

**ANTAGONISM POTENCY OF DARK SEPTATE ENDOPHYTES AGAINST *Pyricularia oryzae*
FOR IMPROVING HEALTH OF RICE PLANTS**

**POTENSI ANTAGONISME DARK SEPTATE ENDOPHYTES TERHADAP *Pyricularia oryzae*
UNTUK PENINGKATAN KESEHATAN TANAMAN PADI**

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ABSTRACT

Blast disease caused by *Pyricularia oryzae* (*Po*) is the main disease affecting rice production. *Dark septate endophytes* (DSEs) is known to improve plant performance and suppress disease. This study evaluated DSEs antagonism potency against *P. oryzae* in improving the rice plant's health. The research stages consisted of: (1). DSE and *Po* growth rate; (2). Antagonism of DSEs against *Po*; (3). Chitinase; (4). DSEs Viability; (5). DSEs application to rice seeds in nurseries. The results showed the growth of APDS 3.2 colonies had fulfilled Petri (d = 9 cm) at three days after incubation (DAI), while 4.1 BTG and TKC 2.2.a at 7 DAI. *Po* had slow colony growth required 20 DAI. Inhibition of APDS 3.2 against *Po* was 43.75%, higher than of 4.1 BTG (38.60%) and of TKC 2.2.a (39.76%). The rice plants inoculated with APDS 3.2 had a relatively higher at seedling height, root length, wet weight, and dry weight than those inoculated with TKC 2.2.a and 4.1 BTG. The highest DSEs colonization was found in APDS 3.2 at 50.56%, followed by TKC 2.2.a (46.67%) and 4.1 BTG (40%). DSEs fungus has the potential to suppress rice blast pathogens by improving the health of rice plants, especially APDS 3.2.

Key words: Colonization, Growth Rate, Viability

ABSTRAK

Penyakit blas yang disebabkan oleh *Pyricularia oryzae* (*Po*) merupakan penyakit utama yang memengaruhi produksi padi. *Dark septate endophyte* (DSE) diketahui mampu meningkatkan performa tanaman dan menekan penyakit. Penelitian ini bertujuan untuk mengevaluasi potensi antagonisme DSE terhadap *P. oryzae* dalam meningkatkan kesehatan tanaman padi. Tahapan Penelitian terdiri atas: (1). Kecepatan tumbuh DSE dan *Po*; (2). Antagonisme DSE terhadap *Po*; (3). Kitinase; (4). Viabilitas DSE; (5). Aplikasi DSE pada benih padi di persemaian. Hasil penelitian menunjukkan pertumbuhan koloni APDS 3.2 telah memenuhi petri (d= 9 cm) pada 3 hari setelah inkubasi (HSI), sedangkan 4.1 BTG dan TKC 2.2.a pada 7 HSI. Pertumbuhan koloni *Po* lambat membutuhkan 20 HSI. Penghambatan APDS 3.2 terhadap *Po* sebesar 43,75% lebih tinggi dibandingkan 4.1 BTG (38,60%) maupun TKC 2.2.a (39,76%). Tanaman padi yang diinokulasi APDS 3.2 memiliki tinggi, panjang akar, bobot basah, dan bobot kering relatif lebih tinggi dibandingkan

yang diinokulasi TKC 2.2.a dan 4.1 BTG. Kolonisasi DSE tertinggi dijumpai pada APDS 3.2 sebesar 50,56%, diikuti TKC 2.2.a (46,67%) dan 4.1 BTG (40%). Cendawan DSE memiliki potensi untuk menekan patogen blas padi dengan cara meningkatkan kesehatan tanaman padi, khususnya APDS 3.2.

Kata kunci: Kecepatan tumbuh, Kolonisasi, Viabilitas

INTRODUCTION

Blast disease caused by *Pyricularia oryzae* is one of the main limiting factors affecting rice production in Indonesia (Sudir *et al.*, 2014). The fungus can infect rice plants at various stages of growth, from nurseries to harvest (Hosseini-moghaddam & Soltani, 2013). Blast disease symptoms are characterized by rhombic spots with pointed tips on the leaves, gray or white center spots with brown leaf edges (Siagian, 2016). The shape and colour of the spots vary depending on environmental conditions, age of the spots, and the degree of resistance of rice varieties (Sesma & Osbourn, 2004).

