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Isolation and Functional Characterization of Culture-Dependent Endophytes Associated with *Vicia villosa* Roth

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Abstract: In a natural ecosystem, endophytic fungi in the internal tissues of plants help to improve the growth of the host plants and to decrease the negative effects of biotic and abiotic stresses without having adverse effects. In Japan, *Vicia villosa* (hairy vetch), a legume plant with a high capacity to fix nitrogen, is usually used as a cover crop before soybeans to enhance the fertility and structure of the soil. This study aimed to isolate endophytic fungi associated with different tissues of hairy vetch and to evaluate their potential for growth-promoting and biocontrol effects in plants. Thirty-three fungal endophytes belonging to Ascomycota and Basidiomycota were isolated from the leaf, stem, and root tissues of hairy vetch grown under both greenhouse and field conditions. The highest colonization frequency in both the greenhouse and field-grown hairy vetch plants was obtained from the root tissues. All isolates were checked for indole-3-acetic acid (IAA) and siderophore production. The maximum IAA content in the culture filtrate (4.21 μg mL^{-1}) was produced by the isolate hvef7 (*Cladosporium cladosporioides*), followed by hvef18 (*Penicillium simplicissimum*) (3.02 μg mL^{-1}) and hvef1 (*Cladosporium pseudocladosporioides*) (2.32 μg mL^{-1}). Nineteen isolates among a total of thirty-three isolates produced siderophores. Moreover, some of the isolated strains could solubilize phosphate and potassium. Most of the isolates showed antagonistic potential against *Calonectria ilicicola*. The results of this study show that endophytic fungi isolated from hairy vetch have the potential for application as plant growth promotion fungi (PGPF) to promote plant growth and control disease in sustainable agriculture.

Keywords: endophytic fungi; cover crop; *Vicia villosa* (hairy vetch); functional characterization

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1. Introduction

The demand to improve and increase food resources through agrochemicals such as nitrogen fertilizers and other crop protection chemicals has risen in recent decades due to the rapid growth of the global population [1]. However, the excessive and often indiscriminate use of agrochemicals has resulted in several negative impacts on our environment, such as soil and water pollution and the destruction of the soil microbiome, threatening our food security [2,3]. Therefore, it is essential to innovate alternatives to minimize the over-reliance on agrochemicals, which may have negative impacts on the ecosystem.

Vicia villosa Roth subsp. *villosa*, commonly known as hairy vetch, is a winter annual legume cultivated for high-quality animal fodder [4,5]. Hairy vetch does not show any

domestication characteristics and is widely used as green manure and cover crop in fruit, vegetable, and rice farms in Japan [6]. Due to its remarkable potential to withstand biotic and abiotic stresses, hairy vetch can be used in a variety of climatic conditions [7]. Hairy vetch is ranked as the second most utilized vetch in agricultural systems in Japan. This unique cover crop is generally utilized in the rotation system as a pre-crop not only for soybeans, but also for other crops in Japan to control weeds and increase the soil organic chemicals for the succeeding crops [4–6]. As a legume plant, it can fix nitrogen from the air, and this nitrogen can be utilized by the plant itself [8–10]. It can also promote the growth of other species in the system after the decomposition of the plant [11–15]. In particular, hairy vetch has a significant effect on the management of plant diseases such as controlling *Fusarium oxysporum* (the main reason for Fusarium wilt of watermelon) [16] and *Thielaviopsis basicola* (important disease of cotton) [17]. In addition, hairy vetch has been reported to be a good candidate for weed suppression due to the production and release of unique allelochemicals such as cyanamide [18–20] and L-canavanine [21]. However, these two chemicals are degraded in a few days or weeks [21]. Other factors responsible for the long-lasting effects of hairy vetch as a weed and disease suppressant species have recently been identified. A new chemical, okaramine, was identified in the rhizosphere soil of hairy vetch grown in the field of Tokyo University of Agriculture and Technology (TUAT) in Japan, as well as in the rhizosphere soil of soybean plants that succeeded hairy vetch in the field of Ibaraki University (Japan), using untargeted and targeted metabolome analyses [22]. The metabolite, okaramine, has been known to be biosynthesized in the soybean pulp (okara) medium using *Penicillium simplicissimum* AK-40 and has a potent insecticidal activity [23].

Plants and endophytic fungi have interacted for more than a million years [24], and evidence from more than 100 years of research indicates that plants in our natural environment have a symbiotic relationship with these microorganisms [25–28]. Studies on endophytic fungi have increased since the 1990s when de Barry, a German scientist, described them as internal organisms of plant tissues. This lifestyle has been established based on the impacts between macroscopic hosts and microscopic symbionts [29]. Generally, an endophyte is defined as any microorganism found inside plant tissues without showing any obvious sign of disease in their host plants [30,31]. In this symbiotic relationship, plants provide diverse accommodations for endophytes [32], and in return endophytic fungi protect their host plant against biotic and abiotic stresses through the regulation of various defense hormones and also by releasing anti-microbial substances. Moreover, many of these fungi have different mechanisms for increasing plant growth. Plant-growth-promoting fungi (PGPFs) directly promote growth by producing different types of plant growth, promoting hormones such as indole-3-acetic acid (IAA) and gibberellic acid [33]. Additionally, some of these fungi promote the growth of host plants through the solubilization of phosphate and potassium, which are important for plant growth [34] to increase the yield of host plants [35–37]. Moreover, Sakurai et al. [22] suggested the indirect plant defense system through the plant-associated fungus. However, the knowledge related to the beneficial fungus associated with *Vicia villosa* is still very limited. To realize the utilization of these symbiotic relationships for crop improvement, it is necessary to increase our knowledge.

Consequently, this study focused on the isolation and identification of culture-dependent endophytic fungal communities associated with different tissues of *Vicia villosa* (hairy vetch). Plant growth properties, as well as inorganic phosphate and potassium solubilization, siderophore production, and qualitative and quantitative indole-3-acetic acid (IAA) production levels, were evaluated. Afterwards, the antimicrobial potential of all isolated fungi against *Calonectria ilicicola* known as red crown rot was tested.

2. Materials and Methods

2.1. Plant Sample Collection

Healthy, fresh, mature samples of *Vicia villosa* (n = 3) were collected separately from the greenhouse and experimental field of the Tokyo University of Agriculture and Technology.

The representative parts (leaf, stem, and root) of the collected plant samples were initially selected with the criteria of being healthy or diseased plants before collection. To avoid desiccation, plant samples were wrapped in moist paper towels and sealed in Ziplock bags. No flowers were observed at the time of sample collection. All of the collected plant materials were processed within 72 h of collection. The soil samples were sent to Vegetech Physical and Chemical Center, Japan, for the analysis of the physicochemical properties of the soil.

