Screening of endophytic \textit{Trichoderma} isolates to improve the growth and health of \textit{Eucalyptus pellita} seedlings

B A Siregar, D Liantiqomah, Halimah, A Gafur and B Tjahjono

Sinarmas Forestry Corporate Research and Development, Perawang 28772, Indonesia

Corresponding author email: gafur@uwalumni.com

Abstract. Since its first introduction to the Indonesian forest plantations, eucalyptus has been associated with pests and diseases. As a component of integrated disease management, some biocontrol agents have been developed to manage eucalyptus diseases. Application of endophytic \textit{Trichoderma} is a critical option in this effort; it has been demonstrated in other pathosystems that the fungus can improve seedling growth and health. This study aims to screen and evaluate the effect of endophytic \textit{Trichoderma} isolates on the growth and health of \textit{E. pellita} seedlings. Field isolation resulted in 43 endophytic \textit{Trichoderma} isolates. The isolates have the antagonistic ability with varied percentages of inhibition of radial growth (PIRG) against \textit{Rhizoctonia} sp. (4.2–48.6%); \textit{Cylindrocladium} sp. (4.8–43.5%); and \textit{Fusarium} sp. (3.3–52.2%). Based on the Analytical Hierarchy Process on the variables of the growth rate of the isolates and their ability to inhibit several fungal pathogens, the best six isolates were selected for further tests. In general, the use of single and/or a consortium of the isolates increases seedling height and reduces the mortality rate of the seedlings. In summary, the tested isolates can improve plant vigor, which would later make the plant more resilient against root and foliar diseases in plantations.

Keywords: analytical hierarchy process, consortium of microbes, fungal pathogen, plant vigor

1. Introduction

The establishment of forest plantations in Indonesia has been growing rapidly, especially in the last two decades. Eucalyptus was chosen as one of the main forest plantation trees due to its fast-growing nature, high productivity and adaptability to various environments. To support the short rotation plantations (5-6 years), it is necessary to provide seedlings in large quantities with high quality. Production of low-quality seedlings has been attributed to different factors, one of which is disease attacks that can kill seedlings. Chemical pesticides have been routinely used to maintain nursery productivity and seedling quality. In this perspective, proper managing the nursery in an environmentally friendly manner is essential, primarily through the exploration of biological control agents.

Application of endophytic \textit{Trichoderma} or \textit{Gliocladium} is considered an option to this effort. It has been demonstrated elsewhere that the fungi can improve seedling growth and health in several plants, especially \textit{Acacia mangium} [1, 2]. Secondary metabolites play a pivotal role in the antagonistic activities of some species of \textit{Trichoderma}, resulting in the suppressant of plant pathogens. They may contribute to both plant growth regulation and activation of plant defense responses [3]. In this experiment, we evaluated the ability of the \textit{Trichoderma} isolates to inhibit pathogenic fungi on eucalyptus seedlings such as \textit{Rhizoctonia} sp., \textit{Cylindrocladium} sp. and \textit{Fusarium} sp. [4, 5]. We also investigated the effect of the different endophytic \textit{Trichoderma} on the growth and health of \textit{Eucalyptus}...
pellita tree stocks.

2. Methodology

2.1 Fungal isolates

A total of 43 isolates of endophytic Trichoderma spp. used in this study are the culture collection of PT. Arara Abadi. The Trichoderma isolates were grown in Petri dishes with Potato-Dextrose-Agar (PDA) pH 7 at 28 °C in the incubator [6]. The pathogens, isolated from acacia and eucalyptus seedlings, i.e. Rhizoctonia sp., Cylindrocladium sp. and Fusarium sp., were also grown on PDA medium, pH 7 at 28°C in incubator.

