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# DIVERSITY OF ENDOPHYTIC FUNGI FROM MEDICINAL PLANTS *Dysosma difformis* (HEMSL & E.H. WILSON) T.H. WANG COLLECTED IN HA GIANG AND LAI CHAU

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SUMMARY

Plant endophytes are an interesting group of microorganisms that colonize the internal tissues of living plants and do not cause any disease symptoms in the host plant. It exists in different parts of plants, such as roots, leaves, and stems, and significantly affects the formation of metabolic products in plants by promoting the accumulation of important secondary metabolites. The present study focused on analyzing the diversity and distribution of endophytic fungi related to different tissues from samples of the medicinal plant *Dysosma difformis* (Hemsl & E.H. Wilson) T.H. Wang collected in Ha Giang and Lai Chau, in which the isolates from roots were 27 strains (50.94%), 12 strains from stems (22.64%), and 14 strains from leaves (26.42%). Based on the isolates, we analyzed the fungal diversity through seven different diversity indices. The results showed that isolates' diversity was similar to the endophytic fungal population in tissues of *D. difformis* distributed in different vegetation in Vietnam. Specifically, Shannon's index showed the highest diversity in roots (H′ = 2.673), followed by stems (H′ = 2.162) and leaves (H′ = 2,054). Similarly, species richness was highest in roots (Dmg = 4,551; Dmn = 3,079) and stem (Dmg = 4,024; Dmn = 3,175) and lowest in leaves (Dmg = 3.41; Dmn = 2,405). However, the Simpson diversity index showed that the endophytic fungal community was most abundance in leaves (1-D = 0.911), followed by stems (1-D = 0.897) and roots (1-D = 0.867). In addition, the Sorensen index of 0.615 shows the average similarity in species composition between the two sites, Ha Giang and Lai Chau. This is the first report on the diversity of endophytic fungi isolated from *D. difformis*, paving a potential way for screening endogenous fungal strains capable of producing important secondary compounds.

**Keywords**: Endophytic fungi, podophyllotoxin, *Dysosma difformis*, diversity, biodiversity evaluation

## INTRODUCTION

*Dysosma difformis* (Hemsl & E.H. Wilson) T.H. Wang or *Podophyllum tonkinense* Gagnep is a rare medicinal plant in Vietnam and well-known for use in the treatment of sore throats, boils, snakebites, breast abscesses, and breast cancer (Bui *et al*., 2022). According to the Checklist of Plant Species of Vietnam, *D. difformis* is distributed mainly in the Northern provinces such as Lai Chau, Lao Cai, Yen Bai, Ha Giang, and Lang Son (Ban, 2003).

Endophytic fungi are defined as those intended for all or part of their life cycle to exist in the healthy tissues of the host plant, in various organs such as leaves, stems, roots, branches, etc., often without causing obvious disease symptoms. Evidence for plant-associated microorganisms detected in fossilized stem and leaf tissues indicates that endophytic microorganisms may have evolved from the time when higher plants first appeared on earth, hundreds of millions of years ago (Taylor *et al*., 2000). Recent studies have shown that endogenous fungi produce biologically active secondary metabolites, including phytohormones, antifungal, antibacterial, antiviral, antitumor, antidiabetic, insecticidal, immunosuppressive, and antidiabetic compounds, *etc.* (Andrea *et al*.,1993; Demain, 1999; Tan, Zou, 2001; Staniek *et al*., 2008). In addition, they can improve the host's growth and could be useful in agriculture (Murphy *et al*., 2014). To date, hundreds of plant species have been studied for endophytic fungi, and most of them have been shown to be rich in endophytic fungi (Schneider *et al*., 2008).

In a previous study, the endophytic fungal community of *D. difformis* (Hemsl & E.H. Wilson) T.H. Wang was isolated. Fifty-three endophytic fungal strains had been isolated and screened for the presence of podophyllotoxin (PTOX) exhibiting antitumor activity, which is also the chemical composition of the species *D. difformis* (Tran *et al*., 2022). However, evaluation of the fungal diversity indices has not been reported. In this study, the diversity of endophytic fungal isolates was for the first time assessed via the analysis of diversity indices: (1) to clarify the diversity of endophytic fungi in *D. difformis* in Vietnam; (2) to analyze changes in the composition and diversity of endophytic fungi in different organs of *D. difformis*. This provides a theoretical basis for using endophytic fungi as an alternative resource of *D. difformis* for producing podophyllotoxin.

