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Enhanced Growth in *Cucurbita maxima* Seedlings Inoculated with Endophytic Fungi isolated from *Rhizophora racemosa* Rhizosphere

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ABSTRACT

Endophytic fungi are considered an eco-friendly and bio-safe alternative to increase agricultural productivity. The study objectives were to isolate and identify endophytic fungi from the roots of *Rhizophora racemosa* and to assess their effect on the growth of *Cucurbita maxima* plants. Molecular identification of the endophytic fungal isolates revealed five fungal species: *Aspergillus aculeatus*, *Aspergillus fumigatus*, *Fusarium equiseti*, *Penicillium citrinum*, and *Talaromyces albobiverticillius*. *A. aculeatus* induced the best improvement rate of plant development with an increase of shoot length (159 %), petiole length (171%), internode length (155%), leaf number (133%) and leaf area (149%) at 84 days after the fungal treatment. This research highlights the importance of *A. aculeatus,* which can be an eco-friendly bio-fertilizer that can enhance the production of *Cucurbita maxima* and improve the agricultural sector. To confirm its effectiveness, experiments must be conducted in fields and greenhouses.

Keywords: Bio-fertilizer, Eco-friendly alternative, Mangrove ecosystem, Plant growth-promotion.

INTRODUCTION

Endophytes live asymptomatically and sometimes systematically within plant tissues without causing symptoms or disease, making them an important group of widespread and diverse plant symbionts (Rashmi *et al.,* 2019). Endophytes occupy microniches within plant tissues, and some are plant-growth-promoting (Devi *et al.,* 2020; Da Silva *et al.,* 2021). Endophytic fungi represent an important and quantifiable component of fungal biodiversity in plants that affect plant community diversity and structure (Chen *et al.,* 2020).

Endophytic fungi are considered the main source of bioactive compounds and secondary metabolites that have potential applications in various fields such as agriculture, pharmaceuticals, environmental cleaning, and the food industry (Alam *et al.,* 2021).

Chadha *et al.* (2015) revealed that the characterization, diversity, and distribution of fungal endophytes across large geographical areas is still in the beginning, and only some general aspects can be affirmed, such as the endophytic fungi diversity that is higher in the tropics than latitudes. Besides, tropical environments

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have a higher number of endophytic species belonging to a few classes. The endophytic fungi diversity in a host plant can be characterized by cultivation-dependent methods and can be affected by various biotic and abiotic factors (Rashmi *et al.,* 2019; Chen *et al.,* 2020). In scientific surveys, molecular tools have excelled in identifying the endophytic community of different plant species. Thus, mass DNA sequencing from plant material has been used to investigate microbiomes, which has led to the identification of a large number of uncharacterized endophytic taxa (Brader *et al.,* 2017; Rhouma *et al.,* 2023).

Endophytic fungi populations have been isolated and characterized in different regions of Mangrove forests around the world including India, Brazil, and China (Costa *et al.,* 2012; Liu *et al.,* 2012). Endophytes are largely understudied components of biodiversity, particularly in Nigerian mangrove forests, and they are constantly exposed to environmental stresses, meaning there is a good opportunity to find new endophytes that colonize the mangrove plants of Akwa Ibom State.

Thus, this study aimed to isolate and characterize endophytic fungi inhabiting the halophytes *Rhizophora racemosa* growing in the mangrove ecosystem of Akwa Ibom State, Nigeria, and evaluate the substantial differences in the response to their symbiotic interaction on *Cucurbita maxima* plants growth and development.

MATERIALS AND METHODS

Rhizophora racemosa Roots Collection and Endophytic Fungi Isolation

Roots of *Rhizophora racemosa* were collected from Okorombokoh, Eastern Obolo Local Government Area, Akwa Ibom State, Nigeria. The root samples were taken to the microbiology laboratory at Akwa Ibom State University to isolate endophytic fungi. The total number of root samples was ten. Endophytic fungi were isolated according to the pour plate method (Germain and Summerbell, 2010; Hrynkiewicz *et al.,* 2010).

Small root pieces (0.5-1 cm) were separated from adherent soil, washed with 2% NaOCl, and dried on sterile filter paper. Root surface sterilization was performed using 70% ethanol (2 min), followed by washing with 2% NaOCl (three times), sterilized with 15% H_2O_2 (5 min), and finally washed with 2% NaOCl (three times). The solutions obtained after the final washing were evaluated for surface sterilization efficiency by plating on agar and monitored for microbial growth. The root material that had been successfully sterilized was the only one used for further analysis. One gram of fresh biomass of sterile roots was stored in 2 mL Eppendorf tubes and lyophilized from each sample (Hrynkiewicz *et al.,* 2010).

