*Int.J.Curr.Microbiol.App.Sci* (2024) *13*(01): 1-9

*International Journal of Current Microbiology and Applied Sciences*
*ISSN: 2319-7706* **Volume 13 Number 1 (2024)**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2024.1301.001

# Utilization of Endophites Fungi in Inhibiting the Growth of *Ustilaginoidea virens* (Cooke) Takahashi Causes False Smut Disease in Rice Plants *invitro*

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**Keywords**

False smut disease, diversity index, evenness dominance and uniformity, inhibition and endophytic fungi

**Article Info**

***Received:***
05 November 2023
***Accepted:***
20 December 2023
***Available Online:***
10 January 2024

**A B S T R A C T**

False smut disease in rice is caused by the fungus *Ustilaginoidea virens* (Cooke) Takahashi, a sporadic pathogen attack in the field, with symptoms of yellow grain which turns black over time. The number of endophytic fungi on the leaves was 15 isolates with the highest prevalence, namely *Aspergillus flavus* of 40%, as well as the highest endophytic on fruit by 80% and the highest endophytic on stem by *Rhizopus* sp. by 55%. The diversity index (H') of endophytic fungi on leaves was 1.332, the evenness uniformity index (E) was 0.272 and the dominance index (D) was 0.72. In fruit endophytes the H' index was 0.500, the E value was 0.273 and the D value was 0.32. For endophytic stems, the H' value was 0.959, the E value was 0.254 and the D value was 0.58. Inhibition test of endophytic fungi on leaves only inhibited *A. flavus* by 80 ± 0.2% and *Rhizopus* sp. by 70 ± 0.3%. The endophytic fungi on fruit were only *A. flavus* which inhibited by 78 ± 0.5%, while the inhibition of endophytic fungi on stems, *A. flavus* and *Rhizopus* sp. can inhibit each by 80 ± 0.4% and 80 ± 1.2%.

## Introduction

Rice (*Oryza sativa* L.) is the most important food crop in developing countries. Among the biotic stresses from false smut is a new disease caused by *Ustilaginoidea virens*. This disease reduces the quality and quantity of rice. Pathogens produce mycotoxins which are harmful to animals and humans. The disease is severe when environmental conditions are favourable such as high humidity (more than 80%) and temperatures ranging from 25 to 30°C, late planting and high soil fertility and use of high amounts of nitrogen. It has acquired the status of a major rice disease and causes yield losses that vary depending on weather conditions during the growing period of the plant and genotype. Therefore, the main concern of farmers is an effective, simple and practical disease control method.

There is no single effective management strategy for false smut, it has been discussed the potential management options available depending on the economic status and adoptive capacity of the farmer. In the view of Plant Pathologists, environmentally friendly disease management methods such as culture, biology

and the use of resistant varieties should be recommended for the sustainability of agriculture and humans (Baite *et al.,* 2021).

Environmentally friendly control by utilizing endophytic fungi has the potential to inhibit the growth of pathogens. Endophytic fungi (EF) are an interesting group of host-associated fungal communities that colonize the intercellular or intracellular spaces of host tissues, conferring beneficial effects on their hosts while gaining an advantage. In recent decades, the accumulation of research on endophytic fungi has revealed their biodiversity, wide ecological distribution, and multidimensional interactions with host plants and other microbiomes in symbiotic sequences (Alam *et al.,* 2021).

Plant-associated fungi (endophytic fungi) are a group of microorganisms rich in biodiversity which are usually found asymptomatic in plant tissues or in the spaces between cells. Endophytic fungi promote host plant growth by directly producing secondary metabolites, which increase plant resistance to biotic and abiotic stresses (Wen *et al.,* 2022). Most of the endophytes associated with infection occur in natural host populations. Although higher plants have developed a variety of generalized resistance mechanisms that prevent infection by most of the opportunistic fungi, endemic symbiotic fungi, including endophytes, have co-evolved with their hosts and adapted to them. Adaptation includes methods for host recognition, means for overcoming complement host defences, mechanisms for host-specific attachment, host-induced spore germination, and diversification of infection structures (White, 2004).

Research on endophytic fungi has proven them to be a promising source of biocontrol agents. These organisms are present in the internal healthy plant tissue for part or/all of their life cycle without causing obvious harm to the host. Endophytic fungi greatly affect the physiological activity of their host plants.

