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Original Research Article

Evaluation of Plant Growth Promoting Production from Endophytic Fungi Isolated from Forage Grass and Grass Weed on *in vitro* propagation of *Paphiopedilum callosum* (Rchb.f.) Stein

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ABSTRACT

Microorganisms known as endophytes inhabit plant tissues produce bioactive secondary metabolites, some of which stimulate plant growth. The objective of this research was to screen plant growth-promoting endophytic fungi isolated from the leaves, stems, and roots of Guinea grass (*Panicum maximum* Jacq.), Napier grass (*Pennisetum purpureum* Schumach.), Ruzi grass (*Brachiaria ruziziensis* R.Germ.), Para grass (*Brachiaria mutica* (Forsk.) Stapt), Pangola grass (*Digitaria eriantha* Steud.), and weed species such as Crowfoot grass (*Dactyloctenium aegyptium* (L.) Willd.), Thatch grass (*Imperata cylindrica* Beauv.), Gold beard grass (*Chrysopogon aciculatus* (Retz.) Trin.), Torpedo grass (*Panicum repens* Linn.), and West Indian marsh grass (*Hymenachne amplexicaulis* (Rudge) Nees.). Fourteen fungal isolates were evaluated for their ability to promote plant growth. Indole 3-acetic acid (IAA) synthesis was observed in all isolates, ranging from 12.70±0.04 to 64.20±0.17 µg/mL. *Epichloë* sp. KN03R was the isolate that made the most IAA (64.20±0.17 µg/mL), ammonia (0.24±0.02 mg/L), and a phosphate solubilization (halo zone ratio of 1.50±0.87 cm). The growth-promoting efficacy of *Epichloë* sp. KN03R was tested on *Paphiopedilum callosum* (Rchb.f.) Stein plantlets under sterile conditions for 8 weeks. The results indicated an increase in fresh weight (104.75±0.35 g/plant), plant height (3.47±0.06 cm), number of shoots (1.88±0.11 shoots/plant), number of leaves (7.98±0.34 leaves/plant), number of roots (4.32±0.40 roots/plant), and root length (3.37±0.21 cm). These results suggest that *Epichloë* grass endophytic fungus has the ability to promote nonhost plant growth *in vitro*.

Keywords: Grass endophytic fungi, plant growth promoting production, *Paphiopedilum callosum* (Rchb.f.) Stein.

Introduction

Endophytic fungi live in plant tissue and grow well. Endophytic fungi do not cause disease or abnormal physiological changes to that type of plant. Fungi in the *Epichloë* family (anamorph: Neotyphodium) are endophytic fungi of plants in the grass family. The fungus significantly contributes to the health of its host plants. It helps promote plant growth and reproduction, increase the ability to resist various types of pests, and increase strength and resistance to biotic stress and abiotic stress, while it can produce alkaloids and other compounds.[1-2] Cibils et al. found that alkaloids cause more harm to insects feeding on plants than to plants without endophytic fungi.[3] Plants often use it as a source for the synthesis of crude extracts and biological secondary metabolites.

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Paphiopedilum callosum (Rchb.f.) Stein, a type of slipper orchid, is found in the mountains of northern, eastern, and southern Thailand, with slightly varying floral characteristics. The natural germination percentage of slipper orchid seeds is very low, making it necessary to cultivate the seeds under sterile conditions, which significantly improves germination compared to natural settings. Several studies have reported methods for growing Lady Slipper orchid seeds, most of which utilize synthetic nutrients. The Murashige and Skoog medium (MS medium), supplemented with the growth regulator 2,4-D along with Thidiazuron (TDZ), has been reported to induce the highest number of shoots and shoots per tissue.[4-5] The highest seed germination rate has been observed when Naphthyl acetic Acid (NAA) is combined with 3-Indolebutyric acid (IBA), Kinetin (Kn), and casein hydrolysate.[6] Perotto et al. (2014) found that protocorm development and orchid growth improved 200 days after sowing seeds in Kessler and Cygan medium (KC medium) containing glucose, coconut water, N6-Benzyladenine (BA), and NAA, which significantly increased the shoot emergence rate of *Paphiopedilum bellatulum* (Rchb. f.) Stein and *Paphiopedilum armeniacum*.[7] Most studies focusing on the relationship between orchids and fungi highlight the crucial role of mycorrhizal fungi in orchid development[8], while fewer studies examine orchid growth influenced by endophytic fungi. These studies often involve placing agar pieces containing fungal colonies in orchid culture media, which may or may not result in fungal growth within the plant tissue. However, volatile substances or metabolites produced by the fungi may promote orchid growth.[9-10]

