Trichoderma virens-Tv4 enhances growth promoter and plant defense-related enzymes of mungbean (*Vigna radiata*) against soil-borne pathogen *Rhizoctonia solani*

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Abstract. *Inayati A, Sulistyowati L, Aini LQ, Yusnawan E. 2020. Trichoderma* virens-*Tv4 enhances growth promoter and plant defense-related enzymes of mungbean (*Vigna radiata*) against soil-borne pathogen* Rhizoctonia solani. *Biodiversitas 21: 2410-2419.* Trichoderma virens has been studied for its ability to control various soil-borne pathogens as well as to induce plant resistance. The ability of *T. virens* control *R. solani* and its capability to induce resistance was evaluated in two different genotypes of mungbean (*Vigna radiata* (L.) R. Wilczek). Plant growth-promoting capability and production of plant defense-related enzymes during plant-pathogen-*Trichoderma* interaction were investigated. Pathogen infection caused the morphological and biochemical changes as well as increased plant defense enzymes activity such as peroxidase, polyphenol oxidase, PAL, phenolics, and flavonoid compared to control uninoculated plants. *T. virens* improved mungbean seedling growth in terms of increased total biomass, root weight, and root length as well as improved chlorophyll content and IAA-synthase from leaves and roots. *T. virens* treatment alone or in the presence of pathogen-induced mungbean defense-related enzymes indicated by the increasing of PO and PPO activity, and higher accumulation of total phenolic and flavonoid content. Although most of plant induced resistance parameters showed low and non-significant in direct single-factor comparison, Pearson's correlation showed there was a positive correlation between plant growth promoter compounds (IAA and chlorophyll) with plant defense-related enzymes (total phenol, and flavonoid content). *T. virens* treatment can induce systemic defense response of mungbean seedling directly by increasing the activity of some defense-related enzymes, and indirectly by improving plant health, and promoting plant growth. In conclusion, *T. virens*-Tv4 has potential to be developed as bio-control agents to control *R. solani* as well as to induce mungbean resistance.

Keywords: Growth promotor, induced resistance, mungbean, plant defense-related enzymes, *Trichoderma virens*

INTRODUCTION

Trichoderma species have been studied for decades as effective bio-control agents against many pathogens through various modes of action (Howell 2003; Harman 2011). Initially, most studies of *Trichoderma* spp. were focused on the ability of these fungi to directly suppress the growth of pathogens with specific mechanisms such as mycoparasitism, production of antibiotics, and competitions for space and nutrition in the rhizosphere (Howell et al. 1993; Shakeri and Foster 2007; Wu et al. 2017). Recently, most of the research is now concentrated on the mechanisms involved in *Trichoderma*-plant-pathogen interaction to further explore the potential of *Trichoderma* as multifunctional bio-control agent (Harman 2011; Singh et al. 2014). Numerous studies indicate the ability of *Trichoderma* spp. to reprogram plant genes expression that change plant proteome and metabolome which alleviate physiological and biochemical change, and improve plant resistance to biotic and abiotic stresses (Woo et al. 2006; Mukherjee et al. 2012; Castillejo et al. 2015; Mazzei et al. 2016).

In plant-pathogen-*Trichoderma* interaction, *Trichoderma* is able to activate plant defense mechanisms mostly for induced systemic resistance (ISR), rarely systemic acquired resistance (SAR) (Hermosa et al. 2012; Martínez-Medina et al. 2014). The latest study proposed mixed ISR/SAR type of resistance called *Trichoderma*-induced systemic resistance (TISR) (Martínez-Medina et al. 2013; Martínez-Medina et al. 2014; Pieterse et al. 2014). However, the mechanism of defense responses induction and the type of resistance induced in plants by *Trichoderma* still remain unclear (Harman et al. 2012).

Trichoderma colonization can trigger plant defense systems not only restricted to the root, but could also be marked in above-ground plant tissues and increasing the spectrum of plant resistance to various pathogens (Martínez-Medina et al. 2013; Shoresh and Harman 2010). Shoresh and Harman (2008) showed that roots colonization with *T. harzianum* T22 induced systemic resistance of maize seedling against soil-borne and foliar diseases as well as promotes overall plant growth. Another study showed that the challenge of cucumber seedlings by pathogen *Pseudomonas syringae* pv. *lachrymans* and *T.asperellum* (T203) increase the levels of plant defense-related enzymes such as peroxidases, chitinases, and β-1, 3-glucanases (Shoresh et al. 2005). Other enzymes related to plant defense such as polyphenol oxidase (PPO),

phenylalanine ammonia-lyase (PAL) that involved in synthesis of phytoalexin and phenolic compounds also reported increase in legume treated by *T.viride* (Surekha et al. 2014). According to the reported study, it is suggested that the induction of plant resistance in different hosts may require different signaling, and the induction was represented in different manner (Martínez-Medina et al. 2013).