Fungal endophytes increase host resistance to abiotic stress, disease, insects and mammalian herbivores by producing various fungal metabolites. Indeed several interesting metabolites isolated from endophytic fungi belong to diverse chemical classes, including: alkaloids, steroids, flavonoids, terpenoids, quinones, and phenols. Since the isolation of paclitaxel in 1993 from the Pacific Yew endophytic fungus, endophytic fungi have received attention as an alternative source of active ingredients for compounds produced by their host plants, however they can be an alternative source of new natural products to be exploited in modern medicine, agriculture and industry (Khiralla *et al.,* 2017).

## Materials and Methods

### Place and time of research

The research was carried out in two places: 1) looking for sick and healthy panicle specimens from rice fields in Penatih Dangin Puri Village, East Denpasar District, Denpasar City. 2) Laboratory of Plant Diseases and Agricultural Biotechnology Laboratory. The research was conducted from January to May 2023.

### Pathogen Macroscopic Identification

Symptoms of the disease are observed to be known macroscopically, then followed by looking at the pathogen under a microscope. The optilab tool is used to help see under a microscope with a magnification of 400 x.

### Endophytic Fungi Isolation

Isolation of endophytic fungi, plant parts such as fruit, leaves and stems were washed with sterile running water, then the plant parts were sterilized with 0.525% sodium hypochlorite for 3 minutes, and 70% alcohol for 2 minutes, then rinsed with sterile water for 1 minute and then placed in PDA media (which was previously given an anti-bacterial antibiotic, namely livoploxacin with a concentration of 0.1% (w/v). The fungus that emerged from the cut leaves was transferred to a test tube containing PDA to be stored and classified by morphospecies.

### Identification of Pathogen and Endophytic Fungi

The stored endophytic fungi were then grown in Petri dishes containing PDA and repeated 5 times. The cultures were incubated in the dark at room temperature (±27oC). The isolates were identified macroscopically after 3 days of age to determine colony colour and growth rate, and microscopically to identify septa on hyphae, spore/conidia forms and sporangiophores. Identification of fungi using the reference book Samson *et al.,* (1981); Pitt and Hocking (1997); Barnett and Hunter (1998) and Indrawati *et al.,* (1999) and identification of actiomyceytes by Miyadoh (1997).

## Test of Inhibited Ability of Endophytic Fungi against Pathogens

The endophytic fungi found were each tested for their inhibition on the growth of pathogenic fungi using the dual culture technique (one pathogenic fungus was grown in one Petri dish each flanked by two endophytic fungi). The inhibition can be calculated as follows (Dolar, 2001; Mojica-Marin *et al.*, 2008):

$$\text{Inhibited ability (\%)} = \frac{A - B}{A} \times 100$$

Where:

A = Pathogen colony diameter in single culture (mm)

B = Pathogen colony in *dual culture* (mm)

## Determine the Diversity and Dominance Index

The diversity and dominance of contaminant fungi can be determined by calculating the Shannon-Wiener diversity index (Odum, 1971) and the dominance of soil microbes is calculated by calculating the Simpson index (Pirzan and Pong-Masak, 2008).

## Relative abundance

Relative abundance according to Odum (1971) is the percentage of the number of individuals of a species to the total number of individuals present in a certain area in a community and is formulated as follows:

KR = ni/N x 100%

Where:

KR = relative abundance
Ni = number of individuals of each i-th species N = total number of individuals

## Microbial diversity index

The diversity index of soil microbes is determined by the Shannon-Wiener diversity index, namely by the formula (Odum, 1971):

$$H' = -\sum_{i=1}^{s} Pi \ln Pi.$$

Where:

H' = Shannon-Wiener diversity index S = Number of genera

Pi = ni/N as the proportion of species i (ni = total number of individuals of total microbial type i, N = number of all individuals in total n).

The criteria for assessing environmental quality can be seen in Table 1.

## Evenness uniformity index (E)

To find out the balance of the community, the uniformity index is used, which is a measure of the similarity in the number of individuals between species in a community. The more similar the number of individuals between species (the more evenly distributed), the greater the degree of balance. The uniformity index formula (e) is obtained from (Insafitri, 2010):

E = H'/ln S

Where:

H' = diversity index

S = Number of species

E = Evenness uniformity index

The smaller the value of the diversity index (H'), the smaller the uniformity index (E), which indicates the dominance of one species over another.

