Acta Scientific NUTRITIONAL HEALTH (ISSN:2582-1423)

Volume 8 Issue 3 March 2024

Research Article

Isolation, Identification and Characterization of Gibberellin GA-3 Phytohormone from Dark Septate Endophyte (DSE) of Sunflower *Helianthus annuus*

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DOI: 10.31080/ASNH.2024.08.1356

Received: February 01, 2024

Published: February 15, 2024

Abstract

Dark Septate Endophyte (DSE) is a group of endophytic fungi that interact in symbiotic mutualism with their host plants and are beneficial in plant growth by facilitating the absorption of nutrients, protecting against biotic and abiotic stress and producing phytohormones such as gibberellins, auxins and cytokinins. This study aims to determine the gibberellin content contained in DSE isolated from sunflower roots (*Helianthus annuus* L.) from Tangerang, Banten-Indonesia, determine the effect of the gibberellin hormone in DSE in stimulating the growth of rice plants (*Oryza sativa*) in the vegetative phase and the activity of DSE isolates. against metal and salt stress. DSE inoculum was made on PDB media, analysis of gibberellin production on DSE using Thin Layer Chromatography, adsorption chromatography and High Performance Liquid Chromatography (HPLC), inoculation of DSE on rice seeds, detection of colonization in rice with a 400 times magnification microscope, and test of the activity of gibberellin isolates on vegetative phase rice growth. The activity of gibberellin isolates and DSE isolates was determined by bio-testing on rice plants (*O. sativa*) by measuring the length and dry weight of the plant shoots. In DSE isolates, gibberellin levels in the growth medium were found to be 0.561 mg/mL. The results showed that in the first week the canopy length increased with an active percentage of 188%. Based on the canopy dry weight parameter, the percentage of activity in the first week was 128.17%. DSE inoculation of rice plants with metal and salt stress can increase the growth tolerance of rice plants (*O. sativa*) in the vegetative phase based on the parameters of shoot dry weight and shoot length.

Keywords: Dark Septate Endophyte (DSE); Gibberellin; *H. annuus* L; *O. sativa*

Introduction

Dark Septate Endophyte (DSE) is an endophytic fungus which can be an innovation in carrying out biological control. Endophytic fungi can have mutually beneficial interactions or mutualistic symbiosis with host plants. DSE plays an important role in ecosystems and plant growth by facilitating the uptake of carbon (C), nitrogen (N), and phosphorus (P) and inducing systemic plant resistance [1]. DSE can also protect host plants from abiotic and biotic stress. Stress produces Reactive Oxygen Species (ROS) which are very cytotoxic and can disrupt normal metabolism through oxidative damage to cellular components. One possible mechanism of ROS binding is the production of antioxidant enzymes including ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). The effect of melanin on DSE can increase fungal survival because of its function as an extracellular redox buffer that can neutralize oxidants produced by environmental stress and increase the production of antioxidant enzymes in plants [2].

Endophytic in relation to the host plant do not show any symptoms of disease so it is called mutualistic symbiosis [3]. Sopialena., *et al*. [4] added that the mutualistic symbiotic relationship between endophytic and their host plants makes endophytic an environmentally friendly alternative for biological control. Endophytic consist of several groups, namely fungi, bacteria, yeast and actinomycetes [5]. In the interaction of endophytic fungi with their host plants,

endophytic fungi have an influence in stimulating plant growth. Endophytic fungi produce secondary metabolites in the form of plant hormones such as auxin, gibberellins and cytokinins [1]. Not only do they produce plant hormones, endophytic fungi can also produce bioactive molecules, siderophores which can bind the element iron (Fe) which functions in growth, and act as a phosphate solvent [6]. These things produced by endophytic fungi can increase plant resistance to disease by utilizing decaying organic matter through dissolving minerals and through antagonistic mechanisms can suppress plant pathogens. The inhibition of pathogen growth is caused by endophytic fungi seizing nutrients from the pathogen, resulting in changes in the pathogen's hyphae [4].

Endophytic fungi secrete an array of bioactive compounds that serve multiple functions, such as stimulating plant growth, inducing defense mechanisms against pathogens, and serving as agents for remediating salt and drought stresses [7-11]. This co-evolution between endophytic fungi and their host plants results in the production of bioactive compounds which contribute in a variety of ways to plant–microbe interactions and can provide fitness benefits to the host plant. Endophytic fungi secrete an array of bioactive compounds that serve multiple functions, such as stimulating plant growth, inducing defense mechanisms against pathogens, and serving as agents for remediating salt and drought stresses [7-11]. This co-evolution between endophytic fungi and their host plants results in the production of bioactive compounds which contribute in a variety of ways to plant–microbe interactions and can provide fitness benefits to the host plant

DSE is a group of endophytic fungi that have potential as biological control agents because they can stimulate plant growth and protect plants from stress, both biotic and abiotic [1]. Ecologically, DSE is a heterogeneous group of fungi and its function overlaps with root saprobe fungi, mycorrhizal fungi, soil fungi, and pathogens. Dark septate endophytic fungi or DSE, are a diverse group of fungi characterized by the growth of hyphae which are generally septate, hyaline or dark in color and melanized and often colonize or associate with plant intracellular roots by colonizing cortical cells to form dense septate intracellular structures (microsclerotia) [10]. Handayani [12] also reported that DSE fungi are abundant in nature, both in tropical areas, the Alps and the polar regions. However, there is still little known and little research carried out. The diversity of DSE can be seen from the occurrence of DSE colonization in around 600 plant species covering 320 genera and 114 families. Generally, DSE does not sporulate, or if it does sporulate, very few conidia are produced. Some strains can only sporulate when given a low temperature stimulus. The advantage of the DSE fungus compared to other biological agents is that the DSE fungus can live in two stress conditions, both biotic and abiotic factors [13].

The effects of DSE fungi on host plants have been reported to be variable and dependent on the host-symbiont combination. DSE can act as a growth promoter and help increase the production and quantity of compounds in plants, especially in medicinal plants. This is caused by the production of plant growth-promoting hormones, such as indole-3-acetic acid (IAA), gibberellins (GA) and cytokinins (CK). In addition, DSE also facilitates C, N, and P uptake, which can protect plants against biotic (pathogens) and abiotic (heavy metals, increased carbon dioxide stress, and drought) [14]. In its role of protecting and suppressing its host plants from disease and increasing tolerance to pathogens, it is directly and indirectly done by producing antifungal metabolites, fungal parasitism or inducing systemic plant resistance [1].

DSE is able to colonize plant roots both intercellularly and intracellularly without causing disease because of the melanin pigment in DSE hyphae which helps defend both the DSE fungus and the host plant [13]. Melanized cell walls can form complexes with oxygen radicals formed during stress and are beneficial for plant survival and growth in stressful environments. Melanin in fungi functions as an extracellular redox buffer that can neutralize oxidants produced by environmental stress [15]. Under stress conditions, DSE inoculation significantly increased the activity of antioxidant enzymes, such as SOD and POD, thereby reducing membrane lipid peroxidation damage caused by metal stress, and reduced leaf malondialdehyde (MDA) concentration more than the non-inoculated treatment. The results showed that DSE increased metal tolerance and improved plant growth, both by reducing metal uptake into roots and shoot accumulation and by increasing antioxidant enzyme activity to eliminate ROS stress caused by excessive metals [16].