2.2. Isolation of Endophytic Fungi

The surface sterilization method proposed by Muller et al. [38] was adopted to study the plant tissues. Plant samples (leaf, stem, and root) were thoroughly cleansed under running tap water to remove the surface adherents followed by being consecutively washed (three times) with sterilized distilled water, then dipped in 70% ethanol (20 s) followed by 1% sodium hypochlorite (20 s), and finally rinsed five times with sterile deionized water to remove the surface sterilant. After air-drying the extra water under the laminar flow, the samples were cut into small pieces (5 mm × 5 mm) under strict aseptic conditions. About 6–8 segments were placed on each Petri dish containing potato dextrose agar medium (PDA) supplemented with 50 μg mL^{-1} of chloramphenicol. The selection of PDA, which supported the maximum endophyte recovery in terms of both the number and types, was made through a literature review. A total of 60 segments of each tissue type from each plant were screened for the presence of endophytes. The Petri dishes were then incubated at 28 ± 2 °C in an incubator (Sanyo MIR-253 Refrigerated Incubator) under dark conditions and monitored every day for fungal emergence for up to one month. Different endophytes emerging from the explants were transferred and maintained on fresh PDA plates as axenic cultures.

2.3. Macroscopic and Microscopic Identification of Recovered Endophytic Fungi

Both morphological and microbial methods were used for the identification. Based on the culture characteristics, such as shape, size, color, texture, growth pattern, and backside color of the colony details, all of the fungal isolates were identified and grouped into different morphotypes or operational taxonomic units (OTUs), and one of them was used as a representative strain for molecular identification. The total genomic DNA of all representative morphotypes was extracted following the DNA plant mini kit (QIAGEN—Hilden, Germany) protocol for high-throughput fungal DNA extraction. Here, 100 mg pure mycelium was pulverized in a plastic microtube for DNA extraction. The concentration and purity of the extracted DNA were measured using NanoDrop instruments (Thermo Fisher Scientific—Waltham, MA, USA). The universal primer pair comprising ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) was used to amplify the fungal 18S rRNA regions of a representative isolate of each morphotype. The PCR amplification (modified) was performed on a PCR 320/350 programmable thermal cycler (ASTEC, Tokyo, Japan) in 25 μL reaction volumes, each containing 12.5 μL of Go Taq DNA polymerase (Promega, Madison, WI, USA), 2 μL of each primer, and 2 μL of extracted DNA template. The rest of the volume was adjusted with 6.5 μL autoclaved deionized distilled water. The optimized conditions used for amplification were the initial denaturation at 95 °C for 5 min followed by 35 cycles of each denaturation at 95 °C for 1 min, primer annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and 1 last cycle for a final extension at 72 °C for 5 min. A non-template negative control was also run each time. The integrity and quality of the resulting PCR products were examined on 1.5% (*w/v*) agarose gel impregnated with ethidium bromide (0.5 μg mL^{-1}) and visualized under a UV transilluminator. The amplified ITS fragments were cleaned using Nucleo-Spin gel and a PCR clean-up kit (Macherey-Nagel, Düren, Germany) by strictly following the manufacturer's manual, and were then sequenced by Eurofins Genomics, Japan, using ITS4 (reverse) primer. The quality of the fungal ITS sequences was checked using Finch TV 1.5 (Geospiza Inc., Seattle, WA, USA; Mac OS X), and any cryptic bases (N) were manually

corrected by matching the chromatogram. For the data analysis, the blast search program (blast.ncbi.nlm.nih.gov, accessed on 11 September 2018) was used to search for nucleotide sequence homology for the 18S region for the fungi. The obtained ITS sequences were submitted to GenBank for accession numbers (www.ncbi.nlm.nih.gov/genbank accessed on 11 September 2018) and then were aligned with the program MEGA6 (Molecular Evolutionary Genetics Analysis, version 6) to derive the evolutionary history by using the maximum likelihood method.

2.4. Colonization and Isolation Frequency

An analysis of fungal occurrence was estimated by calculating the colonization frequency and isolation rate. The colonization frequency (CF) and isolation frequency (IF) were calculated according to the formula below and two-way ANOVA tests were carried out to compare the CF and IF values between the tissue types and growing conditions:

$$\text{CF (\%)} = (\text{Number of segments showing endophyte growth/number of segments plated}) \times 100 \quad (1)$$

$$\text{IF (IF\%)} = (\text{Number of unique taxa isolated/number of segments plated}) \times 100 \quad (2)$$

2.5. Community Structure of Culturable Endophytic Fungi

The α diversity of the endophyte species within the samples was measured by using the Shannon–Wiener diversity index (H') and Simpson dominance. An ANOVA test was performed to compare the diversity index values among the host tissue types and growth conditions. To compare the differences in community composition and structure among the tissue types and growing conditions (β-diversity), an analysis of similarity (ANOSIM) and PERMANOVA were used. The use of two different pairwise similarity measures was attempted because they consider different types of information that can provide different insights for comparing communities: (i) Jaccard's index, which only considers the presence or absence of fungal taxa among samples; (ii) the Bray–Curtis coefficient, which considers the abundance of taxa along with the presence or absence of particular fungal taxa. To analyze the diversity measures (α-diversity and β-diversity), PAST ver. 3.26 were used.

2.6. Plant Growth Promotion Potential of Endophytic Fungi

2.6.1. Phosphate and Potassium Solubilization Activity

A qualitative estimation of the phosphate solubilization activity was performed according to Paul and Rao [39] on a solid medium using PDA plugs from 14-day-old fungal cultures on Pikovskaya medium supplemented with bromophenol blue (0.5 mL/100 mL). The potassium solubilization activity was also tested by using the Alexandrov media [40]. The clear halo around the colony was considered a positive result of the phosphate and potassium solubilization activity. The phosphate and potassium solubilization index values were determined using the following formula:

$$\text{Solubilization Index (SI)} = ((\text{Colony diameter} + \text{Halo zone diameter})/\text{Colony diameter}) \quad (3)$$

2.6.2. Estimation of Siderophore Production

The detection of the siderophore production ability of the fungal isolates was tested based on the CAS half-plate agar plate assay [41]. Each half of the plate was filled with PDA medium and the other half contained a solid CAS autoclaved medium that was prepared according to Schwyn and Neiland [42]. The cube of the fungal isolate inoculum was placed in the PDA half and the plate was sealed with parafilm. All of the plates were incubated at 30 °C under dark conditions for 5–7 days and monitored for a change in the blue color into purplish-red or orange. The CAS liquid assay was used for the quantitative assessment of the siderophore production [43]. The fungal isolates were grown on a liquid PDB medium for 14 days and the fungal culture was centrifuged at 6.00 rpm for 5 min. An equal volume

of CAS reagent was added to the culture supernatant and the O.D. was observed at 630 nm. The following calculation was used to express siderophore units [44]:

$$\% \text{ Siderophores unit} = [(Ar - As)/Ar] \times 100 \quad (4)$$

Where Ar is the reference absorbance at 630 nm (CAS reagent only) and as is the sample absorbance at 630 nm.