Trichoderma virens as well as other *Trichoderma* spp, can induce both localized and systemic resistance in various plant species and pathogens (Howell 2006; Contreras-Cornejo et al. 2009; Angel et al. 2016; Małolepsza et al. 2017). Latest study from Dubey et al. (2018) showed that there were up-regulated expression of some defense-related genes and catalase in response to the presence of *T. virens* and *R. solani*. However, there were still many gaps in the physiological, biochemical, and molecular changes related to *T. virens*-mungbean induced defense responses against soil-borne pathogen *R. solani*. More studies also needed to understand *Trichoderma*-ISR on mungbean-pathogen system which improves efficient formulation for wide application in agriculture. Our previous study showed that *T. virens*-Tv4 strain has relatively high mycoparasitic capacity and able to produce plant hormone IAA and phosphate solubilizer enzyme *in vitro* (data not shown). *T. virens* also increase total phenol and flavonoid content on soybean seedling (Yusnawan et al. 2019). However, the effectiveness of *T. virens* strains in inducing mungbean resistance was not previously tested. In this study, we analyze the ability of *T. virens*-Tv4 to induced mungbean resistance against soil-borne pathogen *R. solani* as well as its ability to promote plant growth and development. *Trichoderma* spp. has been reported to increase plant growth and development through increased nutrient solubility and availability, and nutrient uptake capacity (Zhang et al. 2016). In Arabidopsis, *Trichoderma* spp. also found induced growth of lateral roots through the production of auxin (Contreras-Cornejo et al. 2009). Changes in enzymes related to plant defense such as phenylalanine ammonia-lyase (PAL), Tyrosine ammonia-lyase (TAL) activity, peroxidase, polyphenol oxidase, and total phenolic were evaluated during plant-pathogen-*Trichoderma* interaction. We also observe the accumulation and synthesis of phytohormone (IAA-synthase and IAA-oxidase) and chlorophyll content in the presence of *T. virens, R. solani* or both *T. virens* and *R. solani* compare to uninoculated control plants. Principal component analysis (PCA) was used to explain the influence of the enzymes alteration in plants on the induction of plant resistance by *T. virens*.

MATERIALS AND METHODS

Plant and fungal material

Trichoderma virens strain Tv4 and *R. solani* isolates were obtained from Mycology Laboratory of Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia. *T. virens* strain was cultured in PDA plates for 5 days before use, while *R. solani* was cultured on rice husk according to method proposed by Shrestha et al. (2016) with modification. Briefly, 1000 g of the mixture of rice husk and brand (4: 1 w/w) were moisturized with approximately 200 mL of water containing 20 g/L of glucose. The mixture was then sterilized at 121 °C for 20 min. The sterilized husk mixture medium was inoculated with three to five plugs (diameter 0.5 cm) of *R. solani* mycelia from 7 day-old PDA culture and incubated at room temperature for 7 days. Two different genotypes of mungbean, Sampeong, and Vima-3 which have different resistance levels based on previous study were used in this study.

Greenhouse experiment

Seeds of mungbean were sterilized with 0.5% NaOCl and rinsed twice with sterile distilled water. *Trichoderma* suspension was prepared according to Yusnawan et al. (2019). In general, conidia of *T. virens* were harvested from the 7 days-old culture, and then sterile distilled water was added to adjust the spore concentration approximately 10^6 CFU/mL. The seeds were then dipped into the suspension for 30 minutes to trigger initial imbibition of the seeds before planting. For control treatment, mungbean seeds were dipped in sterile water. Four different treatments were tested: (i) R: plants were non-treated with *T. virens* and challenged with *R. solani,* (ii) TR: plants treated with *T. virens* and challenged with *R. solani*, (iii) T: plants were treated only with *T. virens* and (iv) C: control plants, non-treated with *T. virens*, and non-challenged with *R. solani*, For *R. solani* challenged treatment, *R. solani* from rice husk (10 g/pot) was spread uniformly to the sterile soil 2 days before planted. All plants for sample were taken from 21 days old seedling.

Determination of plant enzymes related to plant growth promoter and plant defense

IAA-synthase

IAA-synthase was determined as per the method given by Phelps and Sequeira (1967) with modification. Enzyme was prepared by finely ground 0.5 g of plant tissue (leaves and roots) then add 5 mL of 0.05 M phosphate buffer saline, pH 5.8. The reaction mixture contained 500 μL extract, 50 μL $MnCl_2$ (0.2 mM), 10 μL $MgSO_4$ (0.01M), 1500 μL PBS, and 500 μL L-tryptophan (125 mg/50mL). The mixture was incubated at 37 °C for 30 min. The reaction was started by added 2.5 mL Salkowski reagent and incubated at room temperature for 15 minutes to developed stable pink color and the absorbance was read at 530 nm. The enzyme activity was expressed as μg IAA produced per minute per gram fresh weight of tissue.

Chlorophyll content

Chlorophyll content was determined according to Lichtenthaler and Buschmann (2001). 0.5 g leaves from 21 days old mungbean seedling were grounded with 5 mL methanol 90% (v/v).The chlorophyll and carotenoid content was quantified by spectroscopic absorbance at three different wave lengths, 470 nm, 652.4 nm, and 665.2 nm. Concentration of chlorophyll and carotenoids were calculated following equation given:

Ch-a (μg/ mL) = 16.82 $A_{665.2}$-9.16 $A_{652.4}$
Ch-b (μg/ mL) = 34.09 $A_{652.4}$-16.54 $A_{665.2}$
Ch (x + c) (μg/mL) = (1000 A_{470}-1.91 Ch-a-95.15 Ch-b)/225

Peroxidase activity (PO) and polyphenol oxidase (PPO)

PO activity was determined following the method Ata et al. (2008) with modification. Samples of was ground in 5 mL of sodium phosphate buffer (pH 6.5) centrifuged for 10 min at 12000 rpm, 4°C. The reaction mixture consisted of 2.9 mL of 100 mM sodium phosphate buffer (pH 6.5) containing 0.25% (v/v) catechol and 100 mM H_2O_2.

PPO activity was determined according to the method described by Malik and Singh (1980). The reaction mixture contained 3.0 mL buffered catechol solution (0.01 m), freshly prepared in 0.1 mL phosphate buffer (pH 6.5). The reaction was started by adding 100 µl of the crude enzyme extract. Changes in the absorbance at 495 nm were recorded every 30-sec intervals for 1 hour. Enzyme activity was expressed as increase in absorbance $min^{-1}g^{-1}$ fresh weight.

Phenylalanine ammonia-lyase (PAL)

PAL and TAL activity was assayed according to methods proposed by Dogbo et al. (2012) with modification. Crude enzyme for PAL assay was prepared by finely ground of 0.5 g plant tissue (leaves or roots) with 5 mL 0.1 M borate buffer, pH 8.8. The solution then sonicated for 10 minutes then centrifuged at 10000 rpm, 4°C for 10 minutes. PAL reaction mixture contained 300 µL extract, 300 µM sodium borate, 30 µM L-phenylalanine, and the mixture than adjusted to 3.5 mL with distilled water. Incubation was done at 30°C for 60 min and the absorbance at 290 nm. The enzyme activity was expressed as millimoles cinnamic acids produced per minute per gram fresh weight of tissue.

