Exploring the Impact of Endophytic Fungus *Aspergillus cejpii* DMKU-R3G3 on Rice: Plant Growth Promotion and Molecular Insights through Proteomic Analysis

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Abstract: Rice is a crucial crop for many people worldwide, especially in regions like Asia, Latin America, and parts of Africa. Thailand is one of the largest exporters of rice. Nowadays, farmers use chemicals to control rice disease, which can have negative effects on humans and the environment. Therefore, the objective of this study was to examine the plant-promoting capabilities of the endophytic fungal strains DMKU-R3G3 in greenhouse settings. The endophytic fungi strain DMKU-R3G3, which was isolated from organic rice root, was identified as *Aspergillus cejpii* based on morphological characteristics and phylogenetic analysis. The production of IAA was detected using Salkowski’s reagent. After 7 days of incubation, the finding revealed that the strain cultivated in PDB supplemented with tryptophan yielded a greater concentration of IAA (25.45 µg/mL). The inoculation with *A. cejpii* DMKU-R3G3 significantly enhanced rice growth, as evidenced by notable increases in shoot height, root length, and fresh weight. Moreover, the chlorophyll content of the rice plants also increased by 1.78 times more than the control group. In addition, proteomic analysis revealed that rice responded toward the colonization of endophytic fungi by producing auxin-responsive proteins to regulate the IAA content in plant tissue and inducing total chlorophyll production due to the up-regulation of proteins in the chlorophyll biosynthesis pathway. The results obtained from this study lead to the conclusion that the *A. cejpii* strain DMKU-R3G3 has the potential to be a promising eco-friendly plant growth promoter for sustainable rice cultivation.

Keywords: chlorophyll content; fungal endophyte; indole-3-actic acid; *Oryza sativa*; phytohormone; pigment parameters; plant growth-promoting fungi

1. Introduction

One of the most important foods for the developing human population is cereal. Wheat, rice, and maize are the primary sources of approximately 50% of the calories consumed by humanity as a whole. Rice (*Oryza sativa* L.) provides 21% of energy and 15% of protein for humans. Rice takes second place because of the planting area, but it serves as the main food crop in Asia, especially in the southeast, where it is cultivated on millions of hectares throughout the region as an economic crop by farmers and workers [1]. Rice serves as a staple food for 2.7 billion individuals across Asia [2]. Historically, rice was first grown in the river valleys of South and Southeast Asia and China 10,000 years ago since it was the...
most significant food for people. Although Asia is the main location of rice cultivation, it is also harvested in other countries, including Latin America, Europe, some areas of Africa, and even the United States [1].

Fungal endophytes, alternatively referred to as endophytic fungi, represent a diverse group of polyphyletic organisms. Constituent taxa can colonize the interior of plant tissues (such as stems, flowers, leaves, fruits, and roots) intracellularly or extracellularly throughout all or a portion of their life cycles [3]. These fungi inhabit plant tissues without inducing any negative effects or diseases in the colonized tissue [4]. Aspergillus, Fusarium, and Penicillium were the dominant endophytic fungi associated with rice plants [5–7]. Endophytic fungi offer various benefits to their hosts, such as promoting germination and shoot growth, bolstering host plant resilience against biotic or abiotic stresses, and stimulating the accumulation of bioactive metabolites in host plants. Additionally, endophytes have been widely employed in agricultural practices, including those involving rice (Oryza sativa L.) and dancing-lady orchids (Oncidium hybridum) [8]. Moreover, endophytic fungi can reduce the intensity of rice blast disease [9].

Fungi of the genus Aspergillus are widespread and can exist either as saprotrophic or endophytic organisms [10]. Lately, various species and strains within this genus have been identified as potential enhancers of plant growth [11–13]. These fungi can stimulate plant growth by synthesizing phytohormones like indole-3-acetic acid (IAA), offering a soil nutrient bioavailability that presents a more environmentally sustainable option compared to conventional agricultural practices [14,15]. Previous studies showed that Aspergillus niger promotes seedling growth in various vegetables, enhancing shoot and root development while increasing indoleacetic acid and gibberellic acid production [16]. Aspergillus spp. also aids cotton growth by enhancing phosphorus and nitrogen absorption [17]. Aspergillus chevalieri and A. egyptiacus produce compounds that boost plant growth and defense, suggesting their eco-friendly potential as biocontrol agents against pathogens [18]. Nevertheless, our understanding of the impact of the Aspergillus genus on plants remains limited.

Proteomics demonstrated its effectiveness in uncovering the molecular intricacies of plants by identifying a wide array of proteins associated with plant growth, development, metabolic processes, and those responsive to environmental stresses, both abiotic and biotic [19,20]. Furthermore, to enhance comprehension of plant–microbe dynamics, proteomics were employed to unravel the responsive pathways between fungi and plants. For instance, the examination of the symbiotic relationship between arbuscular mycorrhizal fungi and A. fruticosa sheds light on the molecular underpinnings of their interaction [21]. Investigations into the proteomes of two varieties of wheat in association with G. mosseae revealed that the fungus aids in alleviating root osmotic stress and preserving cellular structure [22].

The objective of this study was to examine the plant-promoting capabilities of the endophytic fungus DMKU-R3G3 in greenhouse environments. The fungus was isolated from organic rice tissue. This particular isolate was distinguished by its capacity to produce plant growth-promoting attributes, including the synthesis of indole-3-acetic acid (IAA) and the solubilization of phosphate. The potential of the endophytic fungus as a plant growth promoter was evaluated using rice (Oryza sativa L.). In addition, we characterized the proteome of the endophytic fungus DMKU-R3G3 interacting with rice under greenhouse conditions.