Using sterile filter paper, the roots were dried and ground into a fine paste in a mortar. A series of serial dilutions were conducted. One mL of the suspension was mixed with Potato Dextrose Agar (PDA) supplemented with tetracycline (30 µg mL^{-1}). The probability of obtaining most of the culturable endophytic fungi was increased by amending PDA with 100 mM NaCl. For each dilution and plating, three replicates (seven plates/replicate) were done. The plates were incubated at 25±2°C, and observed regularly for fungal growth and isolates were selected based on the colony morphological characteristics such as color, margin, and mycelium form (Germain and Summerbell, 2010).

Molecular Characterization

The molecular analysis was carried out at the Genomics Training Center and Laboratory Limited, Uyo, Akwa Ibom. Molecular characterization includes the following steps: DNA extraction and quantification, Gel Electrophoresis, PCR Amplification, PCR conditions, multiple sequence alignment, and phylogenetic

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analysis. The fungal quantity of molecular characterization was 60 mg.

DNA Extraction and Quantification

The DNA extraction was carried out according to the manufacturer's protocol according to Huzefa *et al.* (2017) (Zymo Research Quick-DNA Fungal and Bacteria Kit). 60 mg of fungal isolate and 750 µL of Bashing bead buffer were added to a ZR Bashing bead lysis tube. A bead beater was used at 10,000 g for not less than 10 min. The ZR Bashing bead lysis tube was centrifuged in a microcentrifuge at 10,000×g for 1 minutes. Then, up to 400 µL of the supernatant was transferred to a zymo-spin filter in a collection tube and centrifuge at 8,000×g for 1 minute. 1,200 µL of genomic lysis buffer was added to the filtrate in the collection tube from the last step. Then, 800 µL of the mixture was transferred to a zymo-spin IICR column in a collection tube and centrifuged at 10,000×g for 1 minute. The flow was discarded, and the last step was repeated. Then, 200 µL DNA pre-wash buffer was added to the zymo-spin ICCR column and centrifuged at 10,000×g in a new collection tube for 1 min. 500 µL g-DNA wash buffer was again added to the zymo-spin ICCR column and centrifuged at 10,000×g for 1 minute. The zymo-spin ICCR column was then transferred to a clean 1.5 mL microcentrifuge tube and 100 µL DNA elution buffer was added directly to the column matrix and centrifuged at 10,000×g for 30 seconds to filter the DNA.

DNA concentration was determined using a Spectrophotometer (Gene Quant Pro). The DNA concentration was 380 ug uL^{-1}. The absorbance of total genomic DNA (gDNA) was quantified by measuring optical density at 260 and 280 nm. DNA concentration was calculated from the absorbance at 260/280 nm as shown in Table 1.

Gel Electrophoresis

The presence and quality of gDNA were also assessedby agarose gel electrophoresis. DNA was quantified on 1.5 agarose gel. Electrophoresis was performedin a 1X TAE (Tris-base glacial Acetic acid, EDTA) gel buffer at 120 volts for 20 minutes. The gel was stained with 7 µL of Safe View dye. After the gel electrophoresis ran for 20 minutes, the TAE buffer was drained from the gel. The gel was then visualized under a trans-UV illuminator.

PCR Amplification

The PCR master mix contains PCR amplification buffer, MgCl, DmSO, DNTPs, and Taq polymerase. Other reagents include forward primer, reverse primer, ddH_2O, and template DNA. The PCR final reaction volume was made up to 25 µL, using 12.5 µL of One Taq master mix, 0.5 µL forward primer, 0.5 µL reverse primer, 2 µL of the sample and 9.5 µL nuclease-free water.

PCR Conditions

PCR reaction conditions conducted at BIO-RAD thermocycler were the following: initial denaturation at 94°C for 30 seconds,

Table 1. DNA concentration and purity of fungi samples.

Sample ID	DNA concentration (ug uL^{-1})	DNA Purity (260/280)
1	337	1.82
2	385	1.89
3	348	1.80
4	351	1.88
5	334	1.90
6	405	1.83

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30 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, initial elongation at 68°C for 1 minutes and final elongation at 68°C for 5 minutes. Amplicons were separated on 1.5% agarose gel electrophoresis for 20 minutes at 120V. DNA ladder of 100 bp was used as a molecular weight pattern. Each amplicon was sequenced on a 3737xl genetic analyzer at Genewiz(USA).