Total phenolic and flavonoid content

Phenolic and flavonoid extract was prepared by dissolving 0.5 g finely ground leaves in 5 mL 80% methanol. Phenolic content was estimated using Folin-Ciocalteu's reagent according to Yusnawan (2016) which expressed as Gallic acid equivalents per gram of sample (mg GAE/g sample). Total flavonoid content was measured with $AlCl_3$ (Heimler et al. 2005) which expressed as catechin equivalents per gram of sample (mg CE/g sample) based on dry basis using the calibration curve of the catechin.

Data analysis

Analyses of variance (ANOVA) from data of plant growth and resistance parameters and enzymes assays were calculated using Microsoft Excel software. Least Significant Difference (LSD) was performed on 0.05 confidence level. The robust analysis of principal component (PCA) was calculated using R-studio statistical environment R version 3.5.3 (Team 2015).

RESULTS AND DISCUSSION

Mungbean growth and resistance against *R. solani* infection and *Trichoderma virens* treatment

Plant-pathogen-*Trichoderma* interaction triggers positive and negative effects on plants. In mungbean-*R. solani*-*T. virens* interaction, there was change in plant defense reactions which directly and indirectly influence the response of mungbean seedling to pathogen infection. *R. solani* infection causes the seeds to rot and abnormality, root rot, and death because of damping-off (Figure 1). Some seedling infected by *R. solani* on their hypocotyls showed the ability to recover and continued to grow by production of new green tissue (Figure 1.D). This indicated the resistant expression of mungbean genotype that could overcome *R. solani* infection. Bengyella et al. (2015) reported that a key feature to plant recovery from pathogen attack is the degree of the host's initial basal immunity response. In plant recovery process, pathogen attack trigger plant response to by-pass the development of severe symptom which leads to partial or complete recovery. The percentage of plant recovery showed significant correlation with the level of resistance (Bengyella et al. 2015).
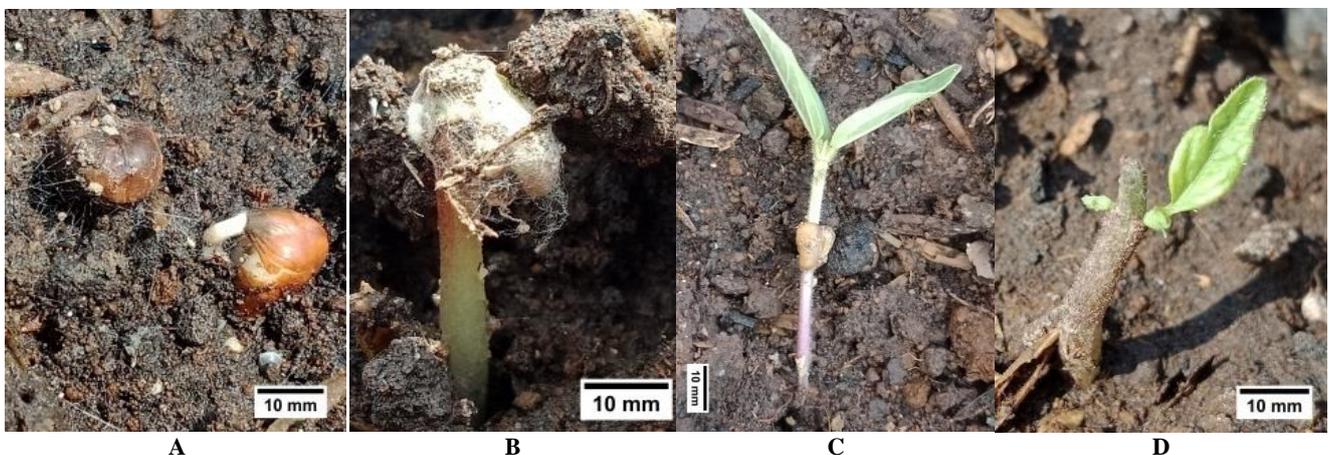
Figure 1. *Rhizoctonia solani* infection symptoms; A. Seed rot, B. Hypocotil rot, C. Damping-off, D. Seedling abnormality caused by *R. solani* infection
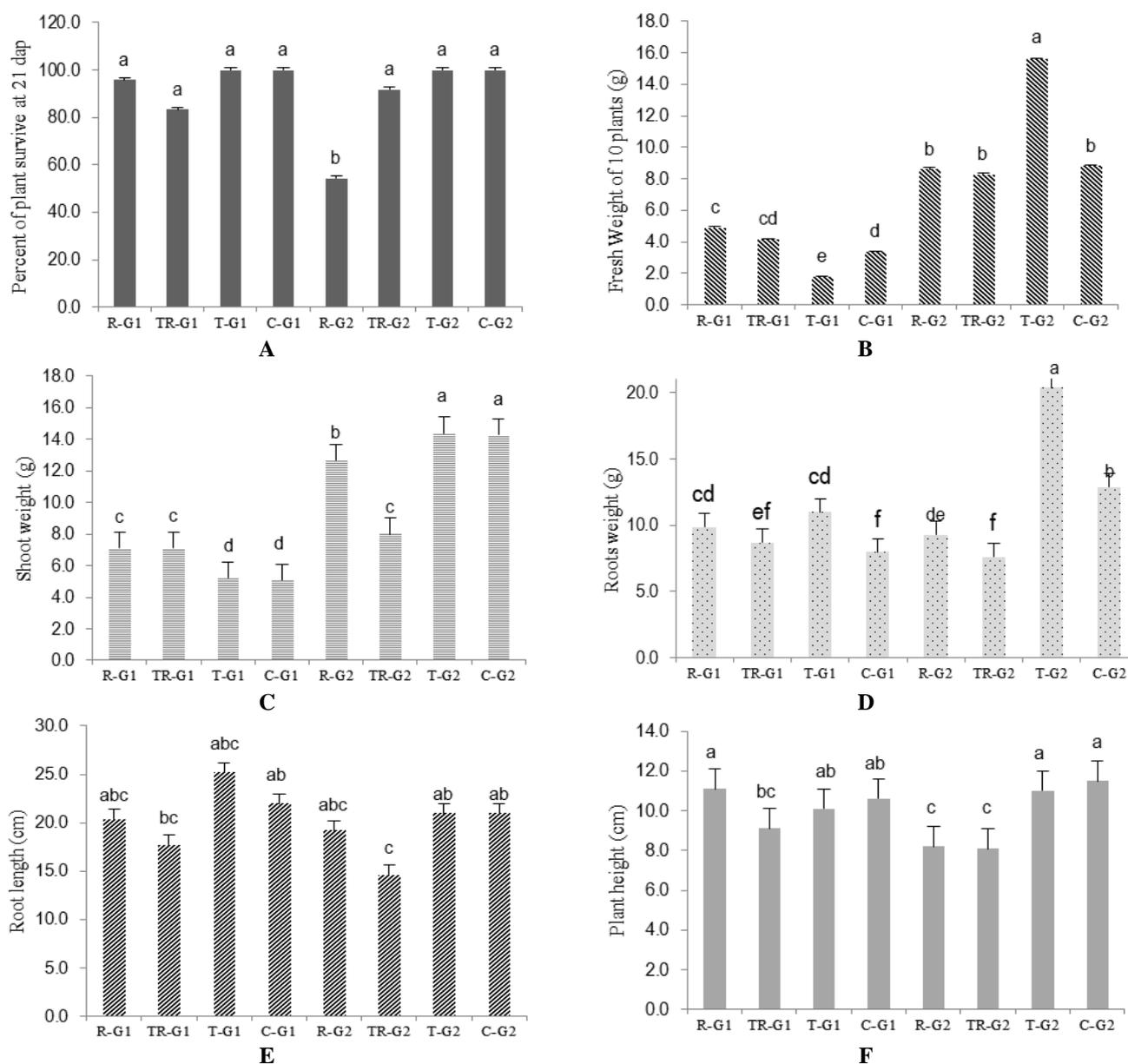
Figure 2. A. Percent survive plants at 21 dap, B. Total fresh weight of 10 plants, C. Fresh weight of shoot, D. Fresh weight of roots, E. Root length, F. Plant height