## MATERIALS AND METHODS

### Fungal source

Fifty-three endogenous fungal strains isolated from *D. difformis* are currently kept at -20°C at the Laboratory of Plant Cell Biotechnology, Institute of Biotechnology, for the evaluation of diversity indexes.

### Fungal culture medium

Endophytic fungi were incubated at 25°C on Potato Glucose Agar (PDA) plates containing 20% (w/v) potatoes, 2% D-glucose, and 1.8% (w/v) agar for 1-2 weeks until fungal mycelia started growing. These growth fungi were further subcultured on new PDA plates to obtain pure hyphae. The fungal endophytes were then coded according to sample location and isolated plant parts (HG and LC encoding for samples from Ha Giang and Lai Chau).

**Table 1.** Endophytic fungi isolated from different tissues of *D. difformis* collected in Ha Giang and Lai Chau, Vietnam.

| Ha Giang | | | Lai Chau | | |
|---|---|---|---|---|---|
| **No.** | **Endophytic fungi** | **Tissues and organs** | **No.** | **Endophytic fungi** | **Tissues and organs** |
| 1 | HGN6.1R | Root | 29 | LCN9R | Root |
| 2 | HGN6.2R | Root | 30 | LCN21T | Stem |
| 3 | HGN13R | Root | 31 | LCN7L | Leaf |
| 4 | HGN1R | Root | 32 | LCN1R | Root |
| 5 | HGN8R | Root | 33 | LCN3L | Leaf |
| 6 | HGN11.1R | Root | 34 | LCN19T | Stem |
| 7 | HGN7.1R | Root | 35 | LCN8.3L | Leaf |
| 8 | HGN7R | Root | 36 | LCN1L | Leaf |
| 9 | HGN5L | Leaf | 37 | LCN6T | Stem |
| 10 | HGN2R | Root | 38 | LCN1T | Stem |
| 11 | HGN3R | Root | 39 | LCN8T | Stem |
| 12 | HGN7.2R | Root | 40 | LCN16L | Leaf |
| 13 | HGN14.1R | Root | 41 | LCN7R | Root |
| 14 | HGN6R | Root | 42 | LCN15L | Leaf |
| 15 | HGN12.1R | Root | 43 | LCN12.1L | Leaf |
| 16 | HGN13.1R | Root | 44 | LCN12T | Stem |
| 17 | HGN5R | Root | 45 | LCN5L | Leaf |
| 18 | HGN1T | Stem | 46 | LCN14L | Leaf |
| 19 | HGN14R | Root | 47 | LCN3T | Stem |
| 20 | HGN3L | Leaf | 48 | LCN13T | Stem |
| 21 | HGN1L | Leaf | 49 | LCN17T | Stem |
| 22 | HGN3T | Stem | 50 | LCN4T | Stem |
| 23 | HGN11R | Root | 51 | LCN11L | Leaf |
| 24 | HGN12.2R | Root | 52 | LCN13L | Leaf |
| 25 | HGN3.1R | Root | 53 | LCN3R | Root |
| 26 | HGN2.1R | Root | | | |
| 27 | HGN5.1R | Root | | | |
| 28 | HGN10R | Root | | | |

**Observation of morphological characteristics**

In order to observe the differences in the fungal mycelium, the PDA medium was prepared and poured into a petri dish to form a 0.5 mm-thick layer. After the medium had solidified, 1x1 cm pieces were cut from it and put on a slide in a separate sterile petri dish. Fungal strains were

cultivated along the edges of the media fragments. Subsequently, the sterile lamella was placed on the surface of the medium. The petri dishes were incubated at 25°C for 7-14 days. Mycelium, spores, sporophytes, and their arrangement were observed through microscopy at 100x magnification.

**Evaluation of endophytic fungal diversity**

The diversity of endophytic fungi associated with *D. difformis* was analyzed using different indices such as Simpson's dominance index (D), Simpson's diversity index (1- D), the Shannon diversity index (H'), Margalef's richness index (Dmg), Menhinick's richness index (Dmn), Camargo's dominance index (1/Dmn), and Pielou evenness index (P). The similarity index for fungal endophytic assemblages among tested tissues and sampling locations were also evaluated using Sorensen's index (S) (Kusari *et al.*, 2013; Shah, Pandit, 2013; Katoch *et al*., 2017; Kanieski *et al*., 2018).