2.6.3. Qualitative and Quantitative Evaluation of Indole-3-Acetic Acid (IAA) Production

The production of indole acetic acid (IAA) was tested colorimetrically according to Gordon and Weber using the Salkowski reagent [45]. Three plugs of 14-day-old cultures were inoculated on YPD broth (1% yeast extract, 2% peptone, 2% glucose, final pH 6.0) and shaken at 120 rpm for 5 days at 28 °C. The cultures were centrifuged at 10,000 g for 1 min and filtered through a 0.2 µm filter (Millipore, Burlington, MA, USA). Finally, 1 mL of the filtered culture was mixed with 1 mL of Salkowski reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$) and incubated at room temperature for 30 min. The development of the pink color was considered a positive result. The quantitative IAA concentration of the samples was measured by checking the absorbance at 530 nm with a microplate reader and then was measured based on a standard curve of known values. The fungal isolate that showed the highest concentration of indole-3-acetic acid (IAA) was further evaluated based on the growth of the soybean (*Glycine max*, Enri Cultivar), which is one of the most important soybean cultivars used in Japan. Surface-disinfected and germinated soybean seeds were transferred into a plant box (CUL-JAR300; Iwaki, Japan) containing sterilized vermiculate and were inoculated with 2 mL of fungal spore suspension (10^7), then the sterilized water was used as a mock treatment. The plants were transferred to a plant growth chamber (25 °C and 70% humidity under a 16/8 h day/night regimen). Sterilized Broughton and Dilworth nitrogen-free solutions (B&D) were used for watering. Disease symptoms, plant height, and plant fresh and dry weights were measured after 25 days in the plant growth chamber.

2.7. In Vitro Antagonistic Potential against Phytopathogenic Fungi

The antagonistic potential of fungal isolates was screened using in vitro dual culture tests for their ability to control the mycelial growth of the fungal soybean pathogen. The identified pathogenic fungus (*Calonectria ilicicola*), known as red crown rot, was used for this experiment. All endophytic fungal strains were tested against the pathogenic fungus. A plug of the pathogen (5 mm) was used and placed on one side of a 9 cm petri dish, which contained Potato dextrose agar. One disc of endophytic fungus (5 mm) was also placed on the opposite side of the petri dish. Both discs were 4 cm apart from each other. In the control plate, only a disc of PDA medium (5 mm) was placed on the opposite side of the pathogen. Three replicates were used for each combination. All the plates were incubated at room temperature and observed every day [46]. When the growth of the pathogenic fungus stopped in the dual culture or the colony filled the whole plate in the control, the antagonistic type, width of the inhibition band, and radius of the pathogen were measured. The antagonism type between the endophytic fungi and pathogen in vitro was estimated in each combination. The endophytic fungal strain was identified to have antagonistic properties if it suppressed the growth of the pathogen, grew faster than the pathogen, or parasitized the pathogen [47]. The growth inhibition rates (CGIR) of the pathogenic fungi were also calculated using the following formula:

$$\text{CGIR} = ((\text{RCK (radius of control colony)} - \text{R test (radius of test colony)})/\text{RCK}) \times 100 \quad (5)$$

2.8. Statistical Analysis

Each result for the test plants was expressed as the mean percentage. Statistical differences in parameters between control and sample groups were evaluated using

a one-factor analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) test. Here, $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Isolation and Identification of Endophytic Fungi

Various studies have shown that plants and beneficial endophytes have a symbiotic relationship that stimulates plant health and growth. In this study, we isolated and identified culturable endophytic fungi from different tissues of *V. villosa* for the first time. To verify the efficacy of the surface disinfection method after surface sterilization of the samples, the sterile water used to rinse the samples was taken for a control experiment to confirm the surface disinfection. The absence of growth of microorganisms on the plate was checked, and this indicated the effectiveness of the protocol to eliminate the epiphytic microorganisms. A total of 1043 isolates (here isolates are defined as completely visible fungal growths from each (5 mm × 5 mm) tissue segment placed on the PDA plates) were recovered from 1080 tissue segments (leaf, stem, and root) of hairy vetch plants collected from the two different growing conditions (experimental field and greenhouse). Based on their morphological characteristics (color and shape), the isolates were grouped into 64 representative fungal isolates. The sixty-four representative fungal isolates were subjected to molecular identification based on ITS rDNA sequences. The BLAST search resulted in a total of 33 different endophytic fungal taxa that belonged to two main phyla, 4 classes, 6 orders, and 7 genera (Table S1, Figure S1, and Table 1).

Table 1. Taxonomic classification of fungal endophyte OTUs from ITS sequences of endophytic fungi recovered from three representative tissues of hairy vetch with NCBI accession numbers.

Isolate No.	Isolates Acc. No.	Class	Closest Match (Genbank)	Query Coverage (%)	Tissue
hvef1	MK036245	Dothideomycetes	*Cladosporium pseudocladosporioides* (MK111597.1)	99.8	R, S
hvef3	MK036247	Eurotiomycetes	*Penicillium* sp. (LC133788.1)	100	L, S, R
hvef6	MK036250	Eurotiomycetes	*Penicillium brefeldianum* (MH858155.1)	100	L, S, R
hvef7	MK036251	Dothideomycetes	*Cladosporium cladosporioides* (MG669180.1)	96.0	R
hvef9	MK036253	Eurotiomycetes	*Penicillium ochrochloron* (MH137639.1)	99.0	L, S, R
hvef10	MK036254	Eurotiomycetes	*Penicillium glaucoroseum* (MH865551.1)	100	L, S, R
hvef12	MK036256	Agaricomycetes	*Trametes versicolor* (MK322281.1)	100	L, S
hvef18	MK036262	Eurotiomycetes	*Penicillium simplicissimum* (MH856014.1)	100	L, S, R
hvef22	MK036266	Eurotiomycetes	*Penicillium cremeogriseum* (MH374608.1)	100	L, S, R
hvef23	MK036267	Eurotiomycetes	*Penicillium* sp. (MK625191.1)	100	L, S, R
hvef26	MK036270	Dothideomycetes	*Cladosporium halotolerans* (MK265717.1)	100	L, S, R
hvef29	MK036273	Eurotiomycetes	*Aspergillus flavus* (MK791661.1)	100	R, L
hvef30	MK036274	Eurotiomycetes	*Aspergillus* sp. (MK817589.1)	100	L, S, R
hvef31	MK036275	Dothideomycetes	*Cladosporium sphaerospermum* (MH482916.1)	100	R, S
hvef32	MK036276	Sordariomycetes	*Eutypella* sp. (KX828160.1)	99.0	L, S, R
hvef37	MK036281	Sordariomycetes	*Chordomyces antarcticum* (KX385856.1)	100	L, S, R

