Mycologia Iranica 2025, Vol. 12 (No. 1): 59–67 JIrMS

Original Article

In vitro investigation of some plant-associated fungi as biological synthesizers of L-asparaginase

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https://doi.org/10.22092/MI.2025.368732.1308

ABSTRACT

Fungi are promising sources of novel bioactive metabolites due to their abundant extracellular enzyme production, easy extraction, and purification. This study aimed to investigate the capability of some plant-associated fungi to produce L-asparaginase enzymes. Twenty plant-associated fungi were inoculated into a modified Czapex Dox broth medium containing 10.0 g/L L-asparagine and 0.3 mL of 2.5% phenol red (pH 7), and maintained at 25 ± 2 °C, for 7 days. The L-asparaginase enzyme activity was evaluated by measuring the amount of free ammonia released through the hydrolysis of L-asparagine through UV-visible spectrophotometry analysis at 450 nm. Seventy percent of the studied fungi could produce the L-asparaginase enzyme. *Trichoderma atroviride*, *Aspergillus flavus,* and *T. harzianum* demonstrated the highest production of L-asparaginase enzyme by 0.47, 0.35, and 0.24 U/mL, respectively. Furthermore, *Cladosporium cladosporioides, C. ramotenellum* and *Verticillium dahliae* with 0.022, 0.009 and 0.015 U/mL, respectively, showed the lowest production of the L-asparaginase enzyme among the others. This research also reports the first successful production of the L-asparaginase enzyme from *Bipolaris oryzae*, *Curvularia trifolii*, *T. atroviride*, *T. harzianum*, and *T. virens*. Plant-associated fungi represent a promising resource for the pharmaceutical industry due to their ability to produce bioactive compounds such as L-asparaginase. While their use may contribute to more sustainable and potentially cost-effective production, successful large-scale application depends on multiple factors beyond enzyme production, including cultivation feasibility, purification processes, and regulatory considerations.

KEYWORDS

Plant pathogenic fungi, Plant endophytic fungi, Rhizospheric fungi, Spectrophotometry.

INTRODUCTION

Microbial agents, including fungi, bacteria, and viruses, are increasingly utilized in agriculture, food production, and pharmaceuticals as eco-friendly alternatives to chemical compounds (Singh et al. 2016). Among these, the enzyme L-asparaginase, derived from eukaryotic and prokaryotic organisms, has gained prominence due to its ability to hydrolyze L-asparagine, an amino acid essential for the growth of certain cells, including cancer cells (Chand et al. 2020). In medicine, L-asparaginase is critical for treating acute lymphoblastic leukemia in children by depleting cancer cells of asparagine, thereby inhibiting their proliferation

Received: 26 Feb. 2025 Revised: 30 Apr. 2025 Accepted: 03 May 2025 Published online: 06 May 2025

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(Van Trimpont et al. 2022). In the food industry, it reduces acrylamide formation during high-temperature cooking, lowering associated cancer risks (Van Trimpont et al. 2022). Additionally, the enzyme shows potential in bioremediation by breaking down environmental pollutants (Darvishi et al. 2022). Current research focuses on identifying novel sources of L-asparaginase with enhanced activity and minimal side effects to maximize its therapeutic and industrial applications (Castro et al. 2021). Fungi have emerged as a promising source of L-asparaginase due to their capacity to produce abundant extracellular enzymes that are easily extractable and purifiable (da Cunha et al. 2019). Due to their eukaryotic nature, fungal enzymes may exhibit greater structural and functional similarity to human enzymes, potentially enhancing their efficacy in cancer treatment when compared to those derived from prokaryotic microbial sources (Naser et al. 2020). Plant-associated fungi, in particular, demonstrate significant potential, with studies indicating that L-asparaginase derived from these sources exhibits superior activity and stability under diverse environmental conditions (da Cunha et al. 2019, Naser et al. 2020). Therefore, this study sought to systematically evaluate the L-asparaginase production potential of twenty plant-associated fungal species.

