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# *Bacillus* and *Trichoderma* Isolate Combination to Suppress *Rigidoporuslignosus*White Root Rot Disease on Cashew Seedling

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***Abstract:*** *White root rot (WRR) disease, one of the important diseases of cashew plants in Indonesia, including Southeast Sulawesi, is caused by fungus* Rigidoporus lignosus. *One method of controlling the disease is the use of biological agents. The current study evaluated* B. subtilis *ST21e and* Trichoderma asperellum *and their combinations in controlling WRR through soil application. The study arranged in a randomized block design with eight treatments, each of which given three replicates. These treatments consisted of* B. subtilis *ST21e,* T. asperellum, *fungicide mancozeb mixture* B. subtilis *ST21e, and* T. asperellum *without inoculation by* R. lignosus, *and same treatments as mentioned above with inoculation by* R. lignosus. *Positive control and negative control also made for comparison. By comparing to the negative control, the treatments of the first group did not offer a significant impact statistically on height and leave several cashew seedlings. While the second group of the treatments provided a significant impact statistically on height and compared to the positive control, this height was increased by 29.9%, 20.7%, 5.4%, and 30.6%, respectively, at 112 days after application. At the same time, the incidence by WRR was respectively 8.3%, 16.7%, 33.3%, and 0.0%, compared to a 75% incidence by WRR on positive control. Therefore, the study results demonstrate the efficacy of biological agents compared to fungicide and the important role of* B. subtilis *ST21e and* T. asperellum *combination in controlling WRR disease.*

***Keywords:****Bacillus subtilis, cashew, mancozeb, Trichoderma asperellum, white root rot disease.*

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## I. Introduction

Cashew nut(*Anacardium occidentale* L.) is an evergreen tropical tree, belongs to the family Anacardiaceae. Specifically, this plant has a characteristic of hardy, fast-growing, drought-tolerant, and can be cultivated even in less endowed environments with minimum care. The cashew tree is native to Brazil's Amazon rain forest, it is grown commercially in the American continent (Salam and Peter, 2010) such as Brazil, Venezuela, *Caatinga* (dry thorn forest), and the *cerrado* vegetation of the savannas of the Amazon basin, Nigeria (Hammed et al., 2008) and Indonesia, i.e., Southeast Sulawesi (Tombe et al., 2014).

With a total area of around 120.263 ha, Southeast Sulawesi is the biggest cashew producer in Indonesia. We calculate the entire production from this area reaching around 40.325 tons (Agency of Plantations and Horticulture, 2009). In terms of Indonesia's average cashew productivity, there are five provinces as East Nusa Tenggara, South Sulawesi, Southeast Sulawesi, West Nusa Tenggara, and East Java give contributed in the amount of 82.91%. East Nusa Tenggara provided the most significant contribution of 33.33%. South Sulawesi contributed 15.05%, Southeast Sulawesi contributed 12.94%, followed by East Nusa Tenggara and East Jave, respectively 11.10% and 10.49% (Data Center and Agricultural Information System, Ministry of Agriculture, 2017). Many factors causing low productivity in Southeast Sulawesi are infection by white root rot fungi diseases (*Rigidoporus lignosus*). The pathogen infects productive cashew plants under ten years old. Above-ground symptoms on the infected tree are leaf yellowing, dieback, and tree death (Supriadi et al., 2004). The wood of infected plants sometimes becomes soft, and white mycelial clumps occur on soil surfaces around the tree (Chang, 1995).

This disease is relatively difficult, and farmers usually abandon their field infested and look for another field for replanting new cashew. On a large scale, chemical control using fungicide is not economical, and cashew resistance to a disease is not available. We are considering using *Trichoderma* sp. and *Bacillus* sp. as a durable, biocontrol alternative for managing white rot disease in short and or in the long term period. *Trichoderma* species are cosmopolitan free-living fungi, colonize the soil and parts of the plant, occupy a physical space, and avoid pathogens multiplication (Monte et al., 2003; Schuster and Schmoll, 2010).Many *Trichoderma* strains have been identified as having potential applications in biological control of soil-borne fungi such as *Armillaria, Fusarium Phytophthora, Pythium, Rhizoctonia, Sclerotium,* and *Verticillium* (Monte, 2001). Another benefit of using Trichoderma is its ability to promote plant growth. A study on cacao seedling and side graft indicates that the application of *Trichoderma asperellum* increases the number of shoots (Rosmana *et al.*2015; Rosmana*et al*., 2016). Besides,*Bacillus* sp. is rhizobacteria that colonize plant roots and well known for their antagonistic effects and their ability to trigger induced systemic resistance (ISR) (Beneduzi et al., 2012).

