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Full Length Research Paper

The antagonistic activity of *Trichoderma virens* strain TvSUT10 against cassava stem rot in Thailand

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In this current study, the beneficial filamentous fungi, *Trichoderma virens*, isolated from cassava field were investigated for antagonistic mode of action against *Lasiodiplodia theobromae*, the causal agent of cassava stem rot in Thailand. *In vitro* screening using the dual culture technique was undertaken to assess the potential of these *Trichoderma* isolates. Our results indicated that fifteen isolates of *T. virens* were collected from various areas of cassava field in Nakhon Ratchasima, Thailand. The *T. virens* isolate, TvSUT10, was the most effective isolate and inhibited *L. theobromae* mycelial growth by 84.12%, due to the antagonistic mechanism. Moreover, *Trichoderma* β-1,3-glucanase activity was determined, the result revealed that the highest activity was recorded in strain of *T. virens* TvSUT10 (25.7 U/ml). In addition, in the greenhouse experiment, the application of the TvSUT10 as a conidial suspension reduced the stem rot disease severity of cassava caused by 53%. The results indicated that the *T. virens* strain TvSUT10 has initial modes of action of biological control to protect cassava crop against *L. theobromae* infections in cassava.

Key words: *Trichoderma*, cassava stem rot disease, growth inhibition, cassava, biocontrol.

INTRODUCTION

Trichoderma spp. is widely used as commercial biofungicides for control of soil-borne and foliar pathogens (Chet and Baker, 1981; Cook and Baker, 1982; Papavizas, 1985; Verma et al., 2007; Buensanteai et al., 2010; Akinbode and Ikotun, 2011). In addition to directly affecting plant pathogens through antibiosis and mycoparasitism, *Trichoderma* spp. can colonize roots and trigger systemic resistance against bacterial and fungal pathogens (Liansheng and Weihua, 2000; Harman et al., 2004). Induced resistance caused by treating plants with a biologically active elicitor is the phenomenon of priming and sensitizing plants to exhibit a more rapid and elevated expression of defense-related responses upon pathogen infection compared to unprimed plants. These responses may include an accumulation of pathogenesis-related (PR) proteins associated with the salicylic acid (SA)-dependent and the pathogen-induced Jasmonic acid (JA)-dependent pathways, as well as phenylalanine ammonia-lyase and redox regulating proteins (Mittler et al., 2004; Fobert and Despres, 2005; Heil and Silva Bueno, 2007; Buensanteai et al., 2010).

Cassava (*Manihot esculenta* Crantz), is a major economic crop in Thailand. It has also mainly been a subsistence crop in Asia and Africa. In Thailand, cassava was the economic crop in terms of production among the

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Abbreviations: PR, Pathogenesis-related; **SA,** salicylic acid; **JA,** Jasmonic acid; **PDA,** potato dextrose agar; **MGI,** mycelial growth inhibition; **MSM,** minimal synthetic medium.

major energy crops. Moreover, cassava has become an industrial crop due to the classification of many industrial processes in which cassava can be industrial raw material. For example, cassava has been found very useful in the production of ethanol and starch for various industrial uses (FAO, 2002). These properties and increased industry demand of the rapidly growing population has led to an expansion of cassava cultivation in many countries (Ubalua and Oti, 2007).

However, cassava production is greatly reduced due to attack by insects and diseases (Harman et al., 1981, 2004). The cassava yield was constantly below the Thai average yield as 1.3 ton per rai and production generally stagnated over the last ten years in Thailand. Cassava is attacked by more than 34 pathogens (Lozano and Nolt, 1993), causing various degrees of losses (Lozano et al., 1981). Among cassava diseases, cassava root and stem rots are most important in Thailand. Cassava root rots are caused by a complex of soilborne pathogens especially *Lasiodiplodia theobromae* which induce damage that eventually reduce the yield. Cassava yield losses of up to 81% due to stem rot diseases have been reported (Theberge, 1985). In some areas, total crop losses have been attributed to rot diseases (Lozano and Nolt, 1993). The aim of this study was to evaluate the efficacy of *Trichoderma virens* strains for antagonistic activity on the fungal pathogen *L. theobromae,* causal agent of cassava stem rot disease.

