# Molecular Identification of *Trichoderma* Isolates and Their Effect in Combination with *Zyngiberaceae* Extracts on Maize Downy Mildew Incidence and Plant Growth

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**Abstracts:** Research was done to molecularly identify *Trichoderma* isolates and to study the effect combination of *Trichoderma* isolates and *Zingiberaceae* extracts on downy mildew intensity as well as the growth of maize. The experiment was arranged in completely randomized design with three replicates. The experiment consisted of combination between *Trichoderma* isolates (no *Trichoderma*, East Lampung isolate, and Plant Clinic isolate) and *Zingiberaceae* extracts: ginger/jahe (*Zingiber officinale*), turmeric/kunyit (*Curcuma longa*), lengkuas (*Alpinia galanga*), galangale/kencur *(Kaempferia galanga),* temulawak (*Curcuma zanthorrhiza*), and temu hitam (*Curcuma aeroginosa*). Variables observed were disease incidence, incubation periode, plant height, and dry matter weight. Data were analyzed using analyses of variance and the difference between means were tested using LSD test at 5% significant level. Based on molecular identification, both *Trichoderma isolates* (East Lampung isolates and Plant Clinic isolate) are *Trichoderma asperrelum.* The results showed that combination of *T. asperellum* isolates and *Zingiberaceae* extracts lowered the disease incidence of maize downy mildew, prolonged the incubation period, increased plant height and dry matter weight. The best result was shown by combination of *T. asperellum* isolate from NTF East Lampung and tumeric extract.

**Keywords:** Downy Mildew, *Peronosclerospora, Trichoderma*, *Zingiberaceae* Extract

## 1. INTRODUCTION

Downy mildew is one of many serious disease in maize production areas worldwide, including Indonesia. Three *Peronosclerospora* species have been reported as the causative agents of corn downy mildew in Indonesia namely, *P. sorghi*, *P. Phillipinensis* or *P. maydis*. It is now confirmed that a species of Peronosclerospora found in Australia i.e. *P. australiensis* [1] is a synonym of *P. maydis* [2]. Recently, a new species of Peronosclerospora namely *P. neglecta,* has been reported to be one of the causative agents of corn downy mildew in Indonesia [3]. The disease incidence caused by this pathogen varies among maize producing areas, depending on the varieties that are grown. Heavy downy mildew may result in total loss, especially in a susceptible variety. In Indonesia, especially in Lampung Province, the disease in 1996 caused 100% of yield loss [4, 5].

So far, disease management of maize downy mildew is administered intergratedly using resistant varieties and application of fungicides, such as metalaxyl as seed dresser [6]. However, the use of metalaxyl is considered causes some negative impact for the environment [7]. The effect of metalaxyl fungicide in the soil is greatly influenced by the soil type and resulted in a change of soil microbial [8]. Beside that, long time usage of methalaxyl has resulted in the occurance of resistance of the fungal pathogens [9, 10, 11]. When resistant fungi found in the field, the effectiveness of metalaxyl against the pathogens will be steadily decrease [12, 10]. [13] stated that metalaxyl that was used to be effective against maize downy mildew caused by *Peronosclerospora sorghi* was not anymore. It is suspected that some downy mildew explosions are caused by the presence of resistant fungal variants of *P. sorghi*, *P. maydis*, and *P. phillipinensis*. In their host, it seemed that the fungi have evolved become new races with higher virulence [14]. Therefore it is very necessary to find an alternatif control method to overcome the problem caused by resistant fungal variants.

Biological control has been considered as one of important component in integrated control. The use of microorganisms such as *Trichoderma* spp. to protect plants is considered risk-free to the environment. Trichoderma is one of the soil microorganisms that has been used as a plant pathogen biocontrol that can also function as a biofertilizer for plants. Most species of the genus Trichoderma are included in plant growth-promoting fungi that produce phytohormones and the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can reduce ethylene (ET) at high concentrations that can inhibit plant growth [15,16]. The mechanisms of biocontrol consisted of toxin production, mycoparasitism, competitor for spaces and resources [17, 18]. *Trichoderma* spp. are also considered as plant growth promoting fungi because of the presence of growth-regulating hormones and formation of iron chelating siderophores that can stimulate tissues in young parts. *Trichoderma* spp. can interact with other microbes in the rhizosphere, form endophytic associations and thereby influencing plant growth, yield, and disease protection. *Trichoderma* spp. has been reported to be able to survive in botanical fungicide such as turmeric powder, galangale powder and clove leaves powder [19]. Application of *Trichoderma* sp. can reduce the incidence of downy mildew in Pacific 105 cultivar maize [20].