Here's the range:

E < 0.4: small population uniformity

0.4 < E < 0.6: moderate population uniformity E > 0.6: high population uniformity

## Dominance index

The soil microbial dominance index is calculated by calculating the Simpson index (Pirzan and Pong-Masak, 2008), with the following formula:

$$C = \sum_{i=1}^{s} Pi2$$

Where:

C = Simpson's index

S = Number of genera

Pi = ni/N, namely the proportion of individuals of type i and all individuals (ni = total number of individuals of type i, N = total number of individuals in total n).

Furthermore, the species dominance index (D) can be calculated with the 1-C formulation (Rad *et al.*, 2009).

The criteria used to interpret the dominance of soil microbial species are: close to 0 = low index or lower domination by one microbial species or no species dominates other species extreme, close to 1 = large index or tends to be dominated by several microbial species (Pirzan and Pong-Masak, 2008).

## Results and Discussion

## Endophytic Fungi

The number of endophytic isolates found was as follows: 6 isolates from *A. flavus* leaf endophytes, while *Curvularia* sp., *Stigmina* sp., and *Rhizopus* sp., 3 isolates each (Table 2; Figure 1). Fruit endophytes were found in 12 isolates of *A. flavus* and *Phytophthora* sp. 3 isolates (Table 2; Figure 2), stem endophytes were found *A. flavus, Miselia sterilia* and *Rhizopus* sp., 5, 12 and 21 isolates respectively (Table 2; Figure 3).

It turned out that in leaf endophytes the highest KR was achieved by *A. flavus* by 40%, similarly for fruit endophytes the highest KR was achieved by the fungus *A. flavus* by 80% and the highest KR stem endophytes were achieved by *Rhizopus* sp. by 55% (Table 2). It means that these microbes dominate in every occupied habitat.

The number of isolates found both on the endophytes will determine the chance of inhibition of the pathogen. The more isolates will provide opportunities the greater the inhibition that occurs. Endophytic fungi will take advantage of space for growth and are able to compete with pathogens in the fruit tissue and produce certain compounds to inhibit the growth of pathogens (Sudarma *et al.*, 2022). Endophytic fungi have been isolated from healthy cocoa pods to control pod rot pathogens, namely *Phytophthora palmivora*, namely *Aspergillus* spp, *Fusarium* sp. and *Ramichloridium* sp. all inhibited *in vitro* (Simamora *et al.*, 2021).

## Diversity and Domination Index of Endophytic Fungi

Leaf endophytic fungi found a diversity index (H') of 1.332, E value of 0.272 and D value of 0.72. Fruit endophytes H value of 0.500, E value of 0.273 and D value of 0.32. Endophytic stems have H' value of 0.959, E value of 0.254, and D value of 0.58 (Table 3). A small diversity index is accompanied by a small evenness uniformity index and dominance above 0.5 (except fruit endophytes) means that there are species that dominate, namely the leaf endophytes by *A. flavus* by 40%, fruit endophytes by *A. flavus* by 80% and stem endophytes by *Rhizopus* sp. by 55% (Table 3, Figure 4).

A very small diversity index means that the individual community is unstable except for leaf endophytes the diversity index is quite stable while the others are bad or even very bad, supported by very small evenness uniformity. This proves that the community is unstable with small population diversity. The domination index for all except for the endophytes on fruit was above 0.5, meaning that there were individuals who dominated, such as *A. flavus* (in endophytic leaves and fruit) and *Rhizopus* sp. on stem endophytes.

## Inhibition of Endophytic Fungi against Pathogen in Vitro

The inhibited ability of leaf endophytic fungi only *A. flavus* inhibited by 80 ± 0.2%, and *Rhizopus* sp. by 70 ± 0.3%. Only A. flavus was able to inhibit fruit endophytes by 78 ± 0.5%, while the stem endophytes of *A. flavus* and *Rhizopus* sp. can inhibit by 80±0.4% and 80±1.2%, respectively (Table 4) (Figure 5). Adebola and Amadi (2010) stated that the results of in vitro screening using multiple culture techniques were carried out to assess the potential of three endophytic fungal species *Aspergillus*, *A. fumigatus, A. repens* and *A. niger* as biological control agents against *Phytophthora palmivora*, a cacao pod disease pathogen. The test organism was isolated from the same cocoa farm where the disease occurred. The results revealed that all the test antagonists effectively checked the growth of the pathogen.