One of the plants that underwent vegetative testing was rice (*O. sativa*) which is a staple food source for almost 40% of the world's population and the main food for the people of Southeast Asia. With the addition of DSE which has the potential to contain phytohormones and melanin as an antioxidant, it can influence the growth of rice plants and protect against biotic and abiotic stress attacks. Vergara., *et al.* [17] conducted research by testing the activ-

ity of DSE isolates by inoculating rice seeds. As a result, DSE isolates can have an effect on increasing canopy length, number of tillers per plant and ability to absorb nutrients.

With these considerations in mind, in this study the effect of the gibberellin hormone contained in DSE isolated from sunflower (*Helianthus annuus* L.) roots on the growth of rice plants (*Oryza sativa*) in the vegetative phase was observed.

Materials and Methods

Isolation of DSE Fungi

In this study, the DSE fungus was isolated from sunflower root samples (*H. annuus* L.) from Kayu Agung Village, Pisangan Jaya, Sepatan, Tangerang Regency, Banten 15520 Indonesia. Surface sterilization of the root samples was carried out using Tween-20, sodium hypochlorite and sterile distilled water. Root samples were surface sterilized, dried with sterile tissue, then grown on PDA agar medium in 9 cm petri dishes.

Preparation of Inoculum and Fermentation of DSE Fungus Isolate

DSE fungus isolates were grown on PDA media. It was taken with a loop needle and an inoculum was made for 2 days in PDB liquid media with a concentration of 24g PDB/L distilled water with a volume of 150 mL. Pipet 5% inoculum into 95% GDP medium for fermentation. Sampling was carried out every day for 14 days, shaking at a speed of 100 rpm. After harvest time, the fresh mycelia were filtered and washed with distilled water and dried in the oven for 24 hours at 80oC. Dry mycelia were weighed by dry weight and a growth curve with dry weight at the optimum time was obtained. Repeat fermentation on dry weight at the optimum time.

Extraction, purification and characterization of DSE gibberellin

Extraction and purification of gibberellin is carried out by adjusting the pH to 8.0 by adding ammonium hydroxide. Extraction was carried out with ethyl acetate (duplo). The water phase was changed back to pH 2.5 by adding 3 N hydrochloric acid, extracted again with ethyl acetate duplo. Next, it was dried by vacuum and added with methanol. Identification and quantitative analysis of gibberellin was carried out using adsorption chromatography and HPLC techniques. The fraction containing gibberellin that had been purified by preparative TLC was analyzed by reverse phase HPLC using a reversed ODS C-18 nucleosyl column, UV detector with λ = 254 nm. The mobile phase was 35% methanol in 20 mM acetate buffer (pH 3.5) with a flow rate of 0.7 mL/min. The reverse phase HPLC results of the samples were compared with gibberellin standards. Pure gibberellin isolates were characterized using infrared (IR) spectrophotometry and UV-Vis spectrophotometry [18,19].

Analytical thin layer chromatography

Analytical thin layer chromatography was carried out using plates coated with GF-254 silica gel. The plate was cut to a size of 6 × 2 cm, then a lower border of 0.5 cm and an upper border of 0.5 cm were made. The sample in methanol was spotted with a capillary tube at the lower boundary line and next to it the standard was spotted. The chromatogram was eluted with a mixture of chloroform : ethyl acetate : acetic acid (6 : 14 : 1) until the surface rose as high as the upper boundary line. The result were viewed with UV lamp λ = 254 n and then Rf was determined [18,19].

Adsorption column chromatography

Four mL of methanol extract was put into a G60 silica gel column, then eluted with a mixture of chloroform: ethyl acetate: acetic acid (6: 14: 1). Then the fractions are accommodated. The stain pattern of each fraction was analyzed by analytical thin layer chromatography and the Rf value of each fraction was compared with the Rf of the Gibberellin standard [18,19].

Preparative thin layer chromatography

Preparative thin layer chromatography was carried out by taking fractions resulting from column chromatography parallel to the GA-3 gibberellin standard. 1 mL of the combined fraction containing gibberellin was taken, then spotted in a straight line on a glass plate (20 × 20 cm) which had been coated with GF-254 silica gel and next to it was spotted with a gibberellin standard with a concentration of 10,000 ppm as a comparison. The sample was eluted with the same solvent as analytical TLC, namely a mixture of chloroform: ethyl acetate: acetic acid (6: 14: 1) until the surface rose as high as the dividing line. From this separation, it can be seen that on the TLC plate there are streak-shaped spots when viewed under UV light. The part of the sample parallel to the standard was scraped and suspended in methanol, then centrifuged and decanted. The supernatant was concentrated using a vacuum evaporator. The residue was then re-concentrated using a vacuum evaporator and methanol added to 1 mL of methanol [18,19].

Analysis of isolate results using the HPLC method

A total of 10 µL of samples containing gibberellin that had been purified by preparative TLC were analyzed with a reverse phase C-18 Nucleosyl ODS column, UV detector with λ = 254 nm. The mo-

bile phase was 35% methanol in acetic acid pH 3.5 with a flow rate of 0.7 mL/min. The HPLC isolate results were compared with the GA-3 standard [18].

Characterization of isolate results using a Uv-V is spectrophotometer.

The UV-Vis spectrophotometer was calibrated using methanol as a blank. Methanol is put into the cuvette until it is 3/4 full and measured at a wavelength of 200-400 nm. After that, a 100 ppm GA-3 standard solution was made and measured at a wavelength of 200-400 nm. Then, the maximum absorbance wavelength of the GA-3 standard is obtained. After that, the GA-3 isolate solution was measured at a wavelength of 200-400 nm. Then, the maximum absorbance wavelength of GA-3 isolate was obtained.

Identification of GA-3 using infrared spectrophotometry

The GA-3 standard solid was placed into the sample holder and its infrared absorption was measured. Then, methanol was dropped into the sample area on an infrared spectrophotometer to calibrate the presence of solvent in the isolate. After that, the GA-3 isolate, whose solvent had been evaporated, was placed onto a sample plate using an infrared spectrophotometer and its infrared absorption was measured. The infrared absorption peaks of the GA-3 isolate were compared with the absorption peaks of the GA-3 standard [18].

DSE inoculation on rice seeds

Rice seeds are soaked in water at 50oC for 20 minutes. Add 3% sodium hypochlorite (for 1 minute), then alcohol (for 1 minute) and sterile water two times (for 1 minute each). Next, soak in DSE for 48 hours assuming a density of (1x105 propagules/mL). Grown on wet cotton for 5 days until germination. At the time of DSE inoculation, DSE levels were carried out (10% v/w).