Table 1. *Cont.*

Isolate No.	Isolates Acc. No.	Class	Closest Match (Genbank)	Query Coverage (%)	Tissue
hvef40	MK036284	Eurotiomycetes	*Penicillium steckii* (MH484016.1)	99.0	S, R
hvef41	MK036285	Eurotiomycetes	*Penicillium expansum* (KC456325.1)	100	L, S, R
hvef43	MK036287	Eurotiomycetes	*Aspergillus westerdijkiae* (MG733714.1)	100	L, S, R
hvef44	MK036288	Eurotiomycetes	*Aspergillus sydowii* (KX898426.1)	99.0	L, S, R
hvef45	MK036289	Eurotiomycetes	*Penicillium italicum* (DQ991463.1)	100	L, S, R
hvef46	MK036290	Eurotiomycetes	*Penicillium svalbardense* (KC346348.1)	93.0	L, S, R
hvef47	MK036291	Eurotiomycetes	*Penicillium* sp. (KY350140.1)	100	L, S, R
hvef48	MK036292	Agaricomycetes	*Tricholoma matsutake* (MF037418.1)	100	R
hvef50	MK036294	Eurotiomycetes	*Penicillium* sp. (MG551581.1)	96.0	L, S, R
hvef52	MK036296	Eurotiomycetes	*Penicillium* sp. (MH325925.1)	98.0	L, S, R
hvef54	MK036298	Eurotiomycetes	*Penicillium* sp. (MH550491.1)	94.0	L, S, R
hvef55	MK036299	Eurotiomycetes	*Penicillium griseopurpureum* (KY678777.1)	100	L, S, R
hvef58	MK036302	Eurotiomycetes	*Penicillium* sp. (KJ921867.1)	99.0	L, R
hvef60	MK036304	Eurotiomycetes	*Penicillium oxalicum* (KF152942.1)	94.0	L, S, R
hvef63	MK036307	Eurotiomycetes	*Penicillium* sp. (KY425713.1)	97.0	L, S, R
hvef64	MK036308	Eurotiomycetes	*Penicillium crustosum* (KT735107.1)	96.0	R
hvef65	MK036309	Eurotiomycetes	*Penicillium* sp. (KT369826.1)	98.0	L, S, R

L: leaf; S: stem; R: root.

The soil type in all experimental sites is volcanic ash soil, with pH and electric conductivity (EC) values of 6.5 and 0.07, respectively. The ANOVA test on colonization frequency (CF) indicated no significant differences between the growing conditions. However, a significant difference was observed among the tissues that were examined in this study (overall colonization frequency = 96.9%). The results showed that the highest colonization frequency was observed in root samples from the field condition, while the stem segments yielded the lowest number of isolates in both growth conditions (Figure 1A).

A similar trend was observed concerning the isolation rate, which expresses the fungal richness of the samples and was calculated by the number of isolates obtained from tissue segments divided by the total number of segments in that sample (Figure 1B). Other studies showed that the colonization rate and species richness of endophytic fungi among root, stem, and leaf samples were the highest in the roots [48], followed by the stems [49,50]. Generally, the isolation frequency in all the tissues of the hairy vetch was high (20–25%). These results suggest that endophytic fungi had extensively colonized many parts of hairy vetch plants, especially the roots. The differences in the assembly of the endophytic fungi in different tissues might be related to the capacity of the root tissue to uptake and utilize nutrients, which provide good conditions for fungal growth. Moreover, other factors such as the structure and chemistry of the tissues might also play a role in this variation. On the contrary, Shreelalitha [51] found a low colonization frequency in the roots compared to other segments in wild legumes.

However, we were not able to identify a few taxa at the genus level (Figure S1). In total, the most dominant phylum in both growth conditions was Ascomycota, with rates of 89% and 92% for field and greenhouse samples, respectively (a total of 91.0%).

However, a few samples in both growing conditions belonged to Basidiomycota (field; 10% and greenhouse; 7%). Those endophytic fungi classified in the Ascomycota phylum were distributed into three classes. The largest part represented Eurotiomycetes (73.5%), followed by Dothideomycetes (15.5%) and Dothideomycetes (6.4%). All Basidiomycota phyla were distributed only in the Agaricomycetes class (Figure S2). The same results were also found in other isolation experiments [49,50,52–54].

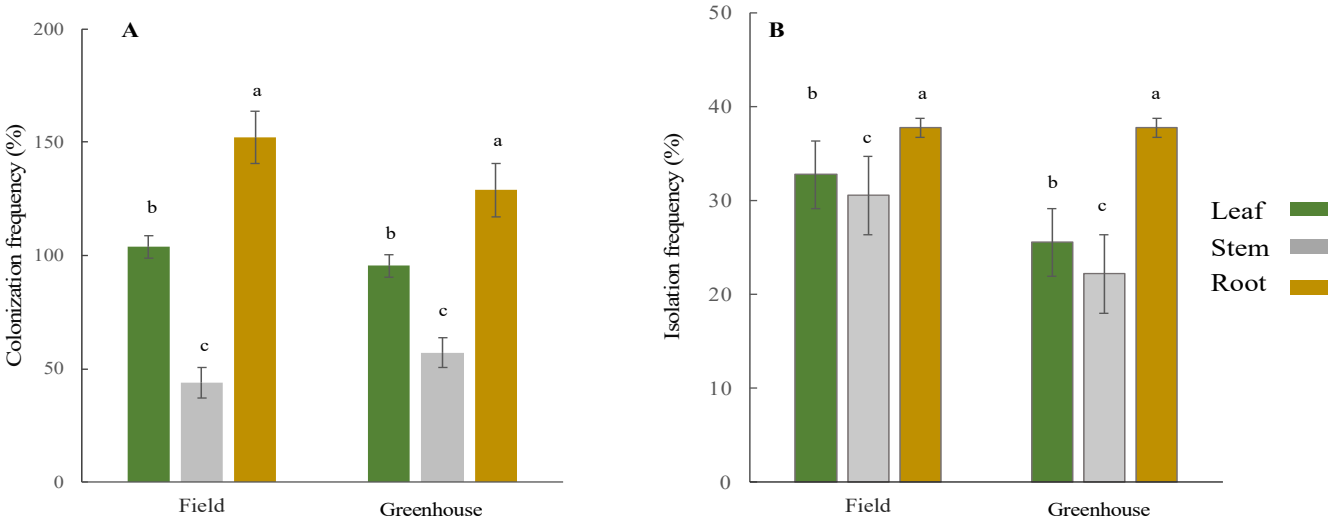

Figure 1. Colonization frequency (**A**) and isolation frequency (**B**) of endophytic fungi common to leaf, stem, and root tissues of hairy vetch (*Vicia villosa*) from two different growing conditions (field and greenhouse). The error bar indicates the standard error (SE) of the mean. Bars with different letters (a, b, c) indicate that the mean values are significantly different at a significance level of $p < 0.05$.

The diversity and species richness of the endophytic fungi (α-diversity) concerning the tissue types and growing conditions were measured using the Shannon–Wiener diversity index (H′) and Simpson dominance index. All indices were calculated with PAST (Version 4) [55]. A higher α-diversity index is defined as higher species diversity and a greater distribution of endophytic fungi. According to the Shannon and Simpson dominance index values for the growing conditions, we did not detect any differences in the α-diversity values of endophytic fungi between the two conditions, but there was significant variation among the tissues based on the results. In total, the root samples showed the highest α-diversity, whereas no significant difference was observed between the leaves and stems in both growing conditions (Figure 2).