2.2 Antagonistic in vitro assessment of Trichoderma spp.

The variables considered for this analysis were the percentage of inhibition of radial growth (PIRG). For in vitro tests, the 43 isolates of Trichoderma spp. were arranged for dual confrontations versus Rhizoctonia sp., Cylindrocladium sp. and Fusarium sp. in a completely randomized experimental design with three replicates. PDA discs (5 mm in diameter) with mycelia of Trichoderma spp. and each of the pathogens was placed at the extremes of Petri plates containing and the cultures were then incubated at 28 °C for 72 h. The mycelial growth was scored every 24 h until the first contact between the mycelia of the Trichoderma isolates and the pathogen [7].

The PIRG was calculated based on the formula proposed by Ezziyyani [8].

\[
PIRG = \frac{R1 - R2}{R1} \times 100\%
\]

PIRG = percent inhibition of radial growth; R1 = radial growth (mm) of pathogens without Trichoderma spp.; R2 = radial growth (mm) of pathogens with Trichoderma spp.

2.3 Selection of six best Trichoderma isolates based on Analytical Hierarchy Process (AHP)

The selection of six best Trichoderma isolates was carried out using the Analytical Hierarchy Process (AHP) method [9]. Determination of the problems in the AHP method in this research case was to assign priorities in selecting Trichoderma isolates. The first step of the AHP method was determining the criteria and sub-criteria and their weighting. The criteria used were Trichoderma growth rate and the inhibition to Rhizoctonia sp., Cylindrocladium sp. and Fusarium sp., while the sub-criteria were 4 class categories based on the quartile value of each of these criteria (Table 1). The rest of the steps are (i) determine a pairwise comparison matrix of the criteria; (ii) determine the normalized eigenvectors; (iii) calculate the consistency ratio of criteria and sub-criteria; and (iv) determine the ranking of each Trichoderma isolates used in the study [9]. The chosen Trichoderma isolates were those with the 6th highest scores. The growth rate character of Trichoderma isolates is very important because Trichoderma suppresses pathogen growth mainly by competition. In addition, the selection of the best isolates was based on the ability to inhibit the three pathogens.

2.4 In-planta application

Inoculum preparation and nursery application. The endophytic Trichoderma isolates were grown on malt extract agar (MEA) medium for seven days. Spores from the sporulating colonies were collected in sterile water. The spore suspension was transferred to hand sprayer. The experiment followed a randomized complete block design (RCBD) with nine treatments and, three replicates for each treatment. The treatments were as follows. (1) T.E039; (2) T.E060; (3) T.E063; (4) T.E069; (5) T.E100; (6) T.E104; (7) T.Mix; (8) Control; (9) Fungicide (Propineb) 1 g/L. Experimental units were plots containing four trays with a total of 384 tubes/tree stocks. Treatment with T.Mix was a consortium of Trichoderma isolates developed from previous experiments (unpublished) as a positive control.

Tree stocks were evenly sprayed with spore suspension (5 ml per tube) at sowing or setting using a hand sprayer. Trichoderma application on seedlings is repeated every two weeks; fungicides for seed
treatments or after sowing or setting were not applied. Other nursery SOPs were strictly followed. An untreated control was nursery SOPs without *Trichoderma* and pesticide application.

**Table 1.** Criteria and sub-criteria and their weighting for selection of *Trichoderma* isolates based on the Analytical Hierarchy Process.

| Criteria                      | Weight of Criteria | Sub-criteria | Weight of Sub-criteria |
|-------------------------------|--------------------|--------------|------------------------|
| Growth rate                   | 3x                 | Very fast    | 4x                     |
|                               |                    | Fast         | 3x                     |
|                               |                    | Medium       | 2x                     |
|                               |                    | Low          | 1x                     |
| Rhizoctonia inhibition        | 2x                 | Very High    | 4x                     |
|                               |                    | High         | 3x                     |
|                               |                    | Medium       | 2x                     |
|                               |                    | Low          | 1x                     |
| Cylindrocladium inhibition    | 2x                 | Very High    | 4x                     |
|                               |                    | High         | 3x                     |
|                               |                    | Medium       | 2x                     |
|                               |                    | Low          | 1x                     |
| Fusarium inhibition           | 1x                 | Very High    | 4x                     |
|                               |                    | High         | 3x                     |
|                               |                    | Medium       | 2x                     |
|                               |                    | Low          | 1x                     |

**Parameter assessment and data analysis.** Parameters assessed in this experiment were seedling mortality, height, root collar diameter (RCD), leaf disease severity and nursery rate. Growth gains were calculated based on the obtained data. The data were analyzed using MS Excel and SAS Programs. Descriptive analysis was used to outline the growth and health data. ANOVA was used to indicate significant differences, followed by further tests using Multiple Comparison Tests to identify the significantly different treatments.

