ORIGINAL ARTICLE

Antioxidant properties and L-asparaginase activities of endophytic fungi from *Cymbidium* orchids

Ru Wei Chua[1] · Keang Peng Song[1] · Adeline Su Yien Ting[1]

Abstract

This study profiled the various endophytic fungi isolated from the orchid *Cymbidium* sp. and their L-asparaginase production and antioxidant potential. The L-asparaginase production was first screened through qualitative plate screening then quantified by the Nesslerization method. The antioxidant potential was quantified via the 2,2-diphenyl-1-picrylhydrazyl assay. A total of 30 endophytic fungi were isolated and all fungal isolates exhibited various degrees of radical scavenging activities (45.28% to 76.4%). Isolate *Lasiodiplodia theobromae* (C11) had the highest antioxidant capacity, represented by the lowest IC_{50} value (5.75 mg/mL) and highest ascorbic acid equivalent antioxidant capacity value (12.17 mg/g). Additionally, 16 isolates produced L-asparaginase (53.33%), which includes primarily species of *Fusarium proliferatum*, *Fusarium fujikuroi*, *Fusarium incarnatum*, and *Fusarium oxysporum*. A new isolate has also been discovered from *Cymbidium* orchid, *Buergenerula spartinae* (C28), which showed the highest L-asparaginase activity (1.736 unit/mL). These findings supported the postulation that medicinal species of Orchidaceae such as *Cymbidium* sp. harbor endophytes that are producers of L-asparaginase and antioxidants with various potential applications.

Keywords Antioxidant · Bioactive compounds · *Cymbidium* · Fungal endophytes · L-asparaginase · Orchids

Introduction

Fungal endophytes are diverse microorganisms that colonize the internal tissues of living plants without causing any symptoms to the host plant (Fouda et al. 2015). Over a long period of co-existence with the host, endophytes garnered the ability to produce a plethora of bioactive compounds with potential applications in medicine, agriculture, and commercial industries (Strobel and Daisy 2003). The bioactive metabolites are involved in plant-endophyte interactions, which include defense mechanisms against biotic and abiotic stresses (Jia et al. 2016). In 1993, the discovery of Taxol, an anticancer drug produced by endophytic *Taxomyces andreanae* from the Pacific yew tree (*Taxus brevifolia*), has paved the way for natural drug research (Stierle et al. 1993). Following this, there was greater emphasis on explorations for anticancer or antitumour agents such as camptothecin, vinblastine, and vincristine, which were progressively isolated from fungal endophytes of medicinal plants (Kumar et al. 2013; Kai et al. 2015). Subsequently, this has extended to novel compounds with antidiabetic, antimicrobial, antioxidant, and immunosuppressive properties (Strobel and Daisy 2003).

One of the most valuable compounds discovered from endophytes is L-asparaginase, an enzyme used to treat cancers, primarily acute lymphoblastic leukemia (Theantana et al. 2009; Hatamzadeh et al. 2020). L-Asparaginase hydrolyzes L-asparagine to L-aspartic acid and ammonia (Theantana et al. 2009). As tumor cells require large amounts of L-asparagine for growth, these compounds are removed from the serum of cancer patients during chemotherapy to diminish and control the development of tumor (Hatamzadeh et al. 2020). *Escherichia coli* and *Erwinia carotovora* are typical sources of L-asparaginase but have been known to cause side effects including chills, abdominal cramps, fever, and fatal hyperthermia (Theantana et al. 2009; Hosamani and Kaliwal 2011). To mitigate this, L-asparaginase is alternatively sourced from eukaryotic sources such as fungi for better compatibility with humans to reduce side effects (Savitri and Azmi 2003; Sarquis et al. 2004). Preliminary reports have indicated that endophytic L-asparaginase producers include species of *Fusarium*,

✉ Adeline Su Yien Ting
   adeline.ting@monash.edu

1   School of Science, Monash University Malaysia,
    Jalan Lagoon Selatan, 47500 Bandar Sunway,
    Selangor Darul Ehsan, Malaysia

Colletotrichum, *Penicillium*, and *Phoma* (Theantana et al. 2009; Chow and Ting 2015; Hatamzadeh et al. 2020).

Endophytic fungi are also valued for their ability to produce antioxidant compounds. Overproduction of reactive oxygen species (ROS) can damage and impair the structure and function of important biological molecules including lipids, proteins, and nucleic acids (Lobo et al. 2010). Accumulation of free radicals also significantly contribute to cancers and neurological, ischemic, and inflammatory diseases (Lobo et al. 2010; Lai and Lim 2011). Antioxidants are therefore valuable as they act as radical scavengers which alleviate and remove harmful free radicals from the human body (Lobo et al. 2010). Synthetic antioxidants such as propyl gallate and butylated hydroxytoluene have been incorporated into food, cosmetics, and pharmaceutical products, but they pose health hazards such as allergy, irritation, and toxicity (Lai and Lim 2011). Thus, it is imperative to seek natural antioxidants with lesser toxicity and side effects. In recent years, endophytes from plants rich with antioxidant potential have been extensively explored (Lai and Lim 2011; Cui et al. 2015). Antioxidant compounds can be sourced from various genera of endophytes such as *Phialophora*, *Aspergillus*, *Fusarium*, *Phialocephala*, *Xylaria*, *Penicillium*, and *Alternaria* (Cui et al. 2015; Gunasekaran et al. 2017; Bhavana et al. 2019). Endophytic antioxidants were reportedly synthesized to protect and defend the host upon exposure to biological or environmental stresses (Cui et al. 2015).