**Table 2.** Indices for biodiversity evaluation.

| | |
|---|---|
| Simpson dominance index (Do) | $\mathbf{Do} = \sum_{k=1}^{S} \mathbf{p_k}^2$ |
| Simpson diversity index (Di) | **Di = 1 - Do** |
| Shannon diversity index (H') | $\mathbf{H'} = -\sum_{k=1}^{S} (\boldsymbol{p_k ln p_k})$ |
| Margalef richness index (Dmg) | Dmg = (S-1)/lnN |
| Menhinick richness index (Dmn) | Dmn = S/√N |
| Camargo dominance index (1/Dmn) | **C = 1/Dmn** |
| Pielou evenness index (P) | P = H'/Hmax |
| Sorensen index(S) | $S = \frac{2c}{a+b}$ |

*S = total number of genera; N = total number of individuals; $p_k$ = proportion of genera in a community ($p_k = N_k/N$); Hmax=lnS; c = number of genera found in both places; a = number of genera found solely in site A; b = number of genera found solely in site B.*

## RESULTS AND DISCUSSION

**Morphological diversity of endophytic fungi**

The diverse morphology of 53 endophytic fungal strains was observed during their growth on PDA (Tran *et al.*, 2022). They are also examined microscopically to determine the structure of mycelium, spores, sporophytes, and their arrangement.