Test the activity of the isolate using the water cultivation method [20]

The test solution, consisting of GA standard and GA-3 isolate, was dropped onto filter paper. Then three different test tubes were prepared and put into two different test tubes, one other test tube was filled with filter paper that did not contain the test solution. Next, the solvent is allowed to evaporate until dry. Then, 3 mL of nutrient solution was added to each test tube and cotton was added. When the rice seeds have started to germinate and have the same size, they are put into a test tube. Next, the test tube is inserted into the laminar, with high humidity and using UV light. Then, measurements were carried out on the 7th and 14th days after planting the sprouts. Then, the average crown length and dry weight of the crown were calculated. The length of the crown is measured from the part of the plant that grows above the planting medium to the highest part of the crown. Meanwhile, the dry weight of the canopy was measured by drying the crown in an oven at a temperature below 40°C until a constant weight was obtained.

Detection of DSE Colonization in Rice Plants

Detection of DSE colonization in rice plants was carried out by cleaning the roots of the rice plants using running water for 10 minutes and cutting them to a size of 2-3 cm. Next, soak it in 10% potassium hydroxide at 90oC for 90 minutes in a water bath. The potassium hydroxide is removed and the roots are rinsed with sterile water three to five times. The roots are soaked in hydrochloric acid overnight (12 hours). Next, the roots are stained with fuchsine acid for 20-30 minutes. Stored in 50% glycerol and observed using a microscope with 400 times magnification.

Results and Discussion

Isolation of dark septate endophyte (DSE) from sunflower roots

In this study, sunflower roots (*H annus* L.), were used to isolate DSE endophytic fungi, because it is known that the Heliannthus group has DSE activity. DSE fungus isolates were taken from samples of sunflower roots (*H. annuus* L.) from Kayu Agung Village, Pisangan Jaya, Sepatan, Tangerang Regency, Banten 15520. Sunflower roots were sterilized using sodium hypochlorite, Tween20 and alcohol.

After 3 days of growing on Potato Dextrose Agar (PDA) media, several endophytic fungi were found to grow. Purification is carried out on each endophytic fungal colony that grows. Purification results obtained pure isolates or single spores (Figure 1). A black endophytic fungus was taken, which is thought to be DSE because DSE has a dark color and was identified based on macroscopic morphology, which includes color and colony shape.

Macroscopically, DSE isolate colonies grow to form filamentous, smoth, convex mold colonies and compact colonies colored gray green, brown green, black, white. Form concentric colonies with white mycelium color and the direction of growth of the mycelium towards the side. Based on the results of the macroscopic characterization of the isolate, it belongs to the fungal group Aspergillus sp. This result can be proven from the color, shape and direction of mycelium growth according to the Trichoderma and Gliocladium

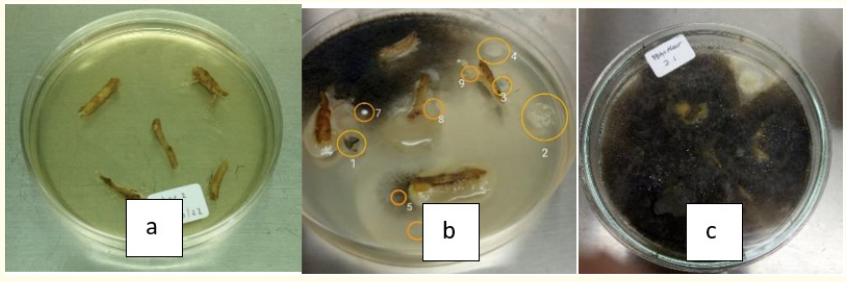


Figure 1: Isolation of DSE from the roots of sunflower plants (L.) on PDA media. (a) Isolation of DSE from sunflower roots. (b) Fungal colonies found on sunflon *H. annuus* wer roots. (c) Isolation of a single isolate.

identification book Volume I [21]. Apart from that, the single isolate was also identified based on its microscopic examination at 100 times magnification.

Microscopic results from DSE show an upright conidiophore shape, a granular fialid shape and green oval conidia which are a group of endophytic fungi Aspergillus sp (Figure 2). In addition, Aspergillus sp hyphae are septate hyphae with branched mycelium. The results of identifying the macroscopic and microscopic characteristics of this fungus are close to the Aspergillus niger group.

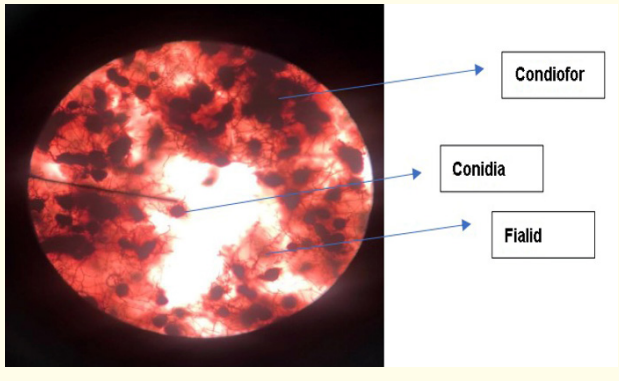


Figure 2: The structure of a single fungus isolated from sunflower roots using a microscope with 100 times magnification.

In terms of DSE characteristics, DSE isolates isolated from sunflower plant roots were similar to the research of He., *et al.* [22], that the DSE structure has septate hyphae and spores or microsclerotia and has color variations from gray to dark brown. Khastini and Jannah [1] who also isolated DSE gave microscopic results in the form of an endophytic fungus that has hyphae and has microsclerotia. Some of them are dark colored with mycelial hyphae that are septate, hyaline and thin-walled when young, become melaninized and thick-walled with age, and can develop into chlamydospores. This was also conveyed by Handayani [12] that the general structure of DSE is microsclerosium and melanized insulated hyphae. This is in accordance with the macroscopic and microscopic results seen from DSE isolates from sunflower plant roots.

Analytical thin layer chromatography

The analytical TLC results of the GA3 standard and the extracted methanol extract were observed for the presence of stains under a UV lamp λ = 254 nm and it was found that they had stains that were parallel to the standard, with an rf value of 0.4 (Figure 3). The Rf value is determined by comparing the distance traveled by the standard with the distance traveled by the solvent. In the analysis of the methanol extract, apart from obtaining stains that were parallel to the standard, there were also other stains that appeared. This indicates that the extracted methanol isolate shows the presence of the target compound, GA3, but were not pure because there were still other compounds. So, further separation process using adsorption column chromatography is needed.

Preparative thin layer chromatography

In TLC, the same mobile phase as analytical TLC is used, namely the solvent system chloroform- ethyl acetate-glacial acetic acid (6: 14: 1), and the stationary phase is silica gel GF-254. After the elution reaches the upper limit of the plate, the stains that appear are observed using ultraviolet light at a wavelength of 254 nm. A stain that is parallel to the standard is obtained, indicating that the stain is gibberellin (Figure 4). Scrape off visible stains parallel to the standard and suspend in methanol. Visible stains are taken to isolate gibberellins to obtain a purer compound.

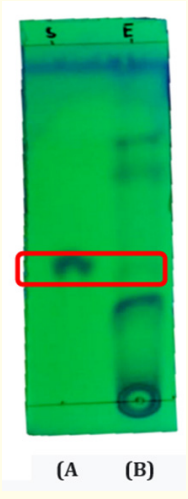


Figure 3: Analytical TLC results (A) of 10,000 ppm gibberellin (GA3) standard and methanol extract (B), with chloroform - ethyl acetate - glacial acetic acid (6:14:1) eluent under a UV lamp at λ = 254 nm.

