European Proceedings of
Social and Behavioural Sciences
EpSBS

www.europeanproceedings.com
e-ISSN: 2357-1330

DOI: 10.15405/epsbs.2020.03.03.8

ICMR 2019
8th International Conference on Multidisciplinary Research

EFFECTIVENESS OF ENDOPHYTIC FUNGI AS BIOLOGICAL CONTROL AGENT ON RUBBER PLANTS

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Abstract

Biological control by using antagonistic microorganisms is an alternative that is currently being carefully studied and used as a control of plant diseases. Endophytic microorganisms also play an important role in controlling plant diseases which are induction-resistant. Endophytic antagonist fungi have high activity in producing enzymes that can be used to control pathogens. The purpose of this study was to determine the effectiveness of rubber plant endophytic fungi as biological agents against fungi *C. gloeosporioides* in rubber (*Hevea brasiliensis* Muell. Arg.) plants. This study was conducted at the Sei Putih Rubber Research Center, Galang, Deli Serdang, North Sumatra Province in February to March 2019. The design used was Factorial Randomized Block Design with 3 replications and Combinations with 5 duplication. The results of the test on the effectiveness of endophytic fungi from rubber leaves with 104 spore treatment and secondary metabolites resulted in different endophytic ability to fall fungi leaf of *Colletotrichum*. Endophytes can improve the plant's defense mechanism against deciduous leaves of *Colletotrichum*, in addition it also produces antibiotic substances that can inhibit growth.

Keywords: Endophytic fungus, biological control, Hevea brasiliensis, Colletotrichum, metabolite, pathogen.

https://doi.org/10.15405/epsbs.2020.03.03.8
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eISSN: 2357-1330

1. Introduction

In Indonesia, the deciduous disease of leaves of *Colletotrichum gloeosporioides* is an important disease in rubber plants. Deciduous leaves cause persistent abortion, especially if the pathogen attacks the period of formation of young leaves after the fall of natural leaves. The formation of new leaves that repeatedly causes dead ends, especially in young plants. Besides being able to cause a decline in production, often the disease can result in the failure of a rubber development program. The attack of *C. gloeosporioides* in nurseries results in delays when grafting the seeds and in severe attacks resulting in defects, stunting, or even death.

The last few years the excavation of microbial resources found in plant tissues has received much attention. Endophytic fungus is also defined as a fungus that consumes all or part of its life cycle between cells and within healthy tissue cells of host plants, usually not causing symptoms of disease. Endophytic fungi are an important component of plant microecosystems and have been found in every plant species that has been studied and it is estimated that there are more than one million endophytic fungi present in nature (Dreyfuss & Chapela, 1994).

Endophytic mushroom groups are able to produce antibiotic compounds that are active against bacteria and pathogenic fungi in humans, animals and plants. Symbiosis between mushrooms and healthy plants in certain tissues are able to produce mycotoxins, enzymes and antibiotics. The association of several endophytic fungi with host plants can protect the latter from several virulent pathogens, both bacteria and fungi (Purwanto, 2008).

Syamsafitri and Hasanuddin (2013) have explored endophytic fungi from different rubber clones resulted in 13 endophytic isolates. The identified endophytic fungi were derived from the genus *Trichoderma*, *Aspergillus*, *Penicillium*, *Gliocladium* and *Pestalotia* whereby each showed the ability to inhibit the growth of *C.gloesporioides* on PDA media. Endophytic fungi can produce many secondary metabolites, some from alkaloids. Alkaloids are very important molecules not only for chemical reasons but also for a variety of biological properties such as antifungal, anticancer and antiviral activities (Wang & Dai, 2011).

2. Problem Statement

Efforts to achieve productivity of rubber plants are currently not optimal. One of the causes is the disruption and declining of secondary leaves caused by the fungus *C. gloeosporioides*. Decrease in production due to an attack of deciduous leaves by *Colletotrichum* can reach 45%. Until now there is no appropriate biological control to suppress or prevent the attack of fungal disease pathogens especially by *Colletotrichum*. An effort that is environment friendly in controlling plant diseases is by utilizing endophytic fungal microbes. These microbes produce secondary metabolites to control fallopus fungal pathogens of *Colletotrichum*. Endophytic fungus screening through antifungal metabolites or toxins produced is expected to control the fallout of *Colletotrichum* infected leaves in rubber plants.