In this study, fungal endophytes were isolated from *Cymbidium* orchids in Malaysia. *Cymbidium* sp. are appreciated as ornamental plants, but they are also cultivated for their therapeutic properties since ancient times, to treat dislocation and fracture of bones, cold, cough, and wounds (Pant 2013). A wide variety of alkaloids, flavonoids, bibenzyls, terpenoids, and phenanthrenes were responsible for the remedial properties of *Cymbidium* orchids (Gutiérrez 2010). It was theorized that endophytes and their host plants often produce similar bioactive constituents, as a result from a long period of co-evolution (Xing et al. 2011). Examples include the initial isolation of taxol and camptothecin from medicinal plants, followed by their endophytic counterpart (Stierle et al. 1993; Gupta et al. 2020). Orchid hosts and their endophytes have also shown similar potential. Dendrobine, a major component of *Dendrobium* orchids, was synthesized by the endophytic *Trichoderma* sp. that is a common orchid resident (Li et al. 2022). *Cymbidium* orchids also harbor endophytic *Colletotrichum* and *Fusarium* species which produced host-associated metabolites such as farnesol and lectins (Khan et al. 2007; Gutiérrez 2010; Ramya et al. 2019; Wary et al. 2022). Therefore, *Cymbidium* orchids can be sourced for beneficial endophytes since antioxidant and antitumor metabolites (cymbidine A, shancidin, gigantol, and lectins) were derived from the orchids (Gutiérrez 2010; Lv et al. 2022).

In recent years, endophytes from orchids have been profiled for their diversity and antimicrobial activities. However, little is known about their antioxidative potential and L-asparaginase production. In addition, studies on endophytes from *Cymbidium* orchids remained limited. Therefore, this study aims to establish the potential antioxidant and L-asparaginase activities of fungal endophytes from the roots of *Cymbidium* orchids. The antioxidant potential was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. For L-asparaginase, production was detected through the qualitative plate screening and quantification via the Nesslerization method. It is expected that the fungal endophytes with antioxidant and L-asparaginase activities could contribute to the ongoing pursuit of novel natural products for applications in biotechnology, pharmacology, food, and medicine.

Materials and methods

Cultivation of endophytic fungi and preparation of extracts

All 30 endophytic fungi used in the current study were isolated from the roots of *Cymbidium* orchids (Chua et al. 2022). Pure cultures were established and maintained at room temperature (26 ± 2 °C) on potato dextrose agar (PDA) (Merck, Germany). All isolates were identified to their most probable species by molecular identification. Sequences were analyzed, compared to the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/) via BLAST search, and deposited in GenBank to obtain the accession numbers (Chua et al. 2022). Ethyl acetate (Systerm, Malaysia) was used to extract the fungal metabolites and the solvent layer was concentrated under a rotary evaporator to collect the crude extracts. Dried extracts were stored in vials at 4 °C and dissolved in dimethyl sulfoxide (DMSO) (Friendemann Schmidt, USA) prior to use.

Testing for antioxidant activities of endophytic fungi

The radical scavenging ability of fungal endophytes was measured using the 2,2-diphenyl-1- 1picrylhydrazyl (DPPH) assay (Bungtongdee et al. 2019). Various dilutions of the fungal crude extracts (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 mg/mL) were prepared. The aliquot of fungal extract (50 μL) was added to 0.004% DPPH solution in methanol (Systerm, Malaysia) (150 μL). The reaction mixture was incubated in the dark for 30 min with the absorbance read at 517 nm thereafter. For control, the fungal extract is substituted with methanol. The inhibition percentage was expressed by the following equation:

$$\text{Radical scavenging activity}(\%) \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \tag{1}$$

where A_{control} represents the absorbance of control and A_{sample} represents the absorbance of test sample. The IC_{50} value (the concentration of extract required to scavenge 50% of the DPPH radicals) was calculated by linear regression analysis. To further express the antioxidant capacities of the fungal extracts, ascorbic acid was employed as a standard to measure the ascorbic acid equivalent antioxidant capacity (AEAC) values according to the following equation (Lai and Lim 2011):

$$AEAC \text{ (mg AA/g)} \frac{IC_{50}(\text{ascorbic acid})}{IC_{50}(\text{sample})} \times 10^3 \tag{2}$$

Screening for L-asparaginase-producing endophytic fungi

Mycelial plug of each fungal endophyte was inoculated onto Modified Czapek Dox (McDox) agar (Conda, Spain) (agar powder (20.0 g/ L), glucose (2.0 g/L), L-asparagine (10.0 g/ L), KH_2PO_4 (1.52 g/L), KCl (0.52 g/L), $MgSO_4 \cdot 7H_2O$ (0.52 g/L), $CuNO_3 \cdot 3H_2O$ (0.001 g/L), $ZnSO_4 \cdot 7H_2O$ (0.001 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L)) supplemented with 0.3 mL of 2.5% phenol red dye as an indicator. Control plates were established by inoculating mycelial plugs onto McDox agar without L-asparagine. All plates were incubated at room temperature (26 ± 2 °C) for 5 days. The diameter of pink zone was observed and measured after incubation (Gulati et al. 1997).

