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Original Research Article

Molecular Identification of Endophytic Fungi from Corn Plant as Antifungal Agent against *Fusarium oxysporum*

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ABSTRACT

Endophytic fungi live in a symbiotic relationship with plants. They produce interesting secondary metabolites that are beneficial to the host plant. This study aims to identify the endophytic fungi of the corn plant and determine their inhibitory activity against the pathogenic fungus *Fusarium oxysporum*. Endophytic fungi were isolated from corn plant using the direct planting method, and the isolates obtained were tested for their antifungal activity against the pathogenic fungus *F. oxysporum using the dual culture method*. The isolates were examined macroscopically and microscopically for the characterization of their morphology. The endophytic fungal isolates were also subjected to molecular identification by DNA amplification and sequencing, followed by polymerase chain reaction (PCR) analysis. Eleven (11) endophytic fungal isolates were isolated from the roots, stems, and leaves of the corn plant. Three of the isolates (Isolates G, I, and J) showed the most promising antifungal activity against *F. oxysporum* with percentage inhibition of 34.60%, 24.40%, and 14.10% for isolates G, I, and J, respectively. Morphological and molecular characterization of the three isolates identified isolate G as the fungus *Fusarium sororula* with a 99.81% similarity, isolate I was identified as *Diaporthe* sp. with 99.64% similarity, and isolate J was identified as *Sarocladium zeae* with 99.64% similarity. The present study has successfully identified endophytic fungi of the corn plant, and the endophytic fungal isolates have shown the potential to be used as antifungal agent against pathogenic fungi.

Keywords: Biological agent, Endophytic fungi, Corn plant, Antifungal, *Fusarium oxysporum*.acid.

Introduction

Endophytic fungi live in plant tissue without causing direct adverse effects or disease to their host plants.[1] Endophytic fungi are beneficial to plants because they produce secondary metabolites that can prevent their host plants from being attacked by pathogenic fungi.[2] Endophytic fungi produce bioactive compounds with numerous pharmacological activities, such as antibacterial,[3] anticancer,[4] antiviral,[5] and antifungal activities.[6] Plant-Endophytic fungi interaction is typical of a symbiotic relationship between plants and microorganisms.[2] The parts of plants that usually harbor, and serve as substrate for endophytic fungi are the roots, stems, and leaves.[7]

The corn plant is a major contributors to the Indonesian economy from the agricultural sector.[8] This is because corn is one of the primary commodities used as staple food by the Indonesian communities.[9] Plant pests such as *Fusarium oxysporum* attack corn plant causing significant infestation that can be transmitted through the plant seeds, and through the soil.[10-15] This pathogen causes rot in corn stems, cobs, and kernels.[16,17] Infestation due to *Fusarium* spp. reduces productivity in corn plant causing significant financial loss to corn farmers.[10,12]

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For this reason, there is continuous search by farmers for measures of eradicating this pest. Biological agents are promising alternative measures of pest eradication.[18] One of such biological agents that can be used as pesticides are endophytic fungi.[6,19,20] The present study aim to isolate and characterize the endophytic fungi of the corn plant, and evaluate its antifungal activity against the fungus *Fusarium oxysporum*.

Materials and Methods

Collection of plant materials and isolation of endophytes

Corn plants were collected from Bulukumba Regency, South Sulawesi, Indonesia (5°26'07"S 120°12'09"E) on 15th April, 2018. Roots, stems, and leaves from healthy and mature corn plants were collected randomly, and wrapped in sterile bags to reduce the possibility of external contamination. Samples were processed within 24 hours after collection. Endophytic fungi were isolated from fresh plant parts following a modified procedure described previously.[21]

Preparation of test microbes Fusarium oxysporum

Cultures of *F. oxysporum* were obtained from the Laboratory Collection Agency Hayati, Food Crop, and Horticulture Protection Center in Maros Regency, Indonesia. The cultures were transferred to Potato Dextrose Agar (PDA). After an incubation period of 3-5 days, the cultures that develop on the PDA media were mixed with 5 mL Tween 80. The fungal mycelium was then collected in a centrifuge tube and centrifuged using a Dynamic Velocity 18R centrifuge at 4000 rpm for 10 min. The supernatant was removed and replaced with glycerol. The tube was kept at -20ºC to preserve the test microbial culture until further experiment.[18,22]

Evaluation of the antifungal activity of the endophytes against Fusarium oxysporum