Asingle biocontrol agent often offers inconsistent performance to suppress a single plant pathogen under any environmental condition. For this reason, in this experiment, we evaluated the use of two microorganisms, i.e.,*Bacillus* and *Trichoderma*,combinedwiththe growth promotion of cashew seedling and biological control of *Rigidoporus lignosus* causal agent of white rot disease.

## II. Materials and Methods

***Preparation Bacillus, Trichoderma, Rigidoporus, cashew seedling for treatment***

A two-day-old biological agent of *Bacillus subtilis* isolates ST21e cultured on Nutrient Agar (NA) medium contain (Distilled water 1 L, beef extract 1 g, yeast extract 2 g, peptone 5 g, NaCl 5 g, and agar 15 g). After that isolates were suspended in Nutrient Broth (NB) medium with $10^9$ CFU/ml. Then, the suspension was incubated for 72 hours. The bacterial suspension was harvested and mixed with a formulated mixture of crushed wood charcoal and organic soil (1:1). The mixture ratio between the suspension and the formulated mixture was 1:2:5 (v/b). The mixture use air-dried for two days, before being used for the application. A pure isolate of *Trichoderma asperellum* fungus was obtained from the laboratory collection, which multiplied on PDA medium. This culture used for further multiplication in rice medium. Rice medium was prepared by suspending rice in water for 24 hours, rinsing with water, and steaming until cooked. The cooked medium (100 g) and put in a heat-resistant plastic bag.

The medium was then sterilized with an autoclave at 100-121$^0$C for 15 minutes. Five agar plugs with spores and mycelia, around 5 mm in diameter from PDA culture, were transferred to rice medium and incubated for ten days, with periodical shaking for a better *Trichoderma* growth.Fungi of *Rigidoporuslignosus* isolate collected from the field was purified onmalt extractmedium. This fungus suspended in sterile water until the concentration of $10^9$CFUbefore soil drenching.Cashew seeds planted in a nursery. Healthy seedlings with good vigor were chosen for the tested plant materials and transplanted into polybags containing a media mixture of topsoil and sterile cowmanure (2:1 ratio). The seedlingsmaintained in a pest-free screen house.

***Greenhouse assessment***

To evaluate the efficacy of *B. subtilis* ST21e and *T. asperellum*, to promote cashew growth and control white root rot of *Rigidoporus lignosus*, we applied these microorganisms in single and in combination, with and without infection of *L. lignosus*, and compared with fungicide. This experiment arranged in a Randomized Block Design with eight treatments, and each treatment repeated three times. Four plants per treatment used. The treatments consist of *B. subtilis* ST21e, *T. asperellum*, mixture of *B. subtilis* ST21e and *T. asperellum*, fungicide mancozeb, *B. subtilis* ST21e with infection of *R. lignosus*, *T. asperellum* with infection of *R. lignosus*, mixture of *B. subtilis* ST21e and *T. asperellum* with infection of *R. lignosus*, fungicide mancozeb with infection of *R. lignosus*. In addition, negative control without any inoculation of microorganisms and positive control which inoculated by *R. lignosus* was done for comparison with the treatments mentioned above. The total seedling used in this experiment, including control, was 96 seedlings of one year old.Pathogen *R. lignosus* was inoculated to cashew seedling through soil drenching with a concentration of 10-15 CFU/ml, to support the infection process, the cashew rootsfirst wounded with a needle. *B. subtilis* ST21eand *T. asperellum* in single and in combination fungicide mancozeb also applied through soil drenching. Application of bacteria and fungus with concentration 10 g per 100 ml in single treatment and 20 g per 200ml in combination treatment were carried out after inoculation by the pathogen, while soil drenching of fungicide done two weeks after pathogen inoculation. The concentration of fungicide used was 2 g/L and from this was taken 5 ml and applied every two weeks. The observation was done on plant height, leaf number, and root rot incidence by *R.lignosus*. The disease incidence was calculated using the formula:

**I = a/b x 100%**

where I: Disease Incidence
a: Number of seedling showing disease symptom
b:Total of seedling observed.

***Analysis***

The data of plant height, leaf number, and disease incidences by*Rigidoporuslignosus*analyzedafter transformation to Log x + 1. Least Significance Different (LSD) used for evaluating significant differences between the treatment means.