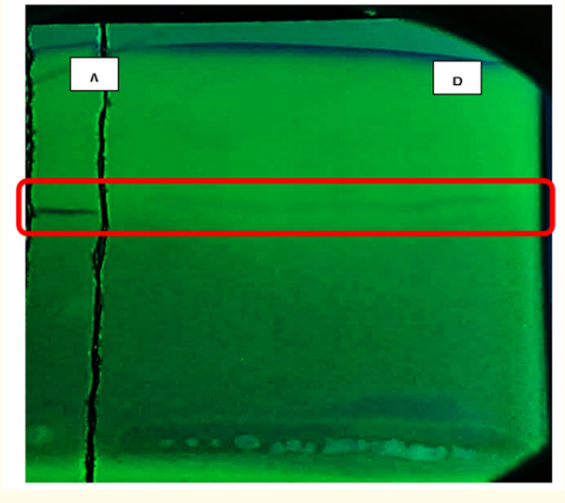


Figure 4: KLTP results of the combined fraction parallel to the GA-3 standard (A) with the solvent system chloroform - ethyl acetate - glacial acetic acid (6:14:1) under a UV lamp at λ = 254 nm. (a) Standard. (b) GA-3 isolate.

Fermentation of DSE fungus isolate

Fermentation of DSE isolates was carried out to produce endophytic cells in large numbers to optimize the secondary metabolite compounds produced. The fermentation process is carried out using a batch or closed fermentation system to minimize contamination, because there is no addition of ingredients or extraction of results during fermentation. The fermentation process in fungi is characterized by a change in the color of the fermentation medium from yellow to orange. This color change is caused by the presence of secondary metabolites that have been produced during the fermentation period and will be released into the media with the color change depending on the secondary metabolites produced.

A standard growth curve was created to determine the growth rate of DSE isolates so that the growth phases of all DSE isolates in the growth medium could be identified. Based on the growth curve of DSE isolates (Figure 5), DSE isolates experienced an exponential phase from day 1 to day 5, this can be seen in the growth curve which shows an increase in cell number with a marked increase in the curve. After day 5 to day 10, the growth curve shows that the growth of endophytic fungi enters a stationary phase. The results showed that DSE growth entered the stationary phase on the 5th day, which indicated that the optimum mycelial number was on the 5th day with an average DSE mycelial content of 0.0124 g/mL.

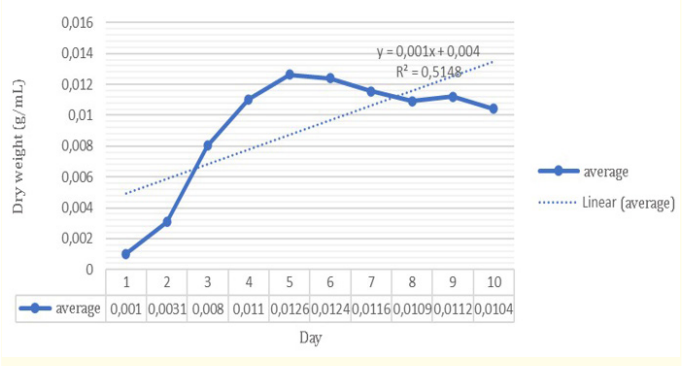


Figure 5: Graph of the growth curve of DSE isolates for 10 days on PDB media.

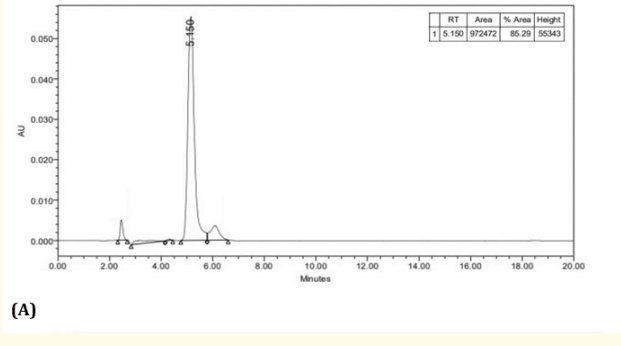
In this study, phytohormone isolation was carried out on the 7th day. This is possible because at stationary conditions, sufficient carbohydrate sources are still available to form secondary metabolites, even though other nutrients have begun to decrease. Apart from that, the most optimum formation of secondary metabolites occurs in the final stationary phase. According to Pelczar and Chan [23], microorganisms will produce secondary metabolites at the end of their growth cycle which is the stationary phase. This is because secondary metabolites are usually synthesized at the end of the cell growth cycle, namely in the stationary phase. At this time,

the number of cells that grow is equal to the number of cells that die. In this study, phytohormone isolation was carried out on the 7th day. This is possible because at stationary conditions, sufficient carbohydrate sources are still available to form secondary metabolites, even though other nutrients have begun to decrease. Apart from that, the most optimum formation of secondary metabolites occurs in the final stationary phase.

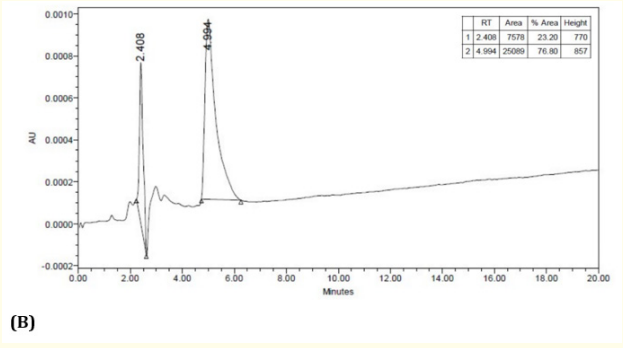
Identification of gibberellin isolates by Reversed-Phase HPLC, UV-Vis and FTIR

Amount of 10 µL fraction containing gibberellin (GA-3 isolate) which had been purified by PTLC, was analyzed by Reversed-Phase (RP) HPLC, using Alltec 8011/2 (C-18 Nucleosyl ODS column) with UV detector at a wavelength of 254 nm. Analyzed by FTIR (Shimadzu 8400) and analyzed by Mass Spec. MS (Waters type Xevo Q-Tof MS). The buffer elution for RP HPLC was performed with an isocratic method using 35% methanol in acetate buffer (pH 3.5) with a flow rate of 0.7 mL/minute. The result of RP-HPLC,Uv-Vis and FTIR were compared to the GA-3 standard [18,19].

In the Figure 6A showed the result of RP-HPLC of GA-3 standard has a retention time of 5,150 minutes. The RP-HPLC result of GA-3 sample has a retention time at 4,994 minutes (Figure 6B), which relatively has the same retention time as the GA-3 standard. This indicates that the sample isolates contain gibberellin type of GA-3. A little difference in retention time could be caused by the presence of other peaks that caused a shift in retention time due to the presence of impurities isolates.



(A)



(B)

Figure 6: Chromatogram of GA-3 standard (A), and GA-3 isolates (B) separated using RP-HPLC method. Column used was C-18 Nucleosyl ODS column and detector used was UV detector at wavelength of 254 nm. The mobile phase used was 35% methanol in acetate buffer (pH 3.5) with a flow rate of 0.7 mL / minute.

