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Tolerance of *Trichoderma asperellum* Isolates to Chemical Fungicide and Their Antagonistic Activity against *Phytophthora infestans*

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Authors' contributions

This work was carried out in collaboration between all authors. Author Elika designed the study, managed the literature searches, wrote the protocol, and wrote the first draft of the manuscript. Author Ni'matuzahroh managed the analyses of the study. Author Kusriningrum performed the statistical analysis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Trichoderma constitute filamentous fungi which are frequently used as biocontrol agents against plant pathogenic fungi. The tolerance of these fungi to chemical fungicides is a prerequisite for biocontrol agents for their application to suppress the growth of some soil-borne pathogens. The objectives of this study were to evaluate tolerance of *Trichoderma asperellum* local isolates TK and TS from Batu-East Java, to *mefenoxam* fungicide, as well as to identify *in-vitro* the antagonistic activity of *T. asperellum* against the plant pathogen *Phytophthora infestans* by using dual-culture method. Thus, an integrated approach of chemical and biological methods was used to control the growth of *P. infestans*. This study was conducted at the Department of Biology, Faculty of Science and Technology Airlangga University, Indonesia, during March through December, 2012. *T. asperellum* isolates TK and TS were evaluated *in-vitro* for their efficacy against *P. infestans* and tolerance to 5000 ppm *mefenoxam*. The results showed that the growth of the two *T. asperellum* isolates on Potato Dextrose Agar (PDA) medium supplemented

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with 5 mL/L *mefenoxam* was up to 67%, while their antagonistic activity against *P. infestans* in dual-culture was 5,08% and 16,37%. The results are expected to help in selection of potential biocontrol agents.

Keywords: Trichoderma; biocontol agents; mefenoxam; Phytophthora infestans.

1. INTRODUCTION

Genus *Trichoderma* are important biocontrol agents (BCAs) against several soil-borne pathogens. *Trichoderma* used different mechanisms to control pathogens includes mycoparasitism, secretion of antibiotics, competition for space and nutrients, as well as secretion of lytic enzymes [1]. According to [2], the activities of *Trichoderma* as BCAs mainly depend on physicochemical factors of environment where they are subjected.

The fact that the individual use of chemical control caused environmental pollution, it triggered the existence of the IPM (Integrated Plant Disease Management) strategy. A part of the IPM strategy is to combine biological control with chemical fungicide. Emerging strategies for plant disease management involve biological and integrated control by applying antagonistic microrganisms alone or in combination with fungicides [3,4,5,6]. Several studies have shown that a large number of plant-beneficial microorganisms e.g. yeasts, yeast-like fungi, filamentous fungi and bacteria, protect plants against postharvest [7,8,5,6] and or soil-borne pathogens [9,10,11,5].

The chemical control is increasingly limited because of environmental and toxicological risks as well as the onset of fungicide-resistant strains of fungal pathogens. Moreover, the legal limits of chemical residues left by pesticides in imported fruit are much lower in some countries, thus discouraging the use of chemical products. Biocontrol by antagonistic microorganisms appears to be a promising tool for fungal disease and minimizing the use of fungicides [12,8]. Nevertheless, BCAs are often insufficient to control diseases when applied alone in field under practical conditions. Therefore, integrated approaches based on the combination of BCAs and fungicides or alternative means have been suggested to prevent a resistance increase in the pathogen population and limit risks due to intensive use of chemicals [13].

For successful biological control of pathogens, BCAs that tolerate chemical fungicides are needed. There is an emerging necessity for *Trichoderma* which would be a prerequisite for their application in combination of chemical and biological control [14]. Some strains of *Trichoderma* show compatibility with fungicides as they are tolerant to fungicides and successfully used in the IPM strategy [15,16]. It was reported that integration of both control strategies showed a positive association, by reducing infection, compared to their individual applications [17].