## III. Results And Discussion

***Preparation Bacillus, Trichoderma, Rigidoporus, cashew seedling for treatment***

Cashew seedling height treated by *B. subtilis* ST21e., *T.asperellum*, and fungicide mancozeb at 112 days after treatment was respectively 47.6 cm, 46.1 cm, and 47.8 cm and by comparing to healthy control seedling having height of 51.75 cm, there was the decrease of height by 8.0%, 10.9%, and 7.6% respectively, while cashew seedling treated by a combination of *B. subtilis* ST21e and *T. asperellum* hasa height of 51.90 cm or increasing of 0.3% (Table 1). Statistically, These differences were not significant ($p \leq 0.05$).

**Table 1.** Cashew seedling height (cm) 98 days and 112 days after treatment by *B. subtilis* ST21e, *T. asperellum*, *B. subtilis* ST21e, and *T. asperellum*, and fungicide mancozeb and inoculation by *Rigidoporus lignosus*

| Treatments and controls | Cashew height in cm (dati)* | |
|---|---|---|
| | 98 | 112 |
| *B. subtilis* ST21e | 38.92±4.42a | 47.58±4.11ab |
| *T. asperellum* | 37.92±3.26a | 46.08±5.49ab |
| *B. Subtilis* ST21e + *T. asperellum* | 40.83±2.55a | 51.92±1.84a |
| Fungicide mancozeb | 38.83±1.38a | 47.83±1.51ab |
| *B. subtilis* ST21e with inoculation *R. lignosus* | 42.17±1.15a | 50.25±4.34ab |
| *T. asperellum* withinoculation *R.lignosus* | 37.42±5.14a | 46.67±5.34ab |
| *B. subtilis* ST21e + *T. asperellum*with inoculation *R. lignosus* | 40.33±4.40a | 50.50±5.58ab |
| Fungicidemancozebwith inoculation *R.lignosus* | 34.42±6.42a | 40.75±6.50ab |
| Negative control | 41.58±2.75a | 51.75±5.63a |
| Positive control | 36.75±5.40a | 38.67±5.02b |

*dati = days after treatment and inoculation.

Means in the same column followed by the same letter are not significantly different according to the least significant difference ($p \leq 0.05$)

The height of cashew seedling with infection of *R. lignosus* at the same as mentioned above was 38.60 cm, and by comparing to healthy control, the height reduced 25.4%. Treatment this cashew inoculatedby *B. subtilis* ST21e, *T. asperellum*, combination of *B. subtilis* ST21e and *T. asperellum*, and mancozeb showed the increase of height respectively 50.25 cm, 46.67 cm, 50.50 cm and 40,75 cm or increasing by 23.2%, 20,9%, 25.6%, and 5.3% respectively, but decreasing by 2.9%, 9.8%, 2,4%, and 21.3% respectively if these heights compared to the contrary or healthy control (Table1). This height of infected seedling or positive control was significantly different ($p \leq 0.05$) with all treatments, while treatments were not.

The average number of cashew seedling leaves treated by *B. subtilis* ST21e, and *T. asperellum* at 112 days after treatment was 21.25, and 21.58, compared to several 22.17 leaves in healthy seedling, there was a reduction of 4.1% and 2.7%. While on that treated by fungicide and combination of *B. subtilis* ST21e and *T. asperellum* the number of leaves was 22.75 and 23.83. There was an increase of 2.6% and 7.5%, respectively (Table 2). However, these differences were not significant statistically ($p \leq 0.05$).

On the cashew seedling infected by *R. lignosus*, the number of leaves was 19.83, and by comparing to the control, the decrease was 10.6%. Treatment this infected cashew by *B. subtilis* ST21e, *T. asperellum*, *B. subtilis* ST21e, and *T. asperellum*, and Fungicide, the number of leaves became 22.83, 21.58, 21.75, and 21.67 respectively or increasing by 15.1%, 8.8%, 9.7% and 9.3% respectively if these compared to infected seedling, but decreasing by 2.9%, 2.7%, 1.9%, and 2.3% respectively if these compared to negative control (Table 2).

**Table 2.** Cashew seedling leaves number 98 days and 112 days after treatment by *B. subtilis* ST21e, *T. asperellum*, *B. subtilis* ST21e and *T. asperellum*, and fungicide mancozeb and inoculation by *Rigidoporus lignosus*

| Treatments and controls | Leaf number (dati)* | |
|---|---|---|
| | 98 | 112 |
| *B. subtilis* ST21e | 18.33±2.04a | 21.25±1.52a |
| *T. asperellum* | 18.58±1.77a | 21.58±2.90a |
| *B. Subtilis* ST21e + *T. asperellum* | 20.17±2.18a | 23.83±3.18a |
| Fungicide mancozeb | 19.25±0.66a | 22.75±0.14a |
| *B. subtilis* ST21e with inoculation *R. Lignosus* | 19.25±0.75a | 22.83±1.50a |
| *T. asperellum* withinoculation *R.lignosus* | 20.42±1.39a | 21.58±2.31a |
| *B. Subtilis* ST21e + *T. asperellum*with inoculation *R. lignosus* | 20.25±1.95a | 21.75±2.95a |
| Mancozebwithinoculation*R. Lignosus* | 21.00±0.38a | 21.67±1.66a |
| Negative control | 18.92±1.66a | 22.17±0.80a |
| Positive control | 18.33±2.05a | 19.83±1.66a |

