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Isolation, Identification, and Characterization Of Indole Acetic Acid (IAA) Phytohormone From Sunflower Root Fungal Isolate Of Dark Septate Endophyte (*Helianthus annuus*)

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Abstract

Endophytic fungi can infect plant tissues without causing disease symptoms and show plants can grow well. One group of endophytic fungi is the dark septate endophyte (DSE) fungus. DSE fungi are reportedly capable of producing phytohormones such as IAA. In plants, the IAA hormone controls physiological processes and regulates cell elongation in stems and roots. This study aims to isolate, identify, and characterize IAA hormones DSE fungi produce from sunflower plants' roots. This research method includes measuring the optimization of IAA hormone production produced by endophytic fungi, including incubation time and the addition of L-tryptophan. In addition, IAA production analysis from DSE fungi was also carried out using thin-layer chromatography, adsorption chromatography, and high-performance liquid chromatography (HPLC). This study showed that DSE fungus isolates produced the highest IAA concentration with the addition of L-Tryptophan precursors as much as 0.5, which was 9.8 ppm, and the incubation time on the eighth day was 25.92 ppm. The results of the analysis of IAA hormone levels in growth media were obtained at 0.679 mgmL-1. There is still a minimal amount of phytohormone compounds obtained from isolation activities in this study, and it is necessary to find an appropriate isolation process so that the maximum amount of compounds is obtained to produce IAA.

Keywords: Dark Septate Endophyte, Endophytic Fungi, Indole Acetic Acid (IAA), Isolation, Sunflower

Introduction

Endophytic fungi can colonize plant tissues without causing disease and show that plants can grow well (Akhir *et al.,* 2023). This occurs due to the symbiotic mutualism between endophytic fungi and host plants (Schulz & Boyle, 2006). Endophytic fungi can be found in almost all parts of plants, including their roots (Huang *et al*., 2001). According to research on *Centella asiatica* by Susilowati *et al*. (2019) using the Shannon-Wiener diversity index, the highest endophytic diversity values were found in roots (1.91), leaves (1.79), stolons (1.75), and petioles (1.29). Likewise, in research conducted by Arifuddin *et al*. (2017) on *Catharanthus roseus*, the frequency of fungal colony growth was 19.23% on leaves and 21.43 on roots. Roots become good growing places for various microorganisms, including endophytic fungi, in sunflower plants (Tamilarasi *et al*., 2008; Akhir *et al.,* 2023).

The sunflower plant (*Helianthus annus*) is essential (oilseed) with global cultivation (Hosni *et al.,* 2022). Sunflower is the third most crucial oil-producing crop, after soybean and rapeseed, and the fourth most essential vegetable oil-producing crop globally (after oil palm, soybean, and rapeseed) (Balogun *et al.,* 2023). Although sunflower plants have enormous economic benefits, they still receive less attention. Sunflower plants have an excellent tolerance to abiotic stress compared to other plants, which is the role of plant hormones. The hormone IAA plays a role in regulating root growth, lateral root formation, and drought tolerance. Increased IAA production can help plants overcome water shortages. In addition, IAA can interact with other hormones, such as abscisic acid (ABA) and jasmonic acid (JA) (Balogun *et al.*, 2023).

IAA hormone is an auxin phytohormone that has exogenous and endogenous IAA. Exogenous IAA is a hormone produced by cells outside plant cells, namely by fungi, such as *Trichoderma viridae, Aspergillus terrieus* (Madasi *et al*., 2021), *Cyanodermella asteris* (Jahn *et al*., 2022), *Aspergillus awamori* (Mehmood *et al*., 2019), and *Penicillium commune* EP-5, *Alternaria tenuissima* EP-13 (Khalil *et al*., 2021). Meanwhile, endogenous IAA is a hormone plant cells produce (Mogea *et al*., 2022). One group of endophytic fungi that have been able to produce phytohormones and help plant resistance to abiotic stress conditions is dark septate endophyte (DSE) fungi. DSE fungi have a common characteristic: forming dark-colored colonies on agar media and experiencing slow growth when grown on PDA media (Surono & Narisawa, 2018). DSE colonizes plant roots with characteristic dark-colored melanized hyphal structures and microsclerotia. DSE colonization is reported to occur in about 600 plant species, covering 320 genera and 114 families, and is widespread from the tropics to the poles and mountains. This study was conducted to isolate, select, measure IAA values, and characterize IAA produced by DSE fungi. The benefits of this research are

expected to provide information about IAA-producing DSE fungi that play an essential role in increasing plant growth.