As for an alternatif of synthetic fungicide, several plant extracts have been known for their effectiveness against plant pathogens. For agricultural disease management, the plant extracts are best suited if freshly used. Several plants have cabability to synthesize and produce some aromatic secondary metabolites such phenolic acids, phenols, quinones, flavonoids, flavones, flavanols, tannins and coumarin as active ingredients against plant pathogens [21]. Many members of the family of *zyngiberaceae* are also known producing active ingredients against fungi or act as a source of natural antifungal. However, their effectiveness has not been studied on downy mildew of maize. This study aimed to molecularly identify trichoderma isolates and to investigate the effect of those *trichoderma* isolates and *zyngiberaceae* extracts combinations on maize downy mildew as well as on maize plant growth. To our knowledge, biocontrol of maize downy mildew using combination of trichoderma and *Zingiberaceae* extract has not been reported under this environmental scenery what makes of this research study a significant contribution to this field.

## 2. MATERIAL AND METHODS

### 2.1. Molecular Identifiation of *Trichoderma* Isolates

Two *Trichoderma* isolates from the collection of Unila Plant Clinics, namely East Lampung (NTF) isolate and Unila Plant Clinics (KTU) isolate used in this study need to be identified. Molecular identification of those *Trichoderma* isolates was carried out based on the ITS1-5.8S-ITS2 rDNA sequence and using universal primer pairs ITS1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse) (5′-TCCTCCGCTTATTGATATGC-3′) according to [22]. The *Trichoderma* spp. fungal DNA genome was extracted with QiAmp DNA mini kit from Germany. The purity of the extracted DNA concentration was tested with a nanophotometer. High-purity DNA was amplified by PCR (Sensodirect Thermocycler from Sensoquest, Germany). All PCR samples were electrophoresed using QIAXCEL ADVANCED with DNA HIGH RESOLUTION KIT. Electrophoresis does not use agarose but uses a KIT containing packaged agarose so that the electrophoresis results band is analyzed directly digitally. The purification as well as the sequencing of PCR results was carried out by PT. Korean Bioner, using the same primer. To determine the species, the nucleotide sequences of the samples were analyzed using the BioEdit Sequence Alignment Editor version 7.0.9.1. ITS segment sequences were aligned using the Clustal Multiple Alignment program [23] and compared to MycoBank DNA data (http://www.mycobank.org) and BLAST data (http://www.blast.ncbi.nlm.nih.gov/blast).

### 2.2. Pot Experiment

The experiment was conducted in the Plant Pests and Diseases Laboratory field, Faculty of Agriculture University of Lampung from April to August 2022. The treatments were arranged in completely randomized design with three replicates. The treatments were 12 combinations of *Trichoderma* sp. isolates (T) and *Zingiberaceae* extract (F). The *Trichoderma* sp. treatments were without *Trichoderma* (T0), *Trichoderma* East Lampung isolate (T1), and *Trichoderma* Plant Clinics isolate (T2), whereas extract of *Zingiberaceae* treatments consisted of no

*Zingiberaceae* (F0), *Zingiber officinale* (jahe/ginger, F1), *Curcuma longa* (kunyit/turmeric, F2), *Alpinia galanga* (lengkuas/galangal, F3), *Kaemferia galangan* (kencur/aromatic ginger, F4), *Curcuma zanthorrhiza* (temulawak, F5), and *Curcuma aeroginosa* (temu hitam, F6). The 12 treatment combinations were T0F0 (control), T1F1 (Trichoderma East Lampung isolate + ginger extract), T1F2 (Trichoderma East Lampung isolate + turmeric extract),, T1F3 (Trichoderma East Lampung isolate + lengkuas extract),, T1F4 (Trichoderma East Lampung isolate + galangale extract), T1F5 Trichoderma East Lampung isolate + temulawak extract), T1F6 (Trichoderma East Lampung isolate + temu hitam extract), T2F1 (*Trichoderma* Plant Clinics isolate + ginger extract), T2F2 (*Trichoderma* Plant Clinics isolate + turmeric extract), T2F3 (*Trichoderma* Plant Clinics isolate + lengkuas extract), T2F4 (*Trichoderma* Plant Clinics isolate + kencur extract), T2F5 (*Trichoderma* Plant Clinics isolate + temulawak extract), and T2F6 (*Trichoderma* Plant Clinics isolate + temu hitam extract). The variables observed were incubation period, disease incidence, plant height, and dry weight of stover, and those data were analyzed using analyses of variance. The difference between means were tested using LSD test at 5% significant level.