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3. Research Questions

- Are there differences in the effectiveness of some endophytic fungi from rubber clones as biological agents in the control of *C. gloeosporioides*?
- Are there differences in the effectiveness of the extraction of endophytic fungi metabolites and spores as biological agents in the control of *C. gloeosporioides*?

4. Purpose of the Study

To determine the effectiveness of several endophytic fungi from rubber clones as biological agents against fungi *C. gloeosporioides* in rubber plants (*Hevea brasiliensis* Muell. Arg.).

5. Research Methods

This research was conducted on an experimental farm at Sungai Putih Rubber Research Center, Galang Subdistrict, Deli Serdang Regency, North Sumatra Province with a site altitude of ± 25 metres above sea level (MASL) from February to March 2019. Samples included endophytic pure isolate clones (RRIM-931, RRIM-901, PB-330 and PB-260) from Bandar Betsy plantation, *C.gloesporioides* isolates from Sungai Putih plantation and *Hevea brasiliensis PB* 260 clone. The research method was factorial randomized block design (RBD) with 3 replications and combinations with 5 replications.

Endophytic factor (E) consisted of 4 treatments which were E1 = RRIM-931 rubber clone from code isolate Bb 1 (endophyte), E2 = rubber clone RRIM-901 from code isolate Bb 2 (endophyte), E3 = PB-330 rubber clone from code isolate Bb 3 (endophyte), and E4 = PB-260 rubber clone from code isolate Bb 4 (endophyte).

Application methods (M) consisted of 4 types which were M0 = control (water treatment) M1 = spores 10^4, M2 = metabolites, and M3 = metabolites + spores. The number of treatment combinations was 16 (4x4), with each treatment being repeated three times.

5.1. Making Endophytic Metabolites

Five isolates were taken from each endophytic type and were placed inside a bottle containing 100 ml of GDP media. This process was carried out in a laminar water flow, followed by a week in shaker (Sungai Putih Rubber Research Center, 2018).

5.2. Endophye Spore and Metabolite Application

An application of 180 ml of distilled water to the upper and lower surfaces of rubber leaves was carried out in the afternoon. The surface of the leaves were then applied with *C.gloesporioides* spores and covered with a plastic bag for 1 day of incubation. The endophytic metabolite application with a construct of 20% was obtained with the amount of 15 ml from one leaf stalk. Tests were carried out following the modified method by Ilyas et al. (2007). The metabolite compounds from each selected endophytic fungi were added to the PDA growing media, so that the growing media were formed with concentrations of 20%, 10% and 5% of the metabolites. This tested the effectiveness of the endophytic fungi with several treatments of spores and metabolites with different concentrations.

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The applications of spores and metabolites with a density of 10^4 were sprayed using a hand sprayer on the leaves of the entire rubber plant. Incubation using *C. gloeosporioides* with a density of 10^4 was for 1 day. Metabolites were applied with a concentration of 20% until the initial patches of diseases or the appearance of symptoms of the disease were seen on the leaves. Observations were done at 2, 4, 6, 8, 10 and 12 days after inoculation.

5.3. Latent Period

The latent period saw the onset of *Colletotrichum* leaf fall symptom one day after incubation of spore spraying. Observation was carried out on 10 leaves on one stem of PB 260 clone rubber plants.

5.4. Disease Indication

Observation of disease occurence was carried out by calculating deciduous plants of *C. gloeosporioides* leaves in each trial plot. Observations were made on the 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day after inoculation (HSI). Data obtained was calculated by the following formula :

$$KP = \frac{n}{z} \times 100\ \%$$

where :

KP : disease incidence, n : number of leaves symptomatic of leaf fall disease, z : number of leaf samples (Inte Agrios, 2005).

5.5. Disease severity

Measurements for the severity of the disease according to the number of days after conidial inoculation were done using the scale of the attack on the leaves. Measurements of the pathogenic disease intensity or scale of attack at the Sungai Putih Rubber Research Institute in the farm with PB 260 clones were followed according to the formula by Towsendt and Hueberger (as cited in Unterstenhover, 1963) :

$$I = \frac{\sum (ni \times vi)}{N \times Z} \times 100\ \%$$

where :

I : disease severity, ni : number of first leaves, vi : scale of attack, N : number of leaves observed, Z : highest value

Symptoms of leaf spots were set on 7 scales (0 – 6), with a spotting diameter of 2 mm.