Quantification of L-asparaginase produced by endophytic fungi

Endophytic fungi with positive L-asparaginase activities from the initial plate screening were subjected to enzyme quantification by the Nesslerization method (Hatamzadeh et al. 2020). Fungal isolates were inoculated in McDox broth (Conda, Spain) for 5 days at room temperature (26 ± 2 °C) with agitation (120 rpm). After incubation, 100 μL of Tris HCL (pH 7), 100 μL of sterile distilled water, and 200 μL of 0.04 M L-asparagine (Merck, Germany) were added to 100 μL of broth containing crude enzyme. The reaction mixture was incubated at 37 ± 2 °C for an hour then added with 100 μL of 1.5 M trichloroacetic acid (TCA) (Merck, Germany) to halt the enzymatic reaction. After 1 h of incubation, 100 μL of the mixture was pipetted into microcentrifuge tubes containing 300 μL of Nessler's reagent (Merck, Germany) and 750 μL of sterile distilled water. The mixture was incubated at 28 ± 2 °C for 20 min. The amount of enzyme activity was

determined by measuring the absorbance of samples at 450 nm using the UV–visible spectrophotometer. One unit of L-asparaginase is expressed as the amount of enzyme that catalyzes the formation of 1 μmol of ammonia per minute at 37 ± 2 °C (Theantana et al. 2009).

Statistical analysis

All assays were conducted in triplicate The data were analyzed using Statistical Package for Social Sciences (SPSS) v. 26.0 (IBM) by one-way analysis of variance (ANOVA), and the means compared with Tukey's honestly significant difference test ($HSD_{(0.01)}$) where $P < 0.01$ indicated significant difference.

Results

Antioxidant activities of endophytic fungi

All 30 fungal isolates have extracts that exhibited varying degrees of radical scavenging activities (RSA) (45.28 to 76.4%) (Fig. 1). The DPPH assay revealed 5 isolates (16.67%) with RSA of > 70%, 16 isolates (53.33%) with RSA of 50–70%, and 9 isolates (30%) with RSA of < 50% (Fig. 1). Isolates with high antioxidant activities (RSA > 70%) were selected for further analysis. These isolates were previously identified by Chua et al. (2022) as *Fusarium fujikuroi* (C1) (MZ496704) (RSA 71.16%), *Fusarium incarnatum* (C4) (MW255305) (RSA 75.29%), *Nigrospora oryzae* (C7) (MW255307) (RSA 71.85%), *Lasiodiplodia theobromae* (C11) (MW255310) (RSA 76.40%), and *Buergenerula spartinae* (C28) (MW255323) (RSA 70.68%) (Fig. 2).

