Exploring *Diaporthe perseae* Isolated from *Monoon longifolium*

Afifa Shaikh*[a], Priyanka Mahanty [b]

a.B. Ed Student, St. Teresa's Institute of Education, Mumbai, Maharashtra, India,

b.Assistant Professor, Department of Environmental Science, SIES College of Arts, Science and Commerce, Mumbai, Maharashtra, India.

shaikhafifa427@gmail.com.

ABSTRACT:

Endophytic fungi, residing within living plant tissues, play a pivotal role in enhancing plant resilience to both abiotic stressors and biotic threats. These fungi also yield a diverse array of novel compounds and secondary metabolites, showcasing antimicrobial, anti-inflammatory, and anti-parasitic properties. In this study, we focused on *Monoon longifolia*, a commonly found avenue tree renowned for its therapeutic uses, to isolate endophytes. One such successfully isolated endophyte, coded as Plen23 and later identified as *Diaporthe perseae*, was subjected to fermentation under two distinct conditions—static and rotatory shaker. Notably, the rotatory shaker method yielded a higher quantity of secondary metabolites. *Diaporthe perseae* demonstrated robust antimicrobial activity against *Escherichia coli*. Additionally, the presence of Phyto-constituents, including Glycosides, flavonoids, alkaloids, and saponins, was identified. This study underscores the vast potential for exploring the intricate relationship between plants and their endophytes, as well as the broader implications of endophytes within various environmental contexts."

KEYWORDS: Endophytic fungi, *Monoon longifolium*, fermentation, secondary metabolites, antimicrobial activity

INTRODUCTION

The term "endophytes" was introduced by Scientist De Bary in 1866 to denote microorganisms residing within plant tissues. Endophytic fungi, a remarkable class of organisms, display the unique ability to thrive within plants without inducing adverse effects on the plant tissue. Ubiquitous across diverse plant species, endophytes play pivotal roles throughout the plant life cycle, contributing to plant adaptation by aiding in the management of both biotic and abiotic stressors. This assistance is facilitated through the production of bioactive substances, encompassing enzymes, phytohormones, nutrients, and minerals, thereby establishing symbiotic relationships with plants (Patil et al., 2016).

Polyalthia longifolia, also known as *Monoon longifolium*, represents an evergreen tree extensively cultivated throughout India, commonly referred to as False Ashoka. Achieving heights of 15 to 20 meters, this evergreen species serves as a prevalent avenue tree in India, possessing notable medicinal properties. Widely utilized in traditional medicine, the bark decoction of *Monoon longifolium* treats ailments such as rheumatism, scorpion stings, diabetes, and other conditions. Furthermore, the plant is employed for addressing issues like mouth ulcers, constipation, and urinary disorders, and exhibits antipyretic activity. Traditional uses extend to the regulation of blood pressure, stimulation of respiration, and alleviation of

conditions such as uterus ailments, leucorrhoea, and Menorrhagia. Beyond its febrifuge properties, *Monoon longifolium* is also believed to mitigate skin diseases, hypertension, and helminthiases, among others (Jothy *et al*., 2013).

Diaporthe, a significant fungal genus classified in the family Diaporthaceae, order Diaporthales, and class Sordariomycetes, holds importance as a plant pathogen. Isolated predominantly from plant hosts globally, many of these strains have been identified as plant pathogens, nonpathogenic endophytes, or saprobes. Additionally, some *Diaporthe* species also pose potential threats as human and other mammalian pathogens.
Diaporthe species is known to generate a variety of secondary metabolites, such as terpenoids, fatty acids, polyketides, steroids, and alkaloids. These diverse compounds display a broad spectrum of biological effects, including cytotoxic, antifungal, antibacterial, antiviral, antioxidant, anti-inflammatory, and phytotoxic activities.

MATERIALS AND METHODS

Sample collection: Fresh leaves of *Monoon longifolium* were collected from SIES College of Arts, Science, and Commerce, Mumbai. The sample comprised mature and healthy leaf specimens, carefully placed in a plastic bag and transported to the laboratory. The leaves were authenticated, followed by surface sterilization and the isolation of endophytic fungi. (Ujam *et al.,* 2020).