Mefenoxam belongs to a class of *phenylamides*, systemic fungicides spesifically for controlling plant pathogens of *Oomycetes* e.g. *Phytophthora, Pythium,* and *Peronospora* [18,19]. According to FRAC *(Fungicide Resistance Action Committee)* Classification, *phenylamides* are a class of high risk fungicides that cause pathogen resistance. Whereas, the effects of *mefenoxam* to BCAs *Trichoderma* existence are still unknown.

The objectives of this study were to assay tolerance of *T. asperellum* isolates TK and TS isolates at 5000 ppm *mefenoxam* as well as to identify *in-vitro* the antagonistic activity of T. *asperellum* isolates TK and TS against *P. infestans* by using the dual-culture method.

2. MATERIALS AND METHODS

2.1 Isolation of *Trichoderma* Species

Six local isolates of *Trichoderma* spp. were used in this study and isolated from rhizosphere soil of horticulture crops including onions, potatoes, banana, carrots, and celery in Batu-East Java, Indonesia and were maintained on PDA medium (Difco^{TM}, USA). Two isolates (TK and TS) were identified as *T. asperellum*, while the other four isolates (TB, TP, TS2, and TW) have not been identified at the species level. Pathogenic fungi of *P. infestans* were obtained from the culture collection of Balai Penelitian Tanaman Hortikultura Bandung-West Java, Indonesia and were maintained on V8 juice Agar (Campbell Soup Co., USA). *Mefenoxam* (PT. Syngenta Indonesia) was obtained as a commercial fungicide.

2.2 Fungistatic Activity

In-vitro fungistatic activity of *mefenoxam* was studied on PDA medium. *Mefenoxam* was dissolved in sterile distilled water and supplemented to autoclaved medium, resulting PDA-*mefenoxam* plates in various concentrations. A mycelial plug of five-day-old *T. asperellum* isolates TK and TS culture were grown on PDA and transferred to PDA-*mefenoxam* plates. Tolerance of *T. asperellum* isolates TK and TS to *mefenoxam* were estimated according to colony diameters and compared to average colony diameters from non-supplemented media after seven days of incubation [20,21,22].

2.3 Dual Culture Technique

For dual-culture, a mycelial plug of *P. infestans* (5mm diameter) from a seven-day-old culture was placed on PDA-supplemented 5000 ppm *mefenoxam*, about one centimeter from the edge of Petri dish. After 72 hours, a mycelial plug of *T. asperellum* isolates TK or TS from the colony margin of a five-day-old culture was placed at an opposite side on the same Petri dish. Similarly, Petri dish inoculated with *P. infestans* alone was used as control. The treatments were replicated three times and incubated at 28°C for seven days. The antagonistic activity of *T. asperellum* isolates TK and TS were estimated according to two criteria, i.e. the pathogen growth inhibition radius and the antagonism class system described by [23]. Radius growth inhibition was calculated in relation to the growth of the control as follows:

$$\% \text{ inhibition of radius mycelial growth} = \frac{X-A}{X} \times 100\%$$

where X was the radius growth of pathogen in the control plate and A was the radius growth of pathogen in presence of *Trichoderma* [24,25,26].

2.4 Statistical Analysis

The data of all experiments were statistically analyzed using ANOVA (analysis of variance) and LSD (Least Significant Difference) test to determine the statistically significant differences [27].

3. RESULTS AND DISCUSSION

3.1 Tolerance *T. asperellum* Isolates TK and TS to 5000 ppm *Mefenoxam*

The two *T. asperellum* isolates TK and TS showed growth greater than 50% on PDA-5000 ppm *mefenoxam* plates (Fig 1). This described that both isolates were able to grow when they were exposed to chemical fungicides.