Multiple Sequence Alignment

Each sequence was blasted against the NCBI database to retrieve a similar sequence. A search result that was ≥ 97% wasselected and their sequences were retrieved from GenBank. The sequences were assigned to taxa based on the BLAST result. 10 sequences from the *Aspergillus* spp. and *Penicillium* spp. were added. ClustalW was used to conduct multiple sequenced alignments on MEGA X software with default parameters.

Phylogenetic Analysis

Multiple sequence alignment was done on all the fragments using Clustal W algorithms. Yarrowia lipolytica was used as the out-group. A maximum likelihood phylogenetic tree was constructed on Mega X according to the Tajima-Nei model. Relative branch support was evaluated by 1,000 bootstrap replicates, the branch lengths were calculated by pairwise comparisons of genetic distances, and the missing data were treated by pairwise deletions of gaps. Phylogenetic studies were carried out using the Clustal W software (Sievers and Higgins, 2017) for sequence alignment and MEGA version 5.2 (Tamura *et al.,* 2013) for phylogenetic tree construction.

Plant Growth Promotion Experiment

Cucurbita maxima seeds were sown in plastic buckets filled with sterilized garden soil. The five endophytic fungal strains [*A. aculeatus* (2-FungiITS4F), *A. fumigatus* (3-FungiITS4F), *F. equiseti* (4-FungiITS4F), *P. citrinum* (6-FungiITS4F_R) and *T. albobiverticillius* (1-FungiITS4F)] were cultivated in malt extract medium (Difco, BD) for 7 days, then, the fungal mycelium was collected and washed in sterile distilled water and crushed using sterile glass beads to obtain dispersed inoculum. The fungal mycelium (500 µL) was inoculated at the root junction of the 1-week-old germinated *C. maxima* seedlings and maintained under controlled conditions. The plants were watered using sterile distilled water twice a week. The non-inoculated plants served as controls, and 3 replicates were maintained for all treatments. Thus, the treated pots were placed in a greenhouse for 84 days.

Measurement of growth parameters such as shoot length, petiole length, leaf number,and internode length were measured every three weeks following seedling emergence. Assessments were on 21, 42, 63, and 84 Days After Fungal Treatment (DAFT). The leaf area (cm^2) was determined at three weekly intervals after sprouting. Measurements were obtained using graph paper (grid method). The area of the leaf was determined by tracing the outlines of the leaves on standard graph paper. The area covered by the outline was then calculated (one small square on the graph represents 1 cm^2). The leaf area for each plant was determined according to the formula of Rhouma *et al.* (2021): LA= L×B×0.72; Where: LA= Leaf Area; L= Leaf Length; B= Leaf width.

Statistical Analysis

The study was conducted using a complete randomized design with six treatments and three replicates for each plant. The entire experiment of plant growth promotion was repeated twice. All data in the present study were subjected to Analysis Of Variance (ANOVA) using GraphPad Prism, and data are presented as the Standard Error of the

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Mean (± SEM) of triplicate experiments. The differences between the means were separated and compared using Duncan's multiple-range tests. However, a probability level of P= 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Endophytic Fungi Isolation and Molecular Characterization

Results obtained from this study showed that fungal samples subjected to genome sequencing for confirmation were identified by obtaining the nucleotide sequences. The fungal-specific ITS4-ITS5 universal primers pair was used in amplifying the Internal Transcribed Spacer (ITS) region from the DNA of all the isolates. The amplicon lengths and purity were estimated by gel electrophoresis and found about 500 bp in size (Figure 1).

PCR provides a sensitive and specific method of identifying a known DNA sequence, and the PCR product is of a lighter band compared to the DNA, hence the flow in Figure 1.

These DNA products showed a bit of diversity on the gel. And there was also variability in the sizes of the DNA. Some had bigger fragments compared to others. Genetic variability analysis based on sequence variation in the ITS region of all the isolates showed the existence of either insertion or deletions of the nucleotides in the ITS region and led to the observed variation in a clade-specific manner.

The sequences were analyzed and compared with available sequences from the NCBI database. The sequences showing 80-100% similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLASTn) program available at the National Center for Biotechnology Information (NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST). The numerical codes before the species names refer to their accession number at the GenBank (NCBI) (Table 2).Two fungal families (*Trichocomaceae* and *Nectriaceae*), belonging to four genera (*Talaromyces*, *Aspergillus*, *Fusarium*, and *Penicillium*) and six species, were isolated from *Rhizophora racemosa* roots. The majority of the isolated endophyte fungi belonged to the phylum *Ascomycota* (Table 2).