Materials and Methods

DSE Fungus Isolate Preparation

The DSE fungus isolates used in this study were isolated from the Biochemistry Laboratory, Department of Chemistry, Padjadjaran University collection, which was isolated from the root part of the sunflower plant. The isolates were cultured on PDA media and incubated for 8-10 days at room temperature before being used for several stages of testing.

Effect of Incubation on Biomass

Optimization of the growth time of DSE fungi was carried out using the Subowo method (2010). Selected DSE mushroom isolates are grown on GDP search media. The culture is then incubated at room temperature for 16 days. Measurement of the weight of fungal biomass is carried out every 24 hours. The mushroom mycelia grown in GDP media is filtered using Whatman No. 1 paper and dried in the oven for 24 hours at 800C. The dry weight of mycelia is determined by calculating the difference in weight between blank dry paper and filter paper containing mycelia.

Effect of Incubation Time

Measure growth time on IAA auxiliary production from DSE fungi using growth on GDP liquid media by adding 0.5 μgml-1 tryptophan for ten days. Furthermore, the DSE mushroom isolate suppression was filtered with two layers of gauze to separate the mycelia daily. The supernatant is taken after the mushroom suspension is centrifuged at 5,000 rpm for 25 minutes. 1 ml of supernatant was added to 2 ml of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$). The suppression mixture is then kept for 30 minutes at room temperature in dark conditions. The pink discoloration of the culture filtrate indicates the ability of endophytic fungus isolates to produce the hormone IAA. The IAA concentration was then measured quantitatively with a spectrophotometer at a wavelength of 530 nm (Dhale *et al.*, 2023).

L-Tryptophan Concentration Effect

Additional variations in L-Tryptophan concentration were measured to determine the effect of tryptophan administration on IAA production. The tryptophan concentration given is

without tryptophan, 0.5 μgml^{-1}, 1 μgml^{-1}, and two μgml^{-1}. The suspension of the fungus isolate is then filtered with two layers of gauze to separate it from the mycelia. The supernatant is taken after the mushroom suspension is centrifuged at 5,000 rpm for 25 minutes. 1 ml of supernatant was added to 2 ml of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$). The suppression mixture is then kept for 30 minutes at room temperature in dark conditions. The pink discoloration of the culture filtrate indicates the ability of endophytic fungus isolates to produce the hormone IAA. The IAA concentration was then measured quantitatively with a spectrophotometer at a wavelength of 530 nm (Dhale *et al.,* 2023).

Extraction, Purification, and Characterization of IAA Auxin Phytohormone from DSE Fungus

IAA auxin extraction and purification is performed by setting the pH to 6.0 by adding 0.5 μg/ml L-Tryptophan. Extraction is carried out with ethyl acetate (Duplo). The aqueous phase is converted to pH 2.5 by adding 3 N hydrochloric acid and extracted with duplo ethyl acetate. Then, it is dried using a vacuum and methanol. IAA auxin was identified and quantitatively analyzed using adsorption chromatography and HPLC techniques. The fraction containing IAA auxin that preparative KLT previously purified was analyzed with reverse-phase HPLC using an inverted ODS C-18 nucleosyl column and UV detector with λ = 254 nm. The mobile phase is 35% methanol in 20 mM acetate buffer (pH 3.5) with a 0.7 mL/min flow rate. The reverse phase HPLC results of the sample were compared with standard auxin.

Thin Layer Chromatography Analytics

This analytical thin-layer chromatography is performed using plates coated with silica gel GF-254. This plate is cut with a size of 5x1 cm, and then a lower border line of 0.5 cm is made. Samples in methanol are tolerated with capillary pipes on the lower boundary line and next to them are tolerated by IAA auxin standards. The chromatogram is eluted with an ethyl acetate-methylene trichloride mixture (6:4) until the surface rises as high as the upper limit line. The results can be seen with UV lamps λ = 254 nm, then determined Rf (Rachman *et al*., 2017; Sari *et al.,* 2022).

Adsorption Column Chromatography

The extract is put into a silica gel column G-60, then eluted with a mixture of ethyl acetate-methylene trichloride (6:4). Then the fractions are accommodated. The stain patterns of each fraction were analyzed using analytical thin-layer chromatography, and the Rf value

of each fraction was compared with the standard Rf of auxin. Standard parallel fractions are combined and evaporated with a vacuum evaporator. The residue was dissolved in 5 mL of methanol (Rachman *et al*., 2017; Sari *et al.,* 2022).