MATERIALS AND METHODS

Twenty plant-associated fungal isolates were sourced from Gorgan University of Agricultural Sciences and Natural Resources. These isolates were obtained and identified in previous studies at Gorgan University of Agricultural Sciences and Natural Resources, and their information is given in Table 1. These isolates were cultured on Potato Dextrose Agar (PDA) medium (Merck 110130) at 25 ± 2 °C for three days under dark conditions to prepare them for subsequent analyses. A rapid screening protocol by Gulati et al. (1997), adapted with slight modifications from Hatamzadeh et al. (2020) was employed to assess L-asparaginase production. Fresh 5 mm mycelial plugs were aseptically transferred to Modified Czapek Dox (McDox) agar medium, composed of agar powder (20.0 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L), glucose (2.0 g/L), KH_2PO_4 (1.52 g/L), L-asparagine (10.0 g/L), KCl (0.52 g/L), $Cu(NO_3)_2 \cdot 3H_2O$ (0.001 g/L), $MgSO_4 \cdot 7H_2O$ (0.52 g/L), $ZnSO_4 \cdot 7H_2O$ (0.001 g/L), and 0.3 mL of 2.5% phenol red as an indicator in 1000 mL distilled water (pH adjusted to 6.8 before autoclaving at 121°C for 15 minutes). Phenol red was added post-autoclaving under sterile conditions. Control treatments involved inoculating mycelial plugs onto Czapek Dox agar medium lacking L-asparagine to confirm enzymatic specificity. Mycelial plugs were placed centrally on each plate, and all treatments were performed in quintuplicate to ensure statistical validity. Plates were incubated at 25 ± 2 °C for five days under dark conditions. L-asparaginase-producing colonies were identified by a distinct color change of the McDox medium from yellow to pink, indicating a rise in pH due to enzymatic activity.

Quantification of L-asparaginase activity was performed using the Direct Nesslerization Method, which determines the free ammonia released during the hydrolysis of asparagine in each treatment (Imada et al. 1973). Fungal isolates exhibiting positive L-asparaginase production were cultured in 200 mL Erlenmeyer flasks containing McDox broth, inoculated with 5 mm fungal mycelial plugs derived from potato dextrose agar (PDA) medium. The cultures were incubated at a controlled temperature of 25 ± 2 °C with agitation at 4 m/s² for 5 days using a shaker incubator (Moxcare MCOS-UPAB). Following incubation, 100 mL of the crude enzyme broth was transferred to 2 mL microcentrifuge tubes, containing a reaction mixture composed of 200 mL of 0.04 M asparagine, 100 mL of Tris-HCl buffer (pH 7), and 100 mL of sterile distilled water. This mixture was incubated at 37 ± 2 °C for 1 hour. To terminate the enzymatic reaction, 100 µL of 1.5 M trichloroacetic acid was added. Subsequently, 100 µL of the resultant mixture was combined with 750 µL of sterile distilled water and 300 µL of freshly prepared Nessler's reagent (5 g KI, 2.2 g $HgCl_2$, and 16 g KOH dissolved in 100 mL of distilled water) (Imada et al. 1973). The prepared tubes were incubated at 28 ± 2 °C for 20 minutes. Enzyme activity was measured by assessing the absorbance at 450 nm using a UV-visible spectrophotometer (Jenway 6315, UK). The experiment was conducted in five replicates to ensure statistical reliability. L-asparaginase activity was expressed as the amount of enzyme required to catalyze the production of 1 mmol of ammonia per minute at 37 ± 2 °C (Imada et al. 1973). To evaluate the efficacy of individual plant-associated fungi in producing L-asparaginase under in vitro conditions, a completely randomized design (CRD) was implemented. The data were analyzed using analysis of variance (ANOVA) performed with SPSS statistical software (version 21.0; SPSS Inc., Chicago, IL, USA). Post-hoc comparisons of treatment means were conducted using Duncan's multiple range test at a significance level of $\alpha = 0.05$. Graphical representations of the results were generated using Microsoft Excel software (version 2210).