**Table.1** Criteria for assessing environmental quality (Tauruslina et al., 2015)

| Diversity index | The condition of the community structure | Catagori | Skala |
|---|---|---|---|
| **>2.41** | Very stable | Very good | 5 |
| **-2.4** | More stable | Good | 4 |
| **1.21 – 1.8** | Stable enough | Currently | 3 |
| **0.61 – 1.2** | Less stable | Bad | 2 |
| **<0.6** | Unstable | Very bad | 1 |

**Table.2** Endophytic fungi of leaves, fruit and stems from healthy plants

| No | Endophytic of leaves | | Endophytic of fruits | | Endophytic of stems | |
|---|---|---|---|---|---|---|
| | Name of fungi | Number of isolate | Name of fungi | Number of isolate | Name of fungi | **Number of isolate** |
| **1** | *A. flavus* | 6 (40%)* | *A. flavus* | 12 (80%) | *A. flavus* | **5 (13%)** |
| **2** | *Curvularia* sp. | 3 (20%) | *Phytophthora* sp. | 3 (20%) | *Miselia sterilia* | **12 (32%)** |
| **3** | *Rhizopus* sp. | 3 (20%) | | | *Rhizopus* sp. | **21 (55%)** |
| **4** | *Stigmina* sp. | 3 (20%) | | | | |
| | **Jumlah** | **15** | | **15** | | **38** |

*Numbers in brackets indicate relative abundance (KR)/prevalence in each isolate.

**Table.3** Diversity, evenness uniformity and domination index of endophytic fungi in leaves, fruits and stems of healthy plants

| Variable (index) | Leaves endophytic | Fruits endophytic | Stems endophytic |
|---|---|---|---|
| **Diversity index (H')** | 1.332 | 0.500 | **0.959** |
| **Evenness uniformity index (E)** | **0.272**<br>**0.720** | **0.273**<br>**0.320** | **0.254**<br>**0.580** |

**Table.4** Endophytic fungi of leaves, fruits and stems from healthy plant

| No | Leaves endophytic | Number of isolates | Fruits endophytic | Number of isolates | Stems endophytic | Number of iaolates |
|---|---|---|---|---|---|---|
| **1** | *A. flavus* | 80±0.2% | *A. flavus* | 78±0.5% | *A. flavus* | **80±0.4%** |
| **2** | *Curvularia* sp. | - | *Phytophthora* sp. | - | *Miselia sterilia* | - |
| **3** | *Rhizopus* sp. | 70±0.3% | | | *Rhizopus* sp. | **80±1.2%** |
| **4** | ***Stigmina* sp.** | - | | | | |