MATERIALS AND METHODS

Collection and isolation of the filamentous fungi *T. virens*

The 105 *Trichoderma* sp. strains isolated from soil samples originating from cassava fields in Nakhon Ratchasima province, Thailand. The isolates were conducted using potato dextrose agar (PDA) and incubate at 28 ± 1°C for 7 days. Colonies were sub-cultured to obtain pure cultures and these isolates were also purified by the single spore culture technique using serial dilution on PDA. *T. virens* were identified according to micro-morphology of sporulation, as well as the color and morphology of their sporulating structures and conidia (Ubalua and Oti, 2007). Cultures of *T. virens* were maintained on PDA slant and stored at 4°C for further use.

Antagonistic activity of *T. virens* isolates against cassava stem rot pathogen using dual culture technique

Out of 105 isolates of *Trichoderma* sp., the fifteen isolates were identified as *T. virens*. These isolates were evaluated for antagonistic activity against *L. theobromae*, by using dual culture techniques as slightly developed by Sobowale et al. (2008, 2010). Mycelial discs of 5 mm diameter of *T. virens* and cassava stem rot pathogens as *L. theobromae* were placed on opposite sides of a petri plate containing PDA. The plates were performed in triplicates with one control set maintained without inoculating the *T. virens* isolates. The plates were incubated at 28 ± 1°C for 7 days. Growth of *L. theobromae* pathogen was measured after 7 days. The data were recorded regularly on the growth of the pathogen and *T. virens* isolates. Percentage of mycelial growth inhibition (MGI) was calculated according to the formula:

$$MGI\ \% = (dc - dt) \times 100/dc$$

Where, dc = Fungal colony diameter in the control plates, and dt = fungal colony diameter in *T. virens* treatment plates.

Measurements from radial growth (cm) in each plate were averaged prior to statistical analysis. Data from each MGI were analyzed separately using Proc Mixed, SAS version 9.1 and means were separated using the LSD test (p= 0.05).

Trichoderma β- 1,3-glucanase enzyme activity assay

For assay of enzyme activity, *T. virens* were grown on minimal synthetic medium (MSM) contained the following components: $MgSO_4.7H_2O$, 0.2 g/L; K_2HPO_4, 0.9 g/L; KCl, 0.2 g/L; NH_4NO_3, 1.0 g/L; $FeSO_4.7H_2O$, 0.002 g/L; $MnSO_4$, 0.002 g/L and ZnSO4, 0.002 g/L. The medium was supplemented with the appropriate carbon source for β-1,3-glucanase assay (Mishra, 2010). The pH was set to 6.3 with 50 mM phosphate buffer. The medium was inoculated with a spore suspension to give a final concentration of approximately 1×10^6 conidia per milliliter and placed on a rotary shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at fourth day of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4°C to get cell-free culture filtrate which were then used as enzyme source (Mishra, 2010). *Trichoderma* β-1,3-glucanase activity was measured by mixing 50 µl of sample with 100 µl of 50 mM acetate buffer (pH 5.0), containing 0.25% laminarin (Sigma). The mixture was incubated at 40°C for 30 min and the reducing sugar produced was determined by the method described by (Buensanteai et al., 2009). One unit (U) of *Trichoderma* β-1,3-glucanase activities was defined as the amount of enzyme that produced 1 µmol of reducing sugar min^{-1} under the conditions. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard (Buensanteai et al., 2009).