Ascorbic acid was employed as a standard to compare the antioxidant potential of the five endophytic fungi. A calibration curve ($R^2 = 0.9977$) was prepared and the IC_{50} value of ascorbic acid was determined as 0.07 mg/mL. Among the selected isolates, extracts from *L. theobromae* (C11) displayed the highest antioxidant activity which was equivalent to 12.17 mg ascorbic acid in a gram of extract, along with the lowest IC_{50} value (5.75 mg/mL) (Table 1). Another isolate with good antioxidant activity was *F. incarnatum* (C4), with antioxidant activity equivalent to 11.76 mg ascorbic acid per gram of extract and an IC_{50} value of 5.95 mg/mL (Table 1). In comparison, *F. fujikuroi* (C1), *N. oryzae* (C7) and *B. spartinae* (C28) extracts exhibited lower antioxidant activities that were equivalent to 10.64 to 10.79 mg of ascorbic acid in a gram of extracts (Table 1). The higher IC_{50} values also corresponded with the lower AEAC values obtained (6.49 to 6.58 mg/mL).
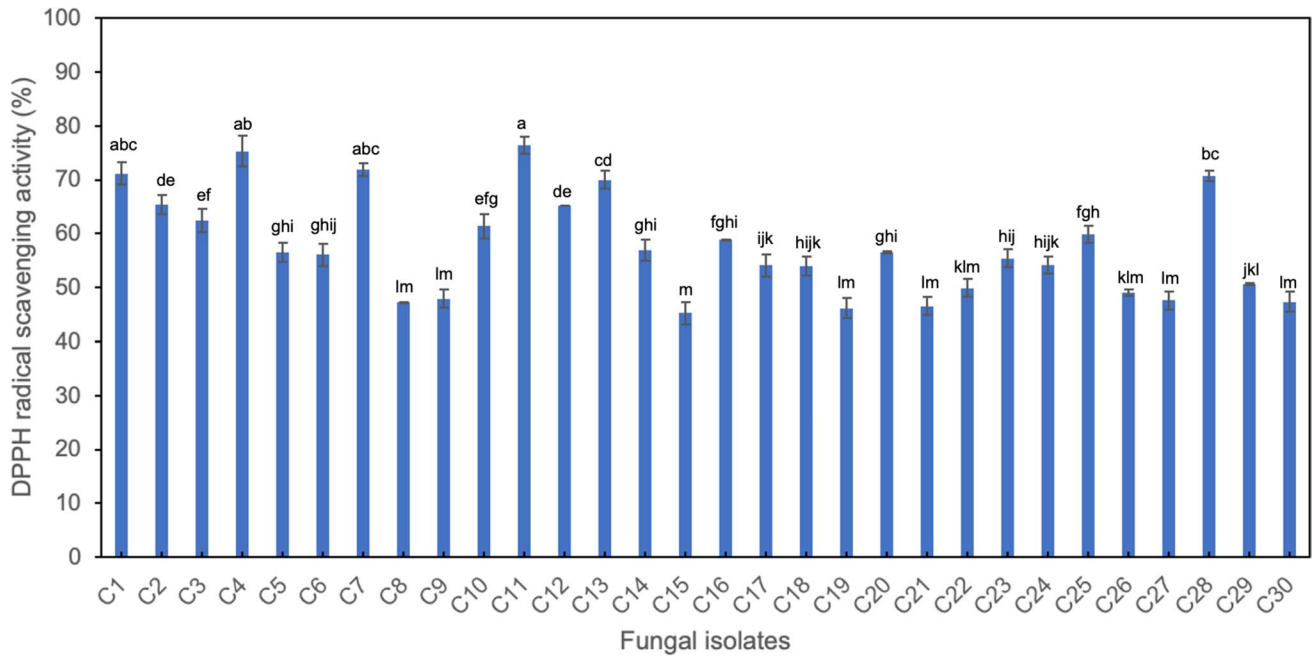
Fig. 1 DPPH free radical scavenging activities of fungal endophytes from *Cymbidium* sp. One-way ANOVA and Tukey's HSD test were employed. Means with the same letters are not significantly different at $P < 0.01$. Error bars represent the standard deviation values of means
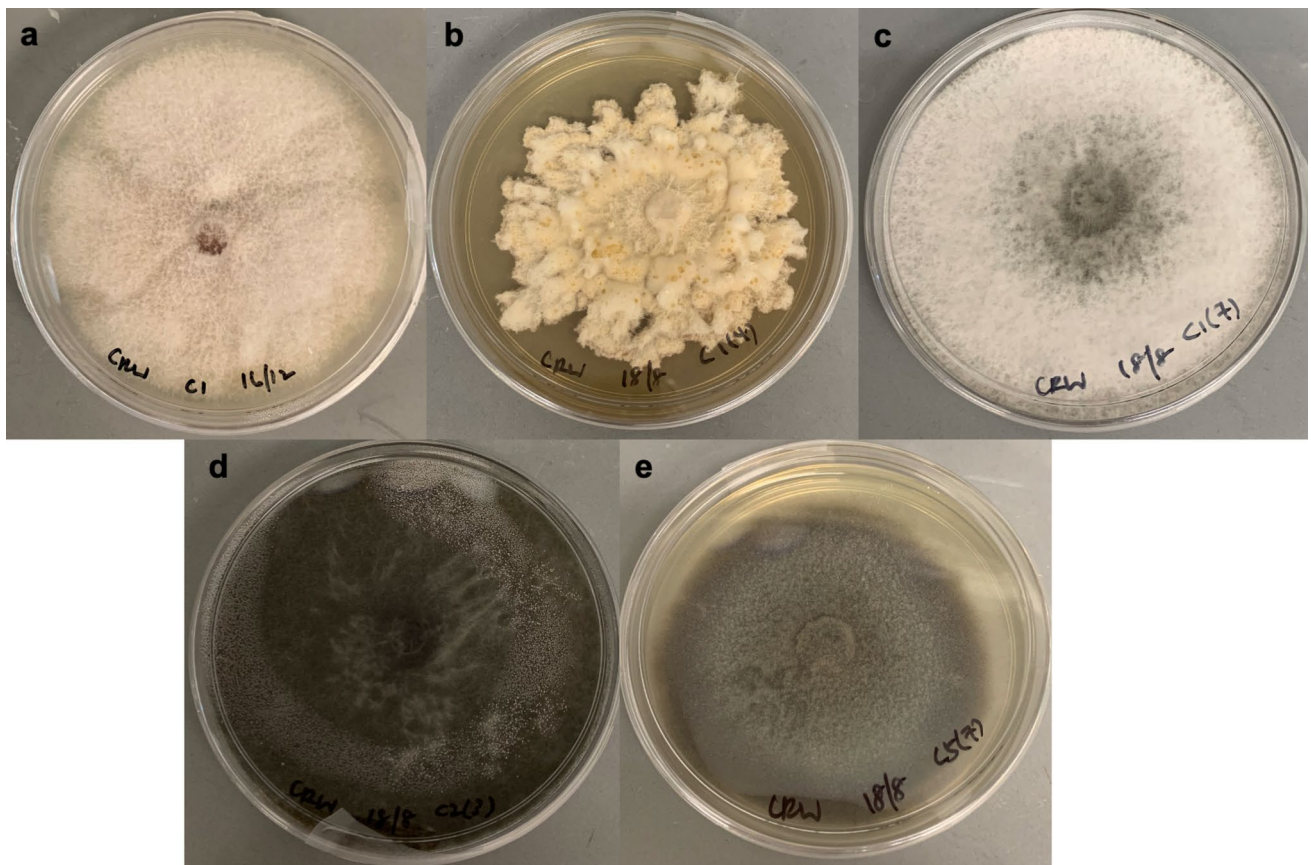

Fig. 2 Cultural characteristics of fungal endophytes with strong DPPH radical scavenging activities. **a** *Fusarium fujikuroi* (C1), **b** *Fusarium incarnatum* (C4), **c** *Nigrospora oryzae* (C7), **d** *Lasiodiplodia theobromae* (C11), and **e** *Buergenerula spartinae* (C28)

Table 1 Antioxidant activities of fungal extracts, expressed in IC_{50} and AEAC values

Fungal isolates	Antioxidant activity	
	IC_{50} (mg/mL)	AEAC (mg AA/g)
C1	6.58	10.64
C4	5.95	11.76
C7	6.58	10.64
C11	5.75	12.17
C28	6.49	10.79

L-asparaginase activities of endophytic fungi

The qualitative plate assay revealed that 16 of the 30 fungal endophytes (53.33%) were positive for L-asparaginase production as evidenced by the formation of pink zones on McDox agar (Fig. 3).