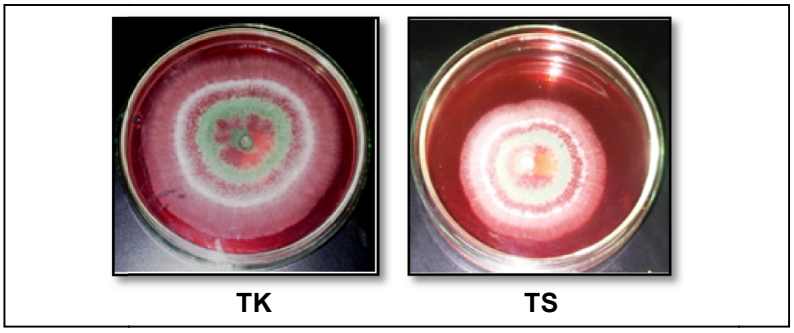

Fig. 1. *In-vitro* growth of *T. asperellum* isolates TK and TS on PDA-5000 ppm *mefenoxam* plates seven days after incubation

While, morphological characters of these isolates that are expressed in the shape and colour of the colony are presented in Table 1. This result indicated that *T. asperellum* isolates TK and TS tolerant to *mefenoxam* and they were considered as resistant isolates to *mefenoxam* fungicide at recommended maximum concentration for field application.

Table 1. Morphological expression of *T. asperellum* isolates TK and TS at 5000 ppm *mefenoxam* seven days after incubation

Isolates	Colony Characteristic	Radial growth (mm)	Tolerance to *mefenoxam* (%)	Category of resistance
TK	colony radial, white-green	62 ^{a}	67.1	Resistant
TP	colony radial, dark green	61^{ab}	65.9	Resistant
TW	colony radial, white-green	59.3^{abc}	63.5	Resistant
TS	colony radial, white-green	57 ^{abcd}	61.2	Resistant
TS_2	coloni spread, dark green	51^{e}	54.1	Resistant
TB	colony spread and radial, dark green	51^{e}	54.1	Resistant

**The number followed by the same letter in the same column were not significantly different at LSD=5%; *Resistant: radial growth on medium supplemented mefenoxam > 50% of that on non-supplemented media, Intermediate: radial growth on medium supplemented mefenoxam >40-50% of that on non-supplemennted media, Sensitive: radial growth on medium-supplemented mefenoxam < 40% of that on non-supplemented media [28,29]*

The ability of *Trichoderma* to withstand chemical stresses including those associated with mycoparasitism has been well studied. The two *T. asperellum* isolates TK and TS used in this study are evidently tolerant to 5000 ppm *mefenoxam*. Both isolates were able to grow when exposed to chemical fungicides without the loss of ability to sporulate and germinate. This result also clarified that *T. asperellum* isolates TK and TS were resistant isolates to *mefenoxam* fungicide which could rapidly grow at recommended maximum concentration for field application. According to [30], the tolerance of *Trichoderma* is one of the keys to ecological success and is a prerequisite for IPM implementation. [31] explained that the ability of *Trichoderma* to tolerate relatively high concentrations of a variety of synthetic and natural toxic compounds, depends on the efficiency of the cell detoxification mechanisms supported by a complex system of membrane pumps. Unfortunately, the mechanism of defence system are still fully unknown.

Tolerant describes a capability of organisms to survive under various surrounding pressure particularly diverse environmental effects [32]. Tolerance of fungi to chemical fungicides related to ABC-transporter gene have been intensively published in the last few years. It was hypothesized that ABC-transporter has an important role in a number of processes such as resistance to environmental toxicants produced either by soil microflora or introduced by human activity [31]. In this study, the rapid growth of *T. asperellum* isolates TK and TS on PDA-5000 ppm *mefenoxam* plates indicated that both isolates developed a tolerance mechanism to chemical fungicide pressure. Fungicide resistance is classified into two types, i.e. qualitative and quantitative resistance [33]. Furthermore, quantitative resistance constitutes a cell physiology reaction which is mediated by keeping the intracellular fungicide concentration still low. Several mechanisms may lead to this type such as: synthesis of efflux transporter secreting drug molecules to the extracellular space; modification of plasma membrane causing reduced fungicide permeability; synthesis of enzymes degrading fungicide molecules; over expression of the gene encoding the fungicide target; and utilization of alternative metabolic pathways.