There was no significant difference between plants survive in control treatment with plant treated by *T. virens* in both genotypes at 21 days after planting (dap) as well as the resistance to *R. solani* infection showed by two genotypes tested (Figure 2). Although *in vitro* assay showed that Tv4 strain has a relatively high ability to suppress *R. solani*, but when interacting with plants, the mycoparasitic ability of *T. virens* is not directly and fully exposed. When plant challenged with pathogen, defense response is not activated directly, but it needs time to accelerated attack upon by pathogen or other pests (Van der Ent et al. 2009).

Trichoderma virens treatment increased the total biomass of G1 seedling as compared to control, however, G2 presented different responses which only seed treatment

with *T. virens* alone increase the fresh weight. Seeds treated with *T. virens* also affect the root weight and length indicated that *T. virens* promotes the root growth, however, *T. virens* treatment showed no effect on the shoot growth. *Trichoderma* spp. colonization has been reported to improve nutrient uptake and availability as well as the allocation of biomass in some plants such as melon (Martínez-Medina et al. 2011) and tomato (Tucci et al. 2011). Since improved in plant nutrition is considered as one of the mechanisms responsible for plant protection against pathogen, root colonization by *Trichoderma* also reported could enhance not only crop yield but also resistance to biotic and abiotic stresses (Harman et al. 2004; Whipps and Lumsden2001). Our study showed there was no significant increase in plant resistance which

represented by number of plant survive with the increase in plant growth parameters such as plant biomass and root growth. It can be suggested that there were other mechanisms involved in the mungbean resistance against *R. solani*. The study conducted by Martínez-Medina et al. (2013) also showed that systemic protection of tomato treated by *T.harzianum* was not related to nutrition improvement or growth promotion.

Determination of plant growth promoter induced in mungbean-*R. solani-T. virens* interaction
IAA-synthase measurement

Plant growth promotion in mungbean seedling represented by the increase in IAA synthase from both roots and leaves of G2 seedling treated with *T. virens* as compared to control treatment (Figure 3). However, G1 showed slightly different responses, only seedling challenged with pathogen and *T. virens* displayed significant increase in IAA synthase from the leaves. The highest IAA-synthase was showed in G1 roots infected by *R. solani* which increase up to 9 fold higher as compare to control plant. In contrast, pathogen infection and *T. virens* treatment decrease the IAA in G2. The correlation between IAA synthase and the growth parameters (root length, plant height, and plant biomass) was not clear, indicated that the capability of *T. virens* synthesizing IAA not directly correlates with promotion of the plant growth. Contreras-Cornejo et al. (2009) reported that *Trichoderma* spp. could induce plant growth by fungal auxin-dependent mechanism even the correlation between them is less convincing. Our study showed, there was negative correlation between IAA-synthase in leaves with root length and plant height, however, positive correlation showed between IAA-synthase in roots with the total biomass, and the plant height. Altogether our results suggest that the mechanisms and molecules involved in plant growth promotion of

mungbean seedling by *T. virens* are multivariable and also affected by the environmental conditions.