Constructing a phylogenetic tree is crucial in molecular identification since BLAST search alone cannot overcome the possibilities of statistical errors. Bootstrap consensus is applied to the constructed tree to read maximum sequence replications. Neighbours joining the tree with bootstrapping gave a clear picture for identifying the six fungal isolates. Since the similarity fell within 96-100% and more, BLAST hits show the samples belonged to the genera, thus strongly recommending the isolates as a member of the groups as shown

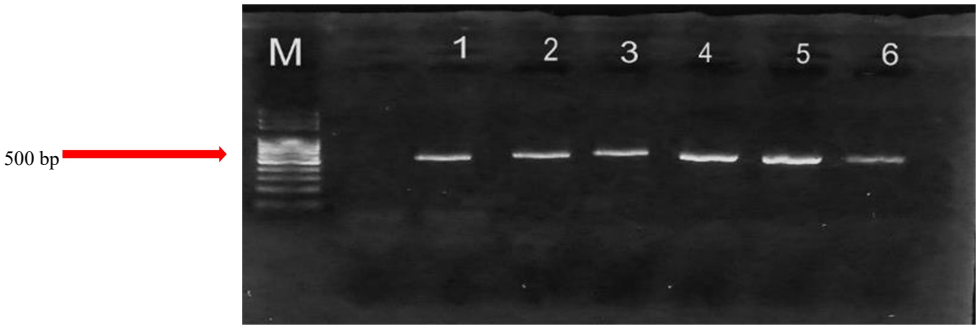

Figure 1. PCR product for fungal isolates (M= 100 bp ladder).

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Table 2. Summary of identified endophytic fungal isolates from the *Rhizophora racemosa* roots.

Samples	Accession number	Name	% Similarity
1-FungiITS4F	MZ227499.1	*Talaromyces albobiverticillius*	98
2-FungiITS4F	MT422091.1	*Aspergillus aculeatus*	97.788
3-FungiITS4F	MH865336.1	*Aspergillus fumigatus*	96.679
4-FungiITS4F	NR_121457.1	*Fusarium equiseti*	97.348
5-FungiITS4F	NR_121224.1	*Penicillium citrinum*	97.714
6-FungiITS4F_R	NR_121224.1	*Penicillium citrinum*	100

in Figure 2.

The tree with the highest log likelihood (-1386.98) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 48 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option) (Figure 2).

The sequences of the genomic regions of ITS, revealed slightly different phylogenetic structures, and all the isolates were grouped into 11 clades in the derived dendrogram. Samples 2 (3-FungiITS4F; *Aspergillus fumigatus*) and 4 (4-FungiITS4F; *Fusarium equiseti*) form a distinct cluster but sample 1 (2-FungiITS4F; *Aspergillus aculeatus*) was found in the same cluster as ascensions of *Aspergillus aculeatus*. Sample 3 (1-FungiITS4F; *Talaromyces albobiverticillius*) belongs to a clade with *Aspergillus flavus*, and *Neosartorya* sp. While samples 5 (5-FungiITS4F; *Penicillium citrinum*) and 6 (6-FungiITS4F_R; *Penicillium citrinum*) belong to the same with *Penicillium* spp. (Figure 2).