2. Materials and Methods

2.1. Endophytic Fungal Isolation

Rice (Oryza sativa) Khao Dawk Mali 105 (KDML105) samples were collected from organic rice fields in Suphanburi province in central Thailand (14°35′34.7″ N 100°08′25.2″ E). The isolation technique for endophytic fungi was based on the method established by Sutthinon et al. [23]. Briefly, healthy rice roots were delicately washed with tap water, followed by immersion in 75% ethanol for 3 min and soaking in 3% sodium hypochlorite
for 5 min. Surface-sterilized plant tissues underwent three rinses with sterile distilled water. The plant tissue was ground in a sterile mortar and then transferred into a potato dextrose agar (PDA) supplement with 0.1 mg/mL streptomycin to prevent bacterial growth. The plates were incubated at 30 °C in darkness. Fungal culture underwent isolation and purification on PDA. The fungus was preserved on PDA at 4 °C and subcultured every 3 months. For long-term preservation, it was stored in 20% glycerol at −80 °C.

2.2. Fungal Identification

Identification of the fungi was conducted through analysis of morphological characteristics combined with phylogenetic assessments. Endophytic fungus DMKU-R3G3 was cultured on PDA for 7–14 days. Macroscopic characteristics of the fungal colony and microscopic characteristics of hypha, ascomata, ascus, and ascospore were recorded [24]. For molecular analysis, endophytic fungus DMKU-R3G3 was cultured on PDA for 3–5 days. The genomic DNA of strain DMKU-R3G3 was extracted by grinding the fungal mycelia with liquid nitrogen, followed by the DNA extraction method [25]. Five hundred microliters of lysis solution (pH 8.0), comprising 400 mM Tris-HCl, 60 mM EDTA, 150 mM NaCl, and 1% SDS, were introduced and allowed to rest on ice for 30 min. Following this, 150 microliters of 5 M potassium acetate (pH 4.8) were included, and the mixture underwent additional cooling on ice for 15 min before being subjected to centrifugation at 12,000 rpm for 2 min. The resultant supernatant was transferred to a fresh tube, and equivalent volume of isopropanol was introduced, followed by centrifugation at 12,000 rpm for 2 min. The genomic DNA underwent cleansing with 70% ethanol, and the desiccated DNA pellets were reconstituted in deionized water.

A sequence of the ITS was determined by PCR amplification using forward ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse ITS-4 (5′-TCCCTCGCTATTGATATGC-3′), while large subunit (LSU) region was determined by PCR amplification using forward (LR0R: 5′-ACCCGCTGAACTTAAGC-3′) and reverse (LR5: 5′-TCCTGAGGGAAACTTCG-3′) primers. Each PCR mixture consisted of 100 ng of fungal genomic DNA in a 50 mL reaction volume alongside a reaction buffer (10 mM Tris-HCl, 3.0 mM magnesium chloride, pH 8.3), 0.4 mM of each primer, and one unit of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR commenced with initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C (ITS) and 60 °C (LSU) for 30 s, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was included. PCR products were observed via 0.9% w/v agarose gel electrophoresis. Purified PCR products underwent sequencing on both strands through a commercial service (Macrogen, Seoul, Republic of Korea).

Sequence alignments and similarity calculations were executed utilizing the Clustal W program. Subsequently, a phylogenetic tree was assembled from LSU sequences employing the MegaX program, employing the maximum likelihood method with 1000 bootstrap resampling replicates [26].

2.3. IAA Production

The endophytic fungus DMKU-R3G3 was determined for IAA production by calorimetric assay using Salkowski reagent [27]. The endophytic fungus DMKU-R3G3 was cultured on potato dextrose agar (PDA) for 3 days. Then, the fungal mycelium was cut by using a sterile cork borer and transferred into potato dextrose broth with a sterile inoculation needle. The fungus was cultured in potato dextrose broth (PDB) supplemented with 100 µg/mL of tryptophan and/or without tryptophan at room temperature and 200 rpm in conditions of darkness for seven days. All experiments were performed in triplicate. The cultured filtrate was kept in a refrigerator at −20 °C. One mL of culture filtrate was mixed with 2 mL of Salkowski reagent (2 mL of 0.5 M FeCl₃ and 98 mL of 35% perchloric acid solution). After incubation in the dark for 30 min, absorbency at 520 nm was determined by a spectrophotometer Multiskan GO (Thermo Scientific, Waltham, MA, USA). IAA quantifications were calculated by comparing the standard curve, which was plotted with standard
IAA concentrations (10, 25, 50, 100, 150, 200, 250, 300 µg/mL) against absorbance ($A_{520}$). The quantity of IAA was measured every day for 7 days.

2.4. Phosphate Solubilization

Phosphate solubilizing activity was performed on Pikovskaya’s agar (PKV) with 1% tricalcium phosphate [28]. The endophytic fungus was cultured on PDA for 3 days. Then, the 5 mm diameter from the growing edge of the mycelia was cut and used as an inoculum. A fungal mycelium plug was then placed on PKV agar. All experiments were performed in triplicate. The halo zone formation around the colony was measured after 7 days of incubation. Three replicates were tested. The solubilization index was calculated following Doilom et al. [29].