Scale 0 = no spots on the leaves were observed

Scale 1 = 1 to 8 spots on the leaves were observed

Scale 2 = 9 to 12 spots on the leaves were observed

Scale 3 = 13 to 16 spots on the leaves were observed

Scale 4 = 17 to 20 spots on the leaves were observed

Scale 5 = 21 to 24 spots on the leaves were observed

Scale 6 = more than 24 spots on the leaves were observed or the leaves fall.

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6. Findings

6.1. Latent period

The results of observations of the time of the appearance of symptoms of a disease attack (latent period) is the fastest is 3th day after inoculation, and the longest is 4 days. This shows that at 3th day after inoculation the penetration of the fungus Colletotrichum into the leaf tissue (Table 01).

Table 01. Percentage of endophytics

Treatment (M)	Endophytic (E)				
	E1	**E2**	**E3**	**E4**	**Mean**
M_0	3.00	3.00	3.00	3.00	3.00
M_1	4.33	3.33	3.00	4.00	3.67
M_2	4.33	5.33	3.00	3.67	4.08
Mean	4.17	4.25	3.17	3.42	4.17

The initial appearance of the symptoms of *C.gloeosporiodes* in several treatments of endophytic isolates either by spore treatment or by the treatment of metabolites was on average seen on the third day. Infection occurred when the inoculum was sprayed on the upper or lower leaf surfaces. The moderate level of clone resistance was reflected by the slow latency period. Vulnerablility was reflected in the latent period by the rate of spots development and the intensity of the attack or severity of the disease.

The incubation period as the initial process of the emergence of symptoms of disease in plants is influenced by environmental factors that are suitable for the development of pathogens, but instead makes plant growth inhibited. In addition to environmental factors, this latent period is also influenced by genetic factors, both from pathogens and host plants to the disease. This is evident from the data generated where the types of endophytic fungi that are applied, each of which has a response to a different disease (**Figure 01**).

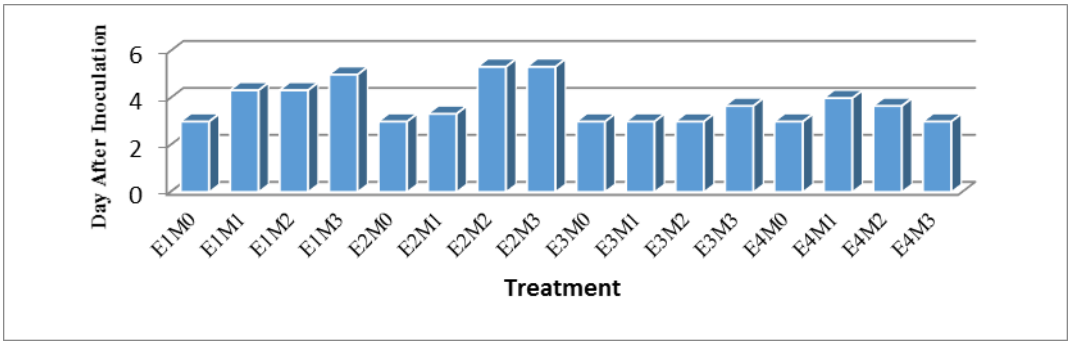

Figure 01. Latent period

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6.2. Disease Incidence (%)

Table 02. Treatments of endophytic species from Bandar Betsy plantation which affect the percentage of *C.gloeosporiodes* disease attack on the rubber plants