Cassava plant material and *T. virens* application

Cassava stem rot-susceptible cassava cultivars Rayong81, were planted in individual pot containing loamy-sand soil. Experiments were conducted at Suranaree University of Technology, Nakhon Ratchasima, Thailand. Cassava stakes were surface disinfested before experiments by treatment with 95% ethanol for 20 s, followed by soaking in 20% bleach for 20 min. Cassava stakes were washed with sterile distilled water five times to remove the bleach and then air dried in a laminar flow hood for 24 h. Disinfected cassava stakes were treated with effective strain TvSUT10 by dipping 10 cassava stakes with 100 ml of the strain TvSUT10 suspension (1×10^6 spore ml^{-1}) for 5 min. The cassava stakes treated with distilled water served as the negative control. Cassava seedling pots were arranged in randomized complete block design (RCBD) in the greenhouse. Cassava was grown in a greenhouse at 28 ± 2°C, with a photoperiod of 12 h of light and a relative humidity (RH) of 70 ± 10% for one month. Cassava was watered daily and supplemented fertilizer every week.

Cassava stem rot pathogen inoculums preparation

The cassava stem rot disease samples were collected from an untreated cassava in the Nakhon Ratchasima. The *L. theobromae* was isolated on water agar medium (WA) and incubate at 28 ± 1°C for 1 day. Mycelia agars were transferred from 7 days old *L. theobromae* grown on WA medium and inoculate into PDA medium (potato 200 g L^{-1}, dextrose 15 g L^{-1}, agar 15 g L^{-1}). To obtained none conidia, the culture were placed in the dark at 100% RH

Table 1. Antagonistic activities of *Trichoderma virens* against *Lasiodiplodia theobromae*, the causal agent of cassava stem rot disease; collected from Nakhon Ratchasima Province, Thailand.

Trichoderma virens isolates	Location	*Lasiodiplodia theobromae* growth inhibition (%)
TvSUT1	SeangSang District	66.50[c]
TvSUT2	SeangSang District	78.34[f]
TvSUT3	SeangSang District	74.18[e]
TvSUT4	Khon Buri District	83.50[h]
TvSUT5	Khon Buri District	81.84[g]
TvSUT6	Khon Buri District	59.43[a]
TvSUT7	Khon Buri District	63.50[b]
TvSUT8	Khon Buri District	82.95[g]
TvSUT9	SeaKeaw District	71.36[d]
TvSUT10	Khon Buri District	84.12[h]
TvSUT11	Khon Buri District	81.32[g]
TvSUT12	Khon Buri District	57.84[a]
TvSUT13	DanKhunTod District	78.94[f]
TvSUT14	DanKhunTod District	62.92[b]
TvSUT15	DanKhunTod District	62.65[b]

Values are average of three replicates ± SD. Values in the column followed by same letter are not significantly different (P< 0.05).

overnight. Conidia then were collected from the petri-dish using a loop and suspended in distilled water. The concentration was adjusted to 10^6 conidia per milliliter using a hemacytometer. Only the *L. theobromae* aggressive strain was selected for the future experiment.

L. theobromae pathogen inoculation and cassava stem rot disease assessment

One week after *T. virens* strain TvSUT10 treatment, stakes of treated and control cassava seedling were challenge inoculated by spraying with aggressive *L. theobromae* onto the plant. Inoculated cassava seedling were kept overnight in the moisture chamber. Cassava stem rot disease severity then was assessed by measuring the cassava stake infection. In each experimental, three cassava stakes were observed per treatment. Disease severity score applied by Buensanteai (2012) method, 1 score = stem rot between 0 to 6%, 2 score = stem rot less than 25%, 3 score = stem rot between 26 to 50%, 4 score = stem rot between 51 to 75% and 5 score = stem rot over 75%.

RESULTS

Isolation and identification of *Trichoderma* isolates

Out of 105 isolates of *Trichoderma* sp., the fifteen isolates were identified as *T. virens* from different soil samples which were collected from different places of cassava field in Nakhon Ratchasima province including SesngSang, Khon Buri, SeaKeaw and DanKhunTod district (Table 1). These isolates were evaluated for antagonistic activity against stem rot disease in cassava.