The concentration of GA-3 isolates (gibberellin isolated from DSE) can be determined by comparing the peak area of the sample with the peak area of the GA-3 standard and multiplied by the concentration of the 10 µL standard injected. After calculation, the concentration of GA-3 in the in the DSE growth medium was 0.516 mg/mL.

Identification of GA-3 using UV-V is spectrophotometry.

In the analysis of gibberellin samples, identification was carried out at ultraviolet wavelengths at a wavelength of 200-400 nm. This is because the sample is colorless, so it does not absorb visible light. The maximum wavelength absorption was found at 206 nm (Figure 7) almost similar with the GA-3 standard, was found at 207 nm (Figure 8).

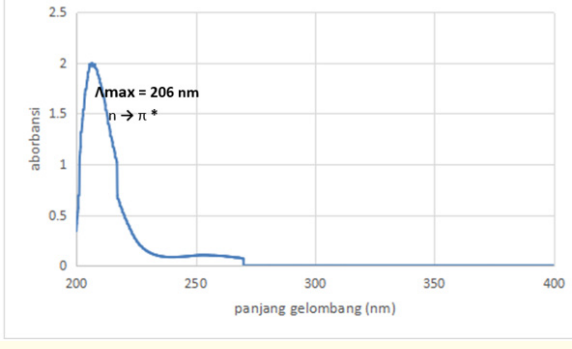


Figure 7: UV spectrum of the GA-3 standard using methanol as a solvent. Obtained λmax at 206 nm.

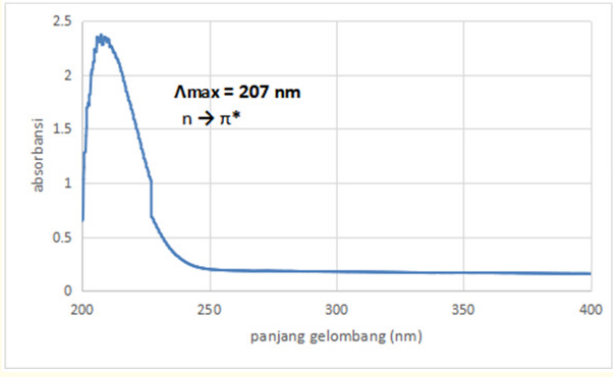


Figure 8: UV spectrum of the GA-3 isolate using methanol as a solvent. Obtained λ max at 207 nm.

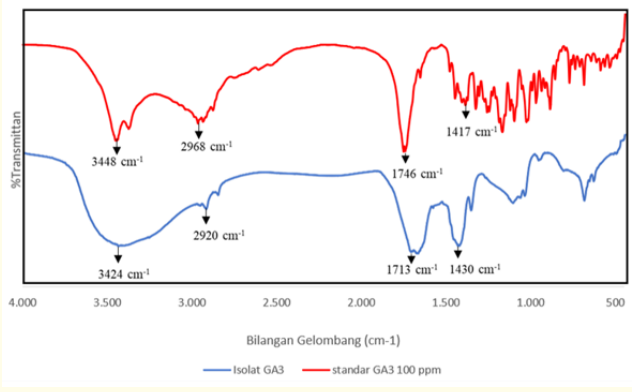


Figure 9: FTIR spectrum of GA-3 standard [red line] (a) and GA-3 isolate [blue line] (b). FTIR spectrum of GA-3 standard and GA3 isolate shows the presence of O-H(s) bonds; C-H sp3 ; C=O ; and C=O(s) at wave numbers 3448 and 3424 cm-1 respectively; 2968 and 2920 cm^{-1}; 1746 and 1713 cm-1; 1417 and 1430 cm^{-1}.

According to Suhartati [24], absorption at a maximum wavelength of 206 nm is absorption from the carbonyl group of carboxylic acids and esters in the R (radical-like) band. This is in accordance with the structure of GA-3 which has carboxylic acid and ester groups. Absorption at a wavelength of 206 nm is thought to be due to the n→ π* transition of the C=O chromophore. Compounds that have non-conjugated carbonyl groups, such as carboxylic acids and esters, have absorption bands originating from the excitation of n→ π* electrons due to the presence of free electrons. The non-conjugated carbonyl group will appear at a lower wavelength compared to the conjugated carbonyl due to the presence of conjugation. The results obtained differ by 1 nm but are still within the tolerance of ±1 nm for the UV area and 2 nm for the visible area. So the results are accurate in indicating the λ max and the shift value is not meaningful.

Identification of GA-3 using Infrared Spectrophotometry

The GA-3 isolates were analyzed to identify isolates based on their functional groups using Fourier-Transform Infrared (FTIR) spectroscopy. The absorbance of GA-3 isolate was compared with the GA-3 standard (Figure 9).

The results of infrared wave absorption measurements show that the standard infrared absorption spectrum of GA-3 is obtained, there is a wide absorption at wave number 3448 cm-1 which is the absorption of the O-H (alcohol) group (stretching, s), the peak at wave number 2968 cm-1 is C-H (s) sp3, C = O (s) (esters and carboxylic acids) at a wave-number of 1746 cm-1, and C = C (s) (alkene) at a wave number of 1417 cm-1 (Figure 9).

The peaks that appeared in the characterization of GA-3 isolates had the same absorbance as the GA-3 standard. However, there are still differences in the shape of the wide peak and a shift in values between the standard and the isolate. This can happen because the isolate still contains impurities which can affect the absorption of IR light during testing which is translated into a spectrum. Hydrogen bonding is one that can influence the shape and frequency of absorption because the free OH group will provide a large frequency band and sharp intensity. Meanwhile, the bound OH group provides lower and wider frequency absorption [25].

Biological Test of GA-3 isolates of DSE on Rice Plants (*O. sativa*)

To determine the effect of the activity of GA-3 from DSE isolates on the growth of rice plants in the vegetative phase using hydroponic media, amount of 8 ppm GA-3 isolate was used and compared with the activity of control (8 ppm of GA-3 standard).

Based on the canopy dry weight parameter, it was found that the active percentage of GA-3 isolates increased by 128.17% and GA-3 standards by 137.32% compared to the control of 100% (Figure 10).

The rice plant canopy length parameter was compared with the control with an active percentage of 100%. The administration of 8 ppm GA-3 isolate showed an activity of 188%, while the addition of 8 ppm GA-3 standard showed an activity of 204.51% (Figure 11). The gibberellin hormone added to plants will provide a response in the form of increasing stem length by increasing cell division and growth [26]. This is in accordance with the activity of gibberel-

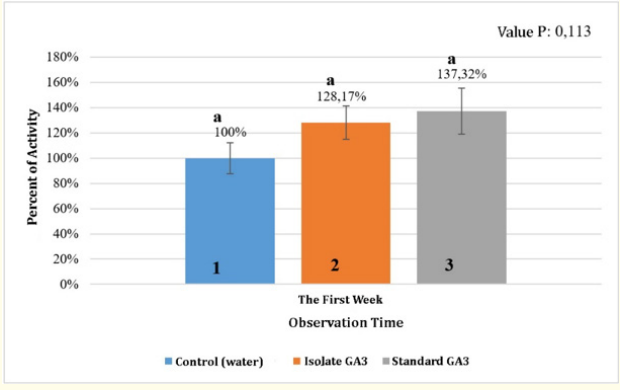


Figure 10: Histogram of dry weight parameters of rice plant shoots against the activity of GA-3 isolate 8 ppm. The treatments observed were 1= control; 2 = 8 ppm of GA-3 isolate, and 3 = 8 ppm of GA-3 standard; in the first week of rice plant growth bio-test.