Chlorophyll content measurement

Change in chlorophyll content from mungbean infected by *R. solani* and treated by *T. virens* was varied among genotypes (Figure 4). There was increase in chlorophyll content in treated G1 plants compared to untreated plants, indicating the growth-promoting ability of *T. virens*. However, pathogen infection cause chlorophyll a, b, and carotenoid decrease in G2 seedling indicating that there was genotype-specific response in chlorophyll content changes. Chlorophyll content is very important in photosynthesis, especially chlorophyll a and b. The decrease in plant chlorophyll content affects photosynthesis and attributed to decrease yield (Durairaj et al. 2018). Plant chlorophyll content also indicates the health of plants and strongly related to its biophysical conditions (Moharana and Dutta 2016). Photosynthesis play critical process in plant physiology, and its regulation play an important role in plant defense (Pérez-Bueno et al. 2019), as a result, the changes in energy source and signaling on photosynthesis process affect plant regulation pathways including defense response to biotic and abiotic challenges (Kangasjärvi et al. 2012). The changes in plant chlorophyll content could be part of mungbean defense mechanisms to limit the nutrient availability to the pathogen.

Determination of plant defense-related enzymes induced in mungbean-*R. solani-T. virens* interaction

Induction of plant defense mechanism was evaluated by determination of plant defense-related enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), total phenols, and flavonoid content. *T. virens* treatment and pathogen infection also trigger plant defense-related enzymes.
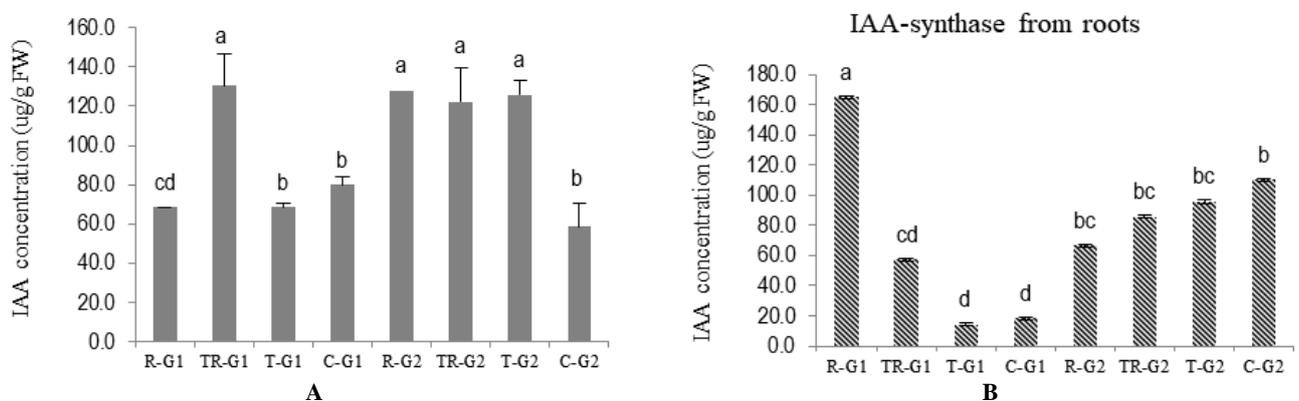
Figure 3. IAA concentration on: A. leaves, B. roots of mungbean seedling. R; plant infected by *Rhizoctonia solani*, TR; plant infected by *R. solani* and treated by *Trichoderma virens*, T; plant treated by *T. virens*, and C; control
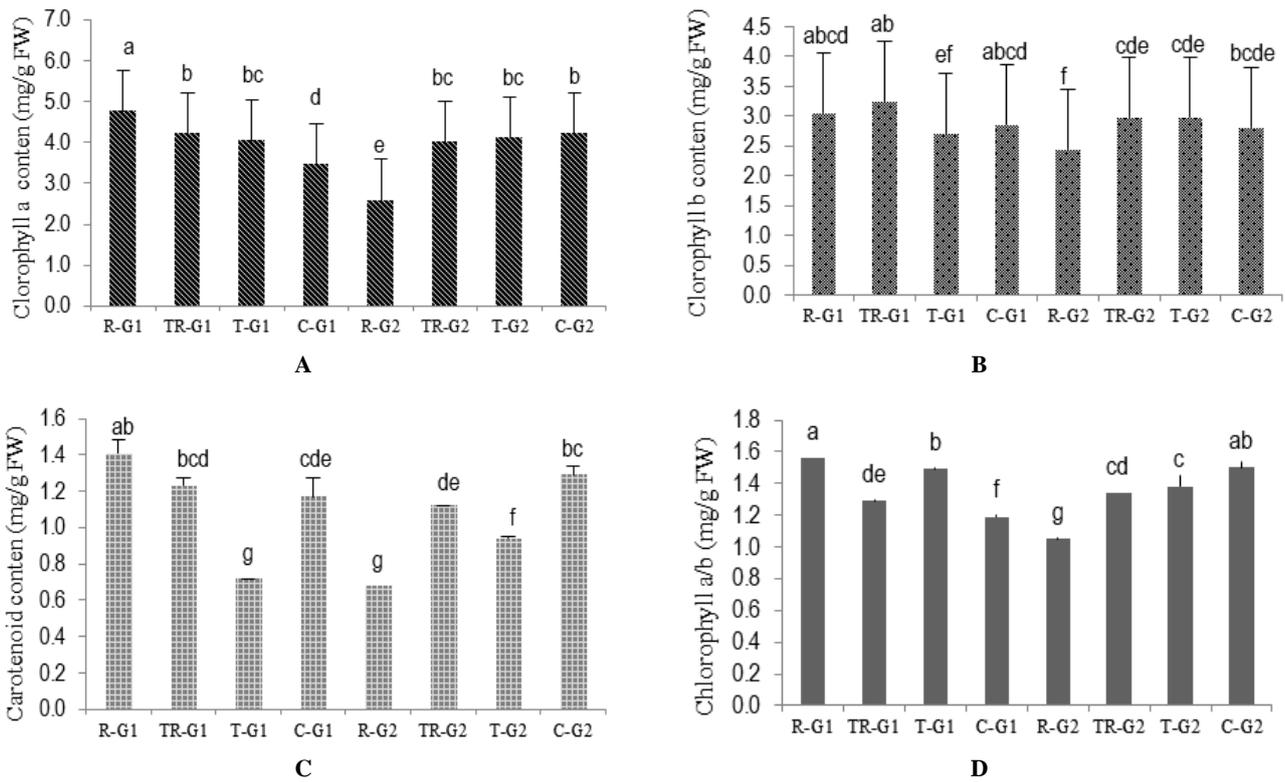
Figure 4. Chlorophyll contents of mungbean plants. A. Chlorophyll a, B. Chlorophyll b, C. Carotenoid, D. Comparison of chlorophyll a/b
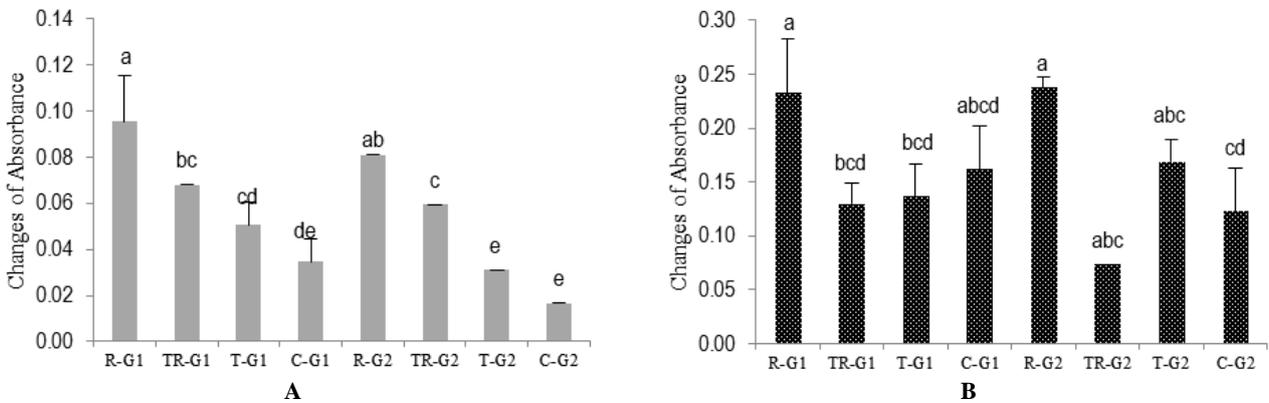
Figure 5. Differences in peroxidase (PO) and Polyphenol oxidase (PPO) activity in mungbean seedling treated by *Trichoderma virens*; A. Peroxidase (PO) activity in mungbean roots, B. Polyphenol oxidase (PPO) activity in mungbean roots