Leaf blast disease can develop rapidly and cause necrotic so that the absorption of nutrients and rice plant growth are disrupted, causing death if environmental conditions are conducive to pathogens (Dewi *et al.*, 2013). Meanwhile, infection in the panicle neck can reach the grain, and the seeds will carry the pathogen (Zhang *et al.*, 2014). This condition causes the rice crop failure in the world and especially Indonesia, to be relatively high. The yield loss due to the blast disease epidemic is around 50-90% in various parts of the world, while in Indonesia, it reaches 61% (Suganda *et al.*, 2016).

The way to control blast disease still relies on the use of synthetic fungicides. Continuous use of synthetic fungicides can become pollutants for the environment and increase pathogen resistance (Zarandi *et al.*, 2009). Control of this disease has not been

optimally successful because *P. oryzae* has cellular development and morphology, which are very adaptive in rice plants to break resistance (Fukuta *et al.*, 2014). *P. oryzae* are also known to have high genetic diversity, making it difficult to control (Ahn *et al.*, 2000).

Microbial antagonists, as biocontrol agents with competition mechanism, antibiosis, parasitism, or induced resistance, are important in controlling plant diseases (Amaria *et al.*, 2015). The use of antagonistic microbes to increase crop yields and protect crops from pests is a promising approach in modern agricultural systems. Endophytes fungi, including biocontrol agents, can complement or even replace the use of synthetic pesticides. Beside, endophytes fungi have some important mechanisms and their ability to reduce disease incidence (Pandya & Saraf, 2010).

Endophytic fungi such as *dark septate endophytes* (DSEs) is an alternative for blast disease control to reduce the impact of synthetic fungicides (Sudha *et al.*, 2016). DSE fungi have the characteristics of melanized hyphae, dark in color, and septated, which show dark pigmentation on agar media and can colonize plant roots inter and intracellularly without causing disease symptoms (Handayani, 2017). DSEs colonizes plant root tissue in the form of septated hyphae and microsclerotial structures. The structure of melanized hyphae and microsclerotia make DSEs tolerant to environmental conditions that are less conducive or abiotic stress, such as

drought or sub-optimal land (Santos *et al.*, 2017).

DSEs is expected to suppress pathogens development, induce resistance, and stimulate plant growth to benefit sustainable agricultural practices. Therefore, the exploration of DSEs, which are the best collections, need to be tested *in-vitro* in suppressing the development of blast pathogens, inducing resistance, and stimulating the growth of rice plants in the nursery so that the antagonist agents with the best ability to control blast disease are obtained. The study aimed to evaluate the *Dark Septate Endophytes*, which can suppress the development of *Pyricularia oryzae* in improving rice plant performance in the nursery.

MATERIALS AND METHODS

Time and Place of Research

The research was carried out at the Laboratory of Indonesian Soil Research Institute, the Laboratory of KP Muara Indonesian Center for Rice Research (ICRR), the Laboratory and paranet house of ICABIOGRAD, Bogor. The research was conducted from September 2019 to June 2020.

Preparation of Selected *Dark Septate Endophytes* Isolates

The DSE isolates used were the three best isolates collected by the Indonesian Soil Research Institute, namely APDS 3.2, 4.1. BTG, and TKC 2.2.a. The three DSE isolates were able to grow at high acidity levels (pH 3, pH 4, and pH 5) as well as Fe concentrations of 500 ppm and 1,000 ppm. All isolates were grown on *Potato Dextrose Agar* (PDA) medium at 25 °C.

Preparation of *Pyricularia oryzae* Isolates

Rice leaf samples were obtained from KP Muara ICRR infected with *P. oryzae*. The single spore isolation of *P. oryzae* refers to the method of Milati *et al.* (2016) with the aid of a microscope, then cultured on water agar media. The germinated spores were transferred to PDA and incubated for seven days. The mycelium was transferred to *oatmeal agar* (OMA). On day 10th, the mycelium on the surface of OMA was rubbed with a sterile brush dipped in sterile distilled water containing *Streptomycin sulfate*. After scrubbing, OMA was incubated for 2 x 24 hours in an incubator equipped with a 10 watt light lamp. *P. oryzae* spores are black in OMA. To collect the spores, OMA media was given a sterile distilled water solution plus Tween 20 (0.01 ml L⁻¹), rubbing the surface with a sterile brush. The spore suspension was put into a sterile bottle and ready to be inoculated.

