plant species are highly recommended. These endophytic fungi have the biotechnological potential to bioremediate effluents containing dyes and should be further developed for such a purpose. The specific mechanisms underlying dye decolorization by endophytic fungi also need further studies.

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