RESULTS

Fourteen fungal species (70%), including *Trichoderma harzianum*, *T. virens*, *T. atroviride*, *Fusarium oxysporum*, *F. solani*, *F. proliferatum*, *Bipolaris oryzae*, *Verticillium dahliae*, *C. trifolii*, *Aspergillus flavus*, *Alternaria alternata*, *Cladosporium ramotenellum*, *C. cladosporioides*, and *C. limoniforme* demonstrated the ability to produce the L-asparaginase enzyme (Fig. 1). Conversely, six strains, namely *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Penicillium digitatum*, *Nigrospora oryzae*, *Aspergillus niger*, and

Pyricularia oryzae, were unable to synthesize the enzyme (Fig. 1).

Among the enzyme producers, *T. atroviride*, *A. flavus*, and *T. harzianum* exhibited the highest enzyme activity levels, with 0.47, 0.35, and 0.24 U/mL, respectively (Fig. 2). Statistical analysis using Duncan's multiple range test revealed significant differences between these groups (Fig. 2). Among the *Cladosporium* species, *C. limoniforme* demonstrated the highest L-asparaginase production, with enzyme levels 2.89 and 7.07 times greater than those of *C. cladosporioides* and *C. ramotenellum*, respectively (Fig.

Table 1. Plant-associated fungal isolates studied in the present research.

Species name	*GAU voucher number	Reported potential	Reference
Alternaria alternata	GAUCC 016 MK-RSB6	A root rot, stem cutting, stem decay, and death agent of young olive trees	(Sanei and Razavi 2012)
Aspergillus flavus	GAUCC 014 MK-RSB28	A destructive pathogen in storage of pistachio fruits; producer of Aflatoxin B1	(Sanei and Razavi 2018)
A. niger	GAUCC 015 MK-RSB7	A destructive pathogen in storage of pistachio fruits; producer of Aflatoxin B1	(Sanei and Razavi 2018)
Bipolaris oryzae	GAUCC 007 ZLA21	A destructive pathogen in rice fields	(Sanei et al. 2012)
Botrytis cinerea	GAUCC 010 MK-RSB24	A root rot, stem cutting, stem decay, and death agent of young olive trees	(Hatamzadeh et al. 2024, Sanei and Razavi 2012)
Cladosporium cladosporioides	GAUCC 019 pc4	A destructive pathogen in poplar nurseries	(Akbari Oghaz et al. 2022)
C. *limoniforme*	GAUCC 020 Br15	Endophyte of yarrow plants; maize plant growth promoter	(Akbari Oghaz et al. 2022, Hatamzadeh et al. 2020, Hatamzadeh et al. 2023)
C. ramotenellum	GAUCC 018 AM55	Endophyte of yarrow plants; maize plant growth promoter	(Akbari Oghaz et al. 2022, Hatamzadeh et al. 2020, Hatamzadeh et al. 2023)
Curvularia trifolii	GAUCC 009 GBRO53	A destructive pathogen in rice fields	(Sanei et al. 2012)
Fusarium oxysporum	GAUCC 004 7391	A destructive root rot and decay pathogen in garlic fields and storage	(Akbari Oghaz et al. 2021)
F. proliferatum	GAUCC 005 pc91	A destructive root rot pathogen in wheat and maize fields	(Maghsoudlou et al. 2007, Noori et al. 2023)
F. solani	GAUCC 006 pc13	A destructive root rot pathogen in wheat fields	(Maghsoudlou et al. 2007)
Nigrospora oryzae	GAUCC 013 SMAG39	A destructive pathogen in rice fields	(Sanei et al. 2012)
Penicillium digitatum	GAUCC 012 J2	A destructive decay pathogen in storage of sweet orange fruits	(Hatamzadeh et al. 2024, Jahantigh et al. 2023)
Pyricularia oryzae	GAUCC 017 NFL70	A destructive pathogen in rice fields	(Sanei et al. 2012)
Sclerotinia sclerotiorum	GAUCC 011 NAO171	A destructive pathogen (white rot) in rapeseed fields	(Vakilizarj et al. 2013)
Trichoderma atroviride	GAUCC 003 6022	Part of the cucumber rhizosphere microbiome; Bio-control agent of plant pathogenic fungi; plant growth-promoting agent.	(Akbari Oghaz et al. 2024, Habibi et al. 2015, Habibi et al. 2018)
T. harzianum	GAUCC 001 Ah90	Part of the cucumber rhizosphere microbiome; Bio-control agent of plant pathogenic fungi; plant growth-promoting agent.	(Akbari Oghaz et al. 2024, Habibi et al. 2015, Habibi et al. 2018)
T. virens	GAUCC 002 6011	Part of the cucumber rhizosphere microbiome; Bio-control agent of plant pathogenic fungi; plant growth-promoting agent.	(Akbari Oghaz et al. 2024, Habibi et al. 2015, Habibi et al. 2018)
Verticillium dahliae	GAUCC 008 BR09	Endophyte of yarrow plants; maize plant growth promoter	(Hatamzadeh et al. 2020, Hatamzadeh et al. 2023)

