Screening of antagonistic fungi against web blight disease and identification of volatile metabolites produced by Trichoderma

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Abstract. Aerial web blight caused by Rhizoctonia solani is one of the main soil borne pathogens which infects soybean crops. Biological control using antagonistic fungi has been reported promising to reduce the incidence of this disease. This study aimed to determine antagonistic activity of Trichoderma virens and T. asperellum against R. solani and to investigate total phenolic changes in soybean plants which interact with those Trichoderma in infected soil. Volatile metabolites produced by Trichoderma were also detected and identified. Antagonistic activity of five isolates of T. virens and five isolates of T. asperellum were comparable; the ranges of in vitro antagonistic activities were 88.7-99.6% and 85.7-91.3%, respectively. Both Trichoderma were able to reduce R. solani infection. Disease intensities of 22-40% and 24-40% for T. virens and T. asperellum were observed lower than that of control (>50%). The increase of total phenolic and flavonoid contents depended on Trichoderma isolates. Volatile compounds were detected in both Trichoderma. Isolates of T. asperellum (F isolate) and T. virens (E isolate) triggered the increase of total phenolic contents which could be potential for biological control agents to induce systemic resistance in soybean.

1. Introduction
Soil borne pathogens are considered one of the main devastating pathogens when their growth is supported by favourable environment. Rhizoctonia solani has many plant hosts including vegetables, rice, corn, and legumes [1-4]. Severe infection of soybean by this soil borne pathogen has been reported in the crops cultivated in tidal swamp area [5]. Cultural practise to control of this pathogen is challenging since the pathogen exists in the soil and R. solani has many host plants.

Antagonistic fungi of several species of Trichoderma are effective to control R. solani. Hyperparasitism by producing lytic enzymes such as chitinase, protease, and β-glucanase, competition of nutrient and space, as well as production of diffusible antibiotics are action modes of Trichoderma to suppress the growth of soil borne pathogens [6-7]. Abundant volatile compounds such as glacial acetic and propyl-benzene produced by T. harzianum and T. viride also played in inhibition of plant pathogens [8].

Previous study reported that Trichoderma spp. isolated from rhizosphere and soils around infected crops are potential to inhibit the growth of R. solani and Fusarium sp. [4]. The inhibition of pathogen growth depends on Trichoderma isolates. Antagonistic activity of Trichoderma spp. in planta in this previous study has not yet been conducted. This further study therefore, aimed to investigate the antagonistic potential of T. virens and T. asperellum isolates against R. solani in planta and to determine
the change of total phenolic content in soybean plants treated with Trichoderma isolates in infected soil. Production of volatile metabolites by these fungi was also detected and identified.

2. Materials and methods

2.1. Antagonistic in-vitro test
Pathogenic fungus of *Rhizoctonia solani* was challenged with *T. virens* (A-E isolates) and *T. asperellum* (F-J isolates) in a Petri dish. All isolates both *R. solani* and *Trichoderma* spp. were obtained from fungal culture collection of mycology laboratory of Indonesian Legumes and Tuber Crops Research Institute [4]. Dual culture in vitro test was performed as [4]. Inhibition of *R. solani* was calculated and compared with negative control which was *R. solani* grown without antagonistic fungi [9].

2.2. Seed treatment using conidia of Trichoderma
Before planting, soybean seeds were surface sterilization using ethanol and sodium hypochlorite as conducted by [10]. After sterilization, the seeds were treated using conidia of *T. virens* and *T. asperellum* (10^6 cfu/mL) and planted in sterile soil separately [10].

2.3. Soybean growth on sterile and infected soil
Seed germination was assessed from 7-14 days after planting according to [11] with modification. The soybean seeds treated with conidia of Trichoderma, fifty seeds of each tray, were sowed in sterile soil (sterilized and without *R. solani* inoculation) as well as in infected soil (sterilized and inoculated with *R. solani*) separately. After 14 days of planting, the number of germinate plants was recorded [4].

2.4. Estimation of total phenolic and flavonoid contents
Total phenolic and flavonoid contents of soybean plants were estimated using Folin-Ciocalteu reagent and AlCl3 solution [12]. Total phenolic content was expressed as chlorogenic acid equivalent (mg ChAE/g sample) and total flavonoid content was expressed as quercetin equivalent (mg QE/g sample).

2.5. Detection of volatile organic compounds (VOCs)
*T. virens* and *T. asperellum* were cultured in headspace (HS) vial containing PDA for 48 h prior to VOCs detection using solid phase micro extraction (SPME) gas chromatography-mass spectrometry (GC-MS). A fiber coated with 65 μm polydimethylsiloxane (PDMS)/divinylbenzene (DVB) was used to absorb the VOCs. GC-MS parameters were set according to Inayati et al. [13].