Thin Layer Chromatography Preparative

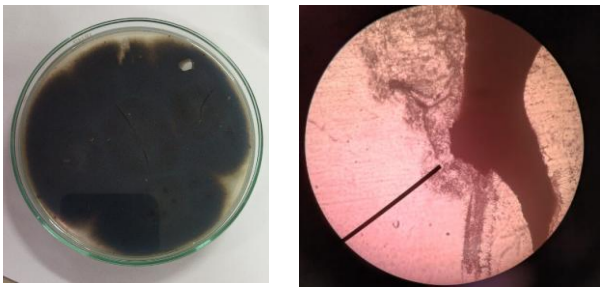
Preparative thin-layer chromatography takes the fractions of column chromatography results parallel to the auxin standard. Furthermore, these fractions are tolerated as a straight line on a glass plate (20x20 cm) coated with silica gel GF-254, and it is tolerated as an auxin standard with a concentration of 100 ppm as a comparison. The chromatogram is eluted with the same solvent as the analytical TLC, an ethyl acetate-methylene trichloride mixture (6:4), until the surface rises as high as the boundary line. The chromatogram region parallel to the auxin standard is scraped and suspended in methanol, after which it is decantated, and the supernatant is concentrated with a vacuum evaporator. The results are in the form of solids, then the residues obtained are dissolved in 1 mL of methanol (Rachman *et al*., 2017; Sari *et al.,* 2022).

High-Performance Liquid Chromatography (HPLC)

A total of 20 μL of auxin-containing fractions purified with preparative TLC were analyzed with reverse phase ODS nucleosyl C-18 column, UV detector with λ = 260 nm. Motion phase 35% methanol in 20 mM acetate buffer (pH 3.5) with a 0.7 mL/min flow velocity. The sample reverse phase HPLC results were compared to the standard (Rachman *et al*., 2017; Sari *et al.,* 2022).

Results and Discussion

The isolates used are DSE fungi identified macroscopically and microscopically (Figure 1). DSE fungi are a group of fungi that colonize the roots of plants and do not cause disease to their host plants. DSE forms structures in the form of microsclerosia. Microsclerosia are small structures that grow inside the cells of the epidermis and cortex of plant roots, have a dark color, and look like tiny grains that fill the cell space. Another morphology of DSE fungi is that they have dark-colored or methylated hyphae. Macroscopic results were characteristic of DSE fungus colonies in dark black PDA media. Dark septate endophyte (DSE) fungus isolate is characterized by forming dark-colored colonies on agar media and experiencing slow growth when grown on PDA media (Surono & Narisawa, 2018).



(a) (b)

Figure 1. DSE fungus isolates on PDA media used in this study. a) macroscopic display; b) Appearance using a microscope with 40x magnification

Growth optimization is carried out to produce DSE fungi in large quantities and optimize the secondary metabolite compounds produced. The fermentation process is carried out with a closed fermentation system to minimize contamination because there is no addition of ingredients or extraction of results at the time of fermentation. The fermentation process in DSE fungi is characterized by a change in the color of the fermentation medium from yellow to orange. This color change is caused by secondary metabolites produced during fermentation, which will be released into the media with color changes depending on the secondary metabolites produced.

The standard growth curve determines the growth rate of DSE fungus isolates so that the growth phase of all DSE fungus isolates in the growth medium can be known. Based on the cultivation optimization curve, the growth of DSE fungus isolates for 14 days consists of the log phase (exponential), stationary phase, and death phase (Figure). In this study, the growth of fungi did not show any lag phase. The lag phase is the growth of fungi adapting to the conditions of the growing medium. The log phase is the phase when the amount of biomass increases. The stationary phase is the growth phase of DSE fungi, which is relatively fixed with the number of dead fungi. In the stationary phase, even though carbon is an essential energy source or nutrient that has been used up, it does not mean growth stops. This happens because lysis in dead cells can be used as a source of nutrition. In addition, waste products can inhibit cell growth or be toxic to cells. Culture enters the last growth of DSE fungus growth, which is the death phase. This phase is seen with a decrease in the amount of biomass. The optimal cultivation time for the growth of isolates of this fungus occurs on the 10th day. During static cultivation, DSE fungi look black, and the mycelia of fungi are visible only on the surface of liquid media and are brownish-yellow—furthermore, the analysis of IAA production produced by DSE fungi.