The antifungal activity of the endophyte was evaluated using the dual culture technique. The endophytic fungal isolates were inoculated

together with *F. oxysporum* on Potato Dextrose Agar (PDA) with a gap of 3 cm between the inoculums. The cultures were incubated at ambient temperature (28 – 30ºC) for 7 days. As a control, *F. oxysporum* was cultured on PDA without exposure to endophytic fungal isolates. The percentage of *F. oxysporum* growth inhibition by the endophytic fungi was calculated using the equation below.[18]

$$PIRG\ (\%) = \frac{R2-R1}{R2} \times 100.$$

Where;
PIRG = Percentage inhibition of radial growth
R1 = Diameter of colonies with antifungal treatment
R2 = Diameter of colonies without antifungal treatment (control)

Morphological identification of endophytic fungi
Fungal colonies with the most significant inhibitory effect were examined microscopically using the microscope (Leica DM500), and the slide culture method. The microscopic examinations are the distinctive features of the fungal colonies as they grow on a culture medium. These features include the shape, colour, surface texture, and edges of the colonies. Hyphal partitions, hyphal growth, hypha colour, the presence or absence of conidia, and the form of conidia were also examined.[21,23,24]

Molecular identification of endophytic fungi
Molecular identification of the fungal strains was done by DNA amplification and sequencing of the internal transcribed spacer (ITS) region using molecular biology protocols.[25] Fungal hyphae sections (0.5-1.0 cm^2) were obtained from petri plates and subjected to lyophilization in a 2 mL Eppendorf tube (Eppendorf, Germany). The freeze-dried fungal mycelia were eradicated. The extraction of fungal DNA was performed following the manufacturer's instructions, utilizing the DN easy Plant Mini Package (QI Agen, USA). The procedure include cell lysis, RNA digestion by RNase A, removal of cell sediments and waste, DNA cutting, precipitation, and purification. The isolated DNA was amplified by polymerase chain reaction (PCR). PCR was carried out using a Master Mix Kit from Hot Star Taq (QI Agen, USA). As primers, ITS 1 (with the base sequence TCCGTAGGTGAACCTGCGG) and ITS4 (with the base sequence TCCTCCGCTTATTGATGATGC) (Invitrogen, USA) were mixed with the Hot Star Taq Master Mix Kit and DNA template with a total volume of 50 µL. The Te mixture was then added into a thermal cycler using programmed PCR (BioRad, USA). The Te-amplified fungal DNA (PCR product) was submitted for sequencing, and the base sequence was compared using the BLAST Algorithm with publicly accessible databases, including GenBank.

Results and Discussion

Endophytic fungal isolates from corn plant
Eleven (11) endophytic fungal isolates were isolated from the roots, stems, and leaves of corn plant. Five of the isolates were found on the root, three were on the stem, and five were on the leaf (Table 1).
There were more isolates in the root and leaf because endophytic fungi prefer to colonize the roots and leaves of plants compared to the stem. According to Alam *et al*. (2021)[1], endophytic fungi enters and interact with plant tissue in two ways, namely; through seeds and vascular bundles. Vascular bundles are found in many root and leaf organs, while stem organs are only intermediaries between the two. Therefore, endophytic fungi are rarely found on stems. This can explain why more endophytic isolates were found on the roots and leaves of corn plant.
Two isolates were found on different organs; isolate A was found on the root and leaf, while isolate D was found on the root and stem. This is because the two isolates are highly adapted to two different habitats.
Antifungal activity of endophytic fungi
The endophytic fungal isolates were assessed for their antifungal activity against the fungus *Fusarium oxysporum*. Table 2 shows the diameter of *Fusarium oxysporum* colonies on treatment with the endophytic fungal isolates. The average of four representative diameter measurements was taken. The percentage inhibition of fungal growth was measured against a control that contains on the test organisms without the endophytic fungi. The fungal colonies showing the antifungal activity of the endophytic fungal isolates are presented in Figure 1.
Among the eleven isolates tested, isolate G showed the highest percentage inhibition against *F. oxysporum* with percentage inhibition of 34.6%. This was followed by isolate I (24.4%), then isolate J (14.1%). Isolates D, K, and L were the least active with percentage inhibition of 6.4% each against *F. oxysporum* (Table 2).
Endophytic fungi have been shown to exhibit growth inhibitory activity against *F. oxysporum* by three antagonistic mechanisms, which are parasitism, competition, and antibiosis.[26] The parasitic mechanism has a distinctive characteristic, involving the growth of endophytic fungi on the surface of the pathogenic fungi, thereby inhibiting their growth and making their colonies smaller than that of the endophytic fungi.[27] For the competitive mechanism, the colonies of the two fungi approach each other and suppress each other's growth, so the colony size is usually almost the same for both organisms. In the antibiosis mechanism, a clear zone of growth inhibition is seen between the endophytic fungi and the pathogenic fungi.
As shown in Figure 1, Isolate G has antagonistic activities of the competitive type. The fungus *F. oxysporum* and endophytic fungus isolate G compete for space, oxygen, and nutrients. This antagonistic mechanism is illustrated by the growth of the endophytic fungi and pathogenic fungi in the same direction and eventually crushes each other. The colony size between the two isolates was almost the same. Isolate I, with an inhibition percentage of 24.4%, also display a competitive antagonistic mechanism by regulating the availability of nutrients, oxygen, and space to outcompete the pathogenic fungus *F. oxysporum.*
On the other hand, isolate J with a percentage inhibition of 14.10% exhibits a distinct antagonistic mechanism from the two previous isolates, specifically through antibiosis. The antibiosis model generates bioactive chemicals that effectively inhibit the growth of *F. oxysporum*. The defining trait of the antagonistic mechanism is the presence of a distinct zone separating endophytic fungi from the pathogenic fungus *F. oxysporum.*