2.5. Co-Cultivation of Endophytic Fungus DMKU-R3G3 with Rice

Rice seeds of Thai Khao Dawk Mali 105 (KDML105) were surface-sterilized in 70% alcohol for 3 min and in 3% sodium hypochlorite solution for 5 min; then, they were rinsed with sterile water repeatedly. Thirty rice seeds were inoculated with 5 mL spore suspension ($10^5$ spore/mL) of endophytic fungus DMKU-R3G3 and cultured in a sterile Petri dish for 7 days under light condition (16 h of light and 8 h of darkness at 30 °C). Non-inoculated rice was used as control. The colonization of fungi was evaluated. The root samples were heated at 100 °C in a 2.5% KOH solution for 5 min, then stained with lactophenol cotton blue. Roots were observed under light microscope (Nikon Instech Co., Ltd., Tokyo, Japan). Then, fifty seedlings were planted into a 32.5 × 40.0 × 9.5 cm plastic pot containing an autoclave commercial soil substrate (Plaipah, Bangkok, Thailand). The plants were grown in a greenhouse with the necessary irrigation to keep them at field capacity. The plants did not receive any fertilizer. After 14 days of cultivation, the growth parameters, including the chlorophyll content, shoot length, root length, fresh weight, and dry weight, were recorded [30]. Ten replications were performed. For dry weight, the rice was incubated in a hot air oven at 65 °C. Samples were dry when the weight was constant for two consecutive readings. The chlorophyll and carotenoid were extracted. A portion of 0.1 g of finely chopped fresh leaves was utilized and blended with 10 mL of 80% acetone. Subsequently, the mixture underwent centrifugation at speeds ranging from 5000 to 10,000 rpm for 5 min, and the resultant supernatant was transferred to a fresh container. This process was reiterated until the residue turned colorless. The optical density of the resulting solution was assessed at wavelengths of 480 nm, 645 nm, and 663 nm in comparison to a blank (80% acetone). The concentrations of chlorophyll A, chlorophyll B, total chlorophyll, and carotenoids were then calculated [31]. To discern significant differences among sample means, a pairwise multiple comparison procedure was implemented post $t$-tests. Statistical probabilities were computed using SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA). A $p$-value of less than 0.05 denoted statistical disparities among the means of chlorophyll content, shoot length, root length, fresh weight, and dry weight, each within a 95% confidence interval.

2.6. Protein Extraction

Procedures for protein preparation and LC-MS/MS analysis were conducted following the methodology established by Leetanasaksakul et al. [32]. In brief, leaf and root samples were finely ground into powder using liquid nitrogen. Each sample weighing 0.2 g was then subjected to extraction with 0.5% sodium dodecyl sulfate (SDS) ($w/v$) while being continuously vortexed for 3 h at room temperature. After extraction, the samples were centrifuged at 8000 rpm for 10 min at room temperature to precipitate them. The resulting supernatant was then combined with 72% trichloroacetic acid (TCA) ($w/v$) and 0.15% deoxycholate ($w/v$) and left overnight at −20 °C to ensure thorough precipitation. Following this, the mixtures were centrifuged at 10,000 rpm for 10 min at room temperature, and the resulting pellets were washed with cold acetone until they became white. The
pellets were resuspended in 0.5% SDS, and the protein concentration was examined using the Lowry method [33].

2.7. Protein Digestion

The samples underwent gel digestion, wherein they were exposed to 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate to break disulfide bonds, followed by incubation at 60 °C for 1 h. Sulphydryl groups were modified by adding 15 mM iodoacetamide in 10 mM ammonium bicarbonate at room temperature for 45 min in the absence of light. Protein samples were digested using 50 ng/µL of sequencing grade trypsin (1:20 ratio) (Promega, Madison, WI, USA) and incubated at 37 °C for 16 h. Peptides were dried using a speed vacuum concentrator and acidified with 0.1% formic acid (v/v) before LC-MS/MS analysis.

2.8. Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

The tryptic peptide samples were prepared for injection into an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, Waltham, MA, USA), which was linked to a SCIEX Zeno TOF 7600 system (Darmstadt, Germany). This system featured a TwinSpray Turbo V ion source and functioned in positive electrospray ionization mode. The peptides underwent enrichment on a micro-Precolumn with a diameter of 300 µm and a length of 5 mm, packed with C18 Pepmap 100 material with particle size of 5 µm and pore size of 100 Å, from Thermo Scientific, based in the United Kingdom. Subsequently, they were separated using a column with a diameter of 75 µm and a length of 15 cm, filled with Acclaim PepMap RSLC C18 material with particle size 2 µm and pore size 100 Å, equipped with nanoViper technology, also from Thermo Scientific, UK. Solvent A consisted of 0.1% formic acid in water, while solvent B comprised 0.1% formic acid in 80% acetonitrile. A gradient ranging from 5% to 55% solvent B was applied to elute the peptides at a steady flow rate of 0.3 µL/min over a duration of 30 min. Electrospray ionization was conducted at 3.3 kilovolts. Mass spectra and MS/MS spectra were recorded in positive-ion mode across the mass-to-charge ratio (m/z) range of 350 to 1800 using SCIEX OS software 2.1.6 (AB Sciex, Framingham, MA, USA).