*Culture Collection of Agricultural Microorganisms of the Gorgan University of Agricultural Science and Natural Resources

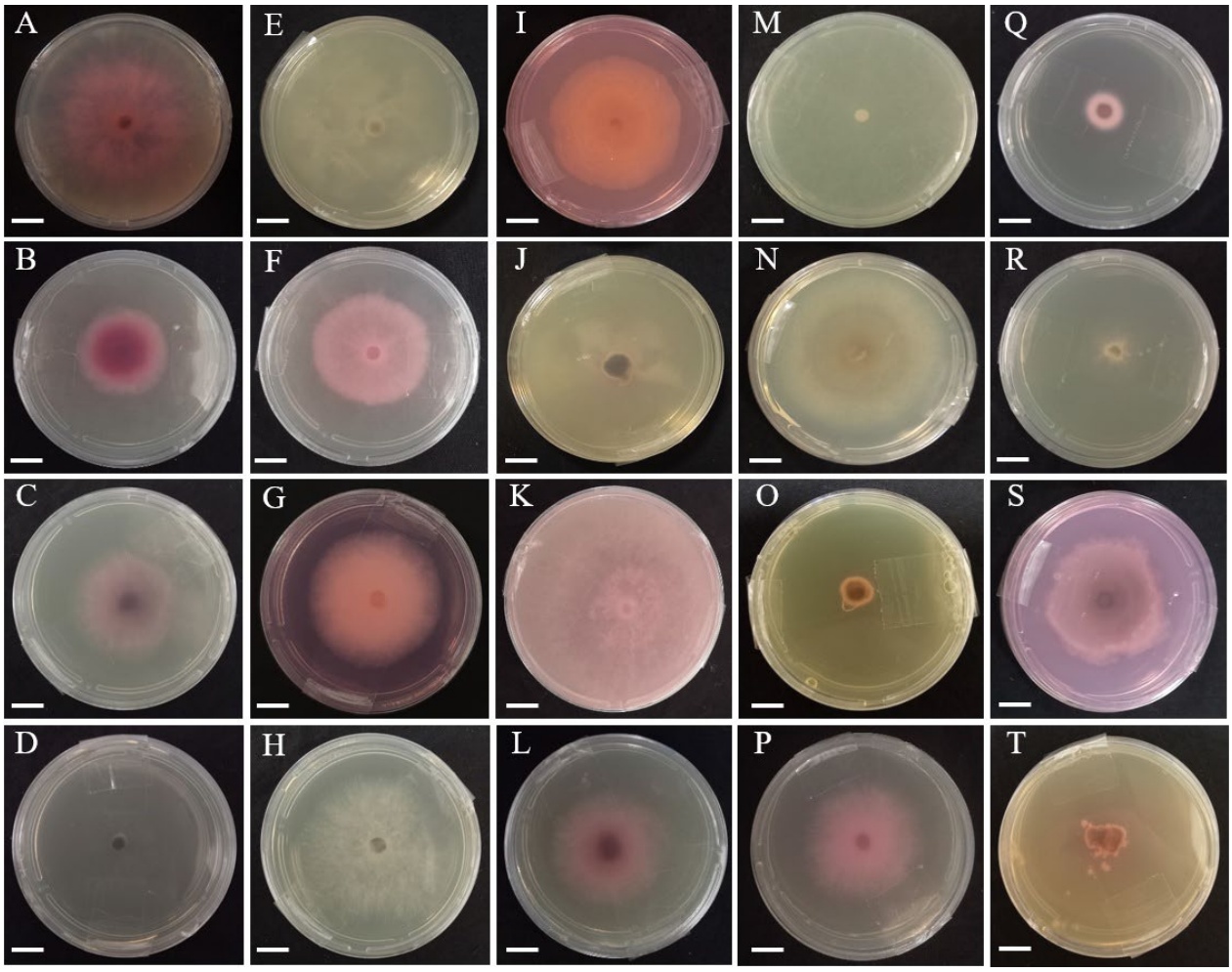

Fig. 1. Preliminary evaluation of L-asparaginase enzyme production by various fungal species. (A) *Trichoderma harzianum*, (B) *Fusarium solani*, © *Cladosporium trifolii*, (D) *Nigrospora oryzae*, (E) *Pyricularia oryzae*, (F) *T. virens*, (G) *Fusarium proliferatum*, (H) *Botrytis cinerea*, (I) *Aspergillus flavus*, (J) *Cladosporium ramotenellum*, (K) *T. atroviride*, (L) *Bipolaris oryzae*, (M) *Sclerotinia sclerotiorum*, (N) *A. niger*, (O) *C. cladosporioides*, (P) *Fusarium oxysporum*, (Q) *Verticillium dahliae*, (R) *Penicillium digitatum*, (S) *Alternaria alternata*, (T) *Cladosporium limoniforme*. The scale bar in the lower left corner of each panel represents 1 cm. Microorganisms that exhibit a red color spectrum in their culture medium are indicative of L-asparaginase production capability.

2). In contrast, *C. cladosporioides*, *C. ramotenellum*, and *V. dahliae* exhibited the lowest L-asparaginase production, with enzyme activities of 0.022, 0.009, and 0.015 U/mL, respectively (Fig. 2). Statistical analysis using Duncan's multiple range test showed no significant difference in L-asparaginase production among *C. cladosporioides*, *V. dahliae*, and *C. ramotenellum*. However, a significant difference was observed between *C. limoniforme* and the other *Cladosporium* species (Fig. 2). Among the *Fusarium* species, *F. proliferatum* exhibited significantly higher L-asparaginase production, with levels 1.58 and 1.75 times greater than those of *F. oxysporum* and *F. solani*, respectively (Fig. 2). The Duncan range test did not reveal significant differences between *F. oxysporum* and *F. solani*, while a significant difference was detected between *F. proliferatum* and the other *Fusarium* species (Fig. 2). For the *Trichoderma* species, all three exhibited significant differences in L-asparaginase production according to the Duncan range test (Fig. 2). *T. atroviride* displayed the highest enzyme production, with levels 1.91 and 2.37 times higher than those of *T. harzianum* and *T. virens*, respectively (Fig. 2). Among the fungal pathogens of rice, both *B. oryzae* and *C. trifolii* were successful in producing L-asparaginase. The Duncan range test indicated no significant difference in enzyme production between these two species (Fig. 2).

DISCUSSION

The findings of this study revealed that the majority of plant-associated fungi possessed the capability to produce L-asparaginase, although the overall enzyme yield remained relatively low. *B. oryzae* and *C. trifolii* were found to possess both qualitative and quantitative abilities to produce the L-asparaginase enzyme. To the