3. Results and discussion

3.1. Antagonistic activity of *T. virens* and *T. asperellum*
Antagonistic activity ranges of five isolates of *T. virens* against *R. solani* (47.8-63.1%) were similar inhibition range of *T. asperellum* against the same pathogen (41.2-57.0%) after two days of incubation (Table 1). The same antagonistic activity trend against *R. solani* was also observed after 3 days of incubation, which were 88.7-99.6% for *T. virens* and 85.7-91.3% for *T. asperellum*, respectively. The effective ability of *Trichoderma* spp. to inhibit plant fungal pathogens specifically soil borne pathogens has been reported [14-15]. Several mechanisms involved in the antagonistic activity such as hyperparasitism, competition of nutrient and space, as well as antibiosis by producing enzymes and toxins [16-18]. A-E isolates were *T. virens* and F-J isolates were *T. asperellum*. DAI = day of incubation. Numbers followed the same letter in the same column were not significantly different (LSD, α = 5%).

3.2. Application of Trichoderma as seed treatment
Application of *T. virens* and *T. asperellum* conidia as seed coating did not affect the number of soybean seed germination or no adverse effect was observed (Table 2). The range of germinate soybean seeds were from 84.0 to 90.0% and from 86.0 to 98.0% for seeds treated with *T. virens* and *T. asperellum*, respectively. No effect on soybean seed germination was also observed after application of two isolates
of *Trichoderma* spp. [19]. Singh et al. [20] reported that seed germination of vegetable crops including tomato, guar, chilli, ridge gourd, okra and brinjal were influenced by spore dose. In their study, higher spore concentration inhibited seed germination on tomato and brinjal.

### Table 1. *In vitro* antagonistic activity of *T. virens* and *T. asperellum*.

| Isolate* | Inhibition (%) |
|----------|----------------|
|          | 2 DAI | 3 DAI |
| A        | 47.8 cde | 88.7 b |
| B        | 63.1 a | 98.7 a |
| C        | 53.9 abcd | 92.2 ab |
| D        | 48.5 cde | 90.0 b |
| E        | 59.7 ab | 99.6 a |
| F        | 49.3 bcde | 85.7 b |
| G        | 41.2 e | 86.1 b |
| H        | 57.0 abc | 91.3 ab |
| I        | 51.6 bcde | 89.6 b |
| J        | 43.9 de | 86.1 b |

Table 2. Soybean growth affected by application of *T. virens* and *T. asperellum*.

| Isolate | Seed germination (%) | Germinate | Ungerminate |
|---------|----------------------|-----------|-------------|
| A       | 86.0 a               | 14.0 a    |
| B       | 90.0 a               | 10.0 a    |
| C       | 88.0 a               | 12.0 a    |
| D       | 86.0 a               | 14.0 a    |
| E       | 84.0 a               | 16.0 a    |
| F       | 86.0 a               | 14.0 a    |
| G       | 90.0 a               | 10.0 a    |
| H       | 92.0 a               | 8.0 a     |
| I       | 98.0 a               | 2.0 a     |
| J       | 94.0 a               | 6.0 a     |
| Co      | 96.0 a               | 4.0 a     |

Numbers followed the same letter in the same column were not significantly different (LSD, α = 5%). A-E isolates were *T. virens* and F-J isolates were *T. asperellum*. Co = control (without seed coating).

*T. virens* and *T. asperellum* applications on soybean seeds were able to reduce soil borne pathogen infection compared to the control (Table 3). No difference of the number of germinate plants was found on plants treated with both *T. virens* (74-88%) and *T. asperellum* (70-92%). The same trend of no difference in disease intensity was also observed in soybeans treated with *T. virens* (22-40%) and *T. asperellum* (24-40%), which was less than 50%. Without *Trichoderma* application, the disease intensity was more than 50% of the population.

The potential antagonistic activity of *T. virens* and *T. asperellum* to suppress the growth of the soil borne pathogen was not surprising. Apart from hyperparasitism by producing enzymes (chitinase, β-glucanase, protease), *Trichoderma* also produced secondary metabolites and volatile compounds which also inhibited the pathogen [6, 13]. *T. asperellum* produced polyketides and alkanes which acted as antimicrobial compounds [21]. Sesquiterpenes, monoterpenes, ketones, aldehydes, lactones, alcohol and sulphides were identified in *T. virens* [22].