Antagonistic activities of *T. viriens* using dual culture method against *L. theobromae*, the cassava stem rot pathogen

In this current study, fifteen *T. virens* strains, isolated from different regions and cassava fields in Nakhon Ratchasima province were selected for screening the mode of action against fungal pathogens as *L. theobromae,* the causal agent of cassava stem rot disease. The result indicated that the isolate TvSUT10 showed excellent antagonistic activity against *L. theobromae*. In dual culture, the inhibition was observed exhibiting antibiosis between pathogen and antagonist of which it was observed that TvSUT10 reduced the growth of *L. theobromae* by 84.12% (Table 1; Figure 1).

Trichoderma β-1,3-glucanase enzyme activity

In this study, *Trichoderma* β-1,3-glucanase enzyme activities were investigated for their ability to fungal cell wall enzyme degradation. Our results found that, the β-1,3-glucanase activity of the strains varied from 12.9 to 25.7 U/mL (Figure 3). The highest activity was recorded in case of *T. virens* strain TvSUT10 (25.7 U/mL). The β-1,3-glucanase activity of *T. virens* strain TvSUT2 (24.3 U/mL) and *T. virens* strain TvSUT9 (24.1 U/mL) was not significantly different ($P < 0.05$). *T. virens* strain TvSUT5 significantly produced minimum β-1,3-glucanase activity

Figure 1. The symptoms of cassava stem rot on susceptible cultivar.

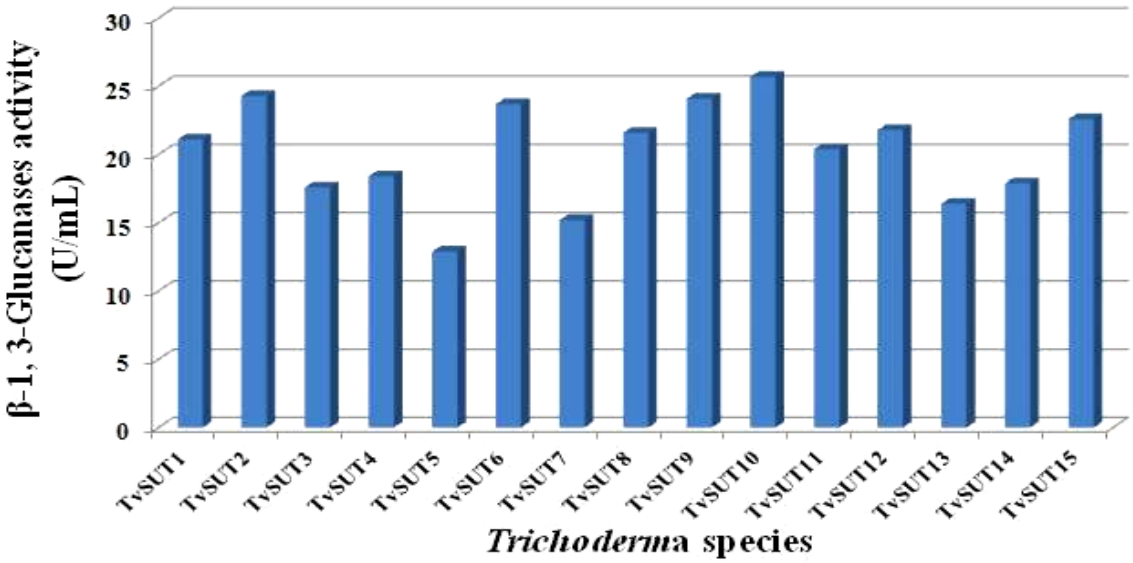

Figure 2. The activity of *Trichoderma* β-1,3-glucanase grown on minimal synthetic medium (MSM) at 150 rpm, 25°C for 4days.

(12.9 U/mL) (Figure 2).

Disease reduction in cassava response to TvSUT10 and *L. theobomae*

In all experiments using cassava cultivars, Rayong81, inoculated with aggressive *L. theobomae*, cassava stakes treatment with *T. virens*, TvSUT10 reduced the severity of stem rot disease on the stem, confirming that induction of resistance had occurred (Figure 3). As illustrated by results from these experiments, treatment with *T. virens* TvSUT10 reduced the severity of stem rot by more than 53% when compared to the negative control (Table 2).