### 2.3. Preparation of Plant Extracts

Tubers of *Zingiberaceae* as much as 200 g were cut into small pieces, dried in oven at $50^{O}$C for 36 hours. The dried tuber then was grinded and sieved to collect fine powder. An alliquot was made by diluting about 10 g of the powder in 100 ml of sterile aquadest, and then sieved with cloth [21]. The filtrate was centrifuged at 3000 rpm as long as 10 minutes, the supernatant was collected and the pellete was discarded.

### 2.4. Application of *Trichoderma* and Inoculation of *Peronosclerospora Australiensis*

Maize seeds (F2 of P27 variety) were planted in polybags containing 5 kg mixture of sterilized soil and goat manure (2:1). Each experimental unit consisted of three polybags, and within each polybag was planted five seeds. Suspension of *Trichoderma (*$2.4 \times 10^6$) as much as 10 ml was poured around the maize plant base at 5 days after planting. Conidia of *P. australiensis* was collected at about 1.00 am from the field in Tegineneng, Pesawaran Regency - Lampung. The conidia were collected from diseased leaves by spraying their lower surface diseased leaves with water, rubbed with a small brush, and put into a beaker glass. The collected spores were homogenized with a rotary mixer before inoculated to the growing point of maize seedlings [24]. Twelve days after planting, artificial inoculation was done at 2.00-3.00 am, by dopping conidia suspension ($4.8 \times 10^5$ spore) into the seedlings growing point.

**2.5. Observation. -** Observation was conducted for five weeks since planting time. Variables observed consisted of incubation period (days), disease incidence (DI, %), plant height (cm), and plant dry weight (g). Disease incidence (DI) of downy mildew was calculated as follows:

$DI = n/N \times 100\%$

DI = Disease Incidence

n = number of diseased plants

N = number of all plants investigated

## 3. RESULTS AND DISCUSSIONS

### 3.1 Molecular Identification

The phylogenetic analysis based on PCR amplification, sequencing, and sequence analysis of internal transcribed spacer (ITS 1 and 2) regions shows that the two identified Trichoderma isolates are found to be in the same group with the reference strains of *Trichoderma asperellum* isolate T2 (MK210562.1), *Trichoderma asperellum* isolate T4 (MK210428.1), and *Trichoderma asperellum* isolate upm12 (MK027315.1 (Figure 1). These

phylogenetic study results support and confirm that the investigated Trichoderma isolates are *Trichoderma asperellum.*