To reduce the use of synthetic nutrients and promote plant growth, reports have indicated the use of endophytic fungi to accelerate growth

through tissue culture in plants such as rice (*Oryza sativa*)[11], tea (*Camellia sinensis*)[12], and *Houttuynia cordata.*[13] This method offers a potential strategy to reduce costs in plant production via tissue culture techniques. In this research, endophytic fungi were isolated from various forage grasses, including guinea grass (*Panicum maximum*), napier grass (*Pennisetum purpureum*), ruzi grass (*Brachiaria ruziziensis*), para grass (*Brachiaria mutica*), pangola grass (*Digitaria eriantha*), and weed species such as crowfoot grass (*Dactyloctenium aegyptium*), thatch grass (*Imperata cylindrica*), gold beard grass (*Chrysopogon aciculatus*), torpedo grass (*Panicum repens*), and west Indian marsh grass (*Hymenachne amplexicaulis*). The effectiveness of these isolated endophytic fungi in promoting the growth of lady slipper orchids propagated via non-host tissue culture was evaluated. The fungi were screened for their ability to synthesize indole-3-acetic acid (IAA), produce ammonia, and solubilize phosphate. The study aimed to assess the efficiency of promoting the growth of *P. callosum* (Rchb.f.). Stein orchid branch shoots using isolated endophytic fungi. The results of this research provide a framework for developing compounds and biosynthesis methods to promote plant growth through endophytic fungi in plant tissue culture. This approach could extend the use of endophytic fungi to non-host plants or other applications in biotechnology.

Materials and Methods

Chemicals and reagents

Ethanol, phenol, sodium nitroprusside, sodium hypochlorite and tricalcium phosphate purchased from Merck (Darmstadt, Germany). Tetracycline, streptomycin, Indole 3-acetic acid standard (IAA), tryptophan and N6-Benzyladenine (BA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potato Dextrose Agar (PDA), Czapek-Dox broth, peptone, Pikovskaya's agar, blue chrome Azurol S agar medium and Murashige and Skoog medium were purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India).

Plant materials

Ten plants (Table 1), *Panicum maximum* Jacq., *Pennisetum purpureum* Schumach., *Brachiaria ruziziensis* R.Germ., *Brachiaria mutica* (Forsk.) Stapt, *Digitaria eriantha* Steud., *Dactyloctenium aegyptium* (L.) Willd., *Imperata cylindrica* Beauv., *Chrysopogon aciculatus* (Retz.) Trin., *Panicum repens* Linn., and *Hymenachne amplexicaulis* (Rudge) Nees. were selected from Thung Song District, Nakhon Si Thammarat Province, Thailand. Voucher specimens were deposited at the herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University.

Table 1: Geographical coordinates of plant sample collection and the corresponding voucher specimen number

Plant Species	Common name	Collection Locality and Coordinates	Herbarium No
Panicum maximum Jacq.	Guinea grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′40.83″N 99°43′38.85″E	BCU014628
Pennisetum purpureum Schumach.	Napier grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′41.98″N 99°43′44.6″E	BCU014631
Brachiaria ruziziensis R.Germ.	Ruzi grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′39.33″N 99°43′46.24″E	BCU014635
Brachiaria mutica (Forsk.) Stapt	Para grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′37.88″N 99°43′45.2″E	BCU014641
Digitaria eriantha Steud.	Pangola grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′41.75″N 99°43′41.09″E	BCU014645
Dactyloctenium aegyptium (L.) Willd.	Crowfoot grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′33.45″N 99°43′41.81″E	BCU014652
Imperata cylindrica Beauv.	Thatch grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′40.26″N 99°43′44.16″E	BCU014658
Chrysopogon aciculatus (Retz.) Trin.	Gold beard grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′45.8″N 99°43′42.52″E	BCU014660
Panicum repens Linn.	Torpedo grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′45.63″N 99°43′45.44″E	BCU014672
Hymenachne amplexicaulis (Rudge) Nees.	West Indian marsh grass	Tham Yai, Thung Song, Nakhon Si Thammarat 8°9′42.93″N 99°43′52.84″E	BCU014683

Isolation of endophytic fungi

Fungal isolation was done using a tissue planting method on PDA medium. For the preparation of inoculate, the preserved leaf, root and stem samples of experimental plants were rinsed in running tap water to remove dust and debris. Leaf pieces were cut to a size of 5 cm, soaked in 1% active chlorine for 10 min, and washed twice with sterile distilled water. Root and stem pieces were cut to lengths of 4-6 cm, disinfected with 70% ethanol for 5 min, followed by soaking in 1% active chlorine for 15 min, and then immersed in 95% ethanol for 2 min, with two rinses in sterile distilled water.[14] After disinfection, the tissue pieces were cut into 4-5 mm sections and placed on PDA medium containing tetracycline and streptomycin (50 µL/mL). The petri dishes were incubated at 25°C. The fungi that grew on the PDA medium were separated, purified, and stored on PDA medium.[15]