### 3. Result and discussion

#### 3.1 Growth and antagonistic ability of *Trichoderma* spp.

The growth rate of *Trichoderma* isolates varied (0.6 – 1.8 cm/day), differed between isolates (P<0.000), and was generally faster than the tested pathogens (Table 2). In all interactions between the *Trichoderma* isolates and the pathogens, the pathogen’s growth was reduced compared to the controls from day 2. The antagonistic ability of *Trichoderma* isolates against pathogens was seen on 2-3 days of incubation with the variation of PIRG against *Rhizoctonia* sp. (4.2 – 48.6 %); *Cylindrocladium* sp. (4.8 – 43.5 %); and *Fusarium* sp. (3.3 – 52.2 %). An inhibition zone was observed around the pathogen colonies, indicating a defense reaction against specific *Trichoderma* isolates (Figure 1). After day 3, most of the *Trichoderma* isolates had overcome these defenses and had colonized the pathogens. Based on Antagonistic Bell’s scale [10], the inhibition activity of *Trichoderma* spp. is categorized as class 1 (*Trichoderma* completely overgrew the pathogen and covered the entire medium surface). An isolate of *Trichoderma* to be antagonistic to the pathogen if categorized as classes 1 and 2.

The dual culture technique is effective for evaluating and selecting superior *Trichoderma* isolates in inhibiting the fungal pathogens in-vitro. In this experiment, several *Trichoderma* isolates effectively inhibit the radial growth or have high inhibition ability against *Rhizoctonia* sp., *Cylindrocladium* sp. and *Fusarium* sp., which is consistent with previous results [11, 12]. Species of the genus *Trichoderma*, well known as green-spored fungi, have been reported widely as biological control agents against plant diseases caused mainly by soil-borne pathogens [13]. It is widely known that the *Trichoderma*
mechanisms mainly rely on mycoparasitism, production of antibiotic and, or hydrolytic enzymes as direct mechanisms against the targeted plant pathogens [14].

Table 2. The growth rate of Trichoderma spp. isolates and their inhibition zone to the pathogens in vitro.