2.9. Protein Identification

The MS/MS data acquired from LC-MS analysis were processed for peptide MS signal intensities using MaxQuant version 2.1.4.0, developed by the Max-Planck Institute for Biochemistry in Martinsried, Germany. For label-free quantification, this analysis utilized the built-in Andromeda search engine [34]. The data were searched against the Uniprot database, dated 28 April 2023, for protein identification. The criteria for database interrogation included taxonomy (Oryza sativa), enzyme (trypsin), diverse alterations (carbamidomethyl, oxidation of methionine residues), mass characteristics (monoisotopic), unbounded protein mass, peptide mass acceptance (1.2 Da), fragment mass acceptance (±0.6 Da), peptide charge states (1+, 2+, and 3+), and the highest permissible overlooked cleavages.

The results were documented within a file denoted as proteinGroup.txt. Subsequent analysis, as outlined by Aguilan et al. [35], aimed to identify alterations in protein levels between the treated endophytic fungus condition and the control group. This was accomplished by evaluating fold-change and associated p-values derived from protein abundance calculations. In essence, the abundance data underwent dual normalization processes subsequent to logarithmic conversion. The initial normalization entailed adjusting each value relative to the mean of all protein abundances within the specific sample. The second normalization approach adapted values based on their spread, considering potential variations in acquisition dynamic range among samples. To handle missing data, the probabilistic minimum imputation model, which involves resampling for low-abundance data, was applied. Low abundance was defined as values exceeding 2 standard deviations less than the distribution of valid values, with resampling variability set at 0.3. Fold-change was cal-
culated as the quotient of altered and standardized protein abundance values between each sample and the reference group. *p*-values were ascertained through the execution of diverse *t*-tests, depending on whether the duplicates for each protein demonstrated homoscedastic or heteroscedastic tendencies, as confirmed by *F*-tests. Uniprot ([http://www.uniprot.org](http://www.uniprot.org), accessed on 11 January 2024) and related exploration utilities were utilized for gene ontology (GO) classification. To facilitate visualization, shared proteins were produced utilizing jvenn [36]. A volcano plot was utilized to illustrate notable alterations in protein expression levels [37].

3. Results

3.1. Isolation and Identification of Endophytic Fungus DMKU-R3G3

Endophytic fungus DMKU-R3G3 was obtained from organic rice root in Suphanburi province, Thailand (Figure 1A). The fungus was identified based on morphological characteristics and phylogenetic analysis. Strain DMKU-R3G3 was observed on potato dextrose agar (PDA), revealing a light yellow-colored floccose colony (Figure 1B). After 2 weeks, ascomata were produced as a cleistothecium. Cleistothecia were spherical and variable in size (Figure 1C). Ascospores were lenticular, with two closely appressed, very thin equatorial crests and smooth convex walls. Eight ascospores in prototunicate asci were observed (Figure 1D). These characteristics indicated that strain DMKU-R3G3 belonged to the fungi in the phylum Ascomycota.

Figure 1. Characters of *Aspergillus cejpii* DMKU-R3G3. (A) Mycelium of endophytic *A. cejpii* DMKU-R3G3 emerging from organic rice root, (B) mycelial growth on potato dextrose agar (PDA) for 7 days, (C) PDA for 14 days, arrowhead indicating cleistothecium, (D) microscopic observation showing ascopores and asci (Scale bar = 10 μm), arrowhead indicating prototunicate ascus with 8 ascospores (E) Phylogenetic tree of *A. cejpii* DMKU-R3G3 and 22 other related species, based on maximum-likelihood analysis of ITS region and 28S ribosomal RNA gene sequences. Numbers at the note indicate the bootstrap values (>50%) from 1000 replications. *Penicillium sacculum* was used as an outgroup. The bar indicates the number of substitutions per position. New isolates from the present study are shown in bold.
Molecular taxonomy based on ITS region and 28S ribosomal RNA gene sequence showed the closest similarity to Aspergillus cejpii. A 100% similarity was observed on the ITS region of A. cejpii CBS157.66, while the 28S ribosomal RNA gene sequence of 926 bp showed a 99.89% similarity to A. cejpii CBS157.66. A phylogenetic tree was constructed using the ITS region and 28S ribosomal RNA gene sequence of the genus Aspergillus. The ITS region and 28S ribosomal RNA gene sequence of Penicillium sacculum was used as the outgroup. The best phylogenetic tree was obtained using a maximum-likelihood analysis (Figure 1E). The result shows that the endophytic fungus DMKU-R3G3 was clustered in the same position as A. cejpii CBS157.66 with high bootstrap support.

Based on both morphology and DNA sequence data, endophytic fungus DMKU-R3G3 isolated from organic rice root was identified as Aspergillus cejpii DMKU-R3G3. DNA sequences of ITS and 28S ribosomal RNA sequence of A. cejpii DMKU-R3G3 were deposited in GenBank under accession numbers LC799730 and LC778432, respectively.

3.2. Indole-3-Acetic Acid (IAA) Production

Endophytic A. cejpii DMKU-R3G3 was grown in potato dextrose broth (PDB) with and without L-tryptophan supplementation. The presence of IAA in the PDB of the strain DMKU-R3G3 was assessed using Salkowski’s reagent. After 7 days, it was observed that the highest concentration of IAA, measuring 25.45 µg/mL, was produced on day 6 when A. cejpii DMKU-R3G3 was cultured in the medium supplemented with L-tryptophan (Figure 2). In contrast, a much lower amount of IAA (4.60 µg/mL) was detected in PDB without L-tryptophan. This result indicates that the strain DMKU-R3G3 produced IAA by utilizing L-tryptophan as a precursor for IAA production; the IAA production was increased by 5.5 times in a medium supplemented with L-tryptophan.