The Growth rate of *Dark Septate Endophytes* and *Pyricularia oryzae*

This activity measures and observes the growth rate of DSE and *Po* colonies. DSE isolates (APDS 3.2; 4.1 BTG; and TKC 2.2.a) and rice blast pathogens (*P. oryzae*) were grown on PDA media in Petri dishes. Isolates were incubated at room temperature and exposed to adequate lighting. The vertical and horizontal growth of the colonies was measured daily until the fungal colonies fulfilled the Petri dishes or the increase in colony diameter stops (Elfina *et al.*, 2013).

Dark Septate Endophytes Antagonism Test against *Pyricularia oryzae*

The antagonism test used a dual culture method using PDA media in a Petri dish with a 9 cm diameter. The *P. oryzae* was planted five days earlier than the DSE fungi. Each fungus was placed opposite to each other at a distance of 3 cm. The treatments were

repeated three times and incubated for seven days. Observations were made on the presence or absence of an inhibition zone. The formula used to calculate inhibition (%) was:

$$E = \frac{x - y}{x} \cdot 100\%$$

Where: E = efficacy (% inhibition), x = *P. oryzae* growth area as a control (cm), y = *P. oryzae* growth area with DSE (cm).

Chitinase Qualitative Test

The qualitative test for chitinase refers to Muharni (2009) method. DSE isolates were grown on solid chitin media and incubated for up to six days at room temperature. The clear zone formed can be observed after the addition of 0.3% Congo red, then rinsed with 0.1% NaCl. The clear zone is the result of the degradation of chitin into N-acetyl-D-glucosamine monomers.

Dark Septate Endophytes Inoculum and Inoculation Preparation

DSEs inoculum preparation refers to the method of Andrade-linares *et al.* (2011). DSE isolates were grown on liquid Potato Dextrose Broth media for two weeks. The seeds of cv Inpara 2 were soaked in hot water (50 °C) for 20 minutes, then surface sterilized with 3% NaOCl (for 1 minute), followed with 70% alcohol (for 1 minute), and rinsed with sterile water two times (Sucipto *et al.*, 2015). Rice seeds were immersed in DSE culture (10% v/w) with a density of 1×10^5 propagules ml⁻¹ for 48 hours. Rice seeds were planted in a nursery medium using soil that has been sterilized twice. After 14 days, the plant height, root length, wet weight, and dry weight were measured for ten plant samples for each DSE treatment as well as of control rice

plants.

Dark Septate Endophytes Viability

The DSEs viability was measured by immersion on PDA media and incubated for 7 days. This test was aimed to see the viability of DSE inoculum which would be inoculated on rice seeds.

Detection of Dark Septate Endophytes Colonization in Rice Plants

Colonization by the DSEs was carried out in the nursery phase. The detection method used a staining technique, which refers to Zhang *et al.* (2011). Root samples were cleaned and cut ± 2 cm. The roots were soaked in a 10% KOH solution at 90 °C in a water bath for 90 minutes. KOH was removed, and the roots were rinsed using distilled water. The roots were immersed in a 1N HCl solution for 24 hours, then the samples were stained with fuchsin acid for 20-30 minutes. The stained roots were stored in a 50% glycerol solution, then observed under a light microscope with a magnification of 400x.

Data Analysis

Data were analyzed using ANOVA with SAS 9.1. When the F test showed significantly different, treatments were compared using Tukey test at the 95% significant level.

RESULTS AND DISCUSSION

Dark Septate Endophytes Growth Rate

APDS 3.2 had the fastest colony growth than other DSE and filled the Petri dishes three days after incubation (DAI), while 4.1 BTG and TKC 2.2.a at 7 DAIs. The fastest growth of APDS 3.2 colonies occurred at 1 DAI, 4.1 BTG at 5 DAI, and TKC 2.2.a at 3 DAI (Table 1). After 7 DAI, the APDS 3.2 color

changes from white to black (Figure 1). The growth of fungal colonies was determined precisely by measuring the colony diameter as an estimator of fungal growth. Colony

diameter is a good choice, given the good correlation between colony diameter and biomass dry weight (Gougouli & Koutsoumanis, 2013).