Evaluation of production efficiency of plant growth promoting substances

Indole 3-acetic acid (IAA) production

The IAA production study was adapted from Mehmood et al.[16] and Rahmad et al.[17] A fungal spore suspension (1 mL, $2x10^6$ spores per mL) was grown in 100 mL of Czapek-Dox broth with tryptophan added at a concentration of 1,000 µg/mL, which had been previously filtered and sterilized. The inoculated broths were incubated in shaker incubator at 25°C and 150 rpm for 2, 4, 6, and 8 days. After incubation, the culture was harvested and centrifuged (Orto Alresa, 2R, Spain) at 5,000 rpm

for 10 min, and the clear supernatant was reacted with Salkowski reagent in a test tube, with the addition of 2 drops of orthophosphoric acid and incubated at room temperature for 25 min in the dark. After the appearance of a pink colour, it was determined by taking the optical density of the mixture against the blank (2 mL blank media containing tryptophan) at 535 nm using a blank medium containing tryptophan and compared with the IAA standard curve.

Ammonia production
The ammonia capacity and production were adapted from the method described by Cappuccino and Sherman and Strickland and Parson using 1 mL of fungal spore suspension ($2x10^6$ spores/mL) grown in agar medium. Liquid peptone water (100 mL) containing 5 g/L of NaCl was shaken at 250 rpm at 35 ℃ for 48 hours. The supernatant was collected via centrifuged (Orto Alresa, 2R, Spain) at 3,000 rpm for 15 min.[18,19] The clear portion was transferred to a test tube, and 2 mL of 10% phenol solution, 2 mL of sodium nitroprusside solution, and 5 mL of oxidizing solution (alkaline hydrochlorite) were added, followed by thorough shaking. The mixture was left at room temperature for about 1 hour until a blue color developed. The absorbance value was measured at a wavelength of 640 nm (Biochrom Ltd., Spectrophotometer, Libra S22, UK) and the amount of ammonium-nitrogen was calculated by comparison to a standard solution.

Phosphate solubility
The phosphate solubility was modified from Lacava et al.[20] by drilling 5- day-old endophytic fungus colonies with a cork borer. The colonies were cultured on Pikovskaya's agar with 0.5% insoluble tricalcium phosphate ($Ca_3(PO_4)_2$) and left to cure at room temperature. The size of the colony diameter and the clear ring around the agar piece were recorded. The agar surrounding the colony's edge changed from opaque to a clear circle (halo zone) after 14 days of incubation, indicating the phosphate-dissolving activity of the fungal isolate. The solubilization index was then evaluated and reported as the ratio of the clear zone diameter to the colony diameter (halo zone).

Ability to form siderophores
The study of siderophore formation was modified from the method of Schwyn and Neilands by using a cork borer to drill colonies of 5-day-old endophytic fungi, which were then placed on blue chrome Azurol S agar medium and incubated in a dark place at room temperature for 5 days.[21] The formation of yellow to orange coloration around the colony was checked after the 5-day incubation period**.**

Morphological characteristics of fungi
The fungal morphology study was adapted from the methods of White, Leuchtmann et al., and Leuchtmann and Schardl by using endophytic fungi capable of producing the best plant growth promoters.[22-24] The fungi were grown on PDA medium and incubated at room temperature for 3–7 days. Morphological characteristics, including colony shape, spore color, and hyphae, were studied using a stereomicroscope and compound microscopy (Nikon, ECLIPSE Ei, Japan) and compared with the endophytic fungus *Epichloë*.

Evaluation of the effectiveness in promoting the growth of P. callosum (Rchb.f.) Stein
Planting seeds and preparing branch shoots
The pods of the *P. callosum* (Rchb.f.) Stein were washed under running water, followed by washing with liquid soap and spraying with 70% ethanol for 30 sec. They were then disinfected with 15% sodium hypochlorite (NaOCl), with 1-2 drops of leaf binder added, and left for 15 min. The pods were soaked in 10% sodium hypochlorite for 10 min, rinsed with distilled water, and steamed 3 times for 5 min each. The orchid pods were dipped with 70% ethanol and heated through a flame, repeating the process 2-3 times. The pods were cut in half lengthwise and the tips were cut off so that the seeds could grow on MS medium that had the hormone BA added to it. The seeds were then exposed to 2,000 lux (Shaker incubator, LSI-1005R, Korea) of light, 16 hours of light per day, and a temperature of 25 ± 2℃ for 12 weeks to cause protocorm formation. The protocorms were then transferred to MS medium without growth regulators for 4 weeks to adjust conditions until branch shoots formed.[14] Shoots smaller than 0.5 cm in size were used for further experiments.