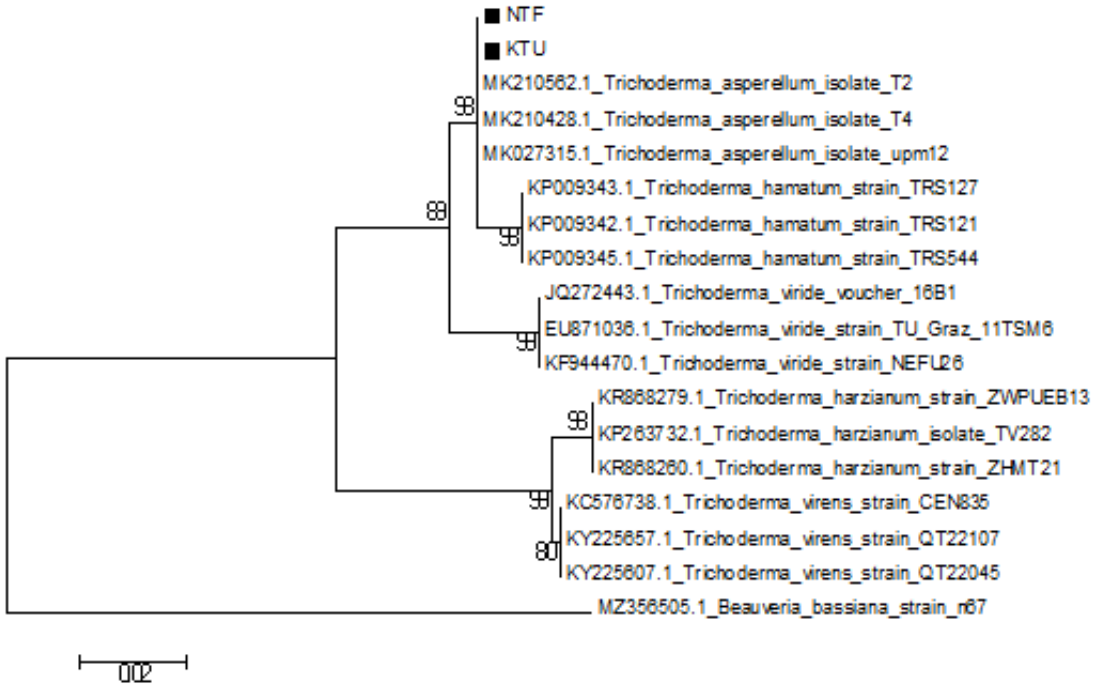


Figure 1. The dendogram of trichoderma isolates based on its analysis using the unweighted pair group method with arithmetic mean (UPGMA) (1000x bootsrap). As the out group, *Beauveria bassiana* strain n67 (Acc. No. Mz356505.1) was used.

### 3.2 Incubation Period

The data showed that combination between *Trichoderma* isolates and *Zingiberaceae* extracts significantly increase the incubation period or delay the progress of downy mildew symptom (Table 1). The prolonged incubation period suggested that the treatments may weaken the pathogen inoculum or delay the infection process. Generally, *Trichoderma spp.* can induce the plant resistance by producing chitinase and glucanase that are able to crush and breakdown the pathogen cell wall. –*Trichoderma asperellum* T-203 (formerly *T. harzianum* T-203) has been reported can provide protection to cucumber plants against *Pseudomonas syringae* pv. *lachrymans* (*Psl*) by inducing systemic resistance [25]. Several mechanisms of action of Trichoderma that are recognized as biocontrol agent are antibiosis, mycoparasitism, competition with the pathogen, enhanced plant-tolerance against abiotic stresses, promotion of plant growth, and stimulation of its defenses against pathogens [26]. Another reaserch proved that in-vitro assays of *T. asperellum* showed their capacity to antagonize *Sclerotium cepivorum*, the causal agent of onion white rot [27]. *Zyngiberaceae* extract was considered as potential biofungicide because contain some active ingredients that inactivates several fungal pathogens such as *Phytophthora infestans*, *Fusarium solani*, and *Pyricularia oryzae* [28].

### 3.3 Disease Incidence

The results of this experiment show that all of the treatments significantly lowered the disease incidence of maize downy mildew (Table 1). The progress of disease incidence of all the treatments tend to increase from time to time, and the fastest is found on the control. The lowest disease incidence is found after the treatment of combination *Trichoderma asperellum* isolate East Lampung and extract of *C. Aeroginosa* (T1F6). The data also showed that *Curcuma longa* (F2), and *Curcuma aeroginosa* (F6) were more synergitic when combined with *Trichoderma* East Lampung isolate (T1) than that of *Trichoderma* Plant Clinics isolate (T2). The potential of all combinations to