There were a total of 178 positions in the

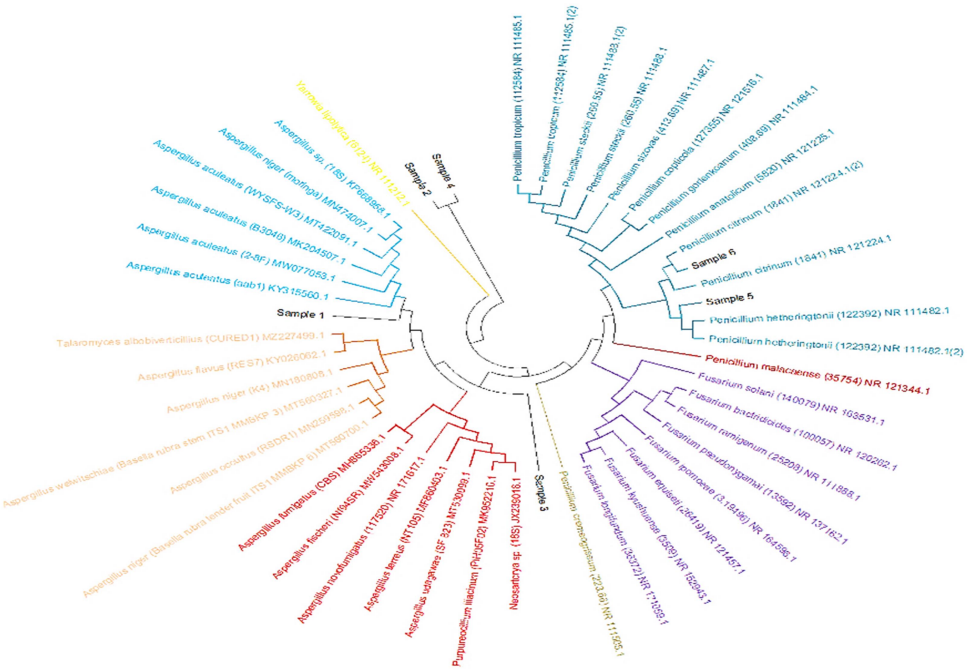

Figure 2. The evolutionary history of the fungal isolates. This was inferred by using the Maximum Likelihood method and the Tamura-Nei model.

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final dataset. Evolutionary analyses were conducted in MEGA X. The tree was rooted to *Yarrowia lipolytica*. Bootstrap replication (1,000 replications) was used for statistical support for the nodes in the phylogenetic tree.Although many phylogenetic trees exist and are used for analysis, the neighbor-joining method was designated the most reliable tree construction method, especially when dealing with closely related strains under varying rates of evolution (Mohamed Zubi *et al.,* 2021).

Costa *et al.* (2012) recovered 25 species from 432 leaf samples of *Rhizophora* sp., *Avicennia* sp., and *Laguncularia* sp. De Souza Sebastianes *et al.* (2013) pointed out that the molecular identification of endophytic fungi revealed that the fungal community isolated from *Rhizophora* sp., *Laguncularia* sp., and *Avicennia* sp. trees is composed of 34 genera. The most frequent were *Xylaria* spp., *Trichoderma* spp., *Fusarium* spp., *Colletotrichum* spp., and *Diaporthe* spp. (De Souza Sebastianes *et al.,* 2013). Koukol *et al.*(2012)and Li *et al.*(2012a, 2012b, 2012c) found that Phylum *Ascomycota*is the most frequently endophytic fungal community. The five most abundant endophytic fungal classes isolated from three Brazilian mangrove species were *Dothideomycetes*, *Eurotiomycetes*, *Tremellomycetes*, *Sordariomycetes*, and *Saccharomycetes*, and all belong to the Phylum *Ascomycota* (Moitinho *et al.,* 2022).

Many species of *Aspergillus* and *Penicillium* were recognized as endophyte fungi (Buommino *et al.,* 2010). Other common endophytes fungi are *Talaromyces* spp., *Phomopsis* spp., *Phoma* spp., *Trichoderma* spp., and many xylariaceous fungi (Alam *et al.,* 2021). Some genera of endophytic fungi isolated in this study may be phytopathogens for mangrove species, such as *Fusarium* spp. (Mohamed Zubi *et al.,* 2021).

Plant Growth Promotion Experiment

The agronomic traits showed that the applications of endophytic fungal species and sampling moments differed significantly ($P< 0.01$), whereas, for petiole length, leaf number, internode length, and leaf area no difference was observed between 21 DAFT ($P\geq 0.05$). The highest values of agronomic traits were recorded at the last sampling moment (84 DAFT) (Tables 3, 4, 5, 6, and 7).

Our results showed that the shoot length increased significantly ($P< 0.01$) after applications with the different endophytic fungal species at different sampling times. *A. Aculeatus* induced the best results with an increase of the shoot length (31.22 cm at 84 DAFT) (the improvement rate is 159.29%) (Table 3; Figure 3).

Treatment of *Cucurbita maxima* roots with *A. aculeatus* exhibited the highest petiole length (3.88 cm at 84 DAFT). *A. fumigatus* was also effective with 3.20 cm, at 84 DAFT with an improvement rate of 170.93 and 140.97%, respectively (Table 4; Figure 3).