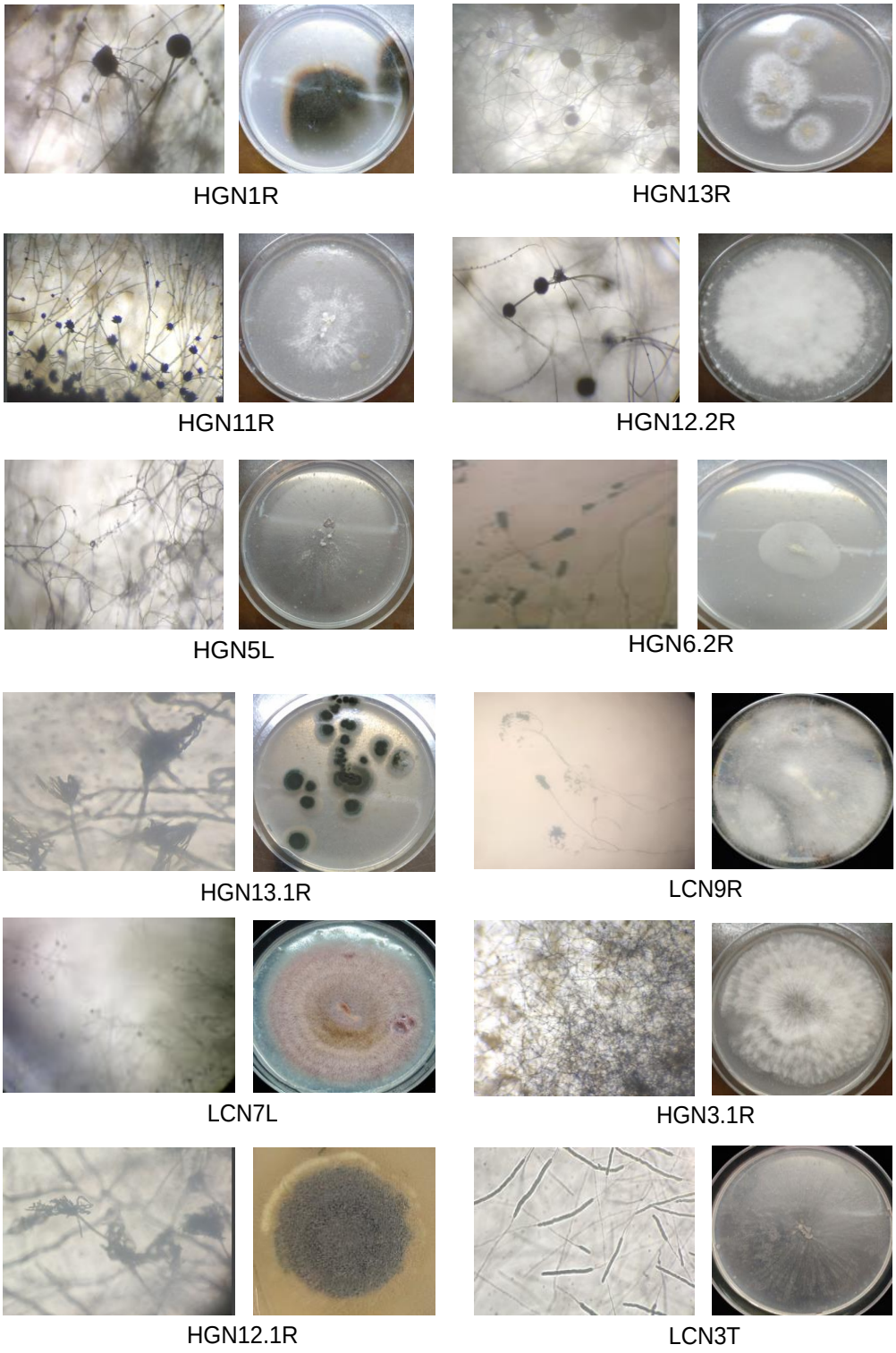


**Figure 1.** Colony and microscopically morphology of endophytes isolated from *D. difformis*

In general, the diversity of endophytic fungi isolated in the two locations clearly indicated the adaptation of the isolates to the host plant. The number of fungi obtained depended on both certain plants and plant tissues. The highest rate of isolates was in the roots, which accounted for 50.94% of total isolates, followed by 26.42% in leaves and 22.64% in stems. From colony morphology, spore-forming stalk, and mycelium structure (Figure 1), the diversity of fungal species isolated from *D. differmis* in a previous study, Tran *et al*. (2022), reported the diversity of fungal genera at these two sampling sites, a total of 27 genera. The population frequency of each isolate was calculated, showing that *Fusarium* was the most common genus, followed by *Trametes* and *Penicillium*. According to a previous study (Dhayanithy *et al.*, 2019), 20 strains of endophytic fungi were isolated from the tissues of leaves, stems, bark, and roots of *Catharanthus roseus*, of which 40% were from bark, 30% from leaves, 25% from stems, and 5% from the roots. In the study of Katoch (Katoch *et al.*, 2017), 28 endophytic fungal strains were isolated from leaves, flowers, and roots of *Monarda citriodora* L., with the highest number of endophytic fungi in the leaves (18 strains), followed by 8 strains in flowers, and 2 strains in roots. All these data serve for endogenous fungal diversity assessment and biodiversity assessment.

**Evaluation of endophytic fungal diversity**

The diversity of fungal endophytes was investigated through the concept of different sampling locations and specific tissues. In general, a high diversity of the endophytic fungal population associated with *D. differmis* was revealed in all of the studied tissues and both sampling locations. In particular, Shannon's index revealed the highest diversity of fungi was found in the roots (H′ = 2.673), followed by stems (H′ = 2.162) and leaves (H′ = 2.054). In the same way, species richness was highest in roots (Dmg = 4.551; Dmn = 3.079) and stems (Dmg = 4.024; Dmn = 3.175), and lowest in leaves (Dmg = 3.41; Dmn = 2.405). The Simpson abundance index showed the endophytic fungal community in roots was the most abundant (D =0.133), followed by that in stems (D = 0.897) and leaves (D = 0.103). The dominance was slightly low, and species evenness was uniform in roots, leaves, and stems (Table 3). Finally, the similarity index was the highest for the leaves versus roots comparison (S = 0.538), indicating that the number of endophytic fungal species shared by the leaves and roots was higher than leaves and stems (0.286) or stems and roots (0.148) (Table 4). In terms of sampling locations, the endophytic fungal population in Lai Chau was more diverse than that in Ha Giang, as depicted by higher diversity indices, higher species richness indices, and lower dominant indices of the former in comparison to the latter. However, the Sorensen index was 0.615, implying medium similarity in species composition between the two locations (Table 5).