Peroxidase (PO) and polyphenol oxidase (PPO)

Peroxidase activity was higher on mungbean seedling when interacted with microorganisms both pathogenic and non-pathogenic. Pathogen infection increases the peroxidase activity as well as *T. virens* treatment (Figure 5.A). There was significant increase in PO activity due to pathogen infection; however, seed treatment with *T. virens* alone didn't show significant effect on PO activity. This fact showed that pathogen triggers stronger mungbean defense signals than *T. virens*. Peroxidase and peroxidase activity has been reported to play important role to initiate plant defense response against pathogens through establishment of structural barriers (lignin accumulation) or generation of highly toxic environments by massively producing ROS, and synthesis of phenolic compounds (Mahanta 2012; Thakker et al. 2012; Adhilakshmi et al. 2014). PO also actively involved in the self-regulation associated with growth regulation including respiration and photosynthesis (Kavitha and Umesha 2008).

Pathogen infection also increases polyphenol oxidase, however *T. virens* pretreatment on mungbean seed did not show adequate evidence whether *T. virens* treatment plays significant role in increasing PPO activity of mungbean seedling. There was significantly different in PPO changes in plant infected by *R. solani* in both genotypes, but only G2 treated with *T. virens* showed increase in PPO activity while others were decreased (Figure 5.B). PPO has been suggested to play important role in disease resistance due

to its ability to catalyze oxidation of phenolic compounds into quinones and lignin biosynthesis (Constabel and Barbehenn 2008; Kavitha and Umesha 2008). However, another study showed that PPO activity not directly correlated in plant defense response (Hoagland 1990). Pearson's correlation showed, there was negative correlation between PPO activity and number of surviving plant, and has no correlation with total phenol indicate PPO activity not directly involved in induced resistance of mungbean against *R. solani*.