Adding fungal cultures using the spore suspension method
The inoculum of modified fungi was prepared following the method of Wijesooriya and Deshappriya by creating a fungal spore solution. Five milliliters of sterile distilled water were added to a Petri dish containing selected endophytic fungi growing on 10-day-old PDA medium.[25] A glass spreader was used to scrape the spores, and the spore solution was aspirated into a sterile bottle. There were 1×10^6 spores/ mL, 1×10^3 spores/ mL, and 40 mL of sterile distilled water used as a control in the endophytic spore solution. The tops of the branches were soaked in all of them. Each branch top was soaked in the spore solution overnight at room temperature. After adding the seeds to the tops of Stein orchid branches, they were grown on MS medium for 8 weeks at a temperature of 25 ± 2℃ and a light intensity of 2,000 lux. The branches were exposed to light for 14 hours and darkness for 10 hours. Growth parameters such as weight, height, number of shoots, shoot length, number of roots, and root length were measured for the orchid branch shoots.

Statistical analysis
The experiment was performed three times. The average value was shown together with the mean ± standard deviation (S.D.). A completely randomized experimental design used was Duncan's Multiple Range Test (DMRT). Analysis of variance (ANOVA) was performed using SPSS Statistics 17.0 (IBM SPSS, IBM Corp., Armonk, New York) at a statistical significance level of $p< 0.05$.

Results and Discussion

Separation of endophytic fungi from grasses and weeds
From a study on the isolation of endophytic fungi from grasses and weeds, 14 isolates of endophytic fungi were obtained from the leaves, stems, and roots of guinea grass, napier grass, rusi grass, feather grass, pangola grass, buffalo beak grass, thatch grass, burdock grass, chankasa grass, and joint grass on PDA medium. The leaves of all types of grass yielded the highest number of isolates, 10 (71.5%), followed by 1 isolate each (21.5%) from the roots of guinea grass, buffalo grass, and cattail grass, and 1 isolate (7%) from the stems of pangola grass. These findings are consistent with the research of Absalan et al., who isolated endophytic fungi from the leaves, branches, and roots of rice (*Oryza sativa* L.).[26] The presence of xylem and phloem tissues, the plant's water and food transport systems, likely contributed to the isolation of most endophytic fungi from the leaves, creating favorable conditions for endophytic fungal growth. The isolation of fungi may also depend on the fungus's living behavior in different environments or the age of the plant tissue used for fungal isolation.[27] Younger plant tissues, which are less diseased, are more suitable for the isolation of endophytic fungi and can yield a greater number of isolates compared to older tissues.[28]

Selection of endophytic fungi that produce plant growth-promoting substances
IAA production
All 14 isolates were able to make IAA in Czapek-Dox broth with 1000 µg/mL of L-tryptophan, as shown in Table 2. This means that the endophytic fungi were able to make IAA. The results revealed that the top three endophytic fungi producing the highest IAA on day 6 of culture were isolate KN03R, which were isolated from guinea grass roots, followed by isolate PL01L, which were isolated from pangola grass stems, and isolate PL02S, which were isolated from pangola grass leaves, with IAA levels of 95.18±0.16, 75.08±0.04, and 74.58±0.05 µg/mL, respectively. These differences were statistically significant. In a study by Nath et al.[29], endophytic fungi were found in the roots, stems, and leaves of tea (*Camellia sinensis*). Eight out of ten isolates produced IAA, with production peaking on the sixth day and then going down. Compared to IAA production in endophytic fungi isolated from native jasmine rice, which produced a maximum of 2.651 µg/mL[30], the isolates in this study produced significantly higher amounts of IAA.

Ammonia production

Table 3 shows how much ammonia 14 different types of endophytic fungi made in peptone water medium with 5 g of NaCl added to it over the course of 48 hours. Isolate KN03R, which came from the roots of guinea grass, made the most ammonia. It was followed by isolate KA01L, which came from thatch grass leaves, and isolate KO01L, which came from Guinea grass leaves. Their ammonia levels were 0.24±0.02, 0.23±0.02, and 0.17±0.05 µg/mL, respectively. These differences were statistically significant ($p<0.05$), indicating that the isolated endophytic fungi possess nitrogen-fixing abilities. The fungi converted nitrogen to ammonia. The results of this study agree with those of Xie et al.[31], who found that the endophytic fungus *Phobosis liquidambaris* can fix nitrogen in the roots of peanuts. This helps the plants absorb phosphorus and zinc better, boosts hormone production, and makes them more resistant to plant diseases. Agriculture can apply endophytic fungi with nitrogen-fixing abilities, which synthesize ammonia, to not only promote and stimulate plant growth but also enhance nutrient absorption.[32]

Table 2: The amount of IAA synthesized by endophytic fungi isolated from weeds and forage grasses