decrease downy mildew incidence were consistent since the early observation through the last observation. Application of *Trichoderma* together with *Zingiberaceae* plant extracts decrease the incidence of maize downy mildew for some reasons [29]. Plant extracts that were used in this experiment was obtained from the family of *zyngiberaceae* that was known produce substances that inactive microbes included several fungal plant pathogens. For examples, turmeric (*Curcuma longa* Linn.) and ginger (*Zingiber officinale* Rosc.) can inactivate *Phytophthora infestans*, *Fusarium solani*, and *Pyricularia oryzae* [28]. [30] reported that *A. galanga* can control stem rot disease of vanilla. Furthermore, *Trichoderma* spp. have also been reported induce systemic resistance in plants if applied in soil [26, 31]. Moreover, [20] showed that application of *Trichoderma* sp. isolate 14 resulted in the decreasing of maize downey mildew incidence on cultivar Pacific 105. This result is in line with the reults from other researchers [32, 33]. Recently it was reported that application of *T. asperellum* and *C. aeroginosa* separately also significantly reduced the disease severity and disease incidence of maize downey mildew [34], *C. aeroginosa* also reduced the percentage of *P. sclerospora* sp. spore germination and prolonged the incubation period [35].

### 3.4 Plant Height and Plant Dry Weight

In general, the data showed that all the treatments significantly increased the plant height (Table 1). The plant dry weight was also significantly increased after the treatments, except for the treatments of *Trichoderma asperellum* isolate East Lampung and extract of galangale (T1F4). The application of *Trichoderma* and plant extracts increased plant growth that might be related to the ability of plant extracts that directly inactivate pathogen inoculum and keep the growth of the maize plants. *Trichoderma* spp. capable of increasing root growth and crop yield, as well as proliferation of secondary roots, seedling fresh weight and foliar area [36]. Colonization of maize rhizosphere by *Trichoderma virens* also can induce higher photosynthetic rates and systemically increase the $CO_2$ uptake in leaves [37].

**Table 1. The effect of *T. asperellum* isolates and *zyngiberaceae* extract combinations on the observed variables**

| Treatments | Incubation period (days) | Disease Incidence (%) | Plant height (cm) | Dry Weight (g) |
|---|---|---|---|---|
| T0F0 | 12 .28 a | 94.44 d | 57.41 a | 9.14 a |
| T1F1 | 22.00 b | 44.45 ab | 97.49 c | 14.18 b |
| T1F2 | 23.83 c | 33.33 ab | 96.36 c | 18.27 c |
| T1F3 | 24.39 c | 33.33 ab | 93.14 bc | 18.86 c |
| T1F4 | 23.11 bc | 33.34 ab | 94.40 c | 13.40 ab |
| T1F5 | 19.44 b | 55.56 c | 87.32 b | 14.07 b |
| T1F6 | 26.89 c | 16.67 a | 92.34 b | 14.12 b |
| T2F1 | 25.50 c | 27.78 a | 94.71 c | 14.84 bc |
| T2F2 | 21.28 b | 50.00 bc | 91.48 b | 21.20 d |
| T2F3 | 25.11 c | 33.33 ab | 100.17 d | 22.26 d |
| T2F4 | 22.39 b | 38.89 ab | 98.51 cd | 18.99 cd |
| T2F5 | 24.44 c | 33.33 ab | 99.10 cd | 18.72 c |
| T2F6 | 19.39 b | 61.11 c | 88.97 b | 20.03 d |
| BNT 5% | 4.05 | 20.88 | 6.17 | 4.70 |

Note: Number in the same column followed by the same letter is not significantly different according to LSD test α=5%. T0: No *Trichoderma*, T1: *Trichoderma asperellum* isolate East Lampung, T2: *Trichoderma asperellum* isolate Unila Plant Clinic; F0: no *Zingiberaceae* extract, F1: *Zingiber officinale*, F2: *Curcuma longa*, F3: *Alpinia galanga*, F4: *Kaemfira galanga*, F5: *Curcuma zanthorrhiza* , F6: *Curcuma aeroginosa* extract.

## CONCLUSIONS

The phylogenetic analysis based on an internal transcribed spacer region (ITS) showed that the two tested Trichoderma isolates were identified as *Trichoderma asperellum.* All treatments of *T. asperellum* isolates and *Zingiberaceae* extracts prolonged the incubation period, decreased the disease incidence of downy mildew, and increased plant height as well as dry matter weight of maize plant. The best treatment was shown by combination of *Trichoderma* isolate from East Lampung (NTF isolate) with tumeric extract. The application of *T. asperellum* was shown to improve plant growth as indicated by higher plant dry weight and plant height compared to the control.

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