3.2 Antagonistic Activity of *T. asperellum* Isolates TK and TS to *P. infestans*

T. asperellum isolates TK and TS had a high mycelial growth rate on both PDA-supplemented *mefenoxam* and PDA-non supplemented which are associated with the ability to control pathogenic fungi. The following data (Table 2) shows the mycelial growth of *T. asperellum* isolates TK and TS which were isolated from Batu-East Java, Indonesia.

Table 2. The mycelial growth of *T. asperellum* isolates TK and TS on both PDA-supplemented *mefenoxam* and PDA-non supplemented

***Trichoderma* isolates**	**Diameter colony (mm)**	
	PDA	**PDA+mefenoxam**
TB	90	51
TS2	90	51
TS	90	57
TW	90	59
TP	90	61
TK	90	62

These isolates were assessed for their antagonistic activity against *P. infestans* on PDA-5000 ppm *mefenoxam*, as presented in Table 3. The result showed that TS isolate exhibited

a better inhibition to mycelial growth of the pathogen (16,37 %) than TK isolate (5,08%). The following antagonisms degree in dual culture was scored on scale of 1-5 as proposed by [23]:

1. Antagonist completely overgrew the pathogen and covered the entire medium surface.
2. Antagonist overgrew at least two third of the medium surface.
3. Antagonist and the pathogen each colonized one half of the medium surface (more than one third and less than two third) and neither organism appeared to dominate each other.
4. The pathogen colonized at least two third of the medium surface and appeared to with stand encroachment.
5. The pathogen completely overgrew the antagonist and occupied the entire medium surface.

Table 3. The antagonistic activity of *T. asperellum* isolates TK and TS against *P. infestans* on PDA-5000 ppm *mefenoxam* seven days after incubation

***Trichoderma* Isolates**	**Radial growth of pathogen (mm)**	**Growth inhibition of pathogen (%)**	**Antagonism score**
TS	24.67 ^{e}	16.37	4
TK	28.00 ^{bcd}	5.08	4
TS_2	28/00 ^{bcd}	5.08	4
TB	28.67 ^{bc}	2.81	4
TP	30.00 ^{ab}	0	5
Tw	32.00 ^{a}	0	5

**The numbers followed by the same letter in the same column were not significantly different at LSD=5%*

**Antagonism score according to [23]*

In dual-culture, the two T. asperellum isolates TK and TS grew rapidly and covered the entire agar surface plates (Fig. 2). It indicated that its rapid growth gave an important advantage in the competition for space and nutrients against pathogen.

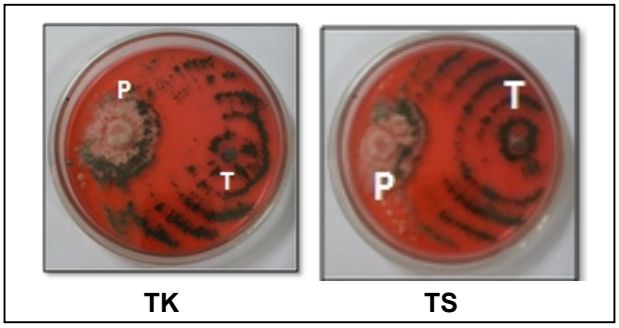

Fig. 2. Antagonistic activity of *T. asperellum* isolates TK and TS against *P. infestans* on PDA-5000 ppm *mefenoxam* seven days after incubation

Inhibition of plant pathogenic fungi by different species of *Trichoderma* was studied under *in vitro* conditions by many researchers [34,35,36]. *T. asperellum* are used as BCAs against a wide spectrum of plant disease-causing organisms, including fungi and nematodes