Endophytic fungal Isolates	IAA Concentration (µg/mL)			
	Day 2	Day 4	Day 6	Day 8
CK01L	9.50±0.02^{fg}	13.96±0.02^{ef}	20.58±0.08^{cd}	6.76±0.02^{gh}
JC01L	11.51±0.01^{fg}	28.10±0.01^{ab}	39.11±0.02^{e}	7.39±0.02^{gh}
KA01L	20.58±0.02^{cd}	25.79±0.01^{bc}	38.22±0.03^{e}	20.58±0.04^{cd}
KA03R	25.79±0.01^{bc}	27.15±0.01^{bc}	38.22±0.03^{e}	19.71±0.03^{de}
KN01L	8.20±0.01^{fg}	23.02±0.01^{cd}	28.10±0.01^{ab}	5.65±0.02^{gh}
KN03R	70.00±0.20^{c}	72.93±0.18^{b}	95.18±0.16^{a}	48.71±0.12^{ab}
KO01L	18.27±0.04^{de}	22.01±0.03^{cd}	26.74±0.03^{bc}	10.07±0.03^{fg}
NP01L	17.67±0.01^{de}	24.46±0.01^{bc}	29.46±0.01^{ab}	11.08±0.03^{fg}
PK01L	23.45±0.04^{cd}	26.33±0.04^{bc}	37.18±0.03^{e}	18.27±0.02^{de}
PK03R	24.43±0.01^{bc}	28.10±0.01^{ab}	32.17±0.03^{f}	17.70±0.01^{de}
PL01L	27.56±0.04^{bc}	39.11±0.01^{e}	75.08±0.04^{b}	22.01±0.10^{cd}
PL02S	45.89±0.04^{d}	67.53±0.01^{c}	74.58±0.05^{b}	30.27±0.10^{ab}
PP01L	13.38±0.02^{ef}	19.14±0.01^{de}	21.58±0.03^{cd}	7.19±0.04^{gh}
RZ01L	6.76±0.02^{gh}	26.20±0.02^{bc}	39.11±0.02^{e}	4.35±0.00^{gh}

Mean ± standard deviation for 3 replicates. $^{a-h}$The numerical values followed by the same letter in the vertical direction were not statistically different at the $p<0.05$ level by Duncan's Multiple Range Test (DMRT).

Phosphate solubility

The phosphate-solubilizing ability of 11 endophytic fungal isolates on Pikovskaya's agar is shown in Table 3. The results indicate that the endophytic fungi were able to produce phosphatase enzymes or organic acids that dissolve phosphates, promoting plant growth. Research reports suggest that fungi from the genera *Aspergillus* spp. and *Penicillium* spp. can produce organic acids to solubilize phosphates. Additionally, reports suggest that endophytic fungi produce phosphatase enzymes, which digest phosphate rock and serve as a phosphorus source for plants.[33] Microorganisms break down phosphate into inorganic compounds that settle in the soil, releasing phosphorus for plant utilization. This process helps promote plant growth and increases the availability of beneficial phosphorus in the soil.[34] According to Nipuni et al.[35], growing plants with phosphate rock and microorganisms like *Penicillium* spp. that make phosphate-solubilizing biofertilizers led to better plant growth and yield. Feng et al. also found that phosphate-solubilizing microorganisms release organic and inorganic acids, leading to a decrease in pH and an increase in phosphorus solubility.[36]

Ability to form siderophores

The study of siderophore formation found that none of the 14 endophytic fungal isolates were able to grow on chrome azurol S (CAS) agar, the test medium for siderophore production (Table 3), indicating that all isolates were unable to produce siderophores. This finding is consistent with Pablo et al. report[37], which revealed that the endophytic fungus *Epichloë festucae* did not produce siderophores due to a disruption in the NRPS gene group responsible for siderophore synthesis. However, Prathyusha et al. found that endophytic fungi isolated from the leaves of *Terminalia bellirica* could make siderophores, and that the fungus *Acemonium sclerotigenum* could also make siderophores.[38] In 2017, Dolatabad et al. found endophytic fungi in pistachios (*Pistacia vera* L.). *Epicoccum nigrum* and *Quambalaria cyanescens* produced the most siderophore.[39] Additionally, fungi produce siderophores, substances that bind iron surrounding plant roots, thereby preventing pathogen growth due to iron deficiency.[40] The siderophores produced by microorganisms help maintain iron in its water-soluble form, enabling microorganisms to use it. Siderophores improve plant iron solubility and transport, contributing to the plant's immune system, hormone regulation, and activities that promote lytic functions.[41]

From the results of evaluating the production of plant growth-promoting substances by endophytic fungi isolated from grasses, it was observed that the endophytic fungus isolate KN03R exhibited the highest potential for producing plant growth-promoting substances. Therefore, we selected isolate KN03R for further study of its morphological characteristics, comparison with other types of endophytic fungi, and evaluation of its efficiency in promoting the growth of tissue-cultured slipper orchids.