Surface Sterilization: Surface sterilization of the leaves was done to preclude unwanted microbial interference. Initially, the leaves were thoroughly washed under running tap water and rinsed with sterile distilled water through multiple cycles. The leaves were cut into small pieces approximately 1x2cm, and subjected to a sequential treatment involving 70% ethanol for 30 seconds, 2% sodium hypochlorite for 2 minutes, and an additional 30% ethanol for 2 minutes. Following this, leaves were rinsed 2-3 times with sterile distilled water and air-dried in aseptic conditions. The water from the final rinse was utilized to check the efficacy of surface sterilization. (Ujam *et al.,* 2020).

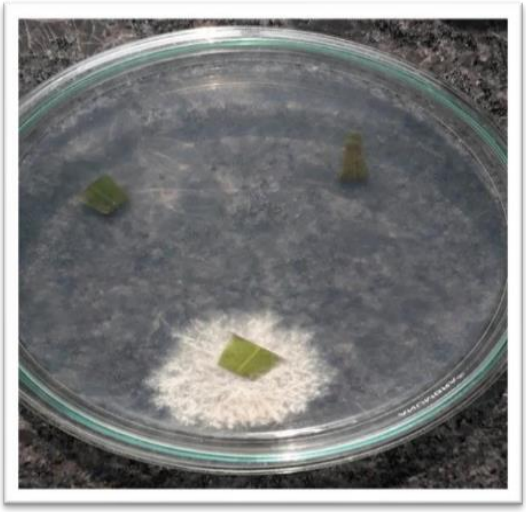


Fig:1 - Isolation of endophytic fungi (*Diaporthe perseae*) from the leaves of *Monoon longifolium.*

Isolation and identification of endophytic fungi:

After surface sterilization, the plant material was inoculated on Czapek Dox Agar (CDA) supplemented with ampicillin (100mL/L) to prevent the growth of unwanted bacteria. The petri plates were sealed with masking tapes and incubated at 25 ±1°C for 15-30 days. During the period of incubation, the petri plates were checked regularly for fungal growth. The fungal hyphal tips observed were then reinoculated to a fresh CDA medium to obtain pure cultures (fig: 2 & 3). The endophyte obtained was sent for identification to Agharkar Research Institute Ujam *et al.,* 2020).

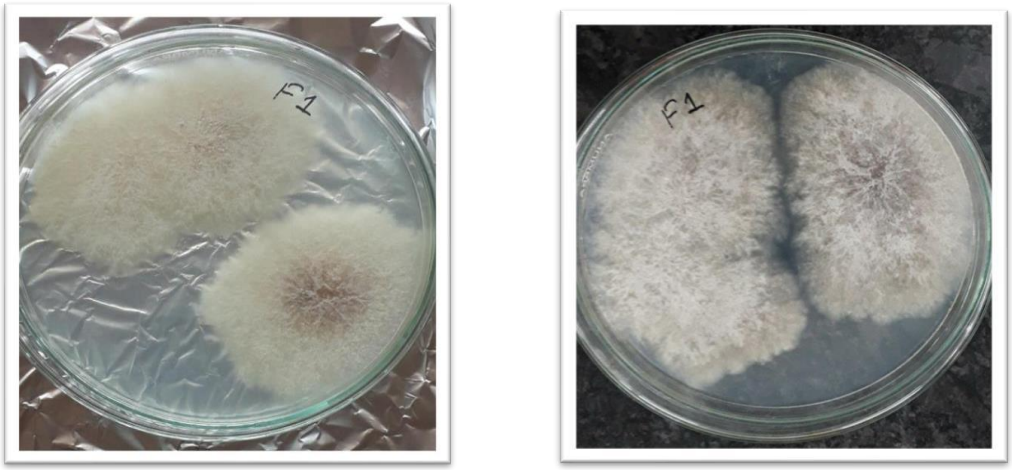


Fig: 2 & 3 - Subsequent culturing

Fig 4- Inoculation of Czapek Dox broth with fungal hyphae

Fermentation:

For the fermentation process, six Erlenmeyer flasks (150 mL) containing 25 mL of Czapek Dox Broth (CDB) were autoclaved at 121°C for 15 minutes. Mycelial plugs were inoculated in these flasks and then kept for incubation for 15 days. Among these, three flasks remained in a stationary state, while the remaining three were placed on a rotary shaker at 150 rpm,

facilitating a comparative assessment of secondary metabolite production under each condition. Following a 15-day incubation period, the fermentation broths were filtered through two layers of muslin cloth, and subsequent extraction procedures were employed for the isolation of secondary metabolites (Sadrati *et al*. 2013).

Extraction of secondary metabolites:
The filtered liquid culture was mixed with ethyl acetate in a ratio of 1:4 (filtered liquid: ethyl acetate) in a separating funnel. The separating funnel was shaken vigorously for about 5 min and then kept steady for separation. Once it got separated, the upper organic phase was removed and the above extraction procedure was repeated 5 times. The obtained organic extracts from the stationary flasks and rotary flasks were measured. This organic solution was concentrated to dryness using a water bath and further subjected to phytochemical screening (Yin *et al.,* 2010).

Phytochemical screening: (Watal *et al.,* 2014)
Following tests were performed to confirm the presence of secondary metabolites.

1.	Test for alkaloids (Hager's Test)	Few ml filtrate + 1-2 ml drangendroff reagent
2.	Test for flavonoid	1ml extract + 1ml Pb $(OAc)_4$ (10%)
3.	Test for tannins (Braymer's Test)	2ml extract + 2ml H_2O + 2-3 drops of $FeCl_3$ (5%)
4.	Test for saponins (Foam Test)	5ml extract + 5ml H_2O + heat
5.	Test for steroids (Salkowski Test)	2ml extract + 2ml $CHCl_3$ + 2ml H_2SO_4 (conc.)
6.	Test for glycoside (Liebermann's Test)	2ml extract + 2ml $CHCl_3$+ 2ml CH_3COOH

Antimicrobial activity
The antimicrobial activity of the secondary metabolites was tested using the disc diffusion method. Sterile nutrient agar plates were prepared and inoculated with the test organism (*E. coli*). The sterile disc was saturated with the extract and placed on the agar plate. The plates were incubated at 37° C for 24 hours. Discs saturated with ampicillin were also placed carefully, as a reference antibiotic. After 24 hours the zone of inhibition was observed (Ananda K. *et al.,* 2012).

RESULTS
An endophytic fungus was obtained from *Monoon longifolium,* identified as *Diaporthe perseae.* The fungus was subjected to fermentation in which the flasks were kept in two different conditions. It was observed that the production of secondary metabolites was 2.7 grams in the flasks kept on the rotary device whereas 1.3 grams of secondary metabolites were produced in flasks kept in stationary conditions (as shown in table 1). The extracted secondary metabolites were screened for phytochemicals such as tannins, alkaloids, flavonoids, saponins, glycosides, and steroids. The presence of glycoside, alkaloids, flavonoids, and saponin was observed (as shown in Table 2 and Fig 5). For antimicrobial activity ampicillin was used as a reference antibiotic, the zone of inhibition by secondary metabolites was close to the zone of inhibition by ampicillin (as shown in Table 3).

Flask No		Condition
	Static (in grams)	**Shaker (in grams)**
1.	0.445	0.670
2.	0.274	0..520
3.	0.588	1.542
Total	1.307	2.732

Table 1: Weight of secondary metabolites in static condition and on rotary shaker

Sr. No.	Phytoconstituents	Result
1.	Tannins	-
2.	Steroids	-
3.	Glycosides	+
4.	Flavonoids	+
5.	Alkaloids	+
6.	Saponins	+