Table 1: Endophytic Fungi isolated from Corn Plant

No.	Isolate Code	Plant Organ		
		Root	Stem	Leaf
1	Isolate A	+	-	+
2	Isolate B	+	-	-
3	Isolate C	+	-	-
4	Isolate D	+	+	-
5	Isolate G	-	+	-
6	Isolate H	-	+	-
7	Isolate I	-	-	+
8	Isolate J	-	-	+
9	Isolate K	-	-	+
10	Isolate L	-	-	+
11	Isolate M	+	-	-

Description: +: Found, - : Not Found

Morphological characteristics of endophytic fungi from corn plant
The isolate with the highest inhibitory activity was identified starting from the morphological characterization. Morphological characterization was carried out macroscopically and microscopically using a microscope and the slide culture method.
.a. Isolate G
Isolate morphological characteristics were examined from a macroscopic and microscopic perspective. Morphologically, isolate G has a round colony shape and comprises two-colour graduations. The colour arrangement consists of white on the outside and brownish yellow on the inside. A cotton-like appearance and a horizontal growth

pattern on the surface of the media characterize the morphology of isolated G colonies. The periphery of the colony exhibits a morphology resembling fibrous filaments. Isolate G exhibited microscopic features such as insulated and branching hyphae, which are translucent (Table 3). Isolate G is a member of a group of fungi that undergo asexual reproduction by producing conidia. The conidia in isolate G exhibit an oval form and possess a green coloration.

Evaluation of the antifungal activity of isolate G shows that the isolate inhibited the proliferation of the pathogenic fungus *F. oxysporum*, and exhibited the highest inhibitory potency compared to other isolates as shown in Table 2 and Figure 1. The process by which G isolates itself from the pathogenic fungus is by competitive antagonism. In the scenario where isolate G and *F. oxysporum* coexist, they compete for space, nutrients, and oxygen.[27] Isolate G exhibited a higher growth rate than *F. oxysporum* and even inhibited the growth of the pathogenic fungus, as demonstrated by the presence of isolate G on top of the fungus *F. oxysporum.*

b. Isolate I

Morphologically, isolate I is characterized as asymmetrical in shape with a cotton-like surface and grows horizontally on the surface of the medium. Isolate I colony show a two-colour gradient with brown colour on the outside and white colour on the inside. The edge of the colony resembles a thread. The microscopic features of isolate I, including the hyphae and conidia types, are shown in Table 3 and Figure 2. Isolate I is characterized by insulated and branching hyphae that are translucent. The conidia generated by the fungal isolate exhibited an oval morphology and possess a green colour.

As indicated in Table 2, Isolate I exhibited an inhibitory activity of 24.40% next to isolate G. Isolate I, in its inhibitory mechanism, is in the form of competitive antagonism. It has been shown that isolate I competes with the pathogenic fungus *F. oxysporum* for nutrients, oxygen, and even space for growth.[27] Isolate I completely covered the growth of pathogenic fungus.