Morphological characteristics and comparisons with endophytic fungus types

The morphological characteristics of the endophytic fungus KN03R, which produced the highest IAA on PDA medium, were observed. As shown in Figure 1, the KN03R colony appeared as white, slow-growing fibers. Under the microscope, the hyphae were colorless (hyaline) with septate walls. Elongated hyphae produced large spores (macroconidia). The morphology of KN03R was found to be similar to *Epichloë bromicola*, and it was compared to *Epichloë** sp. KN03R. Clay and Schardl (2002) said that forage grasses often have endophytic fungi from the genus *Epiichloë* (Clavicipitaceae, Ascomycota).[42]

Effectiveness in promoting the growth of P. callosum (Rchb.f.) Stein

The seeds of *P. callosum* (Rchb.f.) Stein were studied after being cultured on MS medium supplemented with the hormone BA under a light intensity of 2,000 lux, with 16 hours of light per day, at a temperature of 25±2°C for 12 weeks. This induced the formation of protocorms (Figure 2A-B). The protocorms were then transferred to MS medium without the addition of growth regulators for acclimatization over 4 weeks, during which shoot tips emerged. It was possible for protocorm-like bodies (PLBs) to turn into shoots in a medium without growth regulators because of cytokinin in the plant tissue.[43] Subsequently, we tested the branch shoots, no larger than 0.5 cm, for their growth-promoting efficiency when treated with the endophytic fungus *Epichloë* sp. KN03R. The branch shoots (Figure 2C) were

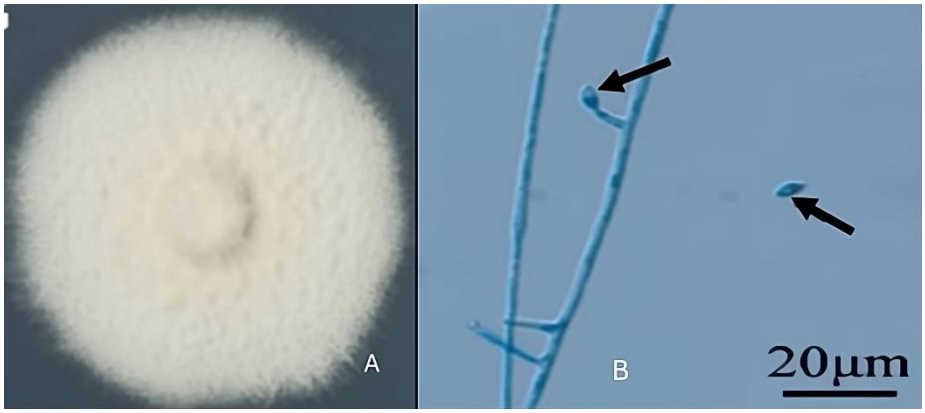

Figure 1: Colony morphology of *Epichloë* sp. KN03R on PDA medium and structure under a microscope at 1,000x magnification.

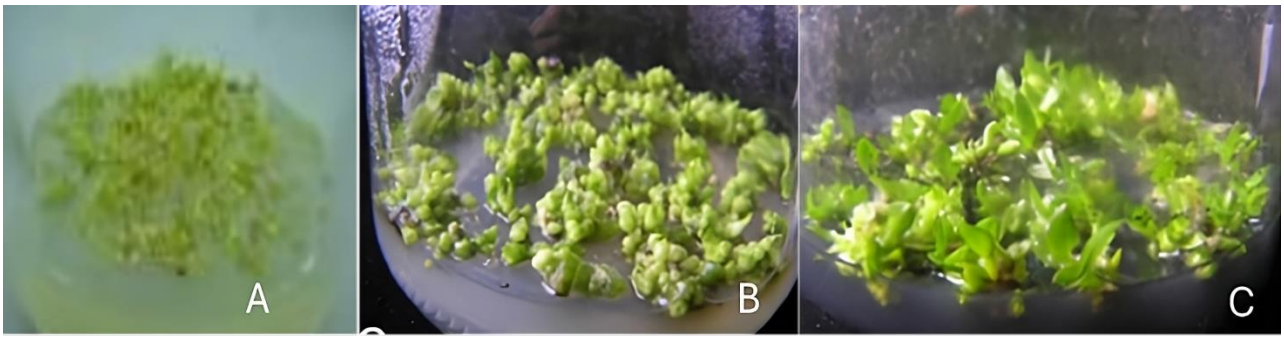

Figure 2: *In vitro* seed culture, seedling development of *Paphiopedilum callosum* (Rchb.f.) Stein (A-B) seed germination on MS medium supplemented with BA for 12 weeks. (B) Protocorm developmental stages (B). (C) Development of seedlings on MS medium without growth regulators for 4 weeks until shoots formed.