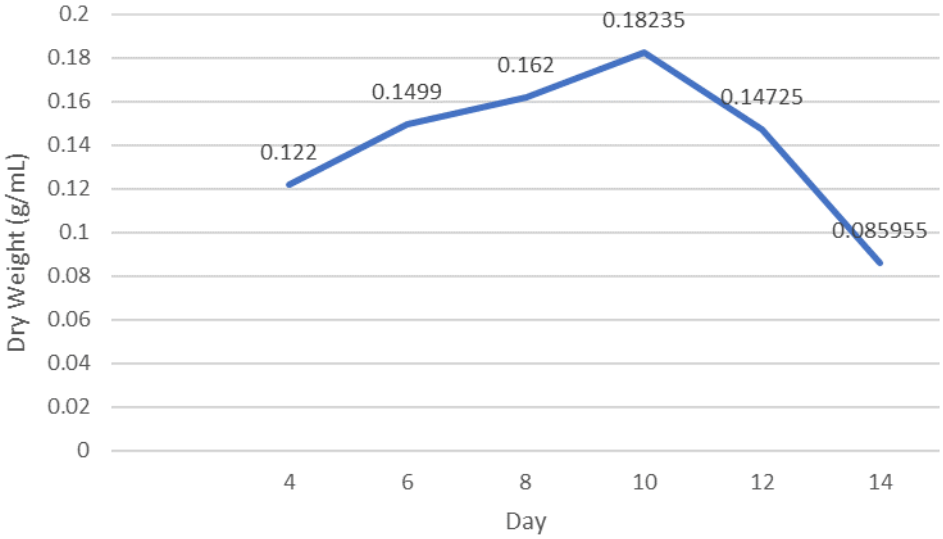


Figure 2. Graph of the growth curve of DSE isolates for days ten on PDB media

IAA hormone is an auxin phytohormone that has exogenous and endogenous IAA. Exogenous IAA is a hormone produced by cells outside plant cells, namely by fungi, and endogenous IAA is produced by plant cells (Mogea *et al.*, 2021). The study's results showed that several factors affect the production of IAA by DSE fungi, including incubation time and L-tryptophan concentration. The results of the growth time test against the IAA concentration obtained on the eighth day showed the highest concentration of 25.92 ppm. The incubation time of endophytic fungi to produce IAA depends on the type of fungus, the growing medium, and the host plant.

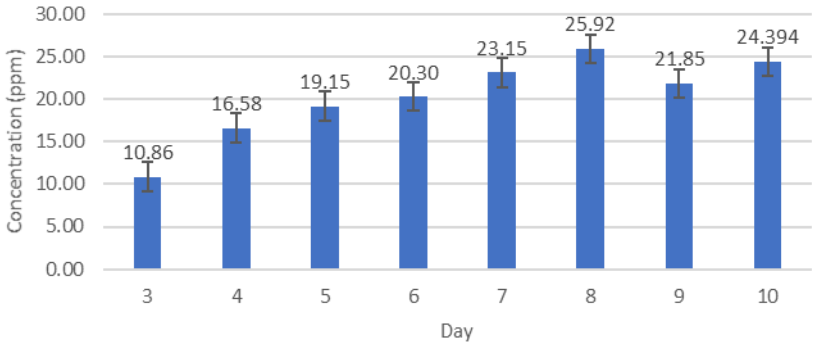


Figure 3. Measurement of absorbance of DSE fungus producing indole acetic acid (IAA) for ten days.