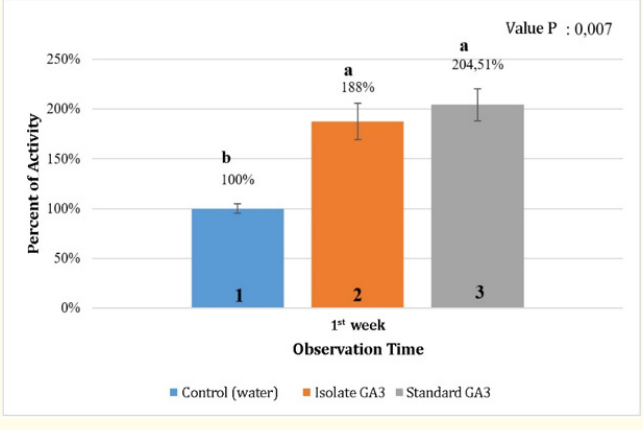


Figure 11: Histogram of rice plant canopy length parameters on the activity of the 8 ppm GA-3 isolate. The treatments observed were 1= control; 2 = 8 ppm of GA-3 isolate; and 3 = 8 ppm of GA-3 standard ; in the first week of rice plant growth bio-test.

lins on plant growth which can help increase the dry weight of the canopy in rice plants and its effect can be seen in the first week of growth.

Biological Test of the Effect Fe2+ Heavy Metals and Salinity Stress of NaCl on the Growth of Rice Plants (O. *sativa*) with DSE Inoculation.

Based on the results of observations of the dry weight of the rice plant canopy, it shows that by administering 300 ppm Fe_{2+} stress, the dry weight of the shoot can be reduced in the first, second and third weeks respectively by 77.48, 97.35, and 96.65% compared to the control 100% without applying stress (Figure 12). This is in accordance with Noor., *et al.* [27], that Fe2+ stress of 300 ppm is at a rather severe level of poisoning. In the research of Sunadi., *et al.* [28], that Ciherang rice experienced a decrease in shoot dry weight with 300 ppm Fe stress of 0.1 gram compared to the control of 0.54 gram. Fe stress is indicated by decreased plant dry weight, much reduced number offspring and little chlorophyll resulting in poor plant appearance.

The effect of DSE inoculation in the presence of Fe stress was observed in protecting plants from the presence of abiotic stress. The results show that the presence of DSE inoculation in Fe stress conditions can further increase the growth of rice plants in the first, second and third weeks by 99.10, 116.37, and 112.55%, respectively compared to rice plants without DSE inoculation (Figure 12). The melanized cell walls of DSE can form complexes with oxygen radicals formed during stress and are beneficial for plant survival and growth in stressful environments. Melanin in fungi functions as an extracellular redox buffer that can neutralize oxidants produced by environmental stress [15].

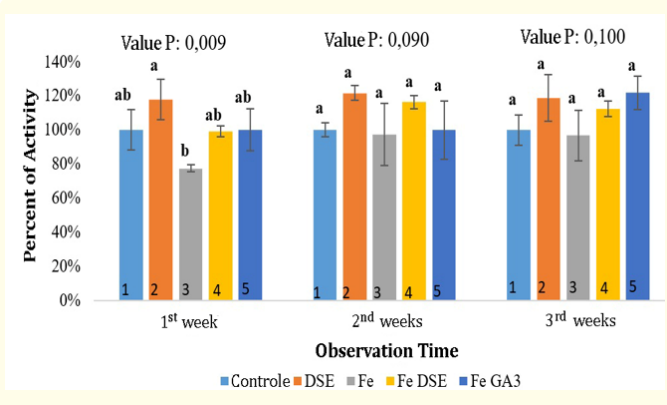


Figure 12: Histogram of the percentage of activity based on the dry weight parameter of rice plant shoots under 300 ppm of Fe stress. The treatments observed were 1 = control; 2 = DSE inoculation; 3 = 300 ppm of Fe stress; 4 = 300 ppm of Fe stress with DSE inoculation; and 5 = 300 ppm of Fe stress with 8 ppm of GA-3 hormone; in the second week of rice plant growth bio-test.

The effect of DSE inoculation on rice plants was also observed on the growth of rice dry weight. Inoculation of DSE can increase shoot dry weight in the first, second and third weeks respectively by 118.02, 121.68, and 118.83% compared to control plants without inoculation (Figure 12). DSE inoculated in plants can have an influence on plant growth because it can produce secondary metabolites, siderophores, and act as a phosphate solvent [29].

In the results of the Tukey test, the dry weight parameter for the first week of shoots showed the results of grouping control (ab), DSE inoculation (a), Fe stress (b), Fe stress with DSE inoculation (ab) and 8 ppm of GA-3 hormone under Fe stress (ab). From the grouping results, it can be concluded that the dry weight parameters of the first week of canopy have visible differences in the grouping of different letters in each treatment. Meanwhile, the results of the Anova test for the canopy dry weight parameter (Figure 13) showed that the P value is smaller than α with a value of 0.009 < 0.05. So it was concluded that if the canopy length parameter H1 was accepted, the addition of isolate GA-3 had a significant difference in the first week of observation. Meanwhile, the second and third weeks showed that the P value was greater than α with values of 0.090 > 0.05 and 0.100 > 0.05 respectively. So it was concluded that the canopy dry weight parameter H0 was accepted and H1 was rejected, which shows that the application of Fe stress to DSE-inoculated rice plants had no significant difference in the week of observation.

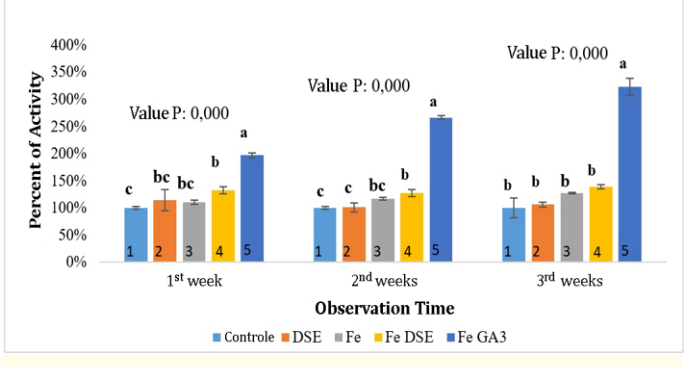


Figure 13: Histogram of the percentage of activity based on the canopy length parameter of rice plants under 300 ppm of Fe stress. The treatments observed were 1 = control; 2 = DSE inoculation; 3 = 300 ppm of Fe stress; 4 = 300 ppm of Fe stress with DSE inoculation; and 5 = 300 ppm of Fe stress with 8 ppm of GA-3 hormone; in the second week of rice plant growth bio-test.