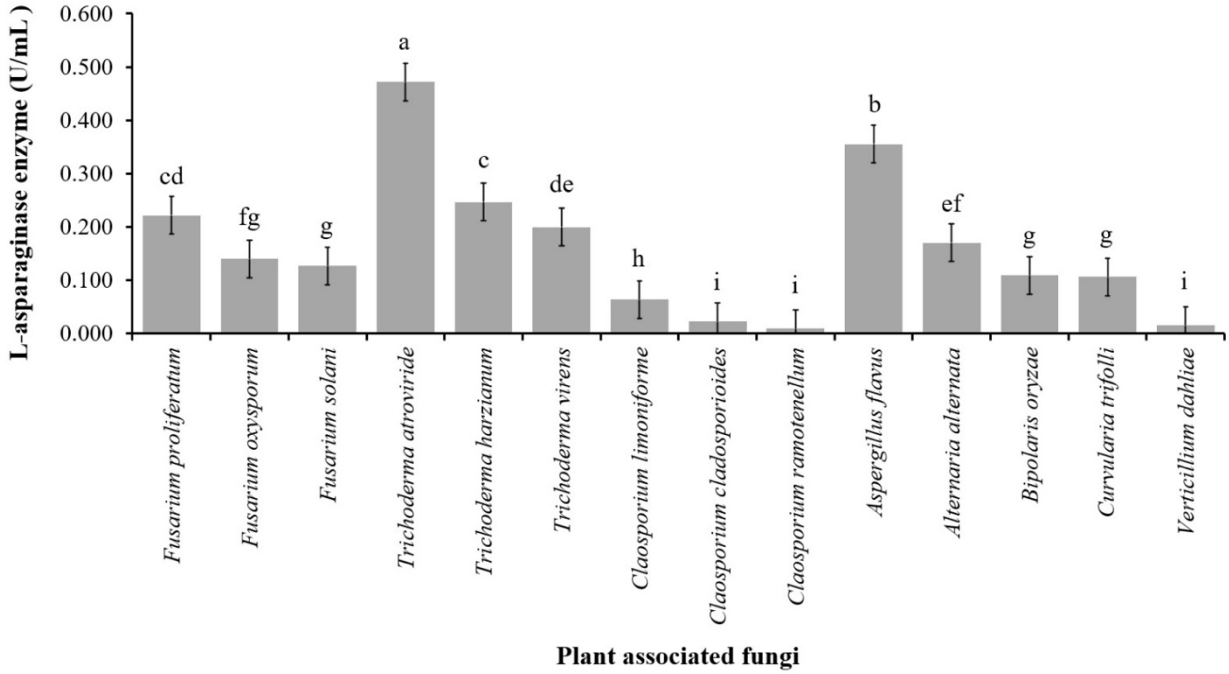

Fig. 2. Clustered column graph depicting the comparison of L-asparaginase production activity among plant-associated fungi. The graph presents mean values derived from five replicate measurements, with statistical comparisons performed using the Duncan Range Test (α = 0.05). Degrees of freedom (df) = 14. The F-value for the test was 125.578, and the P-value was 0.0001, indicating significant differences in enzyme production across the fungal species tested.

best of our knowledge, this is the first report documenting the capacity of these pathogenic fungi to synthesize L-asparaginase. Additionally, *A. alternata* was also found to produce the enzyme, contradicting a recent study by Hatamzadeh et al. (2020), which suggested that endophytic strains of *A. alternata* were incapable of enzyme production. The observed differences between pathogenic strains and plant endophytes may be attributed to genetic and structural variations, as genome analysis is likely to reveal significant distinctions (Möller and Stukenbrock 2017). *C. trifolii*, another rice pathogen examined in this study, also demonstrated the ability to produce L-asparaginase, yet no prior reports exist to support these findings. Moreover, the study by Hatamzadeh et al. (2020) explored the potential of the endophytic fungus *V. dahliae* to produce L-asparaginase after 120 hours of growth at 37 ± 2°C, yielding 0.155 U/mL. In contrast, our experiment involved a 72-hour incubation at 25 ± 2°C to assess enzyme production under more accessible environmental conditions, yielding 0.170 U/mL. This discrepancy is likely due to differences in the applied growth temperatures, as the optimal temperature for *V. dahliae* growth is reported to be 25°C (Klosterman et al. 2009). In our study, the production of L-asparaginase in *C. ramotenellum* and *C. limoniforme* was enhanced compared to the results reported by Hatamzadeh et al. (2020), who found enzyme production levels of 0.232 U/mL and 0.309 U/mL, respectively. The variability in results can be attributed to differences in temperature and growth duration. Additionally, *C. cladosporioides*, a pathogenic species studied by Ali et al. (1993), was also identified as an efficient producer of L-asparaginase, corroborating our findings.