Treatment	Days after inoculation					
	2	4	6	8	10	12
Endophytic type (E)						
E1	0.91	48.53	58.54	63.2	65.16	68.22
E2	0.91	45.06	62.20	62.2	66.88	67.43
E3	0.91	61.30	65.77	67.31	71.06	75.1
E4	0.91	44.74	57.74	60.66	67.03	71.69
Application method (M)						
M_0	0.91	65.67c	78.60c	83.25b	89.09c	89.09b
M_1	0.91	45.09ab	52.08ab	54.10a	54.65a	60.54a
M_2	0.91	34.81a	48.81a	51.25a	58.46ab	62.67a
M_3	0.91	54.05b	64.77a	64.77a	67.91b	70.12a
Interaction (E*M)						
E1M0	0.91	66.14	70.48	83.25	89.09	89.09
E1M1	0.91	34.15	52.86	52.86	52.86	59.00
E1M2	0.91	28.30	43.08	48.93	50.94	57.00
E1M3	0.91	65.55	67.77	67.77	67.77	67.77
E2M0	0.91	53.86	83.25	83.25	89.09	89.09
E2M1	0.91	57.70	57.70	57.70	57.70	57.70
E2M2	0.91	21.75	46.22	46.22	53.15	53.15
E2M3	0.91	46.92	61.62	61.62	67.56	69.77
E3M0	0.91	80.54	83.25	83.25	89.09	89.09
E3M1	0.91	54.78	54.78	57.00	59.21	61.92
E3M2	0.91	53.07	53.07	57.00	61.22	71.99
E3M3	0.91	56.79	71.99	71.99	74.70	77.41
E4M0	0.91	62.17	77.41	83.25	89.09	89.09
E4M1	0.91	33.72	42.99	48.85	48.85	63.54
E4M2	0.91	36.14	52.86	52.86	68.55	68.55
E4M3	0.91	46.92	57.69	57.69	61.62	65.55

*Note: Numbers followed by different letters in the same treatment group are significantly different based on Duncan's multiple distance test at the level of 5 %

Table 02 is the arcsine sine-1√P transformation data showing the variations that range from 0.91% to 89.09%. The administration of endophytic fungi had a very significant effect on the incidence of disease in rubber plants in the farming area. The highest mean of 89.09% was obtained in the treatments E1M0, E2M0, E3M0 and E4M0 whilst the lowest average was obtained in the treatment of E2M2 with 53.15%. The development of deciduous leaves of *C. gloeosporioides* correlated with sensitive rainfall and clones.

https://doi.org/10.15405/epsbs.2020.03.03.8
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eISSN: 2357-1330

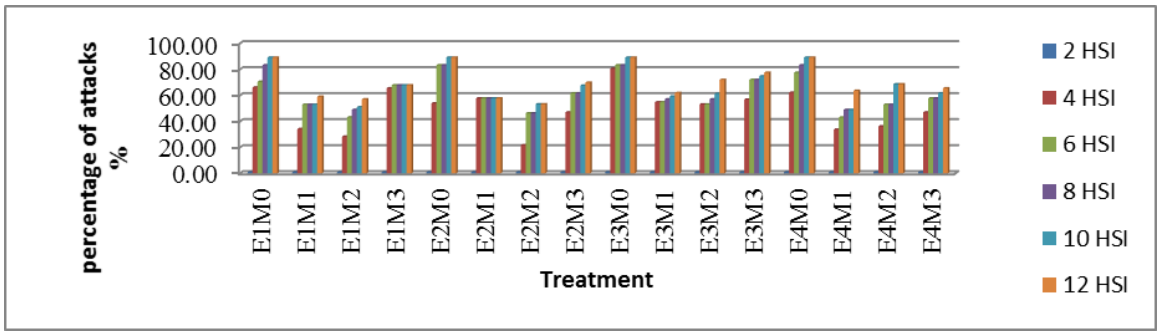

Figure 02. Percentage of disease event

Figure 02 shows that the effectiveness test of endophytic fungi had a very significant effect on the percentage of the disease. The rate of attack of *C.gloeosporiodes* in rubber plants varied and could even reach 100% depending on weather conditions and the vulnerability of the rubber clone

6.3. Disease Inctnsity (%)

The results of analysis of variance showed that the test of endophytic fungi did not significantly affect the intensity of *C.gloeosporioides* disease in observations 2, 4, 6, 8, 10 and 12 days after inoculation (HSI). The application method treatment had no significant effect at 2 and 6 HIS but had very significant effects at 8, 10 and 12 HIS. The interaction between the two treatments however had no significant effect on *C.gloeosporiodes* in all the observations.