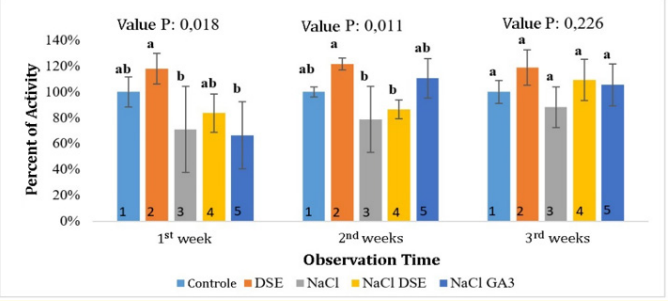


Figure 14: Histogram of the percentage of activity based on the dry weight parameter of rice plant shoots under 5000 ppm of salinity (NaCl) stress. The treatments observed were 1 = control; 2 = DSE inoculation; 3 = 5000 ppm of NaCl stress; 4 = 5000 ppm of salinity (NaCl) stress with DSE inoculation; and 5 = 5000 ppm of salinity (NaCl) stress.

In the results of the Tukey test, the dry weight parameters of shoots with NaCl stress in the first and second weeks (Figure 14), showed the results of grouping control (ab), DSE inoculation (a), NaCl stress (b), NaCl stress with DSE inoculation (ab) in the first week and (b) in the second week, as well as 8 ppm of GA-3 hormone during NaCl stress (b) in the first week and (ab) in the second week. From the results of the grouping, it can be concluded that the dry weight parameters of the first and second weeks of canopy had visible differences in the grouping of the same letters in each treatment. However, the results of the Tukey test, the parameter of dry weight of shoots with the third week of NaCl stress, showed that the grouping results were in the same group for all treatments. From the results of the grouping, it can be concluded that in the dry weight parameters of the third week there was no visible difference when grouping the same letters in each treatment.

The results of the Anova test for canopy dry weight parameters in the presence of NaCl stress (Figure 15), showed that the P value was smaller than α in the first and second weeks with values of 0.018 < 0.05 and 0.011 < 0.05, respectively. So it was concluded that the canopy dry weight parameter H1 was accepted and H0 was rejected, which shows that the application of NaCl stress to DSE-inoculated rice plants had a significant difference in the first and second weeks of observation. Meanwhile, the results of the Anova test for the shoot dry weight parameter in the presence of NaCl stress showed that the P value was greater than α in the third week with a value of 0.226 > 0.05. So it was concluded that the shoot dry weight parameter H0 was accepted and H1 was rejected, which indicates

that NaCl stress was given. There was no significant difference in DSE-inoculated rice plants in the third week of observation.

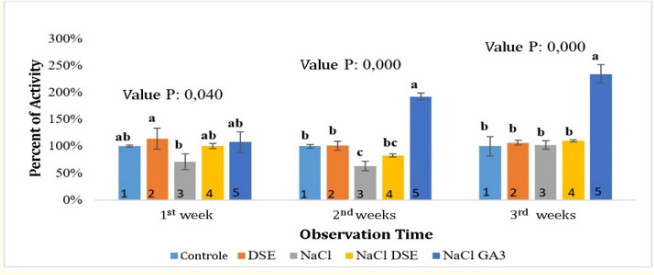


Figure 15: Histogram of the percentage of activity based on the canopy length parameter of rice plants under 5000 ppm of NaCl stress. The treatments observed were 1 = control; 2 =DSE inoculation; 3 = NaCl stress 5000 ppm; 4 = 5000 ppm of NaCl stress with DSE inoculation; and 5 = 5000 ppm of NaCl stress with 8 ppm GA-3 hormone; in the second week of rice plant growth bio-test.

Detection of DSE colonization in rice plants

Microscopic observation of DSE showed that the DSE isolate inoculated in the roots was in the form of microsclerotia and also showed the presence of septate hyphae (Figure 16). In terms of DSE characteristics, DSE isolates isolated from sunflower plant roots were similar to the research of He., *et al.* [22], that the DSE structure has septate hyphae and spores or microsclerosia and has color variations from gray to dark brown. Root-inoculated DSE was also reported by Zhang., *et al*. [30], with the presence of thin, slightly dark melanized hyphae and brand-shaped, peloton-like, brain-like DSE microsclerotia colonize the epidermis and cortex cells of most plants.

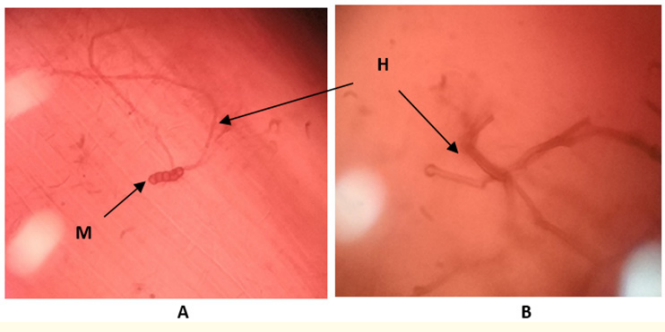


Figure 16: Morphological characteristics of microsclerotia (M) and hyphae (H) from DSE colonized on rice plant roots with 400 times magnification. (A) Microsclerotia and hyphae. (B) Insulated hyphae.

Conclusion

- The gibberellin (GA-3) content in DSE isolates from sunflower roots (H. annuus L.) using high performance liquid chromatography showed gibberellin levels in the DSE growth medium of 0.561 mg/mL.
- GA-3 isolate from DSE isolate can increase the growth of rice plants (O. sativa) in the vegetative phase. Observations based on canopy dry weight parameters in the first week showed an active percentage of 128.17%. Observations based on the canopy length parameter showed that the percentage of activity in the first week was 188%.
- DSE inoculation of rice plants with metal and salt stress can increase the growth of rice plants (O. sativa) in the vegetative phase. In the 300 ppm Fe stress treatment, observations based on shoot dry weight parameters in the first, second and third weeks showed a greater percentage of activity in DSE inoculated rice with Fe stress of 99.10, 116.37 and 112.55% respectively. compared to rice without DSE inoculation with Fe stress of 77.48, 97.35 and 96.65% respectively. Observations based on canopy length parameters in the first, second and third weeks showed a greater percentage of activity in DSE-inoculated rice with Fe stress of 132.56, 126.84, and 139.01% respectively compared to rice without DSE inoculation with Fe stress was 110.60, 116.47, and 127.10% respectively. In the 5000 ppm NaCl stress treatment, observations based on shoot dry weight parameters in the first, second and third weeks showed a greater percentage of activity in DSE inoculated rice with NaCl stress of 83.78, 86.73, and 109.21, respectively, compared to rice without DSE inoculation with NaCl stress of 71.17, 78.76, and 88.28%, respectively. Observations based on canopy length parameters in the first, second and third weeks showed a greater percentage of activity in DSE-inoculated rice with NaCl stress of 100.47, 82.85, and 110.11% compared to rice without DSE inoculation with NaCl stress respectively. Respectively, 71.22, 63.37, and 102.63%.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful for the Laboratory facilities in the Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Padjadjaran, and to the Executive Board of Universitas Padjadjaran, Dean of the Faculty of Mathematics and Natural Sci-

ences, Head of the Department of Chemistry, Head and the entire staff of Biochemistry Laboratory. We would like also to thank all those who have given advice, motivation, and material support.