**Phenylalanine ammonia-lyase (PAL)**

Phenylalanine ammonia-lyase (PAL) has been reported as key enzymes involved in plant defense mechanisms because it synthesizes various phenolic compounds, including flavonoids, hydrocinnamates (Hoagland, 1990), and enters different biosynthetic pathways leading to lignin synthesis (Kavitha and Umesha 2008; Solekha et al. 2019). Our study showed, there was different trend in PAL activity from leaves and roots of mungbean seedling and there also variation of PAL activity among treatment (Figure 6). PAL activity in leaves was relatively higher than in roots. Only G1 seedling treated with *T. virens* showed increase in PAL activity in leaves, while other treatments showed no effect or tend to decrease compared to untreated plants (Figure 6.A). In roots, pathogen infection increase PAL activity of G1 genotype, however, there was no difference in PAL activity in G2 roots (Figure 6.B). Different expression of PAL has been reported in many studies, for example in wheat, the activity of PAL was influenced by deoxynivalenol (DON, *Fusarium* mycotoxin), as a result, downregulation of PAL in susceptible variety (Duba et al. 2019).). Although it is difficult to explain the inhibitory effect of *Trichoderma* on PAL activity in mungbean-pathogen-*Trichoderma* interaction, a possible explanation was biosynthesis of PAL from L-phenylalanine may occur in mungbean system may follow more than one route and PAL reaction is not the only one of defense response pathways.
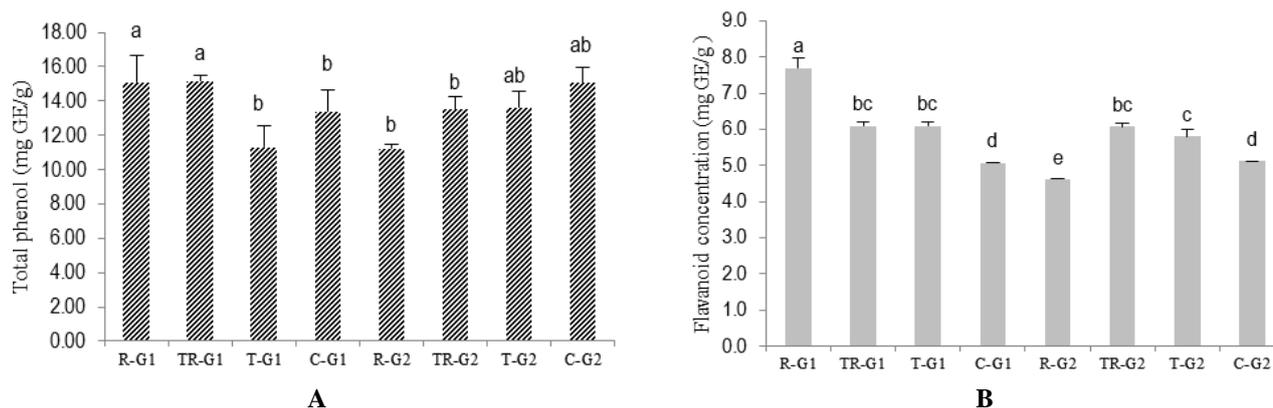
Total phenolic and flavonoid content

Phenolics are one of the largest and most diverse groups of plant active substances involved in the plant growth regulation, and also plays important role in defense responses during pathogen infection and abiotic stress (Kubalt 2016; Mandal et al. 2010). Pathogen infection caused an increase in total phenolic content in G1, however, it's decreased in G2 genotype compared to non-infected plants (Figure 7.A). Seed treatment with *T. virens* has slightly increased the accumulation of total phenol, however, there was no significant increase in phenolic content triggered by *T. virens* when applied in seed without pathogen treatment. This indicated the presence of pathogen was important to trigger *T. virens* capacity to induce phenols accumulation and activate the plant defense mechanism. Phenolics are synthesized when plants recognize potential pathogens (Bhattacharya et al. 2010; Kubalt 2016). However, there are other factors which influence phenol accumulation such as plant genetics or species (Bhattacharya et al. 2010), nutrient status, and environmental factors such as temperature and light (Løvdal et al. 2010) suggest that relationship between elicitor activity and specificity in a plant-microbe interaction is highly complex.

Flavanoids belong to polyphenols compounds play multifunctional roles and involved in plant-microbe interaction (Bekkara et al. 1998). Generally, pathogen infection and the *T. virens* treatment increase the flavonoid content of mungbean seedling except for G2 seedling which showed *R. solani* infection caused a decrease in flavonoid content (Figure 7.B). Most elevated accumulation of flavonoid compounds was showed in G1 genotype infected by *R. solani* while G2 genotype showed the opposite result pointed out the specific response of mungbean genotype to *R. solani* infection. Treutter, (2005) explained that plant defense-related flavonoids can be divided into "performed" and "induced" compounds. *Trichoderma* spp. application and pathogen infection have been reported could induce the accumulation of flavonoid compounds, however, sometimes it is difficult to notice the accumulation of flavonoids in plants induced by external factors or expression of their constitutive flavonoids. There was no difference in constitutive level of flavonoids content of G1 and G2 pointed out the increase/decreased of flavonoid content in treated plants was influenced by the treatment.
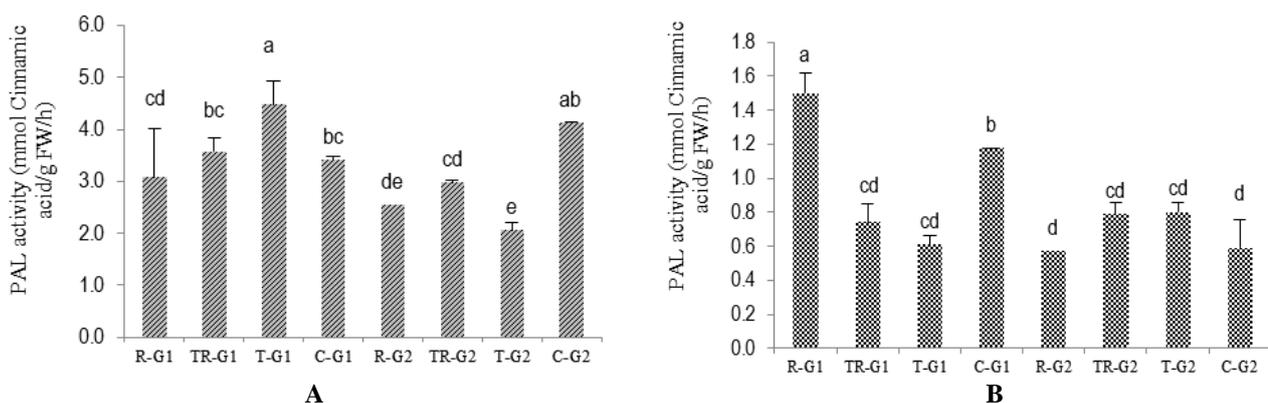
Figure 6. Changes in PAL activity on mungbean seedling in different part of plant; A. PAL activities in leaves, B. PAL activity in roots

Figure 7. Changes in total phenolic and flavonoid content in mungbean seedling infected by *Rhizoctonia solani* and *Trichoderma virens* seed treatment: A. Total phenol in mungbean roots, B. Flavonoid concentration in mungbean roots

Correlation among enzymes related to plant growth and plant defense

In general, most of plant induced resistance parameters showed low and non-significant in direct single-factor comparison. Pearson's correlation was determinate among plant enzymes and resistant parameter to help interpretation of the data variability presented in Table 1. The result showed, there was positive correlation between chlorophyll content with the plant survival, indicating that plant chlorophyll involved in plant defense mechanism of mungbean against *R. solani*. Another plant growth promoter that played important roles was IAA-synthase in roots. IAA-synthase in roots showed positive correlation with chlorophyll-a, total phenol, and flavonoid content. This result indicated that auxin production in the roots can induce the plant defense enzymes in mungbean defense system. Peroxidase and polyphenol oxidase also showed positive correlation with flavonoid content which implied the contribution of those two enzymes in induced mungbean defense response.