Table 1: Results of Phytochemical Screening

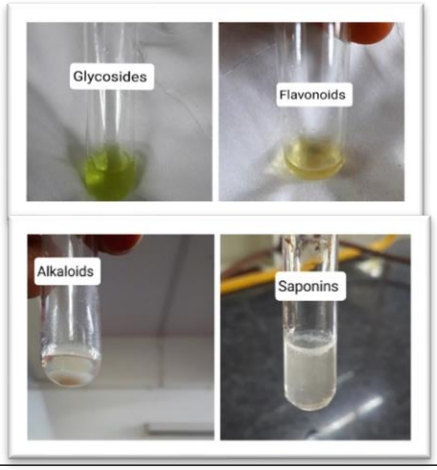
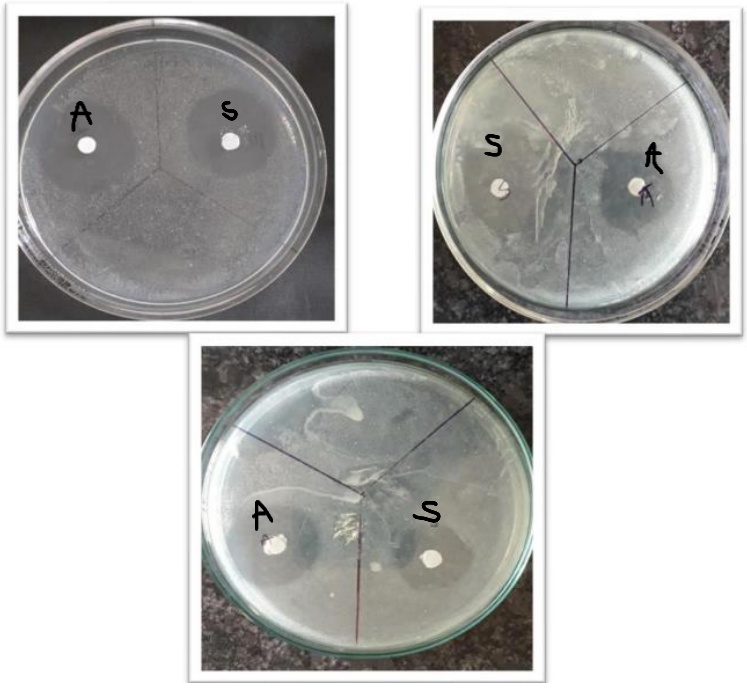


Fig 5: Results of phytochemical screening

Petriplate 1

Petriplate 2



Petriplate 3

Fig 6: Zone of inhibition

Petriplate no.	Zone of Inhibition	
	Secondary metabolites (S)	Ampicillin (A)
1.	27mm ± 0.25	28mm ± 0.25
2.	24mm ± 0.25	25mm ± 0.25
3.	21mm ± 0.25	21mm ± 0.25

Table 3: Antimicrobial activity

DISCUSSION

In the paper published by (Potshangbam *et al*.,2007) endophytes from *Oryza sativa L*. And *Zea mays L.* were isolated using various mediums and Czapek Dox agar was found to be the most suitable medium for endophytes. CDA was used to observe the appearance and growth of endophytic fungi. The endophyte was fermented by inoculating fungal mycelium in broth as mentioned in N. Saleem Basha *et al.,* 2012. As mentioned in the paper (Yin *et al.,* 2010), the culture medium after fermentation was filtered and mixed with ethyl acetate in a ratio of 1:4 (culture medium: ethyl acetate). The upper organic layer was subjected to phytochemical screening. In this study, the presence of Glycosides, Flavonoids, Alkaloids, Saponins, and the absence of steroids, and tannins were seen in secondary metabolites extracted from endophytic fungi using ethyl acetate. Concerning Thenmozhi *et al.,* 2010, the extracted secondary metabolites were subjected to antimicrobial activity against *E.coli* using the disc diffusion method. The zone of inhibition was observed and noted. The zone of inhibition exhibited by secondary metabolites was 25±0.33mm.