| Isolates | Growth rate (cm/day) | Inhibition (%) |
|----------|----------------------|----------------|
|          |                      | Fusarium       | Cylindrocladium | Rhizoctonia   |
| T.E039   | 1.50 k               | 40.8 hijk      | 15.9 abcde      | 22.5 bcdefghi |
| T.E042   | 0.70 abc             | 11.1 abcd      | 18.3 abcd       | 34.7 hijklmn  |
| T.E043   | 1.00 fgh             | 37.3 efghijk   | 25.0 abcde      | 38.9 ijklnmn  |
| T.E044   | 0.95 efg             | 36.5 efghijk   | 9.52 abc        | 31.9 fgijklmn |
| T.E045   | 0.60 a               | 18.8 abcdefg   | 38.4 cde        | 34.7 hijklmn  |
| T.E046   | 0.80 bcde            | 18.2 abcdef    | 25.0 abcd       | 22.5 bcdefghi |
| T.E047   | 0.80 bcde            | 34.1 efghijk   | 17.9 abcd       | 33.8 ghijklmn |
| T.E049   | 0.90 def             | 23.9 bcdefghi  | 18.5 abcd       | 5.6 abc       |
| T.E050   | 0.90 def             | 26.2 cdefghi   | 32.9 bcde       | 43.0 klmn     |
| T.E051   | 0.95 efg             | 36.5 efghijk   | 14.1 abcd       | 47.2 n        |
| T.E052   | 1.10 ghj             | 35.0 efghijk   | 19.0 abcd       | 44.4 lmn      |
| T.E053   | 0.90 def             | 3.3 ab         | 15.7 abcd       | 5.6 abc       |
| T.E054   | 1.00 fgh             | 40.0 fghijk    | 0.0 a           | 5.6 abc       |
| T.E055   | 1.00 fgh             | 41.3 hijk      | 14.3 abcd       | 12.5 abcde    |
| T.E056   | 1.30 j               | 20.8 bcdefgh   | 12.1 abcd       | 33.3 ghijklmn |
| T.E057   | 1.80 l               | 26.9 cdefghi   | 14.6 abcd       | 6.9 abcd      |
| T.E058   | 1.10 ghj             | 51.1 jk        | 11.1 abcd       | 16.7 bcdefgh  |
| T.E059   | 1.10 ghj             | 24.2 cdefghi   | 20.6 abcd       | 5.6 abc       |
| T.E060   | 1.00 fgh             | 28.0 cdefghi   | 32.9 bcde       | 25.0 defghijk |
| T.E061   | 1.10 ghj             | 30.0 cdefghi   | 22.6 abcd       | 0.00 a        |
| T.E062   | 0.80 bcde            | 29.3 cdefghi   | 30.7 abcd       | 45.8 mn       |
| T.E063   | 1.20 ij              | 20.0 bcdefghi  | 13.3 abcd       | 42.0 jklmn    |
| T.E064   | 1.00 fgh             | 25.1 cdefghi   | 38.9 cde        | 16.7 bcdefgh  |
| T.E066   | 0.85 cdef            | 43.1 ijk       | 4.76 ab         | 23.6 cdefghij |
| T.E067   | 0.85 cdef            | 39.2 efghijk   | 4.76 ab         | 27.8 efghijklm|
| T.E068   | 0.75 abcd            | 0.0 a          | 15.9 abcd       | 15.3 bcdefg   |
| T.E069   | 1.15 hij             | 41.7 hijk      | 24.6 abcd       | 13.9 abcdef   |
| T.E071   | 0.75 abcd            | 25.2 cdefghi   | 9.5 abc         | 44.1 lmn      |
| T.E078   | 0.95 efg             | 52.2 k         | 20.6 abcd       | 48.6 n        |
| T.E079   | 0.70 abc             | 31.3 defghijk  | 41.0 cd         | 33.1 ghijklmn |
| T.E082   | 0.70 abc             | 25.8 cdefghi   | 16.7 abcd       | 41.7 jklmn    |
| T.E088   | 1.10 ghj             | 14.4 abcd      | 0.0 a           | 16.7 bcdefgh  |
| T.E089   | 1.00 fgh             | 35.2 efghijk   | 23.8 abcd       | 22.2 bcdefghi |
| T.E090   | 0.70 abc             | 31.3 defghijk  | 38.7 cde        | 12.5 abcd     |
| T.E093   | 1.10 ghj             | 22.2 bcdefghi  | 19.4 abcd       | 47.2 n        |
| T.E094   | 0.90 def             | 22.7 bcdefghi  | 43.5 e          | 22.2 bcdefghi |
### Table 3. The Analytic Hierarchy Process (AHP) total score of the best six *Trichoderma* isolates.

| Isolates | Growth rate | AHP Score | Total Score |
|----------|-------------|-----------|-------------|
|          | (cm/day)    | Fusarium  | Cylindrocladium | Rhizoctonia |
| T.E069   | 0.1457      | 0.08022   | 0.04966       | 0.02964     | 0.30522 |
| T.E060   | 0.1457      | 0.03056   | 0.08022       | 0.04788     | 0.30436 |
| T.E039   | 0.1457      | 0.08022   | 0.03056       | 0.01824     | 0.27472 |
| T.E100   | 0.0868      | 0.04966   | 0.08022       | 0.04788     | 0.26456 |
| T.E104   | 0.0868      | 0.04966   | 0.08022       | 0.04788     | 0.26456 |
| T.E063   | 0.1457      | 0.01910   | 0.04966       | 0.02964     | 0.24410 |

The value of the consistency ratio of the AHP in this study is <0.1000, which indicates that the assessment of the comparison weight of the criteria and sub-criteria is consistent. This method has been previously reported to select endophytic bacteria based on their physiological and antagonistic characteristics in eucalyptus plants [15] and rice plants [16]. This method can be used to select isolates...
of microorganisms with many characters and with the weight of the assessment chosen based on the level of importance.