### 3.3. Total phenolic and flavonoid contents in soybean crops

Total phenolic content and the increase of this compound in soybean plants after application of *T. virens* and *T. asperellum* in infected soil varied significantly (Table 4). One isolate of *T. asperellum* (F isolate)
was able to stimulate the highest increase of phenolic content (18.2%) followed by one isolate of *T. virens* (E isolate) (13.6%). Similar to those of phenolic content, total flavonoid content and the increase of this content were not the same amount in soybean plants treated with each isolate. However, the highest increase of total flavonoid content was also observed in F isolate of *T. asperellum* (15.2%).

| Isolate | Number of plant (%) | Disease intensity (%) |
|---------|---------------------|----------------------|
| A       | 80.0 abc            | 20.0 bcd             | 32.0 b |
| B       | 74.0 bc             | 26.0 bc              | 40.0 b |
| C       | 88.0 ab             | 12.0 cd              | 22.0 b |
| D       | 80.0 abc            | 20.0 bcd             | 34.0 b |
| E       | 84.0 abc            | 16.0 bcd             | 34.0 b |
| F       | 82.0 abc            | 18.0 bcd             | 26.0 b |
| G       | 92.0 a              | 8.0 d                | 24.0 b |
| H       | 70.0 c              | 30.0 b               | 40.0 b |
| I       | 84.0 abc            | 16.0 bcd             | 34.0 b |
| J       | 90.0 a              | 10.0 d               | 24.0 b |
| Co      | 54.0 d              | 46.0 a               | 72.0 a |

Numbers followed the same letter in the same column were not significantly different (LSD, α = 5%). A-E isolates were *T. virens* and F-J isolates were *T. asperellum*. Co = control (without seed coating).

The increase of phenolic as well as flavonoid contents in plants interacted with *Trichoderma* was also reported in tomato plants by [23]. The increase of those compounds indirectly influenced the growth of the pathogen. Defence enzymes such as phenylalanine ammonia lyase, guaiacol peroxidase, and syringaldazine peroxidase had been activated, leading to the enhancement of phenolics and hydrogen peroxidise accumulation. Twenty two phenolic compounds both in free and conjugated forms had been reported increase significantly in plants treated with Trichoderma [23].

| Isolate | Phenolic content (mg ChAE/g) | Increase of phenolic content (%) | Flavonoid content (mg QE/g) | Increase of flavonoid content (%) |
|---------|------------------------------|---------------------------------|----------------------------|----------------------------------|
| A       | 2.30 h                       | -3.97 h                         | 2.13 d                     | 8.12 e                           |
| B       | 2.56 de                      | 6.90 de                         | 2.32 b                     | 17.77 b                          |
| C       | 2.56 de                      | 7.11 de                         | 1.98 e                     | 0.59 fg                          |
| D       | 2.58 de                      | 7.74 de                         | 2.13 d                     | 8.29 e                           |
| E       | 2.72 b                       | 13.60 b                         | 2.27 bc                    | 15.23 bc                         |
| F       | 2.83 a                       | 18.20 a                         | 2.58 a                     | 30.71 a                          |
| G       | 2.49 f                       | 3.97 f                          | 2.22 c                     | 12.69 cd                         |
| H       | 2.64 c                       | 10.39 c                         | 2.19 cd                    | 10.91 de                         |
| I       | 2.61 cd                      | 9.21 cd                         | 1.98 e                     | 0.68 f                           |
| J       | 2.54 ef                      | 6.28 ef                         | 1.90 e                     | -3.55 g                          |
| Co      | 2.42 g                       | 0.00 g                          | 1.97 e                     | 0.00 fg                          |

Numbers followed the same letter in the same column were not significantly different (LSD, α = 5%). A-E isolates were *T. virens* and F-J isolates were *T. asperellum*. Co = control (without seed coating).
3.4. Detection of VOCs using SPME GC-MS

Both *T. virens* and *T. asperellum* produced VOCs after separation and detection with SPME GC-MS. Several compounds acted as antifungal and plant growth promoting, and sesquiterpenes were predominantly found particularly in *T. virens* [13]. Compounds of benzene, (3R)-3-Phenyl-2,3-dihydro-1H-isooindol-1-on, Cyclopentasiloxane, decamethyl-, 3-(4-Isopropylthiazol-2-yl)-6-p-tolyl-[1, 2, 4]triazolo[3,4-b]-[1,3,4]thiadiazole, benzamide, pentadecanoic acid and heptadecanoic acid were detected in both *T. virens* and *T. asperellum*. Further studies are needed to observe the effect of these compounds on soil borne pathogens as well as on soybean crops.

4. Conclusion

In conclusion, treatment of soybean with both *T. virens* and *T. asperellum* reduced disease intensity of *R. solani*. The increase of total phenolic content in soybean crops was *Trichoderma* isolate dependence. Among all isolates tested, one isolate of *T. asperellum* (F isolate) and one isolate of *T. virens* (E isolate) were able to increase total phenolic contents which could be potential for biological control agents and also potential for inducing systemic resistance in soybean.

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