Figure 2. IAA production of Aspergillus cejpii DMKU-R3G3 cultivated in potato dextrose broth (PDB) with L-tryptophan and without L-tryptophan in shake-flask culture at 30 °C and 200 rpm for 7 days.
3.3. Phosphate Solubilization

The halo zone became visible in the endophytic fungus *A. cejpii* DMKU-R3G3 upon 3 days of inoculation. This result indicated the ability in phosphate solubilizing of the endophytic fungus *A. cejpii* DMKU-R3G3 (Figure 3). The phosphate solubilization index on day 7 was 2.16 ± 0.03 cm.

![Figure 3](image)

**Figure 3.** Phosphate-solubilizing ability of *A. cejpii* DMKU-R3G3 on Pikovskaya's agar.

3.4. Evaluation of the Efficacy of Endophytic Fungus DMKU-R3G3 in Promoting Rice Growth

Through the co-cultivation of *A. cejpii* DMKU-R3G3 with rice, notable results were observed. After 7 days, rice inoculated with *A. cejpii* DMKU-R3G3 exhibited a significantly higher density of root hairs compared to the control group (Figure 4A,B). Moreover, a microscopic examination confirmed the colonization of rice roots by the endophytic fungus DMKU-R3G3 (Figure 4D), while the control group displayed clean, non-colonized roots (Figure 4C).

![Figure 4](image)

**Figure 4.** The characterization of rice roots. (A) Control; (B) inoculated with *Aspergillus cejpii* DMKU-R3G3. The colonization of *A. cejpii* DMKU-R3G3 in the rice roots. (C) Control; (D) inoculated with *A. cejpii* DMKU-R3G3. The fungal hypha colonization in roots is represented by black arrows.

After 21 days, it was found that rice seeds inoculated with *A. cejpii* DMKU-R3G3 displayed enhanced growth characteristics compared to the control group (Figure 5). This included significantly increased shoot height, root length, and fresh weight (Figure 6A–C). However, no significant difference was detected in plant dry weight (Figure 6D). Moreover, the presence of endophytic *A. cejpii* DMKU-R3G3 positively impacted the photosynthetic pigmentation of rice leaves. The total chlorophyll content in the leaves also significantly rose by 13.64 mg/mL, as shown in Figure 6E. In addition, chlorophyll A, chlorophyll B, and carotenoid concentration were significantly increased (Figure 6F–H). These findings suggest that endophytic fungus may have a beneficial effect on rice plant photosynthesis due to the increased accumulation of pigments.
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**Figure 5.** Effect of *Aspergillus cejpii* DMKU-R3G3 on the rice growth under greenhouse conditions (Scale bar = 5 cm).

3.5. Proteome Analysis

To identify differentially expressed proteins in leaf and root samples, we utilized a shotgun proteomics approach to analyze peptide sequences. In leaf samples, we initially identified a total of 4613 proteins, and in root samples, 4643 proteins were detected. After filtering the identified proteins, the leaf samples retained 4190 proteins, while the root samples retained 4322 proteins. Subsequently, the protein elucidated in this investigation underwent a series of normalization procedures to transform the intensity output values from mass spectrometry into fold-change measurements for each protein. These measurements facilitated the comparison of protein abundance between the control and inoculated endophytic *A. cejpii* DMKU-R3G3 in both leaf and root samples, as detailed in Tables S1 and S2, respectively [35].

A broad outline of the alteration in fold changes, shown as the proportion of each protein’s prevalence within the circumstance of inoculated endophytic *A. cejpii* DMKU-R3G3 in comparison to the control, plotted alongside their *p*-values, is visible in Figure 7. Proteins exhibiting notably modified levels (log2 fold-change value < -1 or >1) and robust statistical reliability (*p*-value < 0.05) are accentuated and marked in color. In leaf samples, a notable decline in the prevalence of 77 proteins was noted when contrasting inoculated endophytic *A. cejpii* DMKU-R3G3 with the control (depicted as blue dots), whereas 125 proteins demonstrated heightened prevalence (illustrated as red dots). In root samples, 110 proteins showed a significant decrease in abundance, while 99 proteins displayed a significant increase.
Gene ontology (GO) analysis was employed to scrutinize the functions of differentially expressed proteins, both up- and down-regulated, in leaf and root samples. A compilation categorized into three groups, encompassing biological processes (BP), cellular components (CC), and molecular functions (MF), are depicted in Figure S1 and Tables S1 and S2. In leaf samples, regarding up-regulation, the biological process annotation showed that the most representative proteins were classified under gene expression, the cellular component annotation highlighted the nucleus as the most representative location, and for the molecular function, ATP binding was the most characteristic function. In the case of down-regulation, the biological process annotation revealed that the most representative proteins were associated with protein metabolic processes, the cellular component annotation identified the nucleus as the primary location, and for molecular function, ATP binding remained the most representative function.

In root samples, up-regulation was associated with proteins primarily involved in transport in terms of biological process annotation, with cytoplasm as the most representative cellular component and ATP binding as the predominant molecular function. Conversely, down-regulation in biological process annotation led to the classification of the most representative proteins under homeostatic processes. In terms of cellular component annotation, the nucleus remained the most characteristic location, and for molecular function, ATP binding continued to be the most representative function.