3.3 In-planta application

In the in-planta experiment, there were variations in seedling mortality from 2 to 10 weeks after application (WAP) between treatments (P<0.000) from 29.2 – 57.6 % (Figure 2). All Trichoderma treatments can reduce seedling mortality up to 49%. The reduced mortality rate indicates the existence of seedling protection attributed to the Trichoderma treatments. Seedling mortality was caused mainly by dumping-off disease (Rhizoctonia sp., Fusarium sp.), Cylindrocladium blight and bacterial wilt (Ralstonia pseudosolanacearum). The treatments of Trichoderma T.E069, T.E100, T.E104 and T.Mix are better than the chemical fungicide treatment. It implies that endophytic Trichoderma can be used as a substitute for chemical fungicides.

![Figure 2. Effect of endophytic Trichoderma spp. on Eucalyptus pellita seedling mortality.](image)

Consistent results were found in seedling performance at the end of the observation. Two treatments, T.E069 and T.Mix, showed statistically better plant height than the untreated control and chemical fungicide treatments. However, there were no significant differences detected for RCD. Meanwhile, in the nursery rate parameter, T.104 and T.Mix treatments resulted in a higher the nursery rate value than the untreated control and chemical fungicide treatments (Table 4). Nursery rate is the success rate of the nursery in producing seedlings according to predetermined standards. Good seedling standards must be achieved: plant height, RCD, number of leaves, root compactness, and seedling health. In general, using single and/or a consortium of isolates can reduce the seedlings’ mortality rate and increase seedling height and nursery rate. In summary, the tested isolates can improve plant vigor, which would later make the plant more resilient against root and foliar diseases in plantations.

Trichoderma spp. is filamentous fungi that exhibit different modes of action such as growth regulators, competition by space and nutrients, and reduction of the damages caused by pathogens that affect the development of the roots [17]. Plants are protected from numerous plant pathogen classes by responses similar to acquired systemic resistance and rhizobacteria-induced systemic resistance. Root colonization by Trichoderma spp. also frequently enhances root growth and development, productivity, resistance to abiotic stresses and the uptake and use of nutrients [18]. Thus, Trichoderma
has the potential to be developed as a biofertilizer for eucalyptus seedlings because of their ability to enhance nutrient uptake and to improve environment stresses tolerance, as previously reported in Chinese cabbage [19]. In addition, as certain species of Trichoderma have high compatibility with fungicides, this could allow its use in combination with different disease management strategies [20].

Table 4. Eucalyptus pellita seedling height, root collar diameter (RCD) and nursery rate after in-planta application of Trichoderma spp.

| Treatment | Seedling Height (cm)* | RCD (mm)* | Nursery Rate (%) |
|-----------|-----------------------|-----------|-----------------|
|           | 5                     | 9         | 12              | 5     | 9     |         |
| T.E039    | 20.75 b               | 35.49a    | 43.55ab         | 1.57  | 3.70 a | 27.09  |
| T.E060    | 21.42 b               | 37.62a    | 44.20ab         | 2.08  | 3.71 a | 20.83  |
| T.E063    | 20.42 b               | 36.88a    | 43.76ab         | 1.73  | abc   | 29.17  |
| T.E069    | 20.97 b               | 38.80a    | 48.33b          | 2.03  | 3.71 a | 26.39  |
| T.E100    | 20.05 b               | 33.49a    | 42.09a          | 1.97  | c     | 31.60  |
| T.E104    | 19.42 b               | 34.27a    | 39.28 a         | 1.60  | ab    | 38.89  |
| T.Mix     | 21.60 b               | 37.78a    | 47.92 b         | 1.77  | abc   | 40.63  |
| Untreated | 10.77 a               | 34.75a    | 42.22 a         | 2.07  | c     | 31.94  |
| Fungicide 1 g/L | 18.26 b | 36.17a | 48.33b | 1.95  | 3.70 a | 26.39  |