Table 1. The growth rate of *dark septate endophytes* fungi

Days to-	Growth rate of <i>dark septate endophytes</i> fungi					
	APDS 3.2		4.1 BTG		TKC 2.2.a	
	Average diameter (cm)	Difference in growth per day	Average diameter (cm)	Difference in growth per day	Average diameter (cm)	Difference in growth per day
1	4.20	4.20	1.35	1.35	1.31	1.31
2	6.13	1.93	2.45	1.10	2.62	1.31
3	9.00	2.87	4.06	1.61	5.10	2.48
4	-	-	5.71	1.64	6.24	1.14
5	-	-	7.38	1.67	7.40	1.16
6	-	-	8.72	1.48	8.39	0.99
7	-	-	9.00	0.28	9.00	0.61

Colonies usually continue to grow within the same average radius until they encounter obstacles such as the tips of Petri dishes or other colonies (Nurbaya *et al.*, 2010). Fungal growth may be greatly influenced by several physical factors such as temperature, pH, light, aeration, pressure (Maharshi & Thaker, 2012). In this research, *Pyricularia oryzae* has slow colony growth. The fastest growth of *Po* colonies

occurred in 1 DAI. *Po* colony growth was between 0.49-1.35 cm/day in the first ten days. Ten days later, the growth rate of *Po* decreased with increasing incubation time ranging from 0.16 to 0.39 cm day⁻¹. *Po* colonies filled the Petri dishes at 20 DAI (Table 2). Differences in fungal growth may be due to carbon utilization and nutrient consumption (Gayatonde *et al.*, 2016).

Table 2. The growth rate of *Pyricularia oryzae*

Parameter	Growth rate of <i>Pyricularia oryzae</i> at the day of									
	1	2	3	4	5	6	7	8	9	10
Average diameter (cm)	0	1.35	1.92	2.45	3.11	3.68	4.25	4.80	5.30	5.80
Difference in growth per day	0	1.35	0.57	0.53	0.66	0.58	0.56	0.56	0.50	0.49
Parameter	Growth date of <i>Pyricularia oryzae</i> at the day of									
	11	12	13	14	15	16	17	18	19	20
Average diameter (cm)	6.18	6.56	6.94	7.11	7.27	7.93	8.15	8.38	8.61	9.00
Difference in growth per day	0.38	0.38	0.39	0.16	0.16	0.66	0.27	0.23	0.23	0.39

Dark Septate Endophytes Antagonism Test against *Pyricularia oryzae*

APDS 3.2 had a higher percentage of inhibition against *Po* than 4.1 BTG and TKC 2.2.a, but it was not statistically significant except for 5 and 6 DAI. The percentage of inhibition rised with increasing incubation days. Percentage of inhibition by APDS 3.2,

4.1 BTG, and TKC 2.2.a at 7 DAIs was 43.75%; 38.60%; and 39.76%, respectively (Table 3). The role of endophytic fungi in inhibiting the *P. oryzae* was in the form of competition for space and nutrition, reducing the production of toxins produced by pathogens so that they become less pathogenic to plants (Yulianti, 2013).

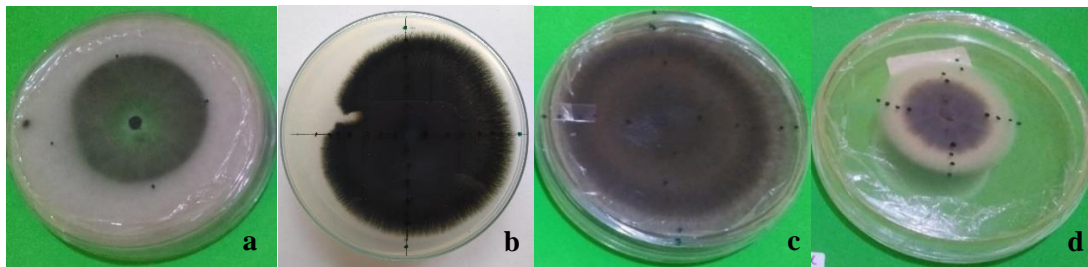


Figure 1. Fungal growth rate at 7 DAI: (a). APDS 3.2; (b). 4.1 BTG; (c). TKC 2.2.a; and (d). *P. oryzae*