Table 2: Antifungal activity of endophytic fungi from Corn Plant

No.	Isolate Code	Isolate Average Diameter (cm) D1D2D3D4	DX	Percentage Inhibition (%)
1	Isolate A	7.74.87.67.5	6.9	11.5
2	Isolate B	8.05.07.57.8	7.1	8.9
3	Isolate C	7.64.57.67.5	6.8	12.8
4	Isolate D	9.04.68.57.0	7.3	6.4
5	Isolate G	5.53.85.55.7	5.1	34.6
6	Isolate H	8.04.68.07.4	7.0	10.2
7	Isolate I	7.54.46.35.4	5.9	24.4
8	Isolate J	7.94.87.17.1	6.7	14.1
9	Isolate K	8.54.78.18.1	7.3	6.4
10	Isolate L	7.95.77.87.8	7.3	6.4
11	Isolate M	7.74.87.17.1	6.8	12.8
12	Control	8.07.87.87.5	7.8	-

c. Isolate J

Isolate J exhibited morphology characterized by asymmetrical or uneven colony shapes, accompanied by variations in colony colours and colour gradients. The colony has a pink colour outside and a white colour within. The colonies of isolate J have a velvety appearance and were horizontally positioned on the surface of the media. The outer edge of the colony exhibited wavy contours. Wavy refers to the presence of a distinct and smooth curve at the outer edges of the colony. Table 3 presents information on the microscopic features of isolate J, which comprises hyphae and conidia. The hyphae in isolate J were insulated and branched and had a clear colour. Isolate J exhibited oval conidia that were green in colour. From the antifungal activity test of isolate J against the pathogenic fungus *F. oxysporum*, isolate J showed an inhibitory activity against *F. oxysporum* with a percentage inhibition of 14.10%. The antagonistic mechanism observed in isolate J was antibiosis. The presence of an inhibitory zone between isolate J and the pathogenic fungus *F. oxysporum* explains this phenomenon. The pathogenic fungus was seen to redirect the growth of its colony away from the endophytic fungal isolate J.[27]

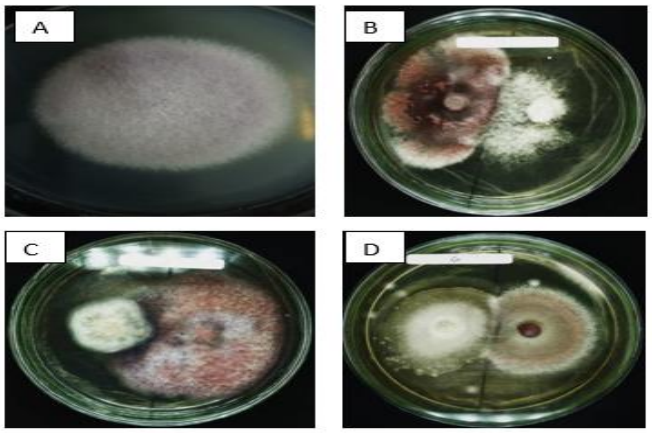

Figure 1: Antifungal activity of endophytic fungal isolates against *F. oxysporum*
(A) *F. oxysporum* (control); (B) Isolate I; (C) Isolate J; (D) Isolate G

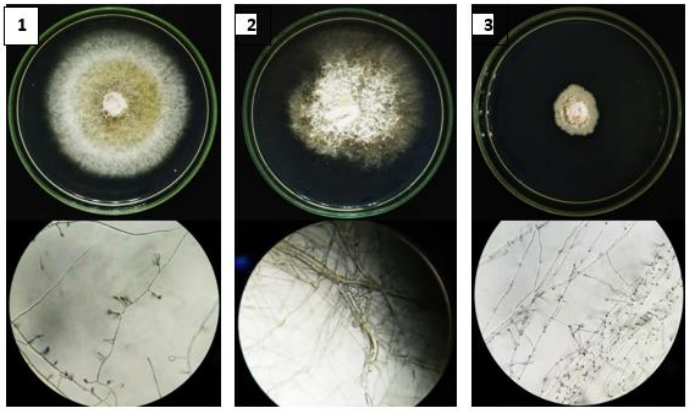

Figure 2: Morphological Characteristics of endophytic fungal Isolates.
(1) Macroscopic and Microscopic Characteristics of Isolate G;
(2) Macroscopic and Microscopic Characteristics of Isolate I;
(3) Macroscopic and Microscopic Characteristics of Isolate J.

Molecular characteristics of endophytic fungi

Molecular characterization of the three isolates with the highest inhibitory activity was also conducted both macroscopically and microscopically**.** The three isolates were characterized morphologically before molecular characterization to ensure that fungal identification was based on the ITS1 and ITS4 sequences in the rDNA gene. Identification of isolates was based on the highest similarity found in BLAST results. Table 4 shows the molecular characteristics of the three endophytic fungal isolates.