[37,38,39]. These species have also an antibacterial activity through the production of trichotoxin peptaibols [40]. The result of this study obviously demonstrated that *T. asperellum* isolates TK and TS were able to suppress the growth of pathogen although their ability was classified as low antagonistic activity. Similarly, [41] reported that *T. asperellum* showed the lowest inhibitory effect to *Fusarium oxysporum* f. sp. *phaseoli* (20.3%), it were compared to *T. reesei* (32.2%) as well as *T. atroviride* and *T. koninngii* both at 28.8%. Likewise, [42] stated that the low suppression observed, in some cases, in peat enriched with *T. asperellum* T-34 is consistent with results obtained for peat enriched with another *Trichoderma* strain combined with *Chryseobacterium*. According to [43], variations in the inhibitory potential may be due to the differences in the quantity and quality of the inhibitory substances produced by the antagonistic agents. Surely, the tested *Trichoderma* isolates also determine the variability of efficacy .

Although, their ability to pressure pathogen was considered low, *T. asperellum* isolates TK and TS used in this study evidently have rapid growth. These would give an important advantage in the competition for space and nutrients against pathogen, since competition constitutes an actual important mechanism of biological control. A competition may lead to pathogens control as long as the growth of BCAs result in reduction or inhibition of pathogen population. It was suggested that competition is most likely to be a successful biological control strategy depending on rapid colonization of *Trichoderma*, before pathogens established [1]. According to [2], the nature of competition is fungistatic or inhibitor. *Trichoderma* with rapid growth will be the winner of the competition for space and nutrients. Furthermore, they can provide colonization of rhizosphere allowing rapid establishment within microbial communities.

The other result of this study showed that tolerance of *T. asperellum* isolates TK and TS to 5000 ppm *mefenoxam* do not have a positive correlation with their antagonistic activity against *P. infestans* (Fig. 3).

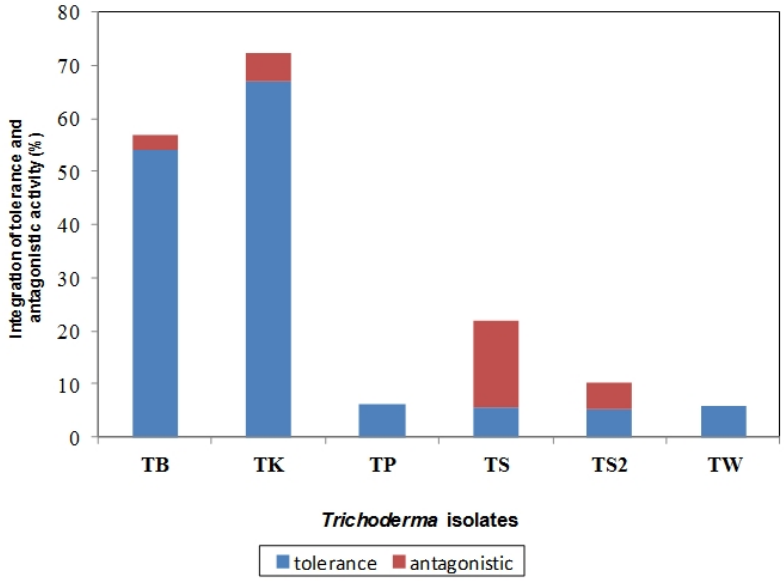

Fig. 3. Integration of tolerance to 5000 ppm *mefenoxam* and antagonistic activity of all *Trichoderma* isolates collected from Batu against *P. infestans*

The TK isolate showed high tolerance to *mefenoxam* but low suppression for pathogen. While, the highest antagonistic activity against *P. infestans* was demonstrated by the TS isolate which had lower tolerance to *mefenoxam* 5000 ppm than TK isolate.

4. CONCLUSION

The local isolates of *T. asperellum* TK and TS can be used as BCAs to protect potato plants from *P. infestans,* since they were tolerant to 5000 ppm *mefenoxam* up to 67%. Moreover, their rapid growth would evidently be a competition support to BCAs against plant pathogens. It is possible to develop *Trichoderma* tolerant of chemical fungicides without the decrease of the antagonistic activity. Nonetheless, further studies of integration on utilization of the local isolates as BCAs as well as chemical fungicide under field conditions is still recommended.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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