Dual culture test results showed that DSE was able to inhibit *Po*. APDS 3.2 has a high-speed mycelial growth. APDS 3.2 at 7 DAI overgrowth *Po*. Meanwhile, 4.1 BTG withstanded the growth of *Po* and TKC 2.2.a restricted the colony growth of *Po* (Figure 2). APDS 3.2 had a high ability to inhibit the development of *Po*. The results of this research was in support with of Dalimunthe

et al. (2019) that APDS 3.2 has high inhibiting capability. Dalimunthe *et al.* (2019) found that an isolate of DSEs that has faster colony growth than the pathogenic colony of *R. microporus* was able to suppress *R. microporus*. It appears that the development of the DSEs colony envelops and suppresses the development of the *R. microporus* colony.

Table 3. Percentage of *dark septate endophytes* inhibition against *Pyricularia oryzae* in dual culture tests.

Treatment	Percentage of Inhibition (%) day of						
	1	2	3	4	5	6	7
APDS 3.2 vs <i>P. oryzae</i>	17.74 a	25.40 a	31.21 a	35.82 a	38.21 a	40.57 a	43.75 a
4.1 BTG vs <i>P. oryzae</i>	14.66 a	21.72 a	27.18 a	29.78 a	31.89 b	33.97 b	38.60 a
TKC 2.2.a vs <i>P. oryzae</i>	15.47 a	22.19 a	27.33 a	31.91 a	34.55 ab	36.78 ab	39.76 a

The mechanisms of pathogens by biocontrol agents according to Agrios (2005); Atugala & Deshappriya (2015) are by directly parasitizing pathogens, producing antibiotics (toxins), and by the ability to compete for space and nutrition.

Endophytes tended to slow the pathogen's growth compared to controls, possibly by producing antibiotics substances. In this study, the growth of pathogen (*P. oryzae*) was inhibited due to competition with DSEs.

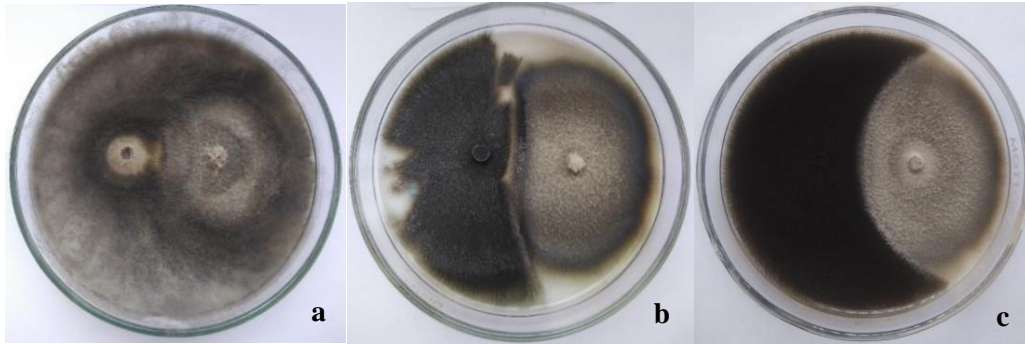


Figure 2. Inhibition of *P. oryzae* (left) by the DSE fungal (right) in a dual culture test at 7 DAI: (a). APDS 3.2; (b). 4.1 BTG; and (c). TKC 2.2.a.

Chitinase Qualitative Test

The three DSE isolates had chitinase enzyme activity as can be seen from the clear zone produced around the fungal colonies. APDS 3.2 had a clear zone larger than 4.1 BTG and TKC 2.2.a (Figure 3). The

chitinase enzyme is a hydrolytic enzyme that degrades chitin. Chitinase can act as an antifungal for biological control of pathogenic fungi (Fadhil *et al.*, 2014). The three DSE isolates were able to degrade the cell walls of pathogenic fungi that have chitin components.