3.2. Variation in Endophyte Community Composition and Assembly

According to the ITS sequence and NCBI database results, the total sequences were characterized into two main phyla, 4 classes, 6 orders, 7 genera, and 33 species. Ascomycota was the most dominant in both growth conditions, with rates of 89% and 92% for field and greenhouse samples, respectively. However, only a few of the samples belonged to Basidiomycota (field = 10%; greenhouse = 7%, respectively). Among the Ascomycota, Eurotiomycetes (78%) were the richest class, whereas only one class was identified in the Basidiomycota phylum.

Overall, the most dominant fungal genera were *Penicillium*, *Cladosporium*, and *Aspergillus*. A previous study showed that several endophytic fungi belonging to the *Penicillium* genus have been reported for their ability to synthesize plant-growth-promoting hormones and antagonize pathogens [56]. Among the tissues of hairy vetch plants evaluated here, the relative abundance of some genera was completely noticeable. The highest abundance of the genus Eutypella was recovered from root samples, followed by stem and leaf samples, respectively, as shown in Figure 3. Meanwhile, the relative abundance levels of the genus Eutypella were significantly different between the two growing conditions

and were mostly recovered from field samples (Figure 3). To evaluate the differences in the community composition of endophytic fungi, a beta diversity analysis of the data was performed. Cluster analyses (two- dimensional NMDS), as well as ANOSIM and PERMANOVA, were performed to understand the effects of the growth conditions and tissue type on the endophytic fungal community patterns, based on Bray–Curtis and Jaccard's similarity tests. The clustering of fungal communities displayed a good representation of the hairy vetch fungal endophytes' community structure.

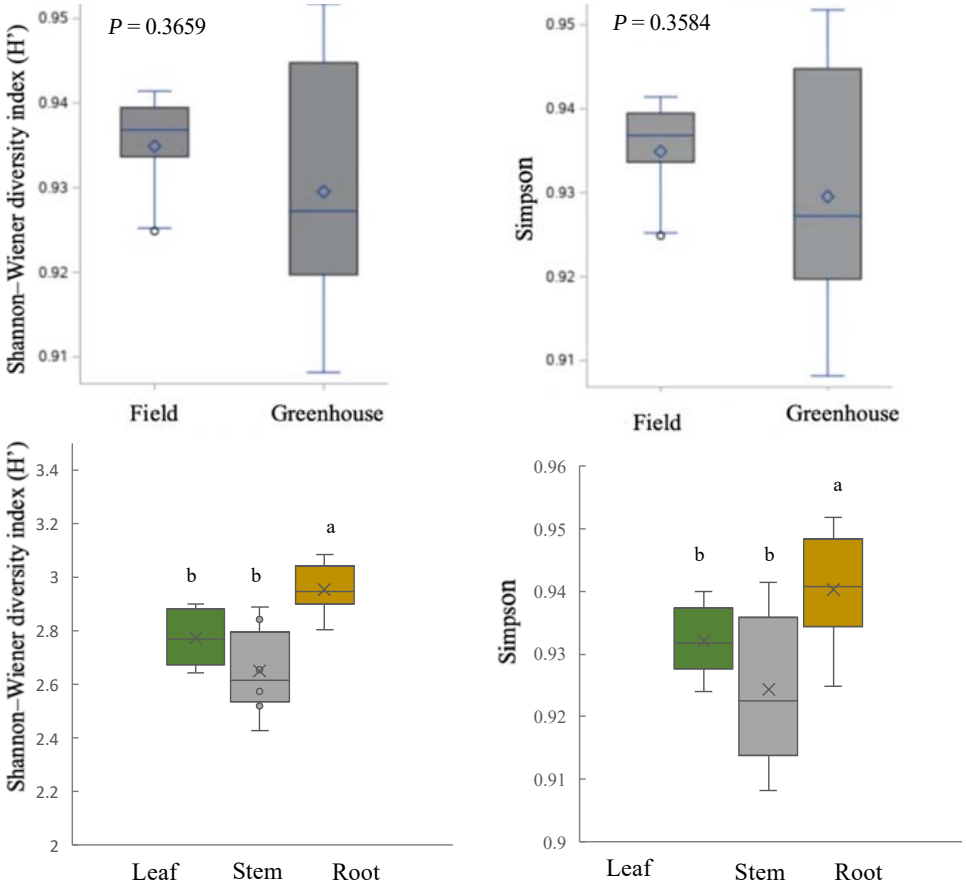

Figure 2. Fungal endophyte biodiversity analysis. The effect of the sampling growing conditions (**top**) and tissue types (**bottom**) on fungal endophyte biodiversity was measured using the Shannon–Wiener diversity (H') and Simpson index. The endophytes were isolated from the leaf, stem, and root tissues of hairy vetch plants from two different growing conditions (field and greenhouse). Bars with different letters (a, b) indicate that the mean values are significantly different at a significance level of $p < 0.05$.

The outcomes of the two-dimensional non-metric multidimensional scale (NMDS) according to the ANOSIM and PERMANOVA tests showed that the growth conditions of the samples did not affect the assembly of the endophyte communities (R = 0.1029, $p = 0.0764$; Figure 4). However, the ANOSIM and PERMANOVA tests suggested that the tissue types significantly influenced the community structure of the fungal endophytes under both growth conditions (Figure S3).

3.3. Plant-Growth-Promoting Traits of Isolated Endophytes

In this study, the plant-growth-promoting potential of all isolates was tested by checking the plant hormone levels (IAA production), which directly stimulates the growth, as well as other factors such as phosphate and potassium solubilization activity and siderophore production levels, which also promote plant growth indirectly.

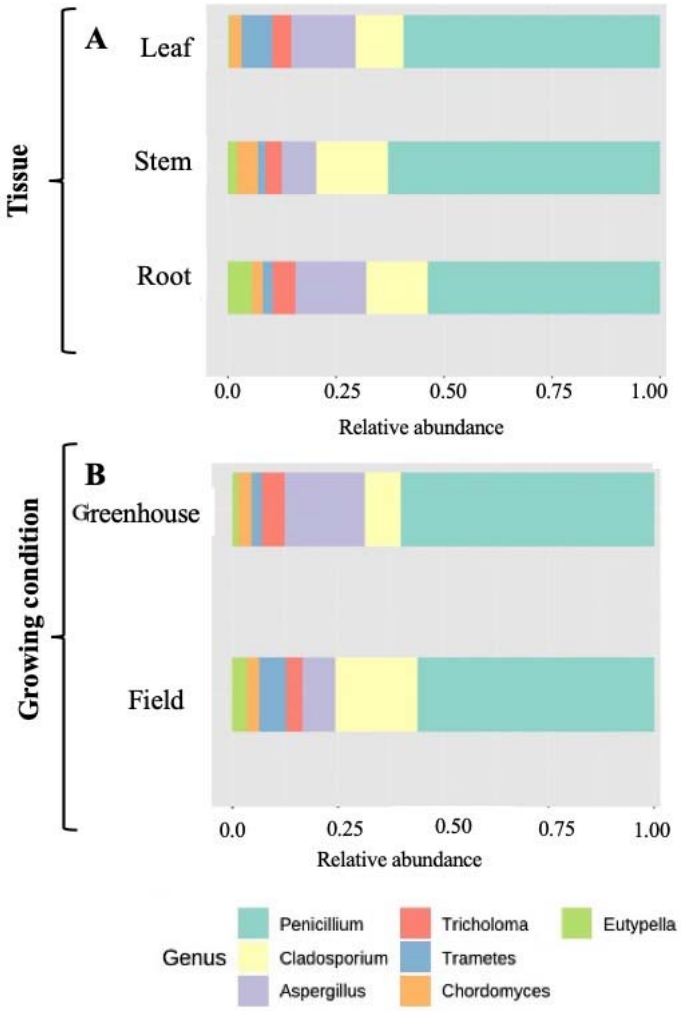

Figure 3. The endophytic community structure of fungal genera in different hairy vetch tissues (**A**) and growth conditions (**B**), showing their relative abundance. The result for each tissue is presented as the sum of that representative tissue, while for the growing condition it is the sum of all tissues.