Furthermore, we identified gene ontology processes related to IAA hormone production in leaf samples, specifically the auxin-activated signaling pathway and auxin polar transport. Additionally, in leaf samples, we found processes associated with photosynthesis, including chlorophyll biosynthesis and granum assembly. In root samples, we also identified processes related to IAA hormone production, including the auxin export across the plasma membrane. Moreover, in root samples, processes associated with photosynthe-
sis were observed, encompassing the chlorophyll catabolic process, thylakoid membrane organization, and chlorophyll biosynthesis (Table 1).

Figure 7. Volcano plots are presented, depicting all identified proteins as grey dots in leaf and root samples following filtering and normalization procedures. The horizontal axis represents the log2 ratio of protein abundance between the inoculated endophytic *A. cejpii* DMKU-R3G3 and the control, whereas the vertical axis denotes the negative log2 transformation of the corresponding *p*-value (indicating statistical significance). Every point on the graph symbolizes a distinct protein. Proteins with a log2 fold-change value below −1 or above 1, coupled with a −log2 (*p*-value) surpassing 4.32 (equating to a raw *p*-value lower than 0.05), are emphasized and labeled in color to signify significant alterations.

Table 1. Identified proteins in leaf and root samples that are associated with GO terms related to IAA hormone production and photosynthesis.
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<th>Protein ID</th>
<th>Function/Component</th>
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<th>Peptide Sequence</th>
<th>p-Value</th>
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<td>Q5W6H5</td>
<td>Chlorophyll biosynthetic process/Chloroplast</td>
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<td>P0C131</td>
<td>Auxin export across the plasma membrane/Endoplasmic reticulum</td>
<td>Putative auxin-responsive protein IAA29 (Indoleacetic acid-induced protein 29)</td>
<td>INLALHNHYDSLSFTLK</td>
<td>0.03</td>
<td>−8.71</td>
</tr>
<tr>
<td>Q6L5F6</td>
<td>Chlorophyll biosynthetic process/Chloroplast</td>
<td>Geranylgeranyl diphosphate reductase, chloroplastic (EC 1.3.1.83) (Geranylgeranyl reductase)</td>
<td>AAASSPK</td>
<td>0.03</td>
<td>−2.5</td>
</tr>
<tr>
<td>Q6Z2T6</td>
<td>Chlorophyll catabolic process/Chloroplast</td>
<td>Red chlorophyll catabolite reductase 1, chloroplastic (OsRCCR1) (RCC reductase) (EC 1.3.7.12)</td>
<td>AFGVQEA</td>
<td>0.03</td>
<td>9.79</td>
</tr>
<tr>
<td>Q8S0J7</td>
<td>Thylakoid membrane organization/Chloroplast inner membrane</td>
<td>Probable membrane-associated 30 kDa protein, chloroplastic</td>
<td>AQLALQK</td>
<td>0.03</td>
<td>2.27</td>
</tr>
</tbody>
</table>

4. Discussion

In the coming years, farming systems are likely to confront challenges such as climate change and restrictive agriculture policies. Addressing these issues will demand greater efforts to enhance crop productivity and promote sustainability [38]. To accomplish this objective, the utilization of beneficial microorganisms for sustainable crop production is emerging as an increasingly promising option [39]. Endophytic fungi hold great promise for enhancing plant growth and improving crop production. They achieve this by producing plant growth-promoting factors, including phytohormones, antioxidants, and nutrients. These factors have a significant impact on plant development, leading to improved growth and various fitness parameters, such as enhanced nutrient acquisition and increased phytohormone levels [40].

In this study, the endophytic fungus *Aspergillus cejpii* DMKU-R3G3 was isolated from the organic root tissue of rice in Suphanburi province in central Thailand. Only one previous report showed that *A. cejpii* was found in the root tissue of common wheat...
(Triticum aestivum) in France [41]. The endophytic fungus Aspergillus cejiyi DMKU-R3G3 employed in this study has demonstrated positive effects, including the promotion of root hair density and the enhancement of rice growth when artificially inoculated as fungal spores. The Aspergillus genus, as a whole, is renowned for its beneficial effects on plants, encompassing plant growth promotion and protection [42]. Certain specific species within the Aspergillus genus, such as A. terreus, were also reported to possess the potential for promoting plant growth [42]. Moreover, Aspergillus species were documented as producers of indole-3-acetic acid (IAA), a plant growth regulator [43]. For instance, Aspergillus PB-7 was found to produce 110 µg/mL and 75 µg/mL of IAA in the presence of glucose and peptone, respectively [44]. Aspergillus niger (01) was reported to produce IAA from tryptophane [35]. Additionally, a study explored the role of indole-3-acetic acid produced by the endophytic fungus Aspergillus awamori and its growth-promoting activities in Zea mays [14].

Endophytic fungi can help plants produce chlorophyll content, which is essential for photosynthesis. Several studies showed that endophytic fungi can enhance chlorophyll content in plants. For example, a study found that endophyte-colonized plants had greater chlorophyll content than non-colonized plants [45]. Another study showed that endophytic fungi can boost gas exchange, chlorophyll content, and photosynthetic rates in a wide range of plant species [46]. The exact mechanisms by which endophytic fungi enhance chlorophyll content are not fully understood, but it is believed that they can improve nutrient uptake and produce plant growth-promoting substances such as auxins, gibberellins, and cytokinins [40].