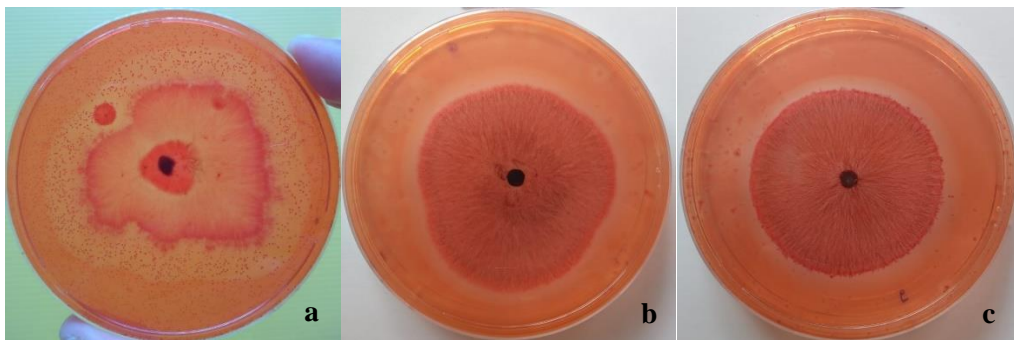


Figure 3. Chitinase test on DSE: (a). APDS 3.2; (b). 4.1 BTG; and (c). TKC 2.2.a.

Dark Septate Endophytes Viability

The DSE inoculum was tested on PDA medium for viability before application to rice seeds. The three DSE grew well from 1 DAI to 7 DAI, to fulfill the PDA media. This test showed that the DSEs inoculum had

good viability (Figure 4). Viability has a positive correlation with the level of fungal infectivity (Prayogo & Santoso, 2013). The same goes for bacteria, *Bacillus* sp. after being formulated, its viability and antagonistic ability are still maintained (Fakhrudin & Nurcahyanti, 2020).



Figure 4. Dark Septate Endophytes viability: (a). APDS 3.2; (b). 4.1 BTG; and (c). TKC 2.2.a.

Agronomic Parameters in the Nursery

The height of rice plants inoculated with APDS 3.2 was relatively higher than the rice plants inoculated with TKC 2.2.a and 4.1 BTG at 1 and 2 weeks after sowing, although statistical analysis was not significantly different. However, the height of rice plants inoculated with DSE was significantly different from the control treatment. Likewise, the root length, wet weight, and

plant dry weight in the APDS 3.2 treatment were relatively better than the other DSE treatments. All DSE fungi gave a significant effect compared to control on rice plants agronomic parameters in the nursery (Table 4). He *et al.*, (2019) showed that DSE inoculation could increase plant biomass by increasing N and P elements absorption by plants. This absorption was consequently depleting these macronutrients in the soil.

Table 4. Average plant height, root length, wet weight and dry weight of rice plants in the nursery.

No.	Treatment	Plant height (cm)		Root length (cm)	Plant wet weight (g)	Plant dry weight (g)
		1 WAS	2 WAS			
1	APDS 3.2	15.90 a	30.80 a	10.96 a	0.160 a	0.125 a
2	4.1 BTG	15.05 a	30.00 a	9.14 ab	0.126 a	0.119 a
3	TKC 2.2.a	15.60 a	30.15 a	9.47 ab	0.155 a	0.119 a
4	Kontrol	13.32 b	24.65 b	7.80 b	0.081 b	0.097 a

Information: WAS= weeks after sowing

Before the rice plants transplanted into plastic buckets, it was seen that the rice plants inoculated with APDS 3.2 had better plant vigour than the other treatments in terms of plant height, root length, and number of rice roots (Figure 5). Endophytes can actively or passively trigger plant

growth through various mechanisms. Endophytes are providing various metabolites enhancements for host plants fitness by increasing plant resistance to biotic and abiotic stresses and increasing plant growth (Sudha *et al.*, 2016).



Figure 5. Growth of rice plants aged 2 weeks after sowing on DSEs and control treatments (left to right): APDS 3.2; 4,1 BTG; TKC 2.2.a; and control.

Endophytes fungi can stimulate plant canopy growth and have a higher wet weight than controls (Syarif *et al.*, 2014). Vergara *et al.* (2018) stated that *dark septate endophytes* fungi could access carbon, nitrogen, and phosphorus from rhizospheres and deliver them to the host. *In-plant* tissue, endophytes will actively protect plants directly or indirectly. Directly through chemical activity produced against pathogens, while indirectly producing some gene regulation of plant resistance to pathogens and increasing plant vigour (Herre *et al.*, 2007).