*dati = days after treatment and inoculation.
Means in the same column followed by the same letter are not significantly different according to the least significant difference ($p \leq 0.05$)

This research showed that *B. subtilis* ST21e, *T. asperellum*, combination *B. subtilis* ST21e, *T. asperellum,* and fungicide applications did not improve plant growth of seeding height and number of leaves. However, they can inhibit the incidence caused by *Rigidoporus lignosus,* the pathogen of white rot disease on cashew seedling.

Cashew seedlings inoculated with *R. lignosus*had significantly retarded growth characterized by the reduction of seedling height. Plants infected by pathogen could exhibit several symptoms, such as color or tissue changesand retarded growth like plant height and leaf number (Agrios, 2005).The symptoms caused by *R. lignosus*were specific with the presence of chloroticspots on the youngest tender leaves and this different from that of caused by *Cryptosporiopsis*, the pathogen of cashew blight. The chlorotic spots occur at both sides of the leaves and varied in size, shape, and color (Menge et al., 2013). In our experiment, these chlorotic spots symptoms were then followed by wilting, and dying of seedling.

Inoculation of both pathogen and *B. subtilis* ST21e and *T. asperellum* isolates into soil permitted these microorganisms to move the roots. The occurrence of roots damage indicatedthat theinfection of*R. lignosus* done through roots and actionof *Bacillus* and *Trichoderma* against started on roots. Beneficial relationships between plants and microorganisms often occur in the rhizosphere (Zamioudis and Pieterse, 2012) and the mode of action of *Bacillus* and *Trichoderma* against *R. lignosus* here suggest direct and indirect actions. Perhaps both isolates can produce hydrolytic enzymes and antibiotics. *Bacillus* and *Trichoderma* immediate action against pathogenic fungi,including *Rigidiporus* has confirmed (Beneduzi et al., 2012; Jayasuriya et al., 2007). *Bacillus* inhibit fungal pathogen by lysis their cells trough production of hydrolytic enzymes such as chitinases, glucanases, proteases, and lipases (Maksimov et al., 2011), also by competition for nutrient and colonization of niches at root surface (Kamilova et al., 2005), and by production of siderophores and antibiotics (Beneduzi et al., 2012). The same with *Trichoderma*, they can also colonize the soil, and part of the plant avoiding multiplication of fungal pathogens and produce cell wall degrading enzymes and antibiotics to killing plant pathogens (Bailey et al., 2008; Bae et al., 2011; Atanasova et al., 2013). In indirect action, *Bacillus* and *Trichoderma* sensitize the plant immune system for enhanced defense without directly activating costly defenses, called induced systemic resistance (ISR) (Beneduzi et al., 2012; Zamioudis and Pieterse, 2012), but further research needs to be done.

Higher in the reduction of disease incidence by *B. subtilis* ST21e or by *T. asperellum* than by mancozeb fungicide seems to have proved the presence of indirect action of these two microorganisms. Mancozeb [[1,2-ethanediylbis-[carbamodithio-ato]](2-)] manganese, mixture with [[1,2-etha-nedi-ylbis-[carbamodithioato]]-(2-)] zinc, is a fungicide from the carbamate group and acts directly on multiple sites in fungal cells (Rasool and Reshi, 2010). A sugarcane study showed that this fungicide could improve root health and plant growth (Magarey and Bull, 2003). In our experiment, the height of cashew seedling after treatment by mancozeb fungicide was comparable to the height after treatment by individual *Bacillus* or *Trichoderma.*

***Greenhouse assessment***


**Figure 1.**Symptom development on cashew seedling infected by *Rigidoporuslignosus*. Yellowingon leaves (A), wilting of seedling (B) anddying of seedling (C).