Table 3: Potential of plant growth promoter production by endophytic fungi isolated from weed and forage grasses

Endophytic fungal Isolates	IAA Concentration (µg/mL)	Ammonia Concentration)mg/mL(	Phosphate solubility)Halo: clear zones ratio) (cm(	Siderophore production
CK01L	12.70±0.04^{fg}	0.15±0.04^{b}	0.20±0.12^{b}	ND
JC01L	21.53±0.02^{cd}	0.10±0.03^{c}	0.20±0.06^{b}	ND
KA01L	26.29±0.03^{bc}	0.23±0.02^{a}	0.00±0.00^{b}	ND
KA03R	27.72±0.02^{bc}	0.03±0.07^{d}	0.60±0.10^{b}	ND
KN01L	16.24±0.01^{de}	0.08±0.03^{d}	0.36±0.21^{b}	ND
KN03R	64.20±0.17^{c}	0.24±0.02^{a}	1.50±0.87^{a}	ND
KO01L	19.28±0.03^{cd}	0.17±0.05^{b}	0.30±0.17^{b}	ND
NP01L	20.67±0.02^{cd}	0.04±0.01^{d}	0.30±0.17^{b}	ND

Endophytic fungal Isolates	IAA Concentration (µg/mL)	Ammonia Concentration)mg/mL(	Phosphate solubility)Halo: clear zones ratio) (cm(	Siderophore production
PK01L	26.31±0.03^{bc}	0.05±0.01^{d}	0.33±0.06^{b}	ND
PK03R	25.60±0.02^{bc}	0.10±0.02^{c}	0.73±0.40^{b}	ND
PL01L	40.94±0.05^{e}	0.09±0.00^{c}	0.00±0.00^{b}	ND
PL02S	54.57±0.05^{d}	0.03±0.01^{d}	1.38±1.15^{a}	ND
PP01L	15.32±0.03^{de}	0.01±0.01^{d}	0.00±0.00^{b}	ND
RZ01L	19.11±0.02^{cd}	0.04±0.04^{d}	1.13±0.15^{a}	ND

ND = Not detected. Mean ± standard deviation for 3 replicates. $^{a-h}$The numerical values followed by the same letter in the vertical direction were not statistically different at the $p<0.05$ level by Duncan's Multiple Range Test (DMRT).

soaked in a solution of endophytic fungal spores overnight and then grown on MS medium for 8 weeks. It was found that the shoots that were soaked in *Epichloë* sp. KN03R spore solution, with a concentration of 1×10^6 spores/mL and a volume of 40 mL, grew the fastest (Table 4 and Figure 3). The average fresh weight was 104.75±0.35 grams/plant, the average height was 3.10±0.06 cm, and the average number of shoots increased to 1.88±0.11 shoots/plant. The average number of leaves rose to 7.98±0.34, the average number of roots rose to 4.32±0.40 roots/plant, and the average root length rose to 3.37±0.21 cm. In addition, the average plant height rose to 3.47±0.06 cm. This was significantly different ($p<0.05$) from the branch shoots that were soaked in a solution with 1×10^3 spores/mL and the control group (Table 4).

A 1,000x magnification microscope (Nikon, ECLIPSE Ei, Japan) examined the root tissue, revealing the growth of fungal hyphae. This indicated that spores of the endophytic fungus *Epichloë* sp. KN03R, originating from Guinea grass roots, were able to penetrate and colonize the roots of slipper orchids, which are not the original host. The fungus successfully produced mycelium and promoted growth by increasing shoot numbers, root length, and root numbers (Figure 4). These results are similar to those of Xie et al.[44], who found that endophytic fungi taken from sow thistle (*Sonchus oleraceus* L.) could help Chinese cabbage seedlings grow in sterile conditions. Similarly, Kedar et al. reported that endophytic fungi from medicinal plants effectively enhanced corn growth, leading to increased shoot height, root length, and weight, as well as the development of root hairs that aid in nutrient absorption, thereby improving plant growth and biomass.[45] Microorganisms capable of producing auxin plant hormones, such as IAA, contribute to promoting plant growth by stimulating cell elongation, cell division, and overall plant development, particularly at root tips. This increases the contact surface area, allowing for improved nutrient uptake and transport.