Isolates *Buergenerula sp.* and *Fusarium sp.* were discovered to produce L-asparaginase activities, with diameter of pink zones of 0.8 to 2.5 cm observed. Most of the *Fusarium* isolates (93.75%) were able to produce L-asparaginase except for *Fusarium annulatum*. The isolate *F. proliferatum* (C9) exhibited the largest diameter of pink zone (2.5 cm), while majority of the isolates presented pink zones in the range of 1 to 1.9 cm. Among the different strains of *Fusarium* isolates, *F. incarnatum* (C4) was deemed to have inferior L-asparaginase production as the diameter of pink zone was the smallest at only 0.8 cm. For non-Fusarium species,

B. spartinae showed L-asparaginase production with a diameter of pink zone of 1.3 cm observed. On the contrary, endophytic fungi of the *Curvularia*, *Exserohilum*, *Lasiodiplodia*, and *Nigrospora* genera did not produce L-asparaginase as no pink zones were observed on the agar from the plate assay.

Fungal endophytes with L-asparaginase production from the initial plate screening were subjected to enzyme quantification by the Nesslerization method. Sixteen positive endophytes revealed L-asparaginase activities in the range of 0.308 to 1.736 unit/mL (Fig. 4). Only four isolates (25%) were considered to have high L-asparaginase activity (1.068 to 1.736 unit/mL), while 12 isolates (75%) were weaker producers of L-asparaginase (0.308 to 0.736 unit/mL) (Fig. 4). Higher enzyme production was detected from *B. spartinae* (C28) (1.736 unit/mL), which was significantly different compared to the other isolates. This was followed by various species of *Fusarium* that are good producers of L-asparaginase, including *F. oxysporum* (C25) (1.237 unit/mL), *F. incarnatum* (C4) (1.192 unit/mL), and *F. proliferatum* (C24) (1.068 unit/mL) (Fig. 4). There is a possibility of variants among the species which influenced their L-asparaginase production, as the lowest production of L-asparaginase was detected in an isolate similar to *F. proliferatum* (C24), which is *F. proliferatum* (C13) (0.308 unit/mL) (Fig. 4).

From both the qualitative (plate assay) and quantitative (Nesslerization assay) assessments, we deduce that the diameter of pink zone from the plate screening did not necessarily reflect the level of enzyme production. For example, *F. proliferatum* (C9) exhibited the largest diameter of pink zone (2.5 cm), but the enzyme activity was lower than anticipated
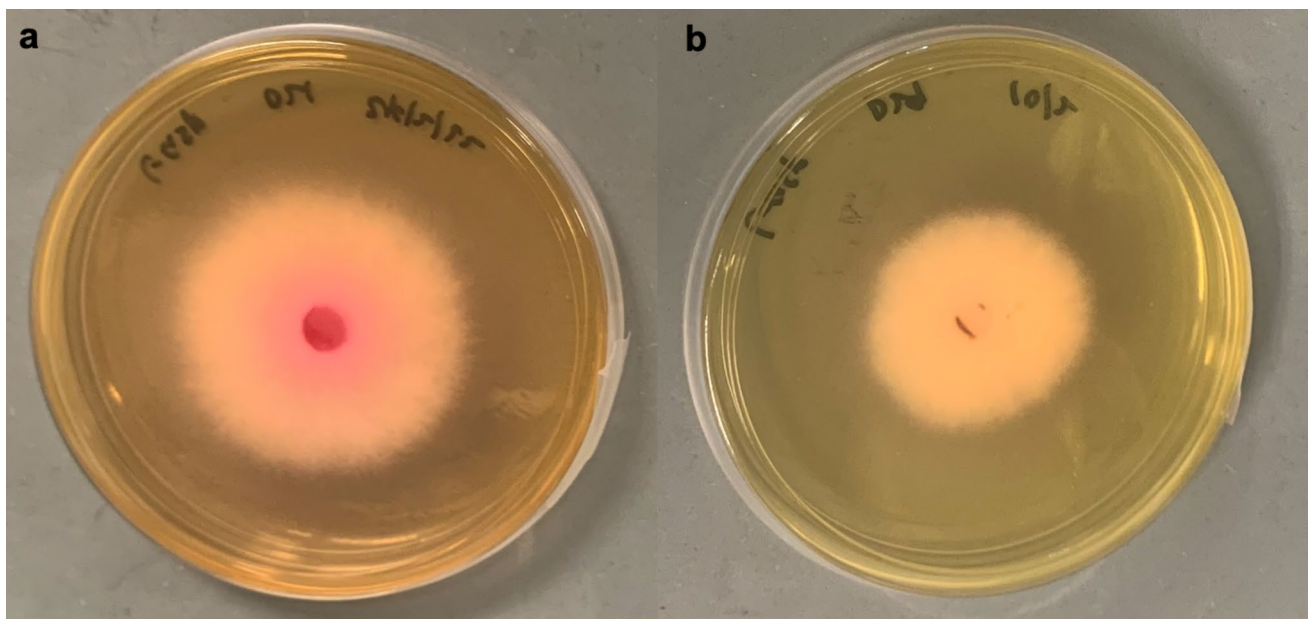