The principal component analysis (PCA) also conducted to all enzymes and growth parameters data. PCA analysis showed that two principal components (PC1 and PC2) explaining 62.9% of the overall variance (Figure 8). The PC1 plot showed the contribution of 34.7% of the explained variance was positively related to the chlorophyll content, total phenolic content, PAL, and IAA-synthase in roots. Whereas, PC2 loading plot that contributed to 28.3% of the explained variance. In PC2 plot, the separation occurred due to peroxidase activity and root length. Based on the genotype grouping, the variables on the plant infected by *R. solani* (R-G1, R-G2) were completely separated from *T. virens* treatment (T-G1, T-G2), indicated that treatment greatly influences the plant response. Though control uninoculated plants grouped together with the plants treated with *T. virens* and pathogen.

Table 1. Pearson's correlation matrix among plant defense-related and growth promoter enzymes with mungbean resistance to *Rhizoctonia solani*

	Surv	FW	RL	IAA-R	IAA-L	Chl-a	Chl-b	PO	PPO	PAL-R	Phe	Flav
Surv	1	0.05	0.37	0.06	-0.58	**0.73**	0.48	-0.46	-0.53	0.33	0.41	-0.46
FW		1	0.12	0.36	0.16	0.03	-0.12	-0.42	0.14	-0.34	0.06	0.02
RL			1	-0.32	-0.64	0.03	-0.34	0.31	-0.33	0.02	-0.33	-0.50
IAA-R				1	-0.11	0.52	0.29	0.42	0.37	0.27	**0.60**	**0.72**
IAA-L					1	-0.39	0.13	-0.03	0.22	-0.31	-0.15	0.29
Chl-a						1	**0.75**	-0.30	0.04	0.41	**0.71**	0.05
Chl-b							1	-0.38	0.07	0.42	**0.80**	0.21
PO								1	0.50	0.36	-0.22	**0.58**
PPO									1	0.37	-0.01	**0.72**
PAL-R										1	0.43	0.51
Phe											1	0.39
Flav												1

Note: Surv number of plant survive, FW total fresh weight, RL roots length, IAA-R IAA-synthase from roots, Chl-a chlorophyll a, Chl-b chlorophyll b, PO peroxidase, PPO polyphenol oxidase, PAL phenylalanine ammonia-lyase, Phe Phenolic content, and Flav flavonoid content
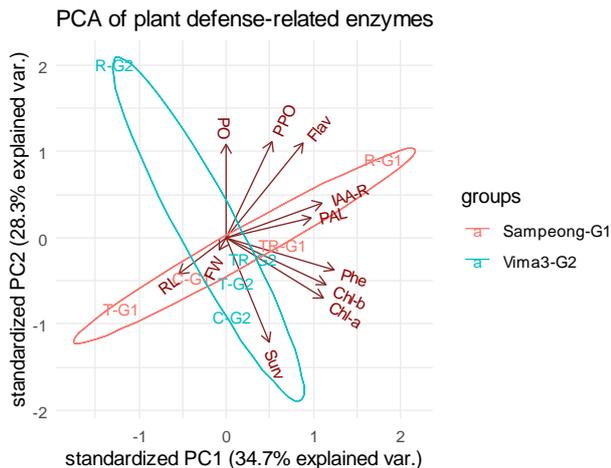
Figure 8. The ggbiplot of principal component analysis of the production of plant defense-related enzymes on mungbean induced resistance against *Rhizoctonia solani*. Surv number of plant survive, RL roots length, FW total fresh weight, PO peroxidase, PPO polyphenol oxidase, PAL phenylalanine ammonia-lyase, Phe Phenolic content, Flav flavonoid content, IAA-R IAA-synthase from roots, Chl-a chlorophyll a, Chl-b chlorophyll b

Mungbean which is pretreated with *T. virens* and together with the exposure to pathogens showed increase in levels of some defense-related compounds such as peroxidase, PAL, phenolic, and flavonoid. Other than direct action, induction of mungbean resistance by *T. virens* involved the synthesis higher plant growth promoter compounds such as phytohormone (IAA), and chlorophyll content. In mungbean-*R. solani-T. virens* interaction, the absence of pathogen has important role in inducing plant defense response. Our study showed that along with induction of IAA-synthase in roots, accumulation of phenolics and flavonoid was observed which indicated that *T. virens* treatment could induce the plant defense response in mungbean seedling, however, it's not fully expressed. Van der Ent et al. (2009) explained sometimes defense responses are not directly activated, but need period of time to accelerate upon attack by pathogens. For example, the resistance of tomato induced by *T. harzianum* to late blight appeared after 90 to 120 days, and over 2 years, the reduction of the disease was up to 80%. In conclusion, *T. virens* strain Tv4 treatment could induce the mungbean defense response against *R. solani* directly and indirectly. Direct induction showed by the increased activity of some defense-related enzymes such as peroxidase, total phenolic, and flavonoid, following the phenylpropanoid pathway. Indirectly, *T. virens* treatment was able to improve photosynthetic performance and promote mungbean growth which positively correlated to the accumulation of plant defense-related enzymes.

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