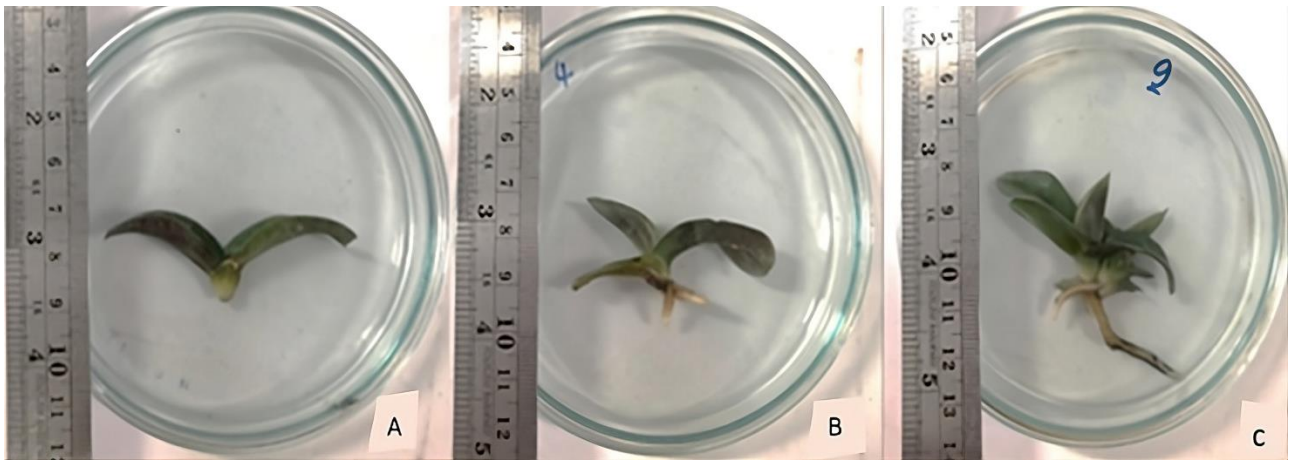

Figure 3: Growth of *P. callosum* (Rchb.f.) plantlet overnight soaked in a spores suspension of *Epichloë* sp. KN03R cultured on MS medium for 8 weeks

A plantlet soaked in 40 mL of distilled water (control)
B plantlet soaked in a spore suspension concentration 1×10^3 spores/mL.
C plantlet soaked in a spore suspension concentration 1×10^6 spores/mL.

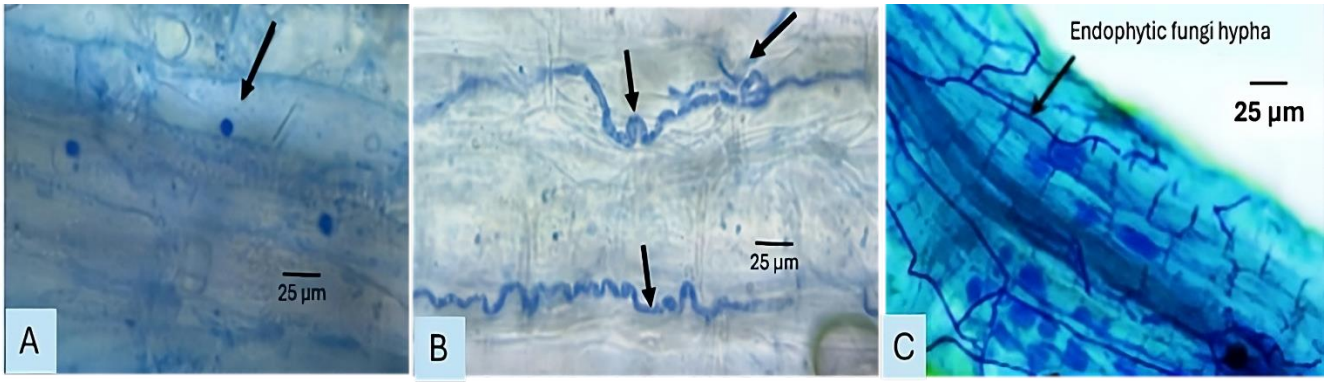

Figure 4: *Epichloë* sp. KN03R spores (A) and hypha (B, C) colonizing roots of *P. callosum* (Rchb.f.) stained with lactophenol cotton blue. (×1000 magnification)

Table 4: Growth of *Paphiopedilum callosum* (Rchb.f.) Stein on MS medium at 8 weeks after soaked overnight in a difference spore concentration of *Epichloë* sp. KN03R

Spores concentration (spores/mL.)	Fresh weight (g/plant)	Shoot number /plant	Leaf number /Plant	Height plant (cm.)	Root number /Plant	Root length (cm.)
0 (control)	44.15±0.41^{c}	0.86±0.04 ^{b}	2.11±0.08^{c}	1.15±0.07^{c}	0.00±0.00^{c}	0.00±0.00^{c}
10x10^{3}	65.50±0.27^{b}	1.25±0.10^{a}	3.47±0.21^{b}	2.78±0.09^{b}	2.18±0.20^{b}	1.56±0.23^{b}
10x10^{6}	104.75±0.35^{a}	1.88±0.11^{a}	7.98±0.34^{a}	3.47±0.06^{a}	4.32±0.40^{a}	3.37±0.21^{a}

Mean ± standard deviation for 3 replicates. $^{a-c}$The numerical values followed by the same letter in the vertical direction were not statistically different at the $p<0.05$ level by Duncan's Multiple Range Test (DMRT).

Conclusion

Endophytic fungi were isolated from the leaves, stems, and roots of forage grass and grass weed in Thung Song District, Nakhon Si Thammarat Province. A total of 14 isolates were obtained from PDA medium. All isolates demonstrated the ability to produce IAA and secrete ammonia. Eleven isolates were able to dissolve phosphate, while none of the isolates were able to form siderophores. The endophytic fungus *Epichloë* sp. KN03R exhibited the highest production of IAA, ammonia, and phosphate solubility. Additionally, it was most effective in promoting the growth of slipper orchids soaked in the spore solution. *Epichloë* sp. KN03R had the most growth. This shows that endophytic fungi found in grasses may be able to help tissue culture plants grow from non-host species.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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