Fig. 3 Detection of L-asparaginase-producing endophytic fungi according to the formation of pink zones. **a** L-Asparaginase-producing fungal endophyte and **b** fungal endophyte without L-asparaginase production. Cultures were 5 days old on McDox agar
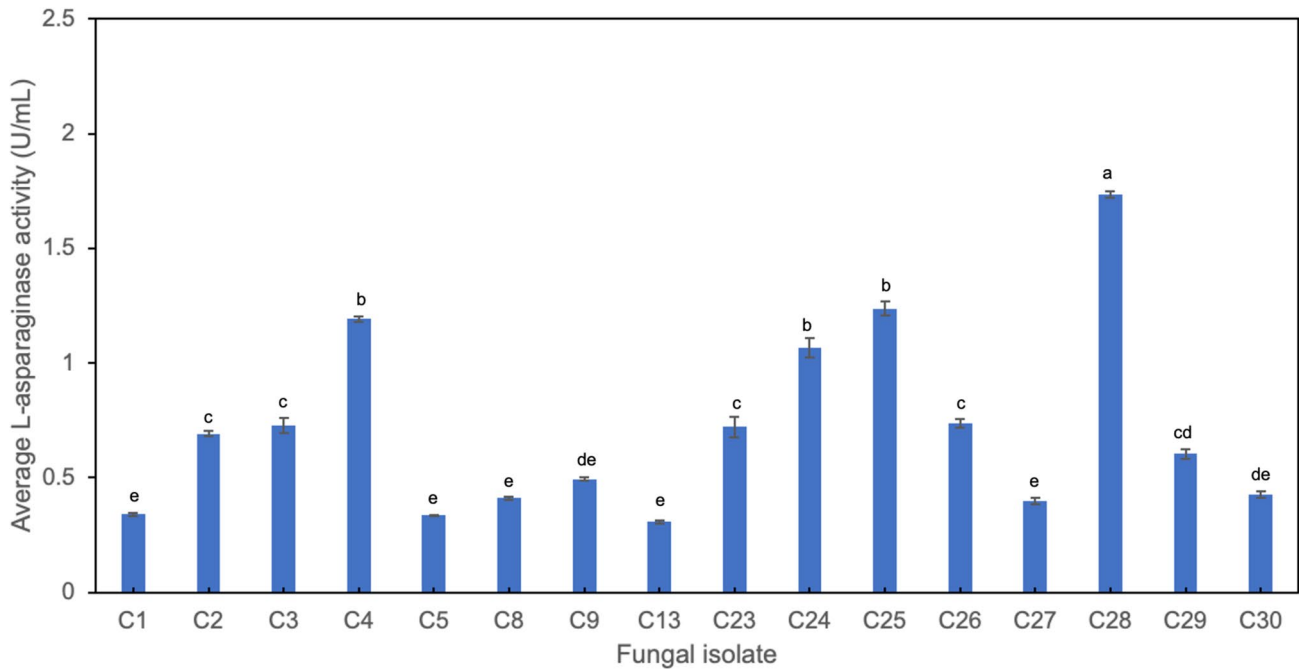
Fig. 4 L-Asparaginase activities of fungal endophytes from *Cymbidium* sp. One-way ANOVA and Tukey's HSD test were employed. Means with the same letters are not significantly different at *P* < 0.01. Error bars represent the standard deviation values of means

(0.494 unit/mL). Similarly, weak enzyme activity (0.426 unit/mL) was displayed by *F. proliferatum* (C30), albeit large pink zones were shown on the plate assay (2.3 cm). On the contrary, *B. spartinae* (C28) with the highest L-asparaginase activity (1.736 unit/mL) did not produce a large pink zone (1.3 cm). This was also observed in *F. incarnatum* (C4), as high L-asparaginase activity (1.192 unit/mL) was observed despite the formation of a small pink zone (0.8 cm). As such, qualitative screening was performed for preliminary detection of L-asparaginase production from endophytic fungi, followed by quantitative determination of enzyme activities by the Nesslerization method for higher accuracy.

Discussion

A large proportion of endophytic fungi from *Cymbidium* sp. were producers of antioxidants and the antileukemic enzyme, L-asparaginase. The findings here are new as existing reports of endophytic fungi from *Cymbidium* orchids were mostly on biodiversity, antimicrobial activities, and the production of extracellular enzymes (amylase, cellulase, laccase, lipase, pectinase, phosphatase, and protease) (Shubha and Srinivas 2017; Chua et al. 2022). The antioxidant activities and L-asparaginase production of endophytic fungi from *Cymbidium* orchids have yet to be documented, which makes this a novel study.

In this study, all endophytic fungi from *Cymbidium* sp. exhibited antioxidant potential. The antioxidant potential in endophytes may perhaps be a relatively common attribute in endophytes. Studies by Pan et al. (2017) and Ibrahim et al. (2021) also reported a high number of endophytic isolates from *Fritillaria unibracteata* and various Nigerian ethnomedicinal plants with high radical scavenging activities. According to Pan et al. (2017), the antioxidant activity of endophytes was established to alleviate and neutralize ROS in host plants to prevent cellular damage. For *Cymbidium* orchids, the high antioxidant activities may be associated with antioxidant effects, attributed by a plethora of alkaloids, phenols, phenanthrenes, dibenzyls, and flavonoids (Axiotis et al. 2022). The root and pseudobulb extracts of the orchid have antioxidant activities (Yoshikawa et al. 2013; Axiotis et al. 2022; Lv et al. 2022); hence, it is also likely that endophytes from the tissues have antioxidative potential as shown in the present study. From this, the postulation that endophytes and the host produce similar biochemicals was also reinforced. Most of the endophytic fungi had moderate antioxidant activities which was consistent with reports by Cui et al. (2015). In their report, fungal endophytes with higher antioxidant activities were from the *Phialophora*, *Aspergillus*, *Penicillium*, and *Lachnum* genera (Cui et al. 2015).