The average percentage intensity of *C.gloeosporioides* disease is presented in Table 03. It can be seen that the treatment of isolates against disease intensity had variations that ranged from 0.00 % to 4.93 %. No leaf deciduous spots appeared at 2 HSI. At 4 HSI, the lowest value was in the treatment of E1M2 (1.39 %) and the highest was in the treatment of E3M0 (2.99 %). At 6 HSI, the lowest value was E1M2 (2.23 %) and the highest value was E3M0 (3.41%) whilst at 8 HIS the lowest value could be seen in the E1M1 and E1M2 treatments (2.57 %) and the highest in the E3M0 treatment (3.41 %). At 10 HIS, the lowest value was in the E1M1 and E1M2 treatments (2.56 %) and the highest value was E2M0 treatment (4.93 %).

Table 03. Average disease intensity (%) of the rubber leaves.

Treatment	Days after inoculation (HSI)					
	2	4	6	8	10	12
Endophytic type E1	0.00	2.18	2.73	2.96	3.40	3.40
E2	0.00	2.05	2.80	2.80	3.59	3.68
E3	0.00	2.68	2.96	3.04	3.60	3.60
E4	0.00	2.09	2.73	2.85	3.55	3.55
Application method M0	0.00	2.71c	3.29c	3.49b	4.80c	4.80c
M1	0.00	2.11ab	2.65ab	2.73a	2.86a	2.96a
M2	0.00	1.67a	2.39a	2.54a	2.94a	2.94a
M3	0.00	2.51bc	2.88bc	2.89a	3.53b	3.53b
Interaction E1M0	0.00	2.76	3.14	3.75	4.90	4.90

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E1M1	0.00	1.76	2.57	2.57	2.57	2.57
E1M2	0.00	1.39	2.23	2.56	2.56	2.56
E1M3	0.00	2.82	2.98	2.98	3.57	3.57
E2M0	0.00	2.37	3.36	3.36	4.93	4.93
E2M1	0.00	2.63	2.70	2.70	2.99	3.37
E2M2	0.00	0.94	2.33	2.33	2.88	2.88
E2M3	0.00	2.24	2.81	2.81	3.54	3.54
E3M0	0.00	2.99	3.41	3.41	4.58	4.58
E3M1	0.00	2.58	2.94	3.00	3.25	3.25
E3M2	0.00	2.50	2.50	2.70	3.05	3.05
E3M3	0.00	2.65	3.00	3.05	3.51	3.51
E4M0	0.00	2.71	3.27	3.46	4.81	4.80
E4M1	0.00	1.48	2.40	2.64	2.64	2.64
E4M2	0.00	1.82	2.51	2.58	3.26	3.26
E4M3	0.00	2.34	2.73	2.73	3.49	3.49

Numbers followed by different letters that in the same treatment group are significantly different based on Duncan's multiple distance test at the level of 5 %.

The effectiveness of endophytic fungi from the Bandar Betsy plantation had a significant effect on the intensity of the disease in the rubber plants in the farm (Figure 03).