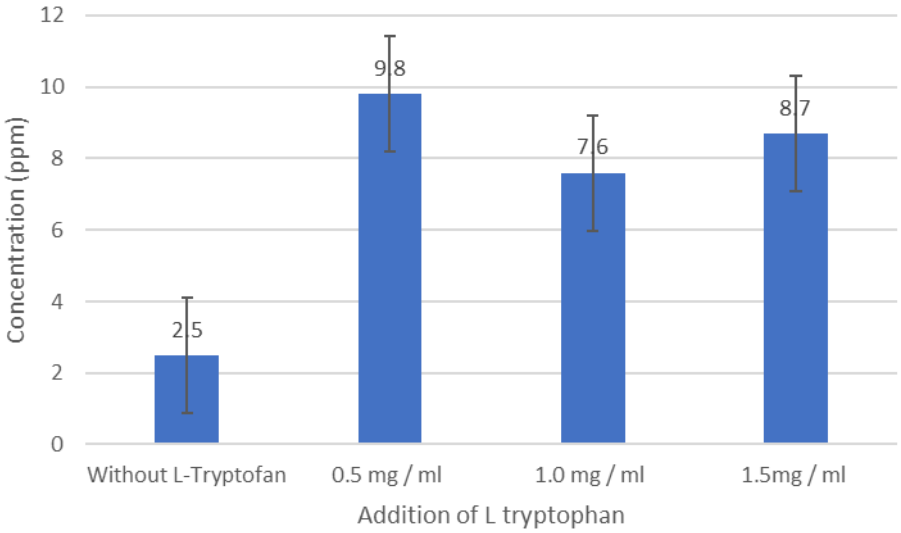


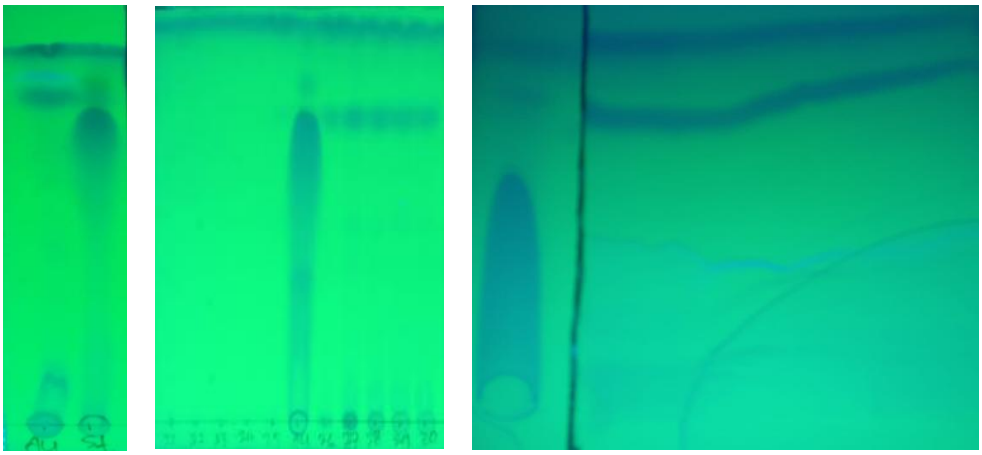
Figure 4. Measurement of absorbance of DSE fungi producing indole acetic acid (IAA) with the addition of variations in L-tryptophan concentration in growth media

Measurement of IAA production with the addition of L-Tryptophan variations to determine the influence of this precursor. Based on the results of the study showed optimization of growing media with a concentration of L-Tryptophan of 0.5 mg $ml^{-1,}$ resulting in high IAA production of 9.8 ppm. One factor in spurring IAA production is the addition of L-tryptophan precursors. This L-tryptophan precursor has a positive effect on fungi in producing IAA hormones. Adding L-tryptophan to DSE mushroom growth media influences the production of IAA through the indole-3-pyruvic acid pathway. The production of IAA through the indole-3-pyruvic acid pathway involves a series of metabolic mechanisms that occur inside DSE fungus cells with the help of enzymes. This follows the results of research by Idris *et al*. (2007), who stated that the increase in IAA production depends on the concentration of L-tryptophan precursors given to growing media.

Analytical TLC results from the auxin standard and the extracted methanol extract were observed for the presence of stains under a UV lamp $\lambda = 254$ nm, and it was found that they had stains parallel to the standard, with an rf value of 0.91 (Figure 5a). The Rf value is determined by comparing the distance traveled by the standard with the distance traveled by the solvent. In the analysis of methanol extract, in addition to obtaining stains that are parallel to the standard, other stains appear. This suggests that the extracted methanol isolate showed the presence of the target compound, auxin, but it was not pure because there were still other compounds. So, a further separation process is needed using adsorption column chromatography.

The same mobile phase as analytical TLC is used in TLC, namely ethyl acetate: methylene trichloride (6:4), and the stationary phase is silica gel GF-254. After the elution

reaches the upper limit of the plate, the visible stain is observed using ultraviolet light at a wavelength of 254 nm. A stain parallel to the standard is obtained, indicating that the stain is auxin (Figure 5c). Scrape away visible stains parallel to the standard and suspend in methanol. Visible stains are taken to isolate auxin to obtain purer compounds.