Based on proteomics analysis comparing inoculated and non-inoculated conditions, it was revealed that in leaf samples, a higher percentage (62.23%) of protein were up-regulated under inoculated conditions compared to down-regulated proteins. Conversely, in root samples, the percentage of down-regulated proteins (11.11%) exceeded that of up-regulated ones. Additionally, the predominant up-regulated protein in leaves corresponds to gene expression, while the predominant down-regulation protein in roots is associated with both gene expression and protein metabolic processes.

Endophytic fungi exert influence on the protein expression of their host plants through gene regulation. Research demonstrated that these fungi can either up-regulate or down-regulate particular genes linked to plant development, productivity, and regulation [47]. Furthermore, the cooperative interaction between endophytic fungi and their host organisms was observed to grant the host plant increased resilience and defense mechanisms against assaults from phytopathogens [48].

In this study, the primary up-regulated proteins in leaf samples were identified within the gene ontology related to gene expression. All proteins in this group play a part in enhancing plant growth and maturation, promoting resilience to a range of environmental pressures, encompassing both abiotic and biotic challenges. One notable example is the transcription factor MYC2 (OsMYC2), also recognized as basic helix-loop-helix protein 9 (OsbHLH009) or bHLH9. This protein acts as a vital controller within the jasmonic acid (JA) communication pathway in plants, serving as a central figure in regulating diverse biological functions, such as defense mechanisms and secondary metabolic processes [49]. The up-regulation of MYC2 can lead to enhanced activation of genetic regulation in reaction to JA, thereby mediating the protective mechanisms of the plant against both biotic and abiotic stress conditions [50].

In the scenario of decreasing expression in proteins within root plants, endophytic fungi possess the capability to modify the activity of genes related to plant physiology and metabolism [51]. To illustrate, the endophytic fungus Glimaniella sp. AL12 was identified as having the capacity to diminish plant immunity, a factor that could potentially enhance the positive interplay between the plant and the endophyte [52].

The intriguing aspect of the rice response lies in its reaction to the characteristics of the endophytic fungi DMKU-R3G3, particularly in auxin production. Differentially expressed proteins in both leaf and root samples were associated with key processes. These processes...
encompassed the auxin-activated signaling pathway, auxin polar transport, and the export of auxin across the plasma membrane (related to IAA production). Additionally, processes such as chlorophyll biosynthesis, granum assembly, chlorophyll catabolic process, and thylakoid membrane organization (linked to photosynthesis) were evident in both leaf and root samples. Previous investigations through transcriptome analysis unveiled that the endophytic *Chaetomium cupreum* can effectively regulate genes engaged in the synthesis and metabolism of auxins. These auxins play a pivotal role in the division, elongation, and differentiation of plant cells [53].

In the leaf, the production of IAA by endophytic fungus can result in elevated levels of auxin concentrations in rice, affecting the expression of key auxin-related proteins. The up-regulation of the auxin transport protein BIG is crucial for auxin distribution and plant growth regulation [54]. Additionally, the auxin-responsive protein IAA3 acts as an active repressor of auxin-responsive gene expression [54,55]. In the presence of high auxin concentration, IAA3 repression may intensify due to its up-regulated expression [56,57]. Similarly, the regulation of auxin-responsive gene expression by OsARF6a enhances its regulatory role [56,57]. On the other hand, the auxin-responsive protein IAA14 acts as an active repressor of auxin-responsive gene expression [55,56]. In the presence of high auxin concentrations, IAA14 repression may diminish due to down-regulation expression [58].

Endophytic fungi additionally contribute to chlorophyll biosynthesis through their impact on the expression of chlorophyll synthase, chloroplastid (EC2.5.1.62) (polyprenyl transferase), which participates in the production of chlorophyll, a critical component for photosynthesis in plants [59]. The diminished expression and functionality of chlorophyll synthase results in the feedback-controlled down-regulation of genes encoding proteins involved in chlorophyll biosynthesis and chlorophyll-binding proteins (Shalygo et al., 2009). Therefore, the up-regulation of the protein expression of chlorophyll synthase, chloroplastid (EC2.5.1.62) (polyprenyl transferase), could enhance ALA synthesizing capacity and increase chelatase activation [60]. Additionally, the magnesium–chelatase subunit ChlD and magnesium–chelatase subunit ChlH are engaged in chlorophyll biosynthesis and facilitate the incorporation of magnesium ions into protoporphyrin IX, resulting in Mg–protoporphyrin IX [61,62]. The regulation of magnesium–chelatase subunit ChlD and magnesium–chelatase subunit ChlH expression is complex and involves various factors, including abscisic acid (ABA) and plastid-to-nucleus retrograde signaling [63]. Therefore, the up-regulation of magnesium–chelatase subunit ChlD and magnesium–chelatase subunit ChlH could lead to enhanced Mg$^{2+}$ insertion into protoporphyrin IX. LHCP translocation defect (LTD) is a protein engaged in aiding the transport of light-harvesting complex proteins (LHCP) and their subsequent conveyance to the cpSRP pathway [64]. Chloroplasts play a pivotal role in photosynthesis, and LHCPs are indispensable for capturing light within chloroplasts [64]. Elevating the expression of the LTD protein could potentially augment the importation of LHCPs and their subsequent delivery to the cpSRP pathway, thereby increasing the efficiency of light harvesting within chloroplasts and potentially enhancing the process of photosynthesis [64]. In the case of *Arabidopsis*, the LTD mutant displays noticeable defects in LHCP biogenesis [65].