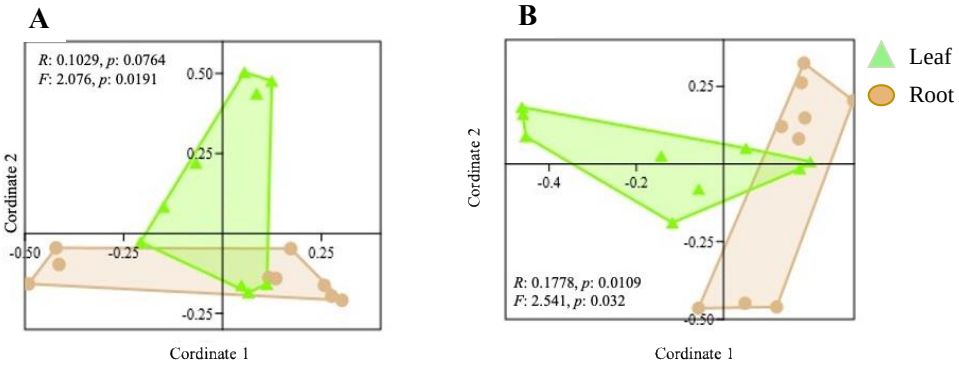

Figure 4. Fungal endophyte communities as influenced by growing conditions. Non-metric multi-dimensional scaling (NMDS) plots for cluster analyses of the fungal endophyte community groups were calculated: (**A**) Bray–Curtis coefficient, which compares the presence or absence of fungal taxa along with the abundance among groups; (**B**) Jaccard's index, which is used to show the presence and absence of data to compare the similarity among groups. The ANOSIM statistic R and PERMANOVA statistic F values and the corresponding p-values indicate the significance of dissimilarities obtained via the permutation of group membership with 9999 replicates.

3.3.1. Phosphate and Potassium Solubilization Activity

Fungal endophytes have various levels of potential to promote the growth of their host plants by harnessing direct and indirect processes. Through an indirect process, these microorganisms can solubilize phosphate and potassium, which are essential for plant growth. [57]. In this study, all the fungal isolates (33 strains) were tested qualitatively on Pikoviskaya media to assess the phosphate solubilization activity (Table 2). Out of all the endophytic fungal samples evaluated, only 5 isolates (hvef7, *Cladosporium cladosporioides*; hvef18, *Penicillium simplicissimum*; hvef29, *Aspergillus flavus*; hvef45, *Penicillium italicum*; hvef60, *Penicillium oxalicum*) showed the potential to solubilize phosphate and produce a clear zone in the Pikoviskaya media supplemented with a pH indicator dye, bromothymol blue. The isolate hvef7 (*Cladosporium cladosporioides*) presented the maximum solubilization index value of 1.36 ± 0.01 (Table 2).

Table 2. Phosphate and potassium solubilizing activities and siderophore production of endophytic fungal strains isolated from *V. villosa*.

Nº	Fugal Strain	Phosphate Solubilization Index	Potassium Solubilization Index	% Siderophore Unit
1	hvef1	0	0	0
2	hvef3	0	0	65.4 ± 0.13
3	hvef6	0	0	0
4	hvef7	1.36 ± 0.01	0	0
5	hvef9	0	0	0
6	hvef10	0	0	0
7	hvef12	0	0	62.0 ± 0.03
8	hvef18	1.13 ± 0.06	0	55.3 ± 0.46
9	hvef22	0	0	0
10	hvef23	0	0	60.7 ± 0.07
11	hvef26	0	0	60.1 ± 0.02
12	hvef29	1.13 ± 0.03	0	74.7 ± 0.16
13	hvef30	0	0	63.0 ± 0.25
14	hvef31	0	0	0
15	hvef32	0	0	64.7 ± 0.23
16	hvef37	0	3.53 ± 0.11	69.4 ± 0.24
17	hvef40	0	0	61.1 ± 0.04
18	hvef41	0	15.9 ± 0.11	61.0 ± 0.05
19	hvef43	0	12.6 ± 0.20	63.1 ± 0.25
20	hvef44	0	0	57.5 ± 0.03
21	hvef45	1.14 ± 0.04	15.1 ± 0.23	0
22	hvef46	0	11.1 ± 0.11	0
23	hvef47	0	4.73 ± 0.25	0
24	hvef48	0	0	56.7 ± 0.05
25	hvef50	0	4.10 ± 0.10	54.7 ± 0.05
26	hvef52	0	0	0
27	hvef54	0	0	54.3 ± 0.08
28	hvef55	0	0	56.3 ± 0.01
29	hvef58	0	3.33 ± 0.30	58.9 ± 0.17
30	hvef60	1.30 ± 0.02	10.8 ± 0.20	61.7 ± 0.24
31	hvef63	0	14.6 ± 0.20	0
32	hvef64	0	0	0
33	hvef65	0	0	0

Values are means ± SD of three replicates.

Moreover, all fungal endophytes were screened for their ability to solubilize potassium by exhibiting a clear zone in the Aleksandrov medium (Figure S4). The potassium solubilization index values ranged from 3 to 15, and the highest solubilization index value was recorded for hvef45 (*Penicillium italicum*) (Table 2). Phosphate and potassium exist in the soil in an insoluble form, but a different type of endophytic fungus has the potential to change them to available forms. In this study, the phosphate and potassium solubilizing

activity levels of all isolates were qualitatively checked, as shown in Table 2. These results are also inconsistent with other reports [58,59], which showed that most *Penicillium* and *Aspergillus* species can convert unavailable phosphate and potassium to available forms for uptake by plants. Phosphate is one of the most vital macronutrients necessary for plant growth and development. However, phosphate in most agricultural soils is in immobile forms, making the isolation and identification of phosphate-solubilizing microbes very essential to modern agriculture.