**Figure.1** Number of isolates found in leaf endophytic fungi

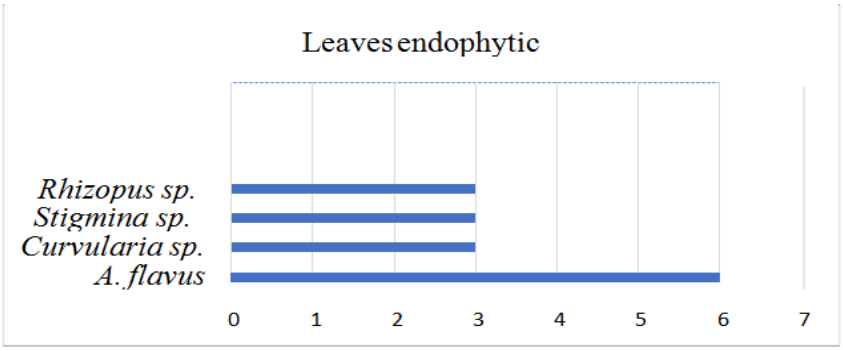



**Figure.2** Number of isolates found in fruits endophytic fungi

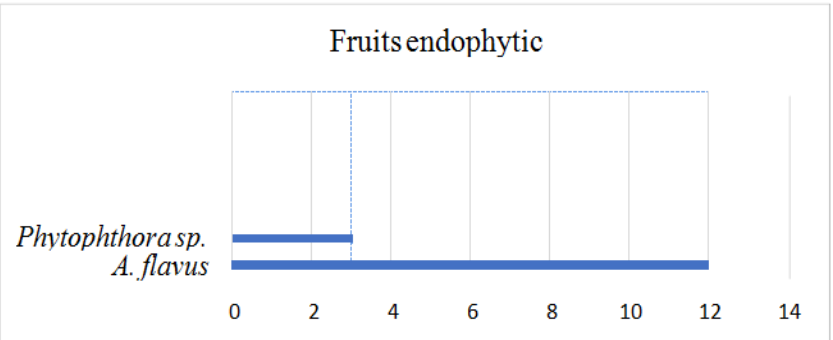



**Figure.3** Number of isolates found in stems endophytic fungi

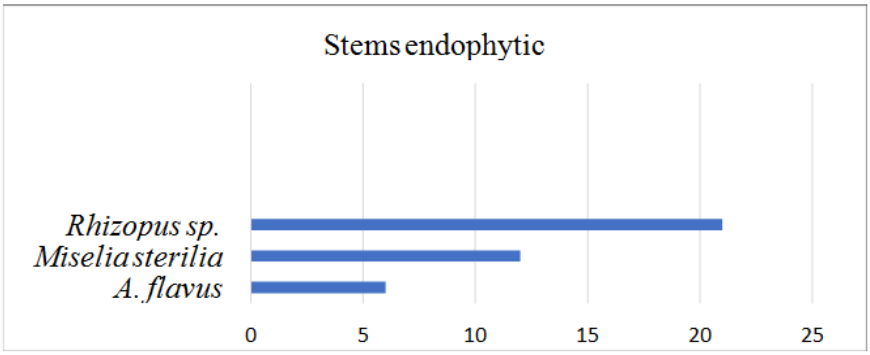
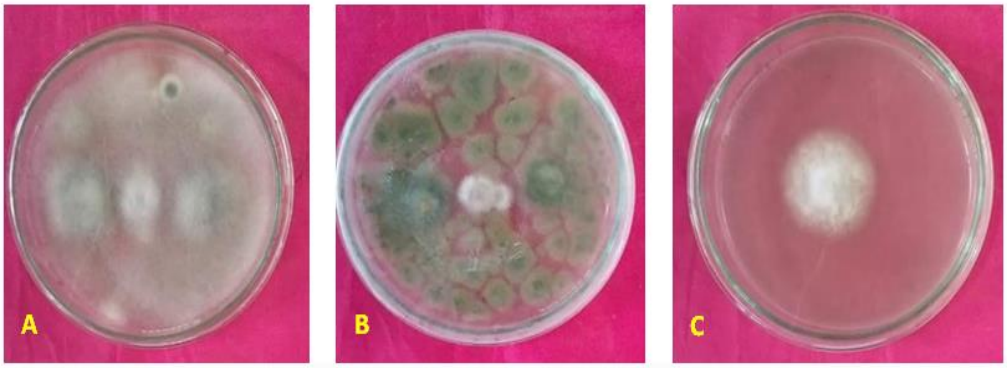


**Figure.4** Inhibited ability of antagonist against pathogen (*Ustilaginoidea virens*)




(A = *Rhizopus* sp., B = *A. flavus* dan C = control/*Ustilaginoidea virens*)

The test antagonist grew faster than the pathogen and produced an inhibition zone thereby limiting the growth of the pathogen. In solid media, *A. repens* was the most antagonistic organism under the conditions of this study. The test mushroom culture filtrate also inhibited the growth of *P. palmivora* with *A. niger* showing the highest inhibition percentage (54%) and *A. repens* the least (44.5%).

It was also discovered the type and effectiveness of endophytic fungi as a biological agent to control cocoa pod rot disease caused by *P. palmivora*. This research includes several steps, namely: isolation of pathogens, isolation of endophytic fungi, identification of endophytic fungi and antagonist test. The results showed that *P. palmivora* was found as the main pathogen causing cocoa pod rot disease. *Neurospora* sp., *Trichoderma* sp., *A. flavus, A. niger, Aspergillus* spp. is a type of endophytic fungus that has the best inhibition power among other endophytic fungi. Endophytic fungi are able to control pathogens with antibiotics. *Trichoderma* sp. is a fungus that is able to control pathogens with antibiotics by releasing antibiotic compounds that are harmful to pathogens (Indrawangsa *et al.*, 2017).