In plant roots, the auxin-responsive protein IAA13 belongs to the Aux/IAA gene family, known as targets of auxin regulation [57,66]. The degree of repression by Aux/IAA proteins is linked to their relative stabilities, which are modulated by auxin [57,59]. In the presence of high auxin concentrations, the repression level by auxin-responsive protein IAA13 may decrease due to down-regulated expression [58,66]. Putative auxin-responsive protein IAA29, another member of the Aux/IAA gene family [58], is associated with auxin-responsive gene expression regulation, although its exact mechanisms remain less understood. Geranylgeranyl reductase (CHL P) triggers the conversion of geranylgeranyl diphosphate to phytyl diphosphate, supplying phytyl for both phylloquinone and chlorophyll biosynthesis [67]. Within chloroplasts, a notable fraction of geranylgeranyl diphosphate undergoes reduction via GGPP reductase to form phytyl diphosphate, which is utilized in the biosynthesis of both phylloquinone and chlorophyll [68]. Therefore, the
down-regulation of geranylgeranyl diphosphate reductase, also known as chloroplastic (EC1.3.1.83) or geranylgeranyl reductase, in plant roots could potentially reduce the production of phytyl diphosphate, leading to reduced chlorophyll biosynthesis and photosynthesis in the plant [60,67]. The genetic sequence responsible for geranylgeranyl reductase in peach (Prunus persica [L.] Batsch) experiences regulation throughout leaf maturation and reacts differently to various stressors [69]. Red chlorophyll catabolite reductase (RCCR) is an enzyme engaged in chlorophyll degradation, facilitating the conversion of red chlorophyll catabolite (RCC) to the primary fluorescent chlorophyll catabolite (pFCC) [70]. The up-regulation of RCCR in roots could potentially lead to the more efficient breakdown of chlorophyll catabolites, enhancing plant growth and photosynthesis [70]. RCCR is essential for the neutralization of chlorophyll breakdown products [70]. The 30 kDa protein associated with the inner membrane (IM30) is indispensable for the photoautotrophic development of cyanobacteria, algae, and higher plants. Although its precise role is not fully understood, it is widely acknowledged that IM30 plays a vital part in the generation, reinforcement, and/or preservation of thylakoid membranes [71]. The chloroplast is crucial for photosynthesis in higher plants, and proteins such as LHCP translocation defect protein (LTD) and STT1/2 participate in protein arrangement within chloroplasts [64]. Moreover, proteins linked with membranes were recognized as membrane proteins responsive to salt stress in soybean roots [72]. Therefore, the up-regulation of probable membrane-associated 30 kDa protein, specifically in plant roots, could potentially affect protein import and sorting within chloroplasts, which could have an impact on photosynthesis and plant growth.

Identifying protein expression during the interaction between endophytic fungi and rice is a pivotal research area within the domain of interactions between plants and microbes. Such studies aim to shed light on the molecular mechanisms underlying how plants react to the interactions. The findings elucidated in this investigation offer an understanding of key proteins that elucidate the impact of endophytic fungi on auxin production. This influences proteins in both the foliage and underground components of rice plants, promoting plant growth and activating the proteins involved in photosynthesis. A broader comprehension of the fundamental process involved in responding to auxin (IAA) production by endophytic fungi is crucial. This understanding is pivotal for the advancement and selection of beneficial endophytic fungi suitable for sustainable crop management practices.

5. Conclusions

The plant growth-promoting abilities of the endophytic fungal strain DMKU-R3G3 were investigated. This isolate had potential in IAA production and phosphate solubilization. A. cejpii DMKU-R3G3 significantly promoted rice growth. Moreover, the chlorophyll content of the rice plants also increased. In addition, proteomic analysis revealed that rice responded to the colonization of endophytic fungi by producing auxin-responsive proteins to regulate the IAA content in plant tissue and inducing total chlorophyll production due to the up-regulation of proteins in the chlorophyll biosynthesis pathway. The study offers valuable insights into the agronomy and plant–microbe interaction fields by examining the growth-promoting potential of Aspergillus cejpii DMKU-R3G3 in rice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030498/s1, Figure S1: Bar graph of the gene ontology (GO) classification of all identified proteins in up- and down-regulation of (A) leaf and (B) root samples in terms of biological processes (red), cellular components (blue), and molecular function (yellow); Table S1: Proteomic analysis in the leaf sample; Table S2: Proteomic analysis in the root sample.

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**References**


12. Yoo, S.-J.; Shin, D.J.; Won, H.Y.; Song, J.; Song, M.K. *Aspergillus terreus* JF27 promotes the growth of tomato plants and induces resistance against *Pseudomonas syringae* pv. *tomato*. *Microbiology* 2018, 46, 147–153. [CrossRef]


53. Ortiz, J.; Soto, J.; Fuentes, A.; Herrera, H.; Meneses, C.; Arriagada, C. The endophytic fungus *Chaetomium cupreum* regulates expression of genes involved in the tolerance to metals and plant growth promotion in *Eucalyptus globulus* roots. *Microorganisms* 2019, 7, 490. [CrossRef]


60. Shalygo, N.; Czarnecki, O.; Peter, E.; Grimm, B. Expression of chlorophyll synthase is also involved in feedback-control of chlorophyll biosynthesis. *Plant Mol. Biol.* 2009, 71, 425–436. [CrossRef] [PubMed]


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