According to Table 5, the highest number of leaves per plant was recorded on *C. maxima* treated with *A. aculeatus* (32 leaves at 84 DAFT with an improvement rate of 133.33%) followed by plant treated separately with *A. fumigatus* (27 leaves at 84 DAFT, with an improvement rate of 112.50%) and *F. equiseti* (27 leaves at 84 DAFT, with an improvement rate of 112.50%) (Table 5; Figure 3).

Root treatment, applied separately with different endophytic fungal strains, significantly enhanced internode length ($P< 0.01$) in comparison with the untreated control. The highest increase in internode length was recorded on plants treated with *A. aculeatus* (4.20 cm at 84 DAFT, with an improvement rate of 154.98%) (Table 6). The same was observed for the leaf area with the strongest value for plants treated with *A. aculeatus* (34.40 cm^2 at 84 DAFT, with an improvement rate of 148.92%) (Table 7).

A considerable number of previous studies have been carried out to isolate and characterize Plant Growth-Promoting (PGP) fungi, including those belonging to the genera of *Aspergillus* and *Penicillium* (Gómez-Muñoz *et al.,* 2018; Javed *et al.,* 2020).

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Table 3. Effect of five endophytic fungal strains on the shoot length (cm) of *Cucurbita maxima*.[a]

Treatments (cm)	21 DAFT[c]	42 DAFT	63 DAFT	84 DAFT
Control	7.17±0.16b[a]	13.67±0.42b	16.93±0.59c	19.60±0.91c
A. aculeatus	12.83±0.53a	19.10±0.72a	25.17±1.73a	31.22±0.37a
A. fumigatus	8.17±1.09b	16.217±0.13a	20.17±0.44b	24.30±0.73b
F. equiseti	11.53±0.32a	18.13±0.47a	21.11±0.56b	25.50±0.49b
P. citrinum	11.87±0.15a	18.10±0.42a	21.53±1.12b	27.30±0.33b
T. albobiverticillius	10.43±0.52b	17.63±0.23a	20.17±1.22b	26.43±0.64b
P value[b]	< 0.01	< 0.01	< 0.01	< 0.01

[a] (a-c): Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤ 0.05. [b] Probabilities associated with individual F tests. [c] DAFT: Days After Fungal Treatment.

Average values±Standard deviation.

Table 4. Effect of five endophytic fungal strains on the petiole length (cm) of *Cucurbita maxima*.[a]

Treatments	21 DAFT[c]	42 DAFT	63 DAFT	84 DAFT
Control	0.60±0.25a[a]	1.03±0.67c	1.70±1.09c	2.27±1.32d
A. aculeatus	0.92±0.12a	2.20±0.70a	3.56±1.33d	3.88±0.98a
A. fumigatus	0.73±0.13a	1.50±0.18b	1.80±0.83c	3.20±1.26b
F. equiseti	0.84±0.30a	1.61±0.27b	2.03±0.42b	2.40±1.16d
P. citrinum	0.90±0.19a	2.13±0.39a	2.50±0.12a	2.72±1.10c
T. albobiverticillius	0.63±0.09a	2.01±0.89a	2.40±1.03a	2.67±1.47c
P value[b]	**≥ 0.05**	**< 0.01**	**< 0.01**	**< 0.01**

[a] (a-d): Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤ 0.05. [b] Probabilities associated with individual F tests. [c] DAFT: Days After Fungal Treatment. Average values±Standard deviation.

Table 5. Effect of five endophytic fungal strains on the leaf number of *Cucurbita maxima*.[a]

Treatments	21 DAFT[c]	42 DAFT	63 DAFT	84 DAFT
Control	4.00±0.43a[a]	7.00±0.32c	13.00±0.45d	24.00±0.52c
A. aculeatus	4.00±0.36a	10.00±0.75a	22.00±0.54a	32.00±0.35a
A. fumigatus	4.00±0.29a	7.00±0.85c	18.00±0.95c	27.00±0.32b
F. equiseti	4.00±0.09a	10.00±0.08a	20.67±0.64b	27.00±0.79b
P. citrinum	4.00±0.48a	10.00±0.58a	18.00±0.59c	25.00±0.99c
T. albobiverticillius	4.00±0.62a	9.00±0.77b	19.00±0.84c	26.00±0.19c
P value[b]	≥ 0.05	< 0.01	< 0.01	< 0.01

[a] (a-d): Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤ 0.05. [b] Probabilities associated with individual F tests. [c] DAFT: Days After Fungal Treatment. Average values±Standard deviation.