Plant-associated fungi (endophytic fungi) are a group of microorganisms rich in biodiversity which are usually found asymptomatic in plant tissues or in the spaces between cells. Endophytic fungi promote host plant growth by directly producing secondary metabolites, which increase plant resistance to biotic and abiotic stresses. In addition, they are capable of biosynthesizing medically important "phytochemicals" that were originally thought to be produced only by their host plants. Endophytic fungi have high potential to be used as an alternative source of secondary metabolites for pharmacological studies (Wen *et al.*, 2022). The positive aspects of endophytic colonization, resultant (acquisition of nutrients and production of phytohormones) and indirect (induced resistance, production of antibiotics and secondary metabolites, production of siderophores and protection against abiotic and biotic stresses) benefit endophytic colonization (Baron and Rigobelo, 2022). The enigmatic endophytic fungus begins to reveal its secrets. Like pathogens, they can manipulate hosts to their own advantage to create their own optimal habitat.

Some endophytic manipulation induces resistance or otherwise defeats the pathogen and thus can be exploited for biological control. Like other pathogens and symbionts, endophytes produce effector proteins and other molecules, ranging from specialized metabolites, phytohormones and microRNAs, to manipulating their hosts and other microorganisms they encounter. There is a compound from endophytes to pathogens: some organisms can infest or cause disease in some hosts, but not in others (Collinge *et al.*, 2022).

The rice false smut pathogen produces mycotoxins, including ustiloxins and ustilaginoidins. Utiloxins contain 13-membered cyclic core structures with phenol ether linkages, including ustiloxins A, B, C, D, E, F and G in *U. virens*.

Utiloxin from *U. virens* inhibits microtubule assembly and the framework of eukaryotic cell formation. Ustilaginoidin is a class of bis-naphtho-γ-pyrones, which

has cytotoxic activity on cancer cells and an inhibitory effect on radicle elongation of rice seeds. Although many mycotoxins have been identified from *U. virens*, at least five aspects still require further exploration, including the activity and toxicity of each mycotoxin in humans or animals, the function of mycotoxins during infection from *U. virens*, differences in mycotoxins in *U. viren* isolates different, the molecular mechanism of mycotoxin synthesis in *U. virens* and a new type of mycotoxin besides ustiloxin and ustilaginoidin (Jiehua *et al.,* 2019). False smut disease in rice is caused by the fungus *Ustilaginoidea virens* with symptoms of yellow powder appearing on some panicles and turning black over time. The highest prevalence of endophytic fungi found on leaves was in *A. flavus* by 40%, likewise the highest fruit endophytes were found in *A. flavus* by 80%, and the highest in stem endophytes was in *Rhizopus* sp. by 55%. The diversity index (H') of leaf, fruit and stem endophytes was 1.332, 0.500 and 0.959, respectively. Evenness uniformity indices on leaf, fruit and stem endophytes were 0.272, 0.273 and 0.254 respectively.

Dominance indices on leaf, fruit and stem endophytes were 0.720, 0.320 and 0.580, respectively. The small diversity index and the large dominance index prove that there are dominating species, namely *A. flavus* and *Rhizopus* sp. The highest inhibition of leaf, fruit and stem endophytic fungi were *A. flavus* and *Rhizopus* sp.

## Acknowledgements

Authors wish to thank to the Rector of Udayana University for their assistance and the opportunity given so that research can be resolved, Dean of the Faculty of Agriculture, Udayana University, and Chairman of the Institute for Research and Community Service Udayana University, for their help and cooperation so that research can be funded to completion.

## Author Contribution

I Made Sudarma: Investigation, formal analysis, writing—original draft. Ni Wayan Suniti: Validation, methodology, writing—reviewing. Ni Nengah Darmiati:—Formal analysis, writing—review and editing.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Research Funding:** Not applicable

**Ethical Approval:** Not applicable.

**Consent to Participate:** Not applicable.

**Consent to Publish:** Not applicable.

**Conflict of Interest:** The authors declare no competing interests.

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**How to cite this article:**

I Made Sudarma, Ni Wayan Suniti and Ni Nengah Darmiati. 2024. Utilization of Endophites Fungi in Inhibiting the Growth of *Ustilaginoidea virens* (Cooke) Takahashi Causes False Smut Disease in Rice Plants *invitro*. *Int.J.Curr.Microbiol.App.Sci.* 13(01): 1-9. **doi:** https://doi.org/10.20546/ijcmas.2024.1301.001