Cashew seedling infected by *R. lignosus* showed the symptoms of roots damage, brownish color on the stem. In severe infection, the stem becomes smaller, and the leaves become yellowing followed leaves senescence. Another possible symptom is the leaf exhibiting dehydrated-like appearance (Figure 1). These symptoms are appear at 98 days after inoculation, and its incidence at this time reached 33.3% and 75.0% at 112 days after application. Disease incidence at the same days after treatment by *B. subtilis* ST21e is respectively 8.3% and 8.3%, by *T. asperellum*is respectively 8.3% and 16.7%, by combination *B. subtilis* ST21e and *T. asperellum*is 0.0% and 0.0%, and by Fungicide is respectively 33.3% and 33.3% (Figure 2). Statistical analysis at 112 days showed a significant difference ($p \leq 0.05$) between positive control and all treatments and also between mancozeb and microorganisms in single and in combination.

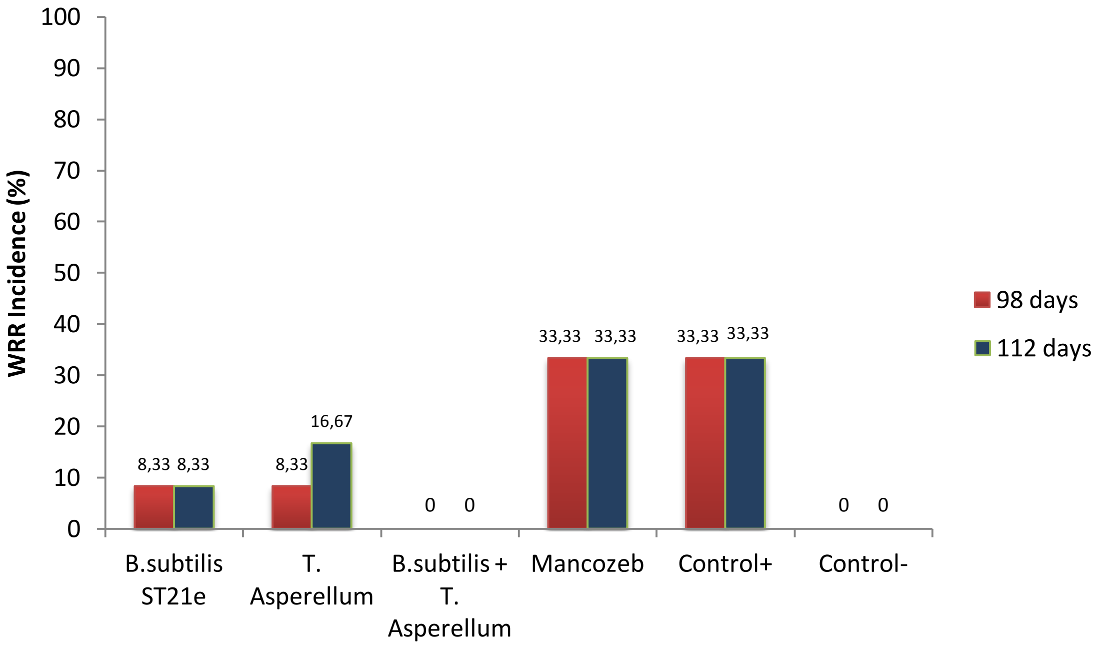


**Figure 2.** The incidence of white root rot (WRR) disease at 98 days and 112 days after inoculation by *Rigidoporus lignosus* and treatment by *B. subtilis*, *T. asperellum*, *B. subtilis*, and *T. asperellum*, fungicide mancozeb, control+ (Inoculated with pathogenic fungi *R. lignosus*) and control– (without inoculated). Means of incidence at the same time, followed by the same letter are not significantly different according to the least significant difference ($p \leq 0.05$).

A combination of *Bacillus* and *Trichoderma* increased the suppression of white root rot incidence. Therefore a synergistic interaction between two microorganisms has occurred. In nature, plants often interact with multiple partners concurrently, and the interactions between these symbionts can influence the dynamics of both host and symbiont populations (Larimer et al., 2010). Rhizobia can interact synergistically with AM fungi in increasing respectively of nitrogen and phosphorus availability (Jia et al., 2004). Another study showed that fungal endophyte infection enhances AM fungal colonization (Novas et al., 2005). These interaction modes were probably the same as that done by *B. subtilis* ST21e and *T. asperellum* in this research.

## IV. Conclusions

We conclude that the application of *B. subtilis* ST21e alone, *T. asperellum* alone, and the combination between *B. subtilis* ST21e and *T. asperellum* through soil drenching can control better WRR disease on cashew seedling than fungicide mancozeb. The use of fungicide, besides costly, also would inhibit the growth and development of useful microorganisms in the soil. Therefore, the application of these microorganisms, notably in the form of combination both in nursery and infield, will support the government of Southeast Sulawesi in the sustainability of cashew production.

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