Aspergillus spp. and *Penicillium* spp. promote plant growth, and they do this in different ways (Khan *et al.,* 2011, 2013). They have been known to release various effective secondary metabolites that can minimize the potential consequences of pathogenic attacks (Gao *et al.,* 2010).

Penicillium spp. and *Aspergillus* spp. produce a wide variety of secondary metabolites (Khan *et al.,* 2013). These fungal endophytes, identified as GA-producers, include *Aspergillus flavus*, *A. niger*, *Penicillium corylophilum*, *P. cyclopium*, *P. funiculosum* (Khan *et al.,* 2011), and *Penicillium* sp., *P. citrinum*

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Table 6. Effect of five endophytic fungal strains on the internode length (cm) of *Cucurbita maxima*.[a]

Treatments	21 DAFT[c]	42 DAFT	63 DAFT	84 DAFT
Control	0.40±0.11a[a]	2.40±1.10b	2.50±1.23b	2.71±0.22c
A. aculeatus	0.87±0.10a	3.50±1.47a	3.93±0.47a	4.20±0.53a
A. fumigatus	0.54±0.30a	3.50±1.55a	3.60±0.44a	3.88±0.97b
F. equiseti	0.53±0.22a	3.23±1.17a	3.47±0.27a	3.50±1.02b
P. citrinum	0.71±0.48a	3.23±1.73a	3.27±0.33a	3.63±1.03b
T. albobiverticillius	0.50±0.30a	3.17±1.10a	3.37±0.17a	3.57±0.47b
P value[b]	≥ 0.05	< 0.01	< 0.01	< 0.01

[a] (a-c): Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤ 0.05. [b] Probabilities associated with individual F tests. [c] DAFT: Days After Fungal Treatment. Average values±Standard deviation.

Table 7. Effect of five endophytic fungal strains on the leaf area (cm²) of *Cucurbita maxima*.[a]

Treatments	21 DAFT[c]	42 DAFT	63 DAFT	84 DAFT
Control	8.77±0.98a[a]	12.41±1.31c	16.20±2.01c	23.10±0.70c
A. aculeatus	10.23±0.75a	21.30±1.76a	25.74±0.86a	34.40±0.41a
A. fumigatus	8.94±0.58a	14.00±0.58c	18.40±1.52c	28.20±1.40b
F. equiseti	9.71±0.67a	16.30±0.43c	20.50±1.17b	28.51±1.14b
P. citrinum	10.14±0.28a	18.40±0.23b	20.00±0.67b	29.10±1.01b
T. albobiverticillius	8.67±0.69a	16.03±0.60b	18.30±0.30a	28.80±1.41b
P value[b]	≥ 0.05	< 0.01	< 0.01	< 0.01

[a] (a-c): Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤ 0.05. [b] Probabilities associated with individual F tests. [c] DAFT: Days After Fungal Treatment. Average values±Standard deviation.

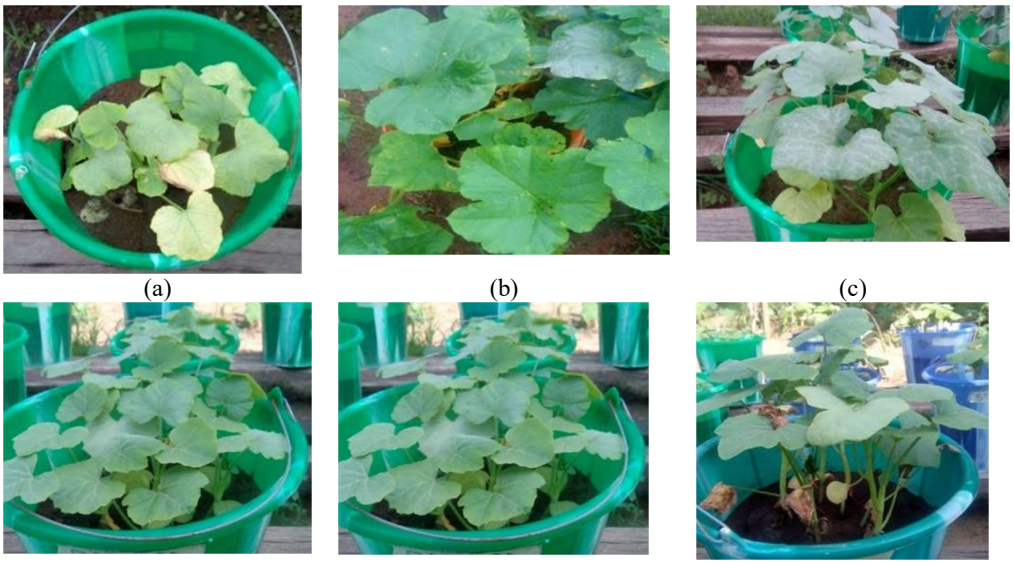

Figure 3. Effect of five endophytic fungal strains on the growth of *Cucurbita maxima*plants. A: Control; B: *A. aculeatus*; C: *F. equiseti*; D: *A. fumigatus*; E: *T. albobiverticillius*; F: *P. citrinum*.