Note: * Week after application

4. Conclusion

Based on the results of the AHP analysis of the Trichoderma growth rate and their efficacy in suppressing the growth of the pathogens, T.E039, T.E063, T.E069, T.E060, T.E100 and T.104 are selected as the best six isolates. The use of single and, or a consortium of the isolates increases seedling height and reduces the mortality rate of the seedlings. The isolates also can improve plant vigor, which would later make the plant more resilient against root and foliar diseases in plantations. They should therefore be considered as environmentally friendly substitutes for chemical fungicides.

Acknowledgements

Research fellowship granted by the Alexander von Humboldt Foundation of the Government of the Federal Republic of Germany to AG is gratefully acknowledged.

References
[1] Gafur A 2021 Environ. Sci. Proc. 3 11
[2] Gafur A 2021 submitted for publication to IOP Conf Series: Earth and Environmental Science
[3] Vinale F, Sivasithamparam K, Ghisalberti E L, Marra R, Barbetti M J, Li H, Woo S L and Lorito M 2008 Physiol. Mol. Plant P. 72 80-6
[4] Sharma J K, Mohanan C and Maria Florence E J 1984 Eur. J. Forest Pathol. 1477-89
[5] Old K M, Wingfield M J and Yuan Z Q 2003 A Manual of Diseases of Eucalypts in South-East Asia (Bogor: Center for International Forestry Research (CIFOR)) p 98
[6] Tseng Y-H, Rouina H, Groten K, Rajani P, Furch A C U, Reichelt M, Baldwin I T, Nataraja K N, Uma Shaanker R and Oelmüller R 2020 Front. Plant Sci. 11 573670
[7] Nawaz K, Shahid A A, Bengyell L, Subhani M N, Alia M, Anwar W, Iftikhar S, and Ali S W 2018 Sci. Hortic. 239 242–52
[8] Ezziyyani M, Requena M E, Egea-Gilabert C and Candela M E 2007 J. Phytopathol. 155 342–9
[9] Saaty T L 2008 Int J Services Sciences. 1 83–98
[10] Bell D K, Wells H D and Markam C R 1982 Phytopathology 72 379–382
[11] Panwar V, Aggarwal A, Singh G, Verma A, Sharma I and Saharan M S 2014 Journal of Wheat Research 6 1-5.
[12] Zhang Y and Zhuang W Y 2020 Biol. Control 142 104151
[13] Adnan M, Islam W, Shabbir A, Khan K A, Ghramh H A, Huang Z, Chen H Y and Lu G D 2019
Microb. Pathog. 129 7–18.

[14] Nusaibah S A and Musa H 2019 in Trichoderma - The Most Widely Used Fungicide. Ed. Shah M M, Sharif U and Buhari T R (London: IntechOpen)

[15] Susanti Y, Giyanto, Sinaga M S, Mutaqin K H and Tjahjono B 2021 Biodiversitas 22 3454-62

[16] Dewi R S, Giyanto G, Sinaga M S, Dadang D and Nuryanto B 2020 Jurnal Fitopatologi Indonesia 16 37-48

[17] Hermosa R, Viterbo A, Chet I and Monte E 2012 Microbiology 158 17–25.

[18] Harman G E, Howell C R, Viterbo A, Chet I and Lorito M 2004 Microbiology 2 43-56.

[19] Shida J, Liu Z, Liu B, Wang Y and Wang J 2020 Scientia Horticulturae 262 109069

[20] Sánchez-Montesinos B, Santos M, Moreno-Gavíra A, Marín-Rodulfo T, Gea F J and Diánez F 2021 J. Fungi 7 598