3.3.2. Siderophore Production Potential of Isolated Fungi

Plants usually suffer from iron deficiencies in the soil, and it is pivotal to solve this problem to increase crop productivity. Some plants have developed certain techniques to overcome the deleterious effects of iron deficiency; for example, monocotyledons produce phytosiderophores. These siderophores are iron carriers that bind with the available ferric iron in the soil with a high affinity [60,61]. Siderophores produced by microorganisms have received more interest because of their potential application in agriculture systems. These small compounds can be synthesized by many fungal species in the rhizosphere soil [62,63]. In this study, nineteen isolates changed the color of the CAS agar plate into a dark purplish-red, as a result of their siderophore production and binding of iron from the medium (Figure S5). The assessment of the siderophore production rates of these nineteen isolates was performed quantitively. Generally, the average siderophore unit production rate of these isolates was 61%. These microbial siderophores play an important role in the transfer of iron to the plants to increase the growth of plants [64]. Moreover, it was reported that siderophores might play a significant role as biocontrol agents [65] because apart from causing the symbiotic relationship between plants and fungi, they also showed hostility against some fungal pathogens [66]. Fungi are generally known for the production of different types of siderophores. Most of them can produce hydroxymate siderophores [67–69]. Other studies have corroborated the results of this study by showing that *Aspergillus* species have a high ability to produce siderophores [40,59,60]. Additionally, the results of this study showed that hvef29 (*Aspergillus flavus*) had the highest rate of siderophore production at 74.7 $\pm$ 0.16%.

3.3.3. Qualitative and Quantitative Evaluation of Indole-3-Acetic Acid (IAA) Production

Phytohormones are essential for the growth and changes in the morphology and structure of plants. The production of phytohormones such as indole acetic acid (IAA), a significant phytohormone, is crucial a technique used by endophytic fungi to stimulate the growth of their host plants. Based on the calorimetric test, 28 isolates were able to produce IAA hormones and showed a pink color when adding the Salkowski reagent. The quantitative amount of IAA concentration of samples was measured by comparing the absorbance of standard pure IAA with culture filtrates of each isolate at 530 nm. The results indicated that the highest IAA content in the culture filtrate (4.21 $\pm$ 0.16 μg mL^{-1}) was produced by isolate hvef7 (*Cladosporium cladosporioides*), followed by hvef18 (*Penicillium simplicissimum*) (3.02 μg mL^{-1}) and hvef1 (*Cladosporium pseudocladosporioides*) (2.32 μg mL^{-1}) (Figure 5).

Indole-3-acetic acid (IAA) is responsible for cell division, differentiation, growth rate control, and root formation [70]. Therefore, the isolation and identification of endophytic fungi capable of producing IAA can significantly promote plant growth by increasing the root length and area, increasing the plant's capacity to uptake nutrients, and helping to regulate the entire metabolism necessary for its life cycle. *Cladosporium* in this study showed the highest amount of IAA production, and these data are similar to the results of previous a study [71].

The strain that produced the highest concentration of indole-3-acetic acid (IAA) was evaluated for its potential as a bio-inoculant for soybean. The results showed that the inoculation of soybean plants with hvef7 (*Cladosporium cladosporioides*) increased the fresh weight of the soybean shoots and roots compared to the non-inoculated plants (control).

Similar results were also observed for the dry weights of the shoots and roots of soybean plants as compared to non-inoculated plants (Figure 6). Moreover, the fungal inoculation did not show any significant differences related to plant height compared to the control plants (data not shown). Phytohormones are essential for plant growth, as well as for changing the morphology and structure of plants. As the result of the current experiment, hvef7 (*Cladosporium cladosporioides*) showed the highest rate of IAA production among all tested isolates. This result is similar to a previous report [72].

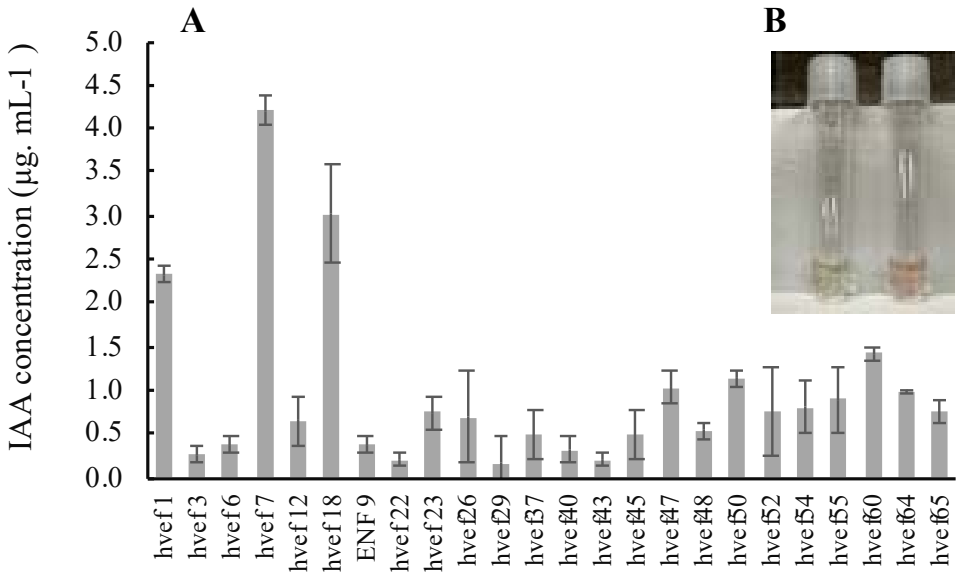

Figure 5. Quantitative (**A**) and qualitative indole-3-acetic acid (IAA) production rates (**B**) caused by 24 isolated fungi after 14 days of incubation. Values are the means of three replicates ± standard deviation.

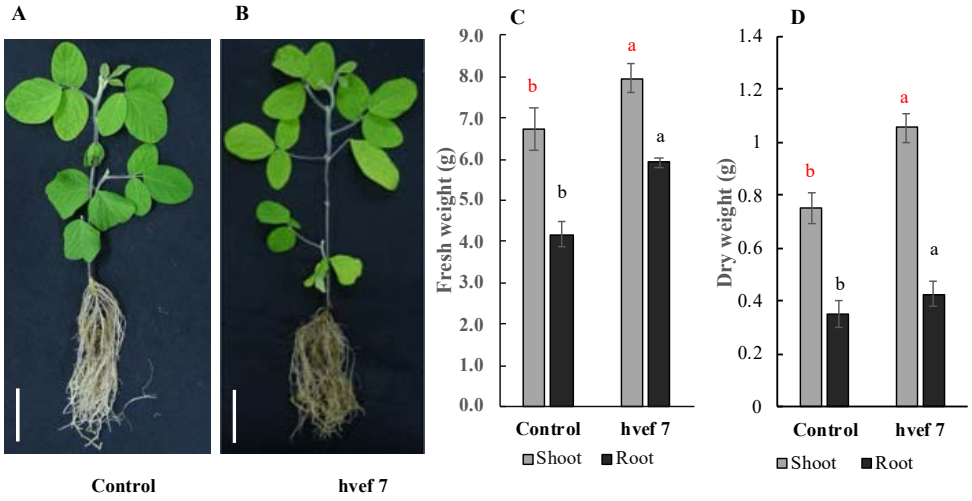

Figure 6. Soybean plant without inoculation (A) and with inoculation of hvef7 (B), soybean shoot and root fresh weights (C), shoot and root dry weights (D) after 25 days inoculation with hvef7 (*Cladosporium cladosporioides*) compared with control plants. Data were statistically different at ($p < 0.05$ as assessed by one-way ANOVA with Tukey's test (n= 5); means ± SD. For each inoculation treatment, bars with different letters (a, b) indicate that the mean values are significantly different at a significance level of $p < 0.05$.