Bibliography

1. Khastini RO and Jannah R. "Potential Contribution of Dark-Septate Endophytic Fungus Isolated from Pulau Dua Nature Reserve, Banten on Growth Promotion of Chinese Cabbage". In Joint proceedings of the 2nd and the 3rd International Conference on Food Security Innovation (ICFSI 2018-2019). Atlantis Press (2019): 83-89.

2. Zhan F., *et al*. "Characterization of melanin isolated from a dark septate endophyte (DSE), *Exophiala pisciphila". World Journal of Microbiology and Biotechnology* 27.10 (2011): 2483-2489.

3. Toding T., *et al*. "Potency of Endophytic Fungi as Biocontrol Agent of Cacao Black Pod Disease Caused by Phytophthora palmivora (Butl.)". *Agron Indonesia* 40.2 (2012): 146-152.

4. Sopialena S., *et al*. "Diversitas Jamur Endofit Pada Tanaman Padi *(Oryza sativa* L.) dan Potensinya Sebagai Pengendali Hama". *Jurnal Agroekoteknologi Tropika Lembab* 2.1 (2019): 44-49.

5. Suswanto I., *et al*. "Penggunaan cendawan endofit sebagai agens pengendali hayati pada lada (*Piper nigrum* L.)". *Jurnal Agroqua* 16.2 (2018): 143-151.

6. Syamsia S., *et al*. "Combination on endophytic fungal as the plant growth-promoting fungi (PGPF) on cucumber (*Cucumis sativus*)". *Biodiversitas* 22.3 (2021): 1194-1202.

7. Digra S and Nonzom S. "An insight into endophytic antimicrobial compounds: An updated analysis". *Plant Biotechnology Reports* (2023): 1-31.

8. Khare E., *et al*. "Multifaceted interactions between endophytes and plant: Developments and prospects". *Frontiers in Microbiology* 9 (2018): 2732.

9. Strobel G. "The emergence of endophytic microbes and their biological promise". *Journal of Fungi* 4 (2018): 57.

10. Meshram V and Gupta M. "Endophytic fungi: A quintessential source of potential bioactive compounds". In *Sustain: Endophytes for a Growing World*; Hodkinson, T., Doohan, F., Saunders, M., Murphy, B., Eds.; Cambridge University Press: Cambridge, UK 13 (2019): 277-309.

11. Meshram V., *et al*. "Endophytic Fusarium clavum confers growth and salt tolerance in Cucumis melo". *Environ. Exp. Bot* 3.206 (2011): 105-153.

12. Handayani, D. 2016. Keberadaan Cendawan Dark Septate Endophyte (DSE) Pada Sistem Perakaran Benih Shorea Salanica". *Eksakta* 1 (2016): 38-44.

13. Dalimunthe CI., *et al*. "Seleksi Dan Uji Potensi Cendawan Dark Septate Endophyte Sebagai Agensia Hayati Penyakit Jamur Akar Putih (Rigidoporus microporus) Pada Tanaman Karet". *Jurnal Penelitian Karet* (2019): 11-20.

14. He C., *et al*. "Characterization of dark septate endophytic fungi and improve the performance of liquorice under organic residue treatment". *Frontiers in Microbiology* 10 (2019).

15. Zhan F., *et al*. "Characterization of melanin isolated from a dark septate endophyte (DSE), Exophiala pisciphila". *World Journal of Microbiology and Biotechnology* 27.10 (2011): 2483-2489.

16. Zhu L., *et al*. "The effects of dark septate endophyte (DSE) inoculation on tomato seedlings under Zn and Cd stress". *Environmental Science and Pollution Research* 25.35 (2018): 35232-35241.

17. Vergara C., *et al*. "Contribution of dark septate fungi to the nutrient uptake and growth of rice plants". *Brazilian Journal of Microbiology* 49.1 (2018): 67-78.

18. Zhilan DF., *et al*. "Isolation and Characterization of Gibberellin (GA-3) from Banana Blossom of *Musa balbisiana* Colla and Effect of The Isolate Addition on The Growth of Soybean (*Glycine max*) Plant". *Research Journal of Chemistry and Environment* 26.11 (2022): 28-37.

19. Sari RP Maksum., *et al*. "Isolation, purification, and characterization of auxin from red algae (*Eucheuma spinosum*) and its application in cayenne pepper (*Capsicum frutescens* L.) plants". *Bulgarian Journal of Agricultural Science* 28.6 (2011).

20. Rachman SD., *et al*. "Alga Merah (Gracilaria coronopifolia) sebagai Sumber Fitohormon Sitokinin yang Potensial". *Chimica et Natura Acta* 5.3 (2017): 124-13. DOI: https://doi.org/10.24198/cna.v5.n3.16060

21. Kubicek CP and Harman GE. "*Trichoderma and Gliocladium"*. Vol. 1, Basic Biology, *Taxonomy and Genetics*. Taylor and Francis, London.

22. He C., *et al*. "Dark Septate Endophytes Isolated From Wild Licorice Roots Grown in the Desert Regions of Northwest China Enhance the Growth of Host Plants Under Water Deficit Stress". *Frontiers in Microbiology* 12 (2021): 1-18.

23. Pelczar MJ Chan., *et al*. "Microbiology Concepts and Applications," Mc Graw-Hill Inc, New York (1993).

24. Suhartati T. "Basics of UV-Vis Spectrophotometry and Mass Spectrometry for Determining the Structure of Organic Compounds. Bandar Lampung: CV". Anugrah Utama Raharja (2017).

25. Supratman U. "Elusidasi Struktur Senyawa Organik. Widya Padjadjaran". Bandung

26. Asra R., *et al*. "Hormon Tumbuhan". 1st edition. I. Jatmoko, ed. Jakarta: UKI Press (2020).

27. Noor A., *et al*. "Pengaruh Konsentrasi Besi dalam Larutan Hara terhadap Gejala Keracunan Besi dan Pertumbuhan Tanaman Padi". *Jurnal Agronomi Indonesia* 40.2 (2012): 91-98

28. Sunadi Wahidi I and Utama MZH. "The Screening of Fe^{2+} Tolerant Rice Variety in New Wetland Field by Using Agronomy and Physiology Indices". *Jurnal Akta Agrosia* 13.1 (2010): 16-23.

29. Syamsia S., *et al*. "Combination on endophytic fungal as the plant .growth-promoting fungi (PGPF) on cucumber (*Cucumis sativus*)". *Biodiversitas* 22.3 (2021): 1194-1202

30. Zhang Y., *et al*. "Colonization Characteristics and Composition of Dark Septate Endophytes (DSE) in a Lead and Zinc Slag Heap in Southwest China". *Soil and Sediment Contamination* 22.5 (2013): 532-545.