The electrophoresis results after PCR analysis showed that the size band was different (Figure 3). From Figure 3, Isolate G (1) showed a length of 500 bp, isolate I (2) had a length of 550 bp, and isolate J (3) had a

length of 500 bp. This agrees with the findings of Porter and Riedman (2023)[28] which stated that the ITS region in the Fungal Kingdom has an average length of 500-600 bp for Ascomycota and Basidiomycota. The MEGA 7.0 program created a phylogenetic tree for endophytic fungal isolates (Figure 4).

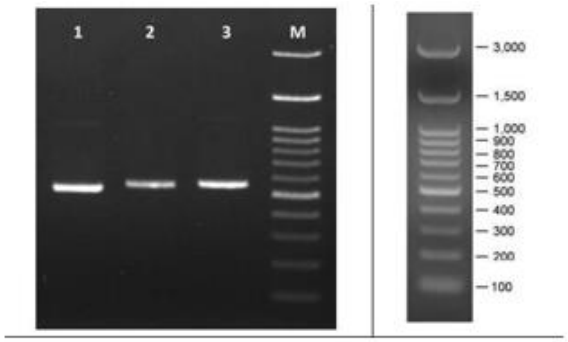

Figure 3: Visualization of PCR amplification (100 Volts, 30 min). (M) marker, (1) Isolate G, (2) Isolate I, and (3) Isolate J.

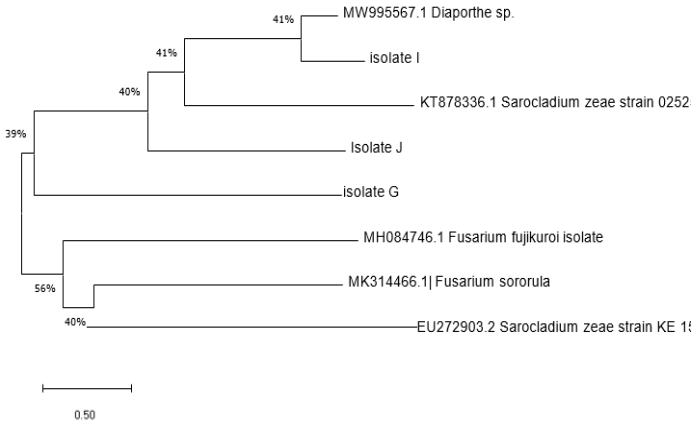

Figure 4: Phylogenetic tree of endophytic fungi

The phylogenetic tree shows that Isolate G, a fungal species *of F. Fujikuroi* was related to the test pathogenic fungus *F. oxysporum*. This is because the two species came from the same genus. *F. Fujikuroi* has a 99.46% identy with isolate G based on the data obtained from the molecular analysis.

Isolate I is a fungal species of the genus *Diaporthe* with a percentage identity of 99.68%. The fungal morphology of *Diaporthe* and that of isolate I as shown in Table 3 are different. The morphology shown in Table 3 is characteristics of the group *Nigrospora*. This discrepancy may be attributed to the challenges associated with the macroscopic and microscopic identification of the fungus of the group *Diaporthe*. Fungi from this group often experience changes in morphological appearance, so determining the characteristics of these isolates will be difficult. Fungi of the group *Diaporthe* lives on terrestrial plants.[29]

Isolate J was identified as the fungal species *Sarocladium zeae based* on the percentage identity of 99.64% obtained from molecular analysis. The name *Acremonium* was initially known as *Sarocladium*,

so the characteristics displayed by the group *Acremonium and Sarocladium* will be the same. Distinctive characteristics of the group *Acremonium*is are the presence of a layered structure resembling a flower, which was also found in the morphology of isolate J, previously designated as belonging to the group *Acremonium*. *Sarocladium* often inhabit the corn plant.[30]