CONCLUSION

In this study, *Diaporthe perseae*, endophytic fungi were isolated from *Monoon longifolium*. It was fermented using Czapek Dox Broth and secondary metabolites were extracted using ethyl acetate. The extractions were subjected to phytochemical screening to see the presence of secondary metabolites. The presence of glycoside, alkaloids, flavonoids, and saponins was observed. The extracted secondary metabolites were also screened for their antimicrobial activity with ampicillin as the reference antibiotic, the zone of inhibition by secondary metabolites was almost the same as the zone of inhibition by ampicillin.

CONFLICT OF INTEREST

The authors declare there are no competing interests associated with the manuscript.

ACKNOWLEDGMENTS

We are extremely obliged to the Principal Dr. Uma Shankar and the Botany department of SIES College for providing me with laboratory and library facilities. We owe our deepest gratitude to the teaching staff and the non-teaching staff for all their guidance and assistance. We would also like to recognize the assistance that we received from the Agharkar Research Institute in the identification of the species.

REFERENCES

1. Akpotu, M. O., Eze, P. M., Abba, C. C., Nwachukwu, C. U., Okoye, F. B., & Esimone, C. O. (2017). Antimicrobial activities of secondary metabolites of endophytic fungi isolated from Catharanthus roseus. Journal of Health Sciences, 7(1), 15-22.
2. Basha, N. S., Ogbaghebriel, A., Yemane, K., & Zenebe, M. (2012). Isolation and screening of endophytic fungi from Eritrean traditional medicinal plant Terminalia brownii leaves for antimicrobial activity. International Journal of Green Pharmacy (IJGP), 6(1).
3. Chutulo, E. C., & Chalannavar, R. K. (2018). Endophytic mycoflora and their bioactive compounds from Azadirachta indica: a comprehensive review. Journal of Fungi, 4(2), 42.
4. Demeni, P. C. E., Betote, P. H. D., Kom, C. W., Tchamgoue, E. N., Ndedi Moni, E. D. F., Foumane Maniepi, J. S., ... & Nyegue, M. A. (2021). Endophytic Fungi from Alstonia boonei
5. Du, W., Yao, Z., Li, J., Sun, C., Xia, J., Wang, B., ... & Ren, L. (2020). Diversity and antimicrobial activity of endophytic fungi isolated from Securinega suffruticosa in the Yellow River Delta. PloS one, 15(3), e0229589.
6. Hsieh, P. C., Mau, J. L., & Huang, S. H. (2001). Antimicrobial effect of various combinations of plant extracts. Food Microbiology, 18(1), 35-43.
7. Jothy, S. L., Choong, Y. S., Saravanan, D., Deivanai, S., Latha, L. Y., Vijayarathna, S., & Sasidharan, S. (2013). Polyalthia longifolia Sonn: An ancient remedy to explore for novel therapeutic agents. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4(1), 714-730.
8. Larran, S., Monaco, C., & Alippi, H. E. (2001). Endophytic fungi in leaves of Lycopersiconesculentum Mill. World Journal of Microbiology and Biotechnology, 17(2), 181184.
9. Larran, S., Perelló, A., Simón, M. R., & Moreno, V. (2007). The endophytic fungi from wheat (Triticum aestivum L.). World Journal of Microbiology and Biotechnology, 23(4), 565-572.
10. Nuankaew, K., Sotome, K., Lumyong, S., & Boonlue, S. (2018). Trichoderma polyalthiae sp. nov., an endophytic fungus from Polyalthia debilis. Phytotaxa, 371(5), 273-282.
11. Patil, R. H., Patil, M. P., & Maheshwari, V. L. (2016). Bioactive secondary metabolites from endophytic fungi: a review of biotechnological production and their potential applications. Studies in natural products chemistry, 49, 189-205.
12. Pavithra, N., Sathish, L., & Ananda, K. (2012). Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi. Journal of Pharmaceutical and Biomedical Sciences (JPBMS), 16(16), 2014.
13. Pavithra, N., Sathish, L., & Ananda, K. (2012). Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi. Journal of Pharmaceutical and Biomedical Sciences (JPBMS), 16(16), 2014.
14. Phongpaichit, S., Rungjindamai, N., Rukachaisirikul, V., & Sakayaroj, J. (2006). Antimicrobial activity in cultures of endophytic fungi isolated from Garcinia species. FEMS Immunology & Medical Microbiology, 48(3), 367-372.