3.4. Antagonistic Potential of Fungal Isolates against Red Crown Rot (Calonectria ilicicola)

In this study, the antagonistic potential of the isolated fungi against *Calonectria ilicicola* was evaluated and classified into three different types. The isolates that showed similar growth with the pathogen were classified as competition for the substance and surfaces.

The growth of both fungi (pathogens and endophytes) will continue until they reach and contact each other. The second group was identified as an antibiotic type, meaning the fungi with antimicrobial potential will inhibit the growth of the pathogen without contact by releasing some antimicrobial metabolites. The last group was classified as mycoparasites. In this group, the rapid growth of endophytic fungi resulted in the coverage of the colony of pathogens, as shown in Figure S6. Most of the isolates showed different abilities to control the growth of *Calonectria ilicicola,* the main cause of red crown rot disease in soybean plants. This result presents the possibility of finding novel metabolites that can be utilized as promising antimicrobial agents. The highest antimicrobial potential was recorded for hvef3 (*Penicillium* sp.) with 95.27 ± 0.06 (Table 3). The antimicrobial potential of endophytic fungi has recently received high attention. Many reports have mentioned the production of some secondary metabolites by endophytic fungi with high potential for antibacterial and antifungal activity, which control the growth of pathogenic microorganisms [65]. The antagonistic potential of the *Penicillium* genus (dominant species in this study) has been reported in other studies [73]. *P. simplicissimum* and *P. simplicissimum* ENF22 isolated from rice have exhibited antagonistic activity against these pathogens, such as *Pyricularia oryzae*, *Rhizoctonia solani*, *Sclerotium oryzae*, and *Pythium ultimum* [74].

Table 3. Inhibitory potential of isolated fungal endophytes against *Calonectria ilicicola*.

N°	Fungal Isolates Code	Growth Inhibition Rates (%)	N°	Fungal Isolates CODE	Growth Inhibition Rates (%)
1	hvef1	16.0 ± 0.25	18	hvef41	49.7 ± 0.29
2	hvef3	95.2 ± 0.06	19	hvef43	93.5 ± 0.13
3	hvef6	89.3 ± 0.17	20	hvef44	56.2 ± 0.15
4	hvef7	62.7 ± 0.26	21	hvef45	1.14 ± 0.04
5	hvef9	92.9 ± 0.01	22	hvef46	43.2 ± 0.20
6	hvef10	67.5 ± 0.29	23	hvef47	92.9 ± 0.10
7	hvef12	59.2± 0.20	24	hvef48	83.4 ± 0.12
8	hvef18	63.3 ± 0.21	25	hvef50	81.1 ± 0.10
9	hvef22	80.5 ± 0.17	26	hvef52	67.5 ± 0.22
10	hvef23	60.4 ± 0.25	27	hvef54	48.5 ± 0.17
11	hvef26	60.3 ± 0.12	28	hvef55	69.8 ± 0.21
12	hvef29	95.9 ± 0.06	29	hvef58	73.4 ± 0.06
13	hvef30	87.0 ± 0.25	30	hvef60	55.6 ± 0.50
14	hvef31	76.3 ± 0.15	31	hvef63	88.7 ± 0.23
15	hvef32	54.4 ± 0.21	32	hvef64	67.5 ± 0.29
16	hvef37	67.5 ± 0.76	33	hvef65	82.2 ± 0.20
17	hvef40	75.7 ± 0.15			

Values are means ± SD of three replicates.

4. Conclusions

Plant diseases and climate change cause major economic losses in crop production and threaten food security. The application of PGPF has been identified as a promising technique to help mitigate crop losses due to diseases and other environmental stresses. The data obtained from this study indicate that besides the well-established weed suppression effect of *Vicia villosa* (hairy vetch), the plant also harbors various kinds of endophytic fungi. Thirty-three strains of fungal endophytes were isolated from different tissues (leaf, stem, and root) of *V. villosa* from different growth conditions based on morphological and molecular analyses. The colonization frequency of fungal endophytes was significantly higher in the root segments than in other tissues. The *Penicillium* genus was found to be the most dominant genus among all isolated fungi. The isolated endophytes showed PGPF potential by producing phytohormones (IAA). Some of these isolates could solubilize phosphate and potassium, which are pivotal for plant growth, and the isolates convert them to the available form for the plants. Moreover, most of the isolated fungi exhibited an antagonistic effect against red crown rot, with the potential to protect plants against

the disease. Although these fungal isolates showed important functional characteristics, further studies are necessary to check their ability to make symbiosis relationships with other target host plants and to evaluate their potential as bioinoculants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102417/s1. Table S1. Mycelium color of the identified fungi (morphological identification. Figure S1. Taxonomic classification of fungal endophytes' operational taxonomic units (OTUs) from ITS sequences of endophytic fungi recovered from three representative tissues from two different growing conditions. Figure S2. Taxonomic classification of fungal endophytes' operational taxonomic units (OTUs) from ITS sequences of endophytic fungi recovered from three representative tissues from two different growing conditions. Figure S3. Fungal endophyte communities as influenced by tissue types from two environmental growing conditions. Non-metric multidimensional scaling (NMDS) plots for cluster analyses of the fungal endophyte community were calculated: (A) Bray–Curtis coefficient, which compares the presence or absence of fungal taxa along with the abundance among groups; (B) Jaccard's index, which is used only to compute presence and absence data to compare fungal community similarity among groups. The ANOSIM statistic R and the PERMANOVA statistic F values and the corresponding p-values indicating the significance of dissimilarity were obtained via the permutation of group membership with 9999 replicates. Figure S4. Potassium solubilization activity of fungal isolates. Figure S5. Identification of the siderophore-producing isolate: (A) control; (B) not able to produce siderophores; (C) siderophore production activity of fungal isolate assessed by changing the blue color of CAS agar into purplish red. Figure S6. (A) The number of antagonistic combinations of endophytic fungi against pathogenic fungi. (B) Antagonistic types of endophytic fungi towards pathogenic fungi.

Author Contributions: Conceptualization, P.T. and Y.F.; methodology, P.T., R.K., K.M.G.D., H.M.K. and S.O.; software, P.T. and K.M.G.D.; validation, P.T., K.M.G.D. and K.T.; formal analysis, P.T., K.M.G.D. and K.T.; investigation, P.T. and Y.F.; data curation, P.T., K.M.G.D. and K.T.; writing-original draft preparation, P.T. and K.S.A.; writing—review and editing, R.K., K.M.G.D., K.S.A., M.Y., K.T., H.M.K., M.A., S.O. and Y.F.; visualization, K.M.G.D., K.S.A. and M.A.; supervision, Y.F.; funding acquisition, Y.F. All authors have read and agreed to the published version of the manuscript.

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