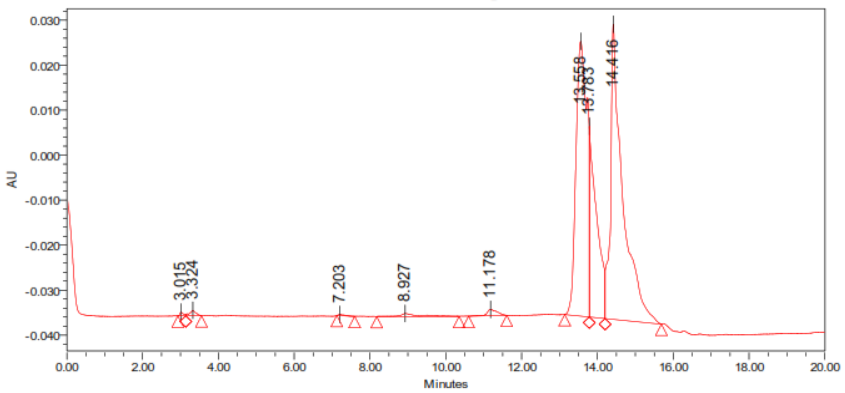


(a) (b) (c)

Figure 5. Analytical TLC results (A) of 10,000 ppm auxin standard and methanol extract (B), with ethyl acetate: methylene trichloride (6:4) eluent under a UV lamp at λ = 254 nm.

The amount of 10 µL fraction containing auxin that PTLC has purified, analyzed with Reversed-Phase (RP) HPLC, using Alltec 8011/2 (column C-18 Nucleosyl ODS) with UV detector at wavelength 254 nm. Buffer solutions for RP HPLC were carried out using an isocratic method using a methanol buffer in acetate at 35% (pH 3.5) with a flow rate of 0.7 mL/min. RP-HPLC results compared to auxin standards (Ikram *et al.*, 2022; Khalil *et al.*, 2021).

a)



b)

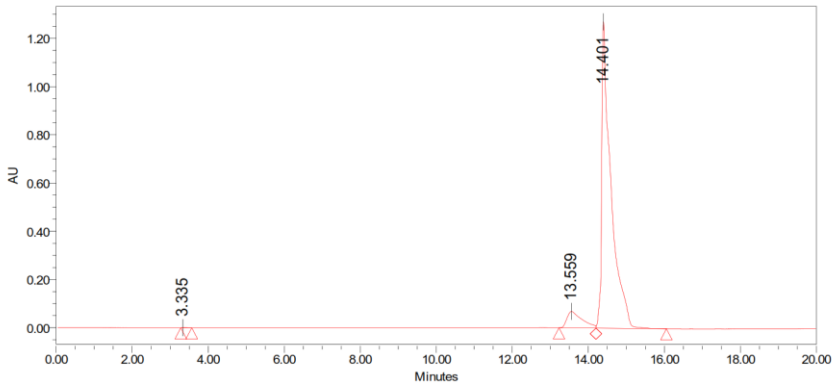


Figure 6. Chromatogram of IAA Isolates (a) and IAA standard (b) separated using RP-HPLC method. The column used was the C-18 Nucleosyl ODS column, and the UV detector used was a UV detector with a wavelength of 254 nm. The mobile phase used was 35% methanol in
acetate buffer (pH 3.5) with a 0.7 mL/minute flow rate.

Figure 6b shows the results of the auxin standard RP-HPLC having a retention time of 14,401 minutes. The RP-HPLC results of the auxin sample had a retention time of 14,416 minutes (Figure 6a), which is relatively the same retention time as the Auxin standard. This indicates that the sample isolate contains auxin. The slight difference in retention time can be caused by the presence of other peaks that cause a shift in retention time due to the presence of impurity isolates. The concentration of auxin isolate (auxin isolated from DSE) can be determined by comparing the peak area of the sample with the standard peak area of auxin and multiplied by the standard concentration of 10 µL injected. After calculation, the auxin concentration in DSE growth media was 0.679 mg/mL.

Conclusion

- Effect of incubation time and addition of L-tryptophan on IAA production produced by DSE fungi
- The auxin content in DSE isolates from sunflower roots (*H. annuus L.*) using high-performance liquid chromatography showed auxin levels in the DSE growth medium of 0.679 $mgmL^{-1}$.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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