15. Potshangbam, M., Devi, S. I., Sahoo, D., & Strobel, G. A. (2017). Functional characterization of endophytic fungal community associated with Oryza sativa L. and Zea mays L. Frontiers in microbiology, 8, 325.
16. Rahman, S., Haque, I., Goswami, R. C., Barooah, P., Sood, K., & Choudhury, B. (2021). Characterization and FPLC analysis of garbage enzyme: biocatalytic and antimicrobial activity. Waste and Biomass Valorization, 12(1), 293-302.
17. Rana, K. L., Kour, D., Sheikh, I., Dhiman, A., Yadav, N., Yadav, A. N., ... & Saxena, A. K. (2019). Endophytic fungi: biodiversity, ecological significance, and potential industrial applications. In Recent advancement in white biotechnology through fungi (pp. 1-62). Springer, Cham.
18. Rodrigues, K. F., Hesse, M., & Werner, C. (2000). Antimicrobial activities of secondary metabolites produced by endophytic fungi from Spondias mombin. Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms, 40(4), 261-267.
19. Sadrati, N., Daoud, H., Zerroug, A., Dahamna, S., & Bouharati, S. (2013). Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (Triticum durum). Journal of plant protection research, 53(2).
20. Sandhu, S. S., Kumar, S., & Aharwal, R. P. (2014). Isolation and identification of endophytic fungi from Ricinus communis Linn. and their antibacterial activity. Int. J. Res. Pharm. Chem, 4(3), 611-618.
21. Schulz, B., Sucker, J., Aust, H. J., Krohn, K., Ludewig, K., Jones, P. G., & Döring, D. (1995). Biologically active secondary metabolites of endophytic Pezicula species. Mycological Research, 99(8), 1007-1015.
22. Shaikh, J. R., & Patil, M. K. (2020). Qualitative tests for preliminary phytochemical screening: An overview. International Journal of Chemical Studies, 8(2), 603-608.
23. Stone, J. K., Polishook, J. D., & White, J. F. (2004). Endophytic fungi. Biodiversity of fungi: inventory and monitoring methods, 241, 270.
24. Tendencia, E. A. (2004). Disk diffusion method. SEAFDEC. Aquaculture Department Japan, 14-29.
25. Thenmozhi, M., & Rajeshwari, S. (2010). Phytochemical analysis and antimicrobial activity of Polyalthia longifolia. Int J Pharm Bio Sci, 1(3), 6288-6299.
26. Torres, M. S., Tadych, M., White Jr, J. F., Bills, G. F., en Andalucía, I., & de Ciencias, P. T. Prospects and Applications for Plant-Associated Microbes. A laboratory Manual, Part B: Fungi" 2. Theory 2.1. Isolation and identification of fungal endophytes 2.2. Experimental procedure:"Dilution-to-extinction cultivation of endophytic fungi".
27. Ujam, N. T., Eze, P. M., Chukwunwejim, C. R., Okoye, F. B., & Esimone, C. O. (2019). Antimicrobial and immunomodulatory activities of secondary metabolites of an endophytic fungus isolated from Ageratum conyzoides. Current Life Sciences, 5(1), 19-27.
28. Varpe, B. D., & Kopargaon, A. M. (2020). Isolation and identification of phylloplane and endophytic fungi from Sapindus mukorossi. Flora and Fauna, 26, 218-220.

29. Yadav, M., Chatterji, S., Gupta, S. K., & Watal, G. (2014). Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm Sci, 6(5), 539-42.
30. Yin, G., Wang, W., Sha, S., Liu, L., & Yu, X. (2010). Inhibition and control effects of the ethyl acetate extract of Trichoderma harzianum fermented broth against Botrytis cinerea. African Journal of Microbiology Research, 4(15), 1647-1653.