Dark Septate Endophytes Colonization Detection on Rice Roots

DSEs microscopic observations on rice roots shows fungal conidia, as shown in Figure 6. APDS 3.2 had hyaline and oval conidia. While 4.1 BTG and TKC 2.2.a had brown conidia with two septate and in the middle was slightly bent on one side, and both ends were tapered like a boomerang.

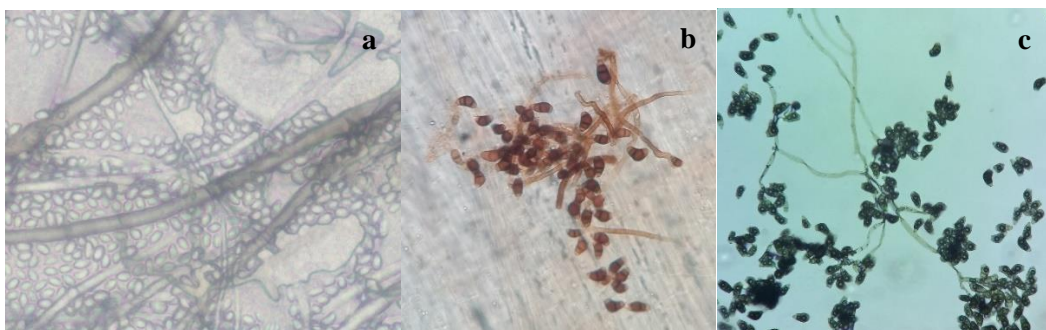


Figure 6. DSE colonization of rice roots at 400x magnification: (a). APDS 3.2; (b). 4.1 BTG; and (c). TKC 2.2.a

The highest DSEs colonization in rice roots in the nursery was found in the APDS

3.2 at 50.56%, followed by TKC 2.2.a and 4.1 BTG (Table 5). The results of Khastini (2007)

study showed that the colonization process of *Aspergillus niger* using the staining method and the GFP marker gene showed that the colonization process began with the penetration of hyphae into the root

epidermal tissue, then an apresorium was formed followed by the formation of intercellular hyphae in the epidermis and root cortex and hyphal formation of swollen structures in the root cortex.

Table 5. *Dark septate endophytes* colonization of rice roots in the nursery phase

No.	Treatment	Colonization (%)
1	APDS 3.2	50.56 a
2	4.1 BTG	40.00 a
3	TKC 2.2.a	46.67 a
4	Kontrol	0.00 b

Plant root tissue provides a conducive habitat for various microbial communities, including endophytes fungi (Bashir *et al.*, 2016). Plant roots are heavily colonized by various endophytes fungi, especially the *dark septate endophytes* group (Knapp *et al.*, 2012). The application of endophytes fungi bioformulation to seeds or aerial parts will be more effective than application in the rhizosphere. The microbes are in the plant tissue and will not face competition with other soil microbes, which generally occurs in the rhizosphere case microbes. Moreover, the benefits are directly transferred to the host plant in a closed system with minimal leakage of metabolites (Khare *et al.*, 2018).

Several DSE isolates obtained from tropical environments can colonize and trigger two rice varieties' growth with and without stress (Santos *et al.*, 2017). DSEs can increase host growth and nutrient uptake in several environmental conditions. Increased growth and increased nutritional status indicate better performance because of the results of DSEs colonization (Andrade-linares *et al.*, 2011).

CONCLUSION

1. APDS 3.2 had the fastest colony growth than the other DSE, at three days after

incubation (DAI) had filled the Petri dishes (d = 9 cm), while 4.1 BTG and TKC 2.2.a at seven DAI. *Pyricularia oryzae* had slow colony growth and required 20 DAI.

2. APDS 3.2 had a percentage of inhibition against *P. oryzae* of 43.75% higher than 4.1 BTG (38.60%) and TKC 2.2.a (39.76%).
3. The three DSE isolates had chitinase enzyme activity and had good viability.
4. The rice plants inoculated by APDS 3.2 had a relatively higher plant at height, root length, wet weight and dry weight than those inoculated by TKC 2.2.a and 4.1 BTG.
5. The highest DSE colonization in rice roots in nurseries found in rice plants inoculated with APDS 3.2 at 50.56%, followed by TKC 2.2.a (46.67%) and 4.1 BTG (40%).
6. DSEs fungi has the potential to suppress rice blast pathogens by improving the health of rice plants, especially APDS 3.2 under *in-vitro* condition.

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