Molecular identity of endophytic fungi

a. Isolate G

Molecular characterization of isolate G showed that isolate G is *Fusarium sororula*. *F. sororula* is one type of fungus from the Ascomycota group of the *Nectriaceae* family. The analysis of isolate G showed that the DNA concentration after DNA extraction was 136.6 ng/µL with a purity level of 1.84, which means that the isolate is pure. The BLAST process on isolate G states that isolate G belongs to the fungal species *F. sororula* with an identity percentage of 99.81%. This was then tested again using the BLAST program on NCBI, and it was found that the percentage level of identity between isolate G and *F. sororula* was indeed 99.81%. Meanwhile, according to the phylogenetic tree, isolate G does have a close kinship with *F. sororula* seen from a distance between isolate G and *F. scrofula. F. scrofula* is one of the five species of fungi included in the *Fusarium fujikuroi Species Complex* (FFSC) group of fungi, which are pathogens of many cultivation variants. *F. sororula* is one of the pathogenic fungi that attack pine plants.[31]

b. Isolate I

Isolate I, based on molecular analysis, belongs to the *Diaporthe* sp. *Diaporthe* is a fungus from the Ascomycota division of the Diaportacheae family. Based on the DNA extraction and quantification of fungal samples, isolate I had a DNA concentration of 30 ng/µL and was the least concentrated isolate compared to the other two isolates. In addition to the DNA concentration, it was also necessary to know the purity of the existing isolates; therefore, it was tested at 260/280 nm wavelength, which showed a value of I.80, so it can be said that the isolate is pure.

Molecular characterization of isolate I showed that it is a fungal species of the genus *Diaporthe* sp., which was indicated by a percentage identity of 99.64%. This means that the level of similarity between isolate I and *Diaporthe* sp is above the minimum threshold of percentage identity. Samples are said to be identical if the percentage identity number is above 97% at the species level and 95% at the genus level. Apart from looking at the percentage identity, paying attention to the kinship distance on the phylogenetic tree is also necessary. Figure 4 showed the phylogenetic tree between the sample and the species similar to the sample, so the kinship distance between the two species is close.

c. Isolate J

Isolate J belongs to the *Sarocladium* group of fungi and the species *Sarocladium zeae*. *S. zeae* is a fungus from the division Ascomycota and the family Sarocladiaceae. *Sarocladium* was previously known as *Acremonium*. Therefore, the characteristics of *Sarocladium* and *Acremonium* are the same.[32] The results of the DNA extraction, amplification, and quantification of isolate J showed that the isolate had a DNA concentration of 62.2 ng/µL. DNA quantification analysis also showed the purity levels of the isolate to know whether or not there are contaminants in the isolate. The quantification results show no contaminants in isolate J at 260/280 nm wavelength, whereas, contaminants were visible at a wavelength of 260/230 nm.

Isolate J, which has been analyzed again using the BLAST program on the NCBI website, showed a percentage identity of 99.64% with the fungus species *S. zeae*. The results shown from the BLAST program were then clarified by using a phylogenetic tree. The kinship between isolate J and *S. zeae* showed a close distance. This means that the two samples still have a close kinship.

Table 3: Morphological characteristics of endophytic fungi from corn plant

No	Macroscopic characteristics	Microscopic characteristics

	Isolate Code	colony colour	Colony Shape and Edges	Colony surface	Hyphae Shape	Hypha Colour	Conidia Shape and Colour
1	Isolate G	White and Yellow	Round and threadlike	Resembles cotton, flat	Divided and branched	Transparent	Elliptical and Green
2	Isolate I	Brown and white	Asymmetry and Thread-like	Resembles cotton, flat	Divided and branched	Transparent	Elliptical and Green
3	Isolate J	Pink and white	Asymmetry and choppy	Resembles velvet, flat embossed	Divided and branched	Transparent	Elliptical and Green

Table 4: Molecular identity of endophytic fungi from corn plant

No.	Isolate code	Concentration (ng/µL)	A260/280	A260/230	Endophytic Fungal Species	Percentage Identity (%)
1	Isolate G	136.6	1.84	0.79	*Fusarium sorolula*	99.81
2	Isolate I	30.0	1.80	0.36	*Diaphorthe* sp.	99.64
3	Isolate J	62.2	1.95	0.57	*Sarocladium Zeae*	99.64

Conclusion

The findings from the present study have shown that the corn plant is rich in endophytic fungi, from which eleven fungal species were isolated. All isolated endophytic fungi inhibited the growth of the pathogenic fungus *F. oxysporum*. Of all the endophytic fungal isolates, isolate G exhibited the highest inhibitory activity against *F. oxysporum*, with percentage inhibition of 34.60%, followed by isolate I with percentage inhibition of 24.40%, and isolate J with percentage inhibition of 14.10%. Molecular analysis revealed isolate G to belong to the genus *Fusarium*, isolate I to the genus *Diaporthe*, and isolate J to the genus *Sarocladium*. Future studies are needed to isolate and identify the substances present in the endophytic fungi which is responsible for the inhibitory activity against *Fusarium oxysporum* for possible use as a biopesticide.

Conflict of Interest

The authors declare 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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