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(Khan *et al.,* 2020). In this study, an evident increase in growth parameters was observed in plants inoculated with *Aspergillus aculeatus*, compared with non-fungi treatment conditions and those inoculated with other fungal isolates. Xie *et al.* (2019) revealed that *A. aculeatus* was reported to stimulate plant growth as evidenced by higher photosynthesis activity, as well as plant length and weight. Leaves treated with *A. aculeatus* mitigated the peroxidation of membrane lipid, lowered the antioxidase activities and salicylic acid production, and increased abscisic acid production and horticultural parameters (plant length and weight, root length and weight, leaf area, etc.) (Li *et al.,* 2019, 2021).

Previous findings suggest endophytic fungi, specifically Aspergillus and Penicillium species, promote plant growth.This can be attributed that can enhance the solubilization of insoluble phosphate compounds (Chowdappa *et al.,* 2020), produce Acetic Indole Acid, and increase amino acid (glycine and proline) and sugar (such as fructose, sorbose, talose, and glucose) (Xie *et al.,* 2019). Increased access to nutrients is one of the mechanisms involved in plant growth promotion by endophytic fungi through the production and transfer of organic acids, siderophores, and nutrients (N, P, K, Fe, Zn, lipids, etc.), and increase water uptake (Osman *et al.,* 2019; Chowdappa *et al.,* 2020). It is evident that the endophytic fungi can improve the production of hydrolytic enzymes (cellulases, pectinases, laccases, xylanases, etc.) and plant growth regulators (abscisic acid, ethylene, gibberellins, cytokinins, auxins, etc.) (Devi *et al.,* 2020; Da Silva *et al.,* 2021). Endophytic fungi can not only enhance nutritional status but also reduce ethylene production through the secretion of 1-aminocyclopropane-1-carboxylate deaminase (Da Silva *et al.,* 2021; Poveda *et al.,* 2021). The induction of plant defense mechanisms against phytopathogens using beneficial fungi (through the expression of defense genes in plants) also contributes to promoting plant growth. These mechanisms enhanced agronomic, biochemical, and physiological parameters in plants (Devi *et al.,* 2020; Da Silva *et al.,* 2021; Argumedo-Delira *et al.,* 2022).

CONCLUSIONS

The isolation and molecular identification of endophytic fungi isolated from *Rhizophora racemosa* showed that the main genera were *Talaromyces*, *Aspergillus*, *Fusarium*, and *Penicillium*. Among the five species screened for their plant growth promotion, *A. aculeatus* achieved the most outstanding growth of *Cucurbita maxima*. Therefore, this species could be recommended as a biostimulant.

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افزایش رشد در نهال های *Cucurbita maxima* تلقیح شده با قارچ اندوفیت جدا شده از ریشه‌گاه *Rhizophora racemosa*

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چکیده

قارچ های اندوفیت به عنوان یک جایگزین سازگار با محیط زیست و زیست‌ایمن (bio-safe) برای افزایش بهره وری کشاورزی در نظر گرفته می شود. هدف از این پژوهش جداسازی و شناسایی قارچ‌های اندوفیت از ریشه *Rhizophora racemosa* و ارزیابی تأثیر آنها بر رشد گیاهان *Cucurbita maxima* بود. شناسایی مولکولی جدایه های قارچ اندوفیت پنج گونه قارچ زیر را نشان داد : *Aspergillus aculeatus*، *Aspergillus fumigatus*، *Fusarium equiseti*، *Penicillium citrinum* و *Talaromyces albobiverticillius*. این تحقیق اهمیت *A. aculeatus* را برجسته می کند، چرا که می تواند یک کود زیستی سازگار با محیط زیست باشد و می‌تواند تولید *Cucurbita maxima* را افزایش داده و بخش کشاورزی را بهبود بخشد. برای تایید اثربخشی آن، باید آزمایش‌هایی در مزارع و گلخانه‌ها انجام شود.

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