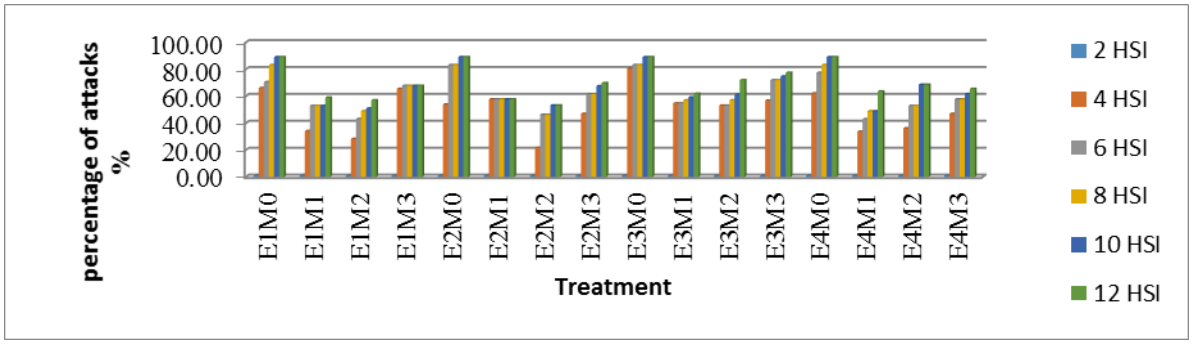

Figure 03. Disease intensity

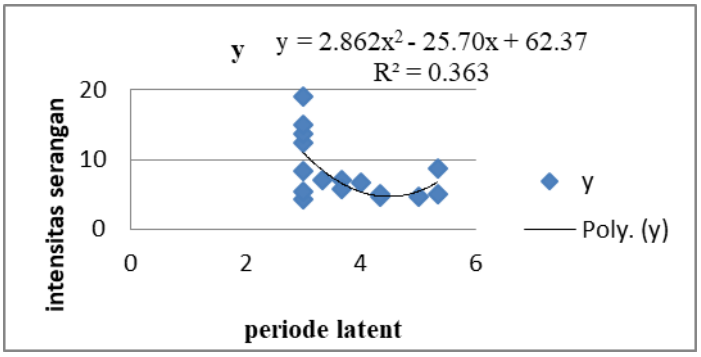

Figure 04. Latent performance

Figure 04 shows that latent performance affected the level of attack intensity by 36% and was influenced by other factors. The results showed that the effectiveness test of metabolites from endophytic fungi from Bandar Betsy plantation significantly affected the intensity of the disease. According to Tan

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eISSN: 2357-1330

and Zou (2001), endophytic microbes can indeed produce bioactive compounds that have similar or equal characteristics to their host. This is due to the evolutionary genetic exchange that takes place between the host and endophytic microbes.

The absence of an interaction in observations 2, 4, 6, 8, 10 and 12 HSI showed that the effect of the application method was very significant to the intensity of the disease in the endophytic application method from the Bandar Betsy Clone RRIM 931 Garden with spore and metabolite treatments.

Deciduous disease of *C.gloeosporiodes* in PB 260 clone rubber plants was tested using spores and endophytic fungal metabolites from Bandar Betsy garden with isolates BB1, BB2, BB3 and BB4 clones with observations at 2 to 12 HSI. Each treatment from 4 isolates had controls with codes KE1, KE2, KE3 and KE4.

Results of the study showed that the severity and resistance of PB 260 rubber clones to *C.gloeosporioides* deciduous disease with endophytic treatment and the application method in all treatments were at 0 - 5%. This percentage was categorised as mild damage-resistant, thus the treatment of endophytic and effective application method as biological control in suppressing the development of *C.gloeosporioides* deciduous disease. Interaction between endophytic fungi from RRIM-901 rubber clone from Bb 2 isolate code (endophytes) and spore and metabolite application method showed the duration of days after inoculation (HSI) and the presence of an influence caused by endophytic fungi in suppressing the development of fungal deciduous leaf *C. gloeosporioides.*

The effectiveness of endophytic fungi from the Betsy Bandar garden with the code of isolates BB1, BB2, BB3 and BB4 showed effective isolates suppressing the development of *C.gloeoporiodes.* BB1 with treatment of endophyte 1 Spores and E1M1 metabolites with an average disease intensity of 2.57 % is said to be able to suppress the fungal development. In the opinion of Debbab, Muller, and Mosaddak (2009), endophytic fungi are microbes that live in the internal tissues of almost all healthy plants without causing direct negative effects on their host plants. Endophytic fungi are found in the leaves, flowers, twigs and roots of plants. They synergize with their host plants through a symbiotic relationship between mutualism and some endophytic fungi are considered useful for plants by producing special substances such as enzymes that can prevent host plants from attacking pathogens such as fungi and pests. Verma, Patel, Pratap, Gangwar, and Nath (2014) said that 80% of endophytic fungi tested showed ability as antibacterial, anti-fungal, anti-allergic or as an herbicide.

7. Conclusion

The treatment of the application method has a significant effect on the 4, 6, 8, 10 and 12 HSI treatments, while the interaction of the two treatments did not significantly affect the development of leaf deciduous *C.gloeoporioides*. Intensity The lowest disease was obtained from endophytic treatment at E1M2 2.56% and the highest value at E2M0 treatment. 4.93%. Effective endophytic fungi suppress and prevent the development of *C.gloeosporioides* deciduous disease in entres.

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