Most of the fungal extracts with antioxidant activities originated from the *Fusarium* genus. *Fusarium* species from various medicinal plants and orchids (*Dendrobium lindleyi*, *Cassia alata* Linn., *Otoba gracilipes*, *Fritillaria*

unibracteata, and *Debregeasia salicifolia*) were acknowledged as producers of antioxidants (Pan et al. 2017; Khan et al. 2018; Bungtongdee et al. 2019; Caicedo et al. 2019; Nisa et al. 2020). Among different strains of *Fusarium* isolates, extracts from *F. incarnatum* (C4) and *F. fujikuroi* (C1) had better antioxidant capacities. The high antioxidant activity of *F. fujikuroi* (C1) in the present study differed from Nisa et al. (2020) where lower antioxidant activities from *F. fujikuroi* were reported. This may presumably be due to the concentration of extracts used by Nisa et al. (2020) which was much lower (1 mg/mL) than our study (10 mg/mL). In addition, stronger antioxidant activity of *F. incarnatum* was reported by Das et al. (2017), with a smaller IC_{50} value compared to the current study. These isolates were not sampled from orchids but other medicinal plants (*Zingiber nimmonii* and *Debregeasia salicifolia*) (Das et al. 2017; Nisa et al. 2020)*,* which may explain the difference in antioxidant activities as the type of host plants could alter the phytochemical composition of fungal endophytes. According to Das et al. (2017) and Gautam et al. (2022), depsidones, phenols, and flavonoids were largely responsible for the antioxidant activities of the fungal extracts. Some of the antioxidant compounds which could be anticipated from endophytic *Fusarium* extracts include quercetin, bostrycoidin, and fusarubin (Das et al. 2017; Khan et al. 2018).

Other strong producers of antioxidants that are non-*Fusarium* species include the *Buergenerula* genus. The antioxidant activity of *B. spartinae* (C28) was revealed for the first time in this study as it is a minor genus of endophyte with no prior reports on its bioactivity. Despite the rare association with orchids, *Buergenerula* endophytes may play profound ecological roles by synthesizing essential molecules for the survival of the host (Duan et al. 2019). Good antioxidant activities were also detected from the *Nigrospora* and *Lasiodiplodia* genera, which echoes findings by Zhou et al. (2018) and Gautam et al. (2022) on the endophytes isolated from *Euphorbia hirta*, *Rhizophora stylosa*, and *Rhizophora mucronata*. In this study, extracts from *L. theobromae* (C11) produced the smallest IC_{50} value, which was in the range reported by Zhou et al. (2018) (3.24 to 14.36 mg/mL) from the same endophyte. It has also been reported that usage of different culture media may have an impact on the production of antioxidant metabolites as *L. theobromae* cultures in grain medium exhibited better radical scavenging capacities with smaller IC_{50} values (3.24 mg/mL) compared to the rice medium cultures (14.36 mg/mL) (Zhou et al. 2018). Similarly, Gautam et al. (2022) reported the highest antioxidant activity from the ethyl acetate extracts of *Nigrospora sphaerica* in potato dextrose broth (RSA 96.8%), but the antioxidant potential weakened (16.22 to 87.82%) when a different medium and extraction solvent was used. Nonetheless, both *Lasiodiplodia* and *Nigrospora* species were producers of phenols, depsidones, flavonoids, and glucosides

that are major antioxidant components (Salvatore et al. 2020; Gautam et al. 2022). Compounds including 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone and 2,4-Di-tert-butylphenol were believed to be accountable for the antioxidant activities of *Lasiodiplodia* and *Nigrospora* spp., respectively (Gautam et al. 2022; Elfita et al. 2023).

Apart from the antioxidant activities, L-asparaginase production was also detected from the fungal endophytes from *Cymbidium* sp. Studies on L-asparaginase production by orchid endophytes were limited; hence, this study is the first to report on the production of endophytic L-asparaginase from *Cymbidium* orchids. Nevertheless, L-asparaginase-producing endophytes have been isolated from other non-orchid hosts, such as from marine algae (*Sargassum* spp., *Turbinaria* spp., *Caulerpa* spp., *Ulva* spp., and *Chaetomorpha* spp.) and medicinal plants (*Cymbopogon citratus*, *Murraya koenigii*, *Oldenlandia diffusa*, *Pereskia bleo*, *Matricaria* spp., *Anthemis* spp., and *Achillea* spp.) (Thirunavukkarasu et al. 2011; Chow and Ting 2015; Hatamzadeh et al. 2020). The number of L-asparaginase-producing isolates from this study (53.33%) was relatively high and similar to the number of isolates positive for L-asparaginase production in existing reports (28.09 to 78.05%) (Thirunavukkarasu et al. 2011; Chow and Ting 2015; Hatamzadeh et al. 2020). Endophytic fungi with L-asparaginase production were predominantly of the *Fusarium* species, with 0.308 to 1.237 unit/mL activities that were comparable to existing reports (0.08 to 3.14 unit/mL) (Nakahama et al. 1973; Manasa and Nalini 2014; Hatamzadeh et al. 2020). The range of L-asparaginase activities may be due to variance between endophytic species. Most *Fusarium* species which include *F. incarnatum*, *F. oxysporum*, *F. fujikuroi*, and *F. proliferatum* are known to produce L-asparaginase (Nakahama et al. 1973; Theantana et al. 2009; Chow and Ting 2015; Ali et al. 2018; Hatamzadeh et al. 2020; Yap et al. 2021). Among different *Fusarium* strains, *F. oxysporum* (C25) (1.237 unit/mL), *F. incarnatum* (C4) (1.192 unit/mL), and *F. proliferatum* (C24) (1.068 unit/mL) exhibited high L-asparaginase activities in this study. Previous literature revealed higher L-asparaginase activities of *F. incarnatum* (2.41 unit/mL) from soil samples and lower enzyme activities of *F. proliferatum* (0.492 unit/mL) and *F. oxysporum* (0.332 unit/mL) from medicinal plants (*Anthemis altissima* and *Achillea millefolium*) (Ali et al. 2018; Hatamzadeh et al. 2020). We also observed that another strain of *F. proliferatum* (C13) was the weakest producer of L-asparaginase, despite stronger enzymatic activities that were expressed by other isolates from the same species. The difference of bioactivities between similar species could be fueled by expression of different genes (Mengistu 2020).