Biological diversity could be assessed via biodiversity indices (Kanieski *et al.*, 2018). Dhayanithy and others (2019) analyzed the diversity of the endophytic fungi associated with *Catharanthus roseus*, which showed that the highest values of the Shannon and Simpson diversity indices as well as the Menhinick richness index for bark were 1.47, 0.85, and 1.89, respectively (Dhayanithy *et al.*, 2019). Katoch and others (2017) reported diversity indices of endophytic fungal populations isolated from

*Monarda citriodora*, revealing the high diversity and species richness in leaves with Shannon, Simpson, Margalef richness, and Menhinick richness indexes of 2.89, 0.944, 5.882, and 4.243, respectively (Katoch *et al.*, 2017). According to Kanieski and others (2018), the Menhinick richness index with a value of 2.05 revealed high diversity, while the Shannon diversity index with a value of 2.13 denoted medium diversity. The Pielou evenness index, with a value of 0.76, and the Simpson dominance index, with a value of 0.21, implied high uniformity and low dominance in species. The Sorensen index, with a value of 0.38, suggested low similarity between floristic groups (Kanieski *et al.*, 2018). In the present study, biodiversity indices of the endophytic fungi community associated with *D. difformis* were also assessed. In comparison to the previous study, our results revealed high values for the Simpson diversity index, the Shannon diversity index, the Margalef richness index, and the Menhinick richness index. These results confirmed the high diversity of endophytic fungi associated with *D. difformis.* Meanwhile, a high value of the Pielou evenness index and a low value of Camargo's dominance index indicated high uniformity in the composition of the endophytic fungi. Interestingly, Simpson and Shannon diversity indexes showed different patterns in the concept of specific tissues. According to Kanieski *et al. (*2018), the Simpson index expressed only the dominance of some species in the sample, while the Shannon index considers species richness and evenness. Therefore, the Shannon Index has been considered to be better than Simpson's index for diversity evaluation (Whitakker, 1965; Kanieski *et al.*, 2018). The coefficient of similarity of Sorensen, with a low value and a medium value, denoted a low and a medium similarity in species composition for different tissues and different locations, respectively. These results implied that: (i) Endophytes exhibited different attractions towards different tissues; and (ii) The difference in the area where the plants were collected could lead to a difference in the microbial community. Our results are consistent with the conclusion of Gupta *et al. (*2020) who reviewed that the site of collection, sample size (size of explants), and localization of fungal endophytes in plant tissues can influence the endophytic fungal community.

**Table 3.** Tissue-specific diversity indices of endophytic fungi isolated from *D. difformis.*

| Diversity index | Roots | Leaves | Stems |
|---|---|---|---|
| Simpson dominance index (D) | 0.133 | 0.089 | 0.103 |
| Simpson diversity index (1-D) | 0.867 | 0.911 | 0.897 |
| Shannon diversity index (H') | 2.673 | 2.054 | 2.162 |
| Margalef richness index (Dmg) | 4.551 | 3.410 | 4.024 |
| Menhinnick richness index (Dmn) | 3.079 | 2.405 | 3.175 |
| Carmago dominance index (Cm) | 0.325 | 0.416 | 0.315 |
| Pielou evenness index (P) | 0.964 | 0.892 | 0.902 |

**Table 4.** Sorenson's similarity indices for endophytic fungi of *D. difformis.*

| Sorensen's index (S) | Roots | Leaves | Stems |
|---|---|---|---|
| Roots | 1.000 | 0.538 | 0.148 |
| Leaves | 0.538 | 1.000 | 0.286 |
| Stems | 0.148 | 0.286 | 1.000 |

**Table 5.** Sampling location-specific diversity indices of endophytic fungi isolated from *D. difformis.*

| Diversity index | Ha Giang | Lai Chau |
|---|---|---|
| Simpson dominance index (D) | 0.064 | 0.059 |
| Simpson diversity index (1-D) | 0.936 | 0.941 |
| Shannon diversity index (H') | 2.849 | 2.921 |
| Margalef richness index (Dmg) | 5.402 | 5.903 |
| Menhinnick richness index (Dmn) | 3.591 | 4.000 |
| Carmago dominance index (Cm) | 0.279 | 0.250 |
| Pielou evenness index (P) | 0.968 | 0.975 |
| Sorensen index (S) | 0.615 | 0.615 |

## CONCLUSION

The present study is the first report on the biodiversity of endophytic fungi inhabiting *D. difformis* based on common diversity indices. The data highlighted a high diversity of endophytic fungi and the adaptation of the species to specific tissues and habitats. The study is also the basis for the use of diversity indices to evaluate the endogenous microbiome of plants, orienting the conservation of microbial biodiversity in medicinal plants.

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