The findings of this study demonstrate that *T. harzianum*, *T. virens*, and *T. atroviride* are capable of producing the L-asparaginase enzyme. These species were isolated from the rhizosphere of cucumber plants (Akbari Oghaz et al. 2024, Habibi et al. 2015, 2018). While no previous reports exist regarding the production of L-asparaginase by these specific *Trichoderma* species, similar studies on other members of the genus support our observations. For example, *T. viride*, isolated from the sea bed, is a significant bio-producer of L-asparaginase (Lincoln et al. 2015). Additionally, *T. asperellum* has been identified as containing genes associated with asparaginase production (Elsaba et al. 2022), and *T. viride* F2 has been documented as a producer of L-asparaginase at pH 7.5 (Elshafei and El-Ghonemy 2021). In this study, *F. oxysporum*, *F. solani*, and *F. proliferatum* were also found to produce L-asparaginase, aligning with existing literature that recognizes *Fusarium* species as important sources for enzyme production. *F. oxysporum* has long been acknowledged for its L-asparaginase production capabilities, with early studies by Nakahama et al. (1973) examining various isolates of this species. More recently, *F. oxysporum* F-S has been reported to exhibit the highest production efficiency, especially at pH levels greater than 7 (Abdel-Hamid et al. 2022). *F. proliferatum* has also gained attention for its enzyme production potential, with (Yap et al. 2021) highlighting its capabilities, and (Hatamzadeh et al. 2020) reporting

its highest L-asparaginase production (0.492 U/mL) among tested fungi. Similarly, *F. solani* has been recognized for its potential in L-asparaginase production (El-Hadi et al. 2017), supporting the findings in this study.

The pathogenic fungi *A. flavus* and *A. niger* are known to be destructive agents, particularly in onions and during the post-harvest storage of agricultural crops (Baltussen et al. 2020). Our findings demonstrated that *A. flavus* possesses the ability to produce the L-asparaginase enzyme, whereas *A. niger* did not exhibit this capability. Despite the widespread use of *Aspergillus* species in the pharmaceutical industry, there is limited research on their potential for L-asparaginase production. A study by Shuker (2019) examined various filamentous fungi isolated from diseased fruits and vegetables to assess their capacity for L-asparaginase production. Among the isolates, *A. flavus* was identified as the most effective producer of the enzyme at a pH of 6.0, utilizing glucose as a carbon source, while *A. niger* also demonstrated notable enzyme production potential. The discrepancy between our findings and those of Shuker (2019), particularly the inability of *A. niger* to produce L-asparaginase in our study, may be attributed to differences in experimental conditions such as variations in host and environmental factors, as well as possible differences between fungal isolates or limitations in species identification. Notably, Shuker (2019) conducted his research in an acidic environment with a pH of 6, while our study was performed under neutral conditions. This variation in growth conditions likely accounts for the observed differences in enzyme production. Thus, the functional discrepancy between *A. flavus* and *A. niger* can be explained by environmental and host-specific factors, suggesting that further research is needed to optimize conditions for enzyme production by these fungi.

CONCLUSION

This study aimed to investigate whether certain plant-associated fungal species, previously unstudied for L-asparaginase production, are capable of synthesizing the enzyme. Although the activity levels detected may not yet support industrial-scale application, our primary objective was to establish the baseline production potential of these fungi. The findings expand current knowledge on the biosynthetic capabilities of plant-associated fungi and suggest they may represent a previously overlooked source of L-asparaginase. This work lays a foundation for future studies aimed at optimizing enzyme yield, elucidating underlying genetic and biochemical pathways, and evaluating potential applications in biotechnology and medicine. Future research should focus on optimizing cultivation conditions, exploring genetic and biochemical mechanisms, and assessing the enzyme's clinical efficacy for broader pharmaceutical applications.

ACKNOWLEDGMENT

This study was conducted as part of an intra-academic research project at Gorgan University of Agricultural Sciences and Natural Resources, under the supervision of Prof. Dr. Kamran Rahnama. The authors express their gratitude to Dr. Hassan Vatandoost, Professor of Medical Entomology and Vector Control at Tehran University of Medical Sciences, for his invaluable scientific guidance and contributions throughout the course of this research.

AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. Kamran Rahnama contributed to funding acquisition, project administration, resources, supervision, writing, review, and editing. Sareh Hatamzadeh contributed to conceptualization, investigation, methodology, validation, visualization, writing, review, and editing. Nima Akbari Oghaz contributed to investigation, data curation, formal analysis, software, validation, visualization, and writing the original draft. All participating authors read, commented, and approved previous versions of the manuscript.

DATA AVAILABILITY

Raw data will be made available on request.

DECLARATION

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

FUNDING

This research received an academic research grant from Gorgan University of Agricultural Sciences and Natural Resources (Grant number: 9943040).

ETHICS APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors. All authors approved to participate in this research work and in the manuscript.

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ارزیابی زیست‌فناورانه تولید آنزیم ال-آسپاراژیناز توسط قارچ‌های هم‌زیست گیاهی در شرایط آزمایشگاهی

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چکیده

قارچ‌ها به دلیل توانایی بالا در تولید آنزیم‌های خارج‌سلولی، استخراج و تصفیه آسان، به‌عنوان منابع نویدبخش متابولیت‌های زیست‌فعال جدید شناخته می‌شوند. هدف این مطالعه، بررسی توانایی برخی قارچ‌های هم‌زیست با گیاهان در تولید آنزیم ال-آسپاراژیناز بود. برای این منظور، بیست جدایه قارچی در محیط کشت مایع اصلاح‌شده Czapex Dox حاوی ۱۰ g/L ال-آسپاراژین و ۰/۳ mL از معرف فنل‌رد ۲/۵٪ درصد (pH = ۷) تلقیح و به مدت ۷ روز در دمای ۲ ± ۲۵ °C انکوبه شدند. فعالیت آنزیم ال-آسپاراژیناز از طریق اندازه‌گیری مقدار آمونیاک آزادشده حاصل از هیدرولیز ال-آسپاراژین با استفاده از طیف‌سنجی مرئی فرابنفش در طول موج ۴۵۰ nm ارزیابی شد. نتایج نشان داد که ۷۰٪ از قارچ‌های مورد بررسی قادر به تولید آنزیم ال-آسپاراژیناز بودند. در میان آن‌ها، گونه قارچ‌های *Trichoderma atroviride*، *Aspergillus flavus* و *T. harzianum* به ترتیب با تولید ۰/۴۷، ۰/۳۵ و ۰/۲۴ U/mL، بالاترین میزان تولید آنزیم را نشان دادند. در مقابل قارچ‌های *Cladosporium cladosporioides*، *C. ramotenellum* و *Verticillium dahlia* به ترتیب با ۰/۰۲۲، ۰/۰۰۹ و ۰/۰۱۵ U/mL، کمترین میزان تولید را دارا بودند. همچنین، این پژوهش نخستین گزارش موفق از تولید آنزیم ال-آسپاراژیناز توسط گونه‌های *Bipolaris oryzae*، *Curvularia trifolii*، *T. atroviride*، *T. harzianum* و *T. virens* را ارائه می‌دهد. قارچ‌های هم‌زیست با گیاهان به‌دلیل توانایی در تولید ترکیبات زیست‌فعال از جمله آنزیم ال-آسپاراژیناز، به‌عنوان منابع بالقوه مطرح هستند. با این حال، کاربرد موفق در مقیاس صنعتی مستلزم بررسی عواملی فراتر از صرفاً تولید آنزیم بوده و شامل قابلیت کشت، فرآیندهای تصفیه، و ملاحظات قانونی نیز می‌شود.

کلمات کلیدی

قارچ‌های بیماری‌زای گیاهی، قارچ‌های اندوفیت گیاهی، قارچ‌های ریزوسفری، اسپکتروفتومتر.

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تاریخ دریافت: ۱۴۰۳/۱۲/۰۸، تاریخ بازنگری: ۱۴۰۴/۰۲/۱۰، تاریخ پذیرش: ۱۴۰۴/۰۲/۱۳، تاریخ انتشار برخط: ۱۴۰۴/۰۲/۱۶