A new L-asparaginase-producing endophyte of the *Buergenerula* genus was isolated. Although no reports of L-asparaginase from *Buergenerula* have been reported, *B. spartinae* (C28) produced the highest L-asparaginase activity

(1.736 unit/mL) which was comparable to other endophytic species of *Fusarium*, *Hypomyces*, and *Nectria* (1.006 to 3.14 unit/mL) with a higher range of L-asparaginase activities before optimization of culture parameters (Nakahama et al. 1973; Manasa and Nalini 2014). Since *B. spartinae* (C28) had the strongest potential, L-asparaginase production could be optimized to maximize enzymatic yield for further testing. In this study, production of L-asparaginase was not detected for endophytic *Curvularia*, *Exserohilum*, *Lasiodiplodia*, and *Nigrospora*. Contrary to what was observed in this study, Thirunavukkarasu et al. (2011), de Pádua et al. (2018), Balbool and Abdel-Azeem (2020), and Moubasher et al. (2022) reported positive L-asparaginase production from *Lasiodiplodia*, *Curvularia*, *Exserohilum*, and *Nigrospora* species found in marine seaweeds (*Sargassum* spp., *Turbinaria* sp., *Caulerpa scalpelliformis*, *Ulva* spp., and *Grateloupia lithophila*) and medicinal plants (*Teucrium polium* and *Myracrodruon urundeuva*). It was hypothesized that the ability to produce L-asparaginase may differ between species of host plants and geographical areas as the metabolite profile of endophytic fungi varied. L-Asparaginase-producing capacity of fungal endophytes could also be dependent on cultivation methods as enzyme production could be induced through specific culture media, addition of elicitors, and optimization of parameters (Savitri and Azmi 2003; Moubasher et al. 2022). Therefore, it is premature to conclude that *Cymbidium* endophytes of the *Lasiodiplodia*, *Curvularia*, *Exserohilum*, and *Nigrospora* genera are non-producers of L-asparaginase as enzyme production could emerge following optimization. Since potato dextrose agar was used for cultivation of endophytic fungi prior to the plate assay, future attempts may include the use of malt extract agar to induce production of L-asparaginase (Moubasher et al. 2022).

As the L-asparaginase activities of endophytic fungi were assessed, we also observed that fungal endophytes with a large diameter of pink zone in the initial plate screening may not exhibit better enzyme activities when tested via the Nesslerization method. Discrepancies could be due to different enzyme production in solid and liquid states, which corroborated with findings by Holker et al. (2004), Theantana et al. (2009), and Hatamzadeh et al. (2020). Thus, plate assays are typically done for preliminary selection of potential isolates, then supported by quantitative assays for greater accuracy (Chow and Ting 2015).

According to Nongkhlaw and Joshi (2015), L-asparaginase-producing endophytes typically possess good antioxidant activities. We found that this was non-species specific as both traits were detected from *Fusarium fujikuroi* (C1), *Fusarium incarnatum* (C4), and *Buergenerula spartinae* (C28) in this study. Similar observations were made by Nongkhlaw and Joshi (2015) and Bhavana et al. (2019), as

endophytic *Penicillium*, *Aspergillus*, *Serratia*, and *Bacillus* spp. demonstrated strong antioxidant and L-asparaginase activities. Apart from the antitumor activities, purified L-asparaginase demonstrated promising RSA in a concentration-dependent manner (Sulistiyani et al. 2016; Amer et al. 2022; Sheltagh and Ali 2023). Therefore, the enzyme could work synergistically with other antioxidants in the fungal extracts although it has yet to be purified, which resulted in stronger inhibition of DPPH radicals. There is also a possibility that potent antitumor and antioxidant activities would emerge once L-asparaginase from endophytic fungi was harvested and purified. However, we noticed that the fungal isolates with high radical scavenging capacities may not synthesize L-asparaginase as seen from *N. oryzae* (C7) and *L. theobromae* (C11). It was plausible that the production of L-asparaginase can be induced; hence, further studies may be required for confirmation.

Conclusion

Diverse endophytic fungi reside in the roots of *Cymbidium* orchids and a vast majority of the isolates possess antioxidative and L-asparaginase potential. *L. theobromae* (C11) exhibited the highest antioxidant capacity while a minor endophyte species, *B. spartinae* (C28), displayed maximum L-asparaginase activity among the isolates. We also discovered that isolates *Fusarium fujikuroi* (C1), *Fusarium incarnatum* (C4), and *Buergenerula spartinae* (C28) have strong antioxidant and L-asparaginase activities. As a rare endophyte, the antioxidant and L-asparaginase activities of *B. spartinae* were revealed for the first time in this study. These isolates were recommended for further studies to harvest and purify the compounds of interest. It is imperative to discover alternative sources of natural antioxidants and L-asparaginase that are cost-effective with minimal toxicity to the human system. As such, our findings would contribute to the discovery and development of novel therapeutic candidates.

Declarations

Conflict of interest The authors declare no competing interests.

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