DISCUSSION

Plant disease reduction effects using *Trichoderma* sp. Strains in different economic crops were clearly

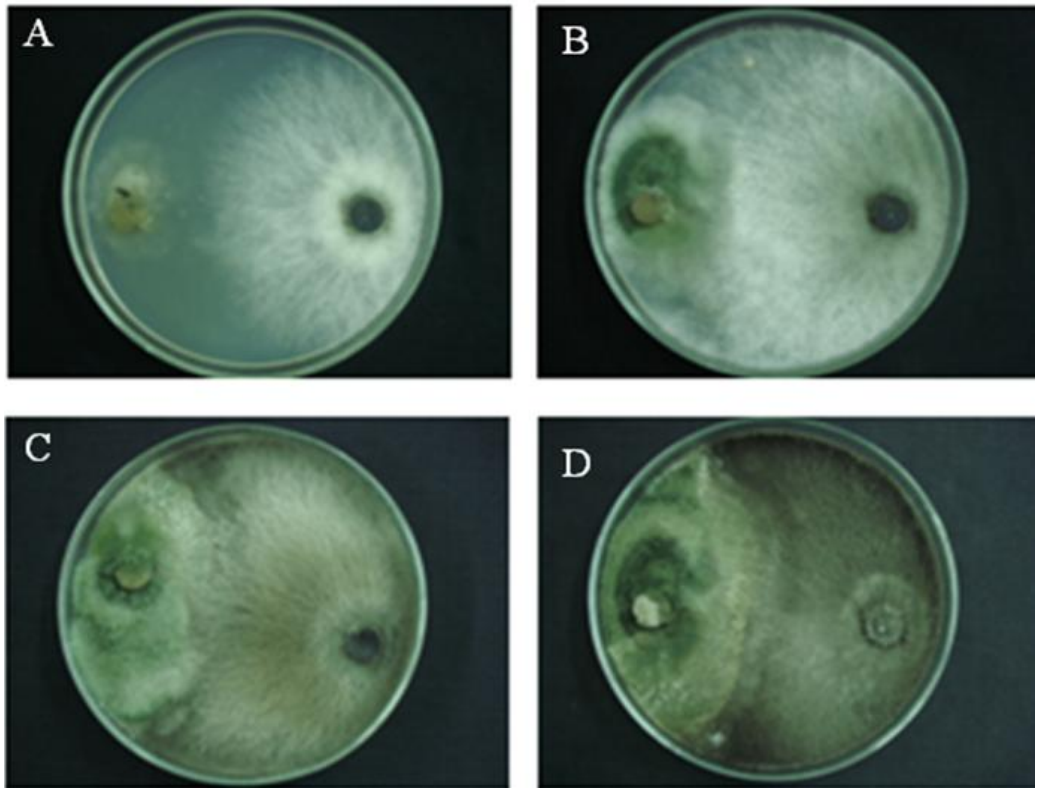

Figure 3. Antagonistic activity of *Trichoderma virens* isolates TvSUT10 against fungal plant pathogens *Lasiodiplodia theobromae* aggressive strain, the causal agent of stem rot disease of cassava at (A) 1, (B) 2, (C) 5 and (D) 7 days after being grown together on potato dextrose agar (PDA).

Table 2. Effects of cassava stake treatment with *Trichoderma virens* strain TvSUT10 on severity of cassava stem rot disease.

Treatment	Cassava stem inoculation[1]	Disease severity[2] (% infected)
Distilled water	*L. theobromae*	74[a]
TvSUT10 (cassava stake treatment)	*L. theobromae*	21[b]
Distilled water	Distilled water	0[c]
TvSUT10 (cassava stake treatment)	Distilled water	0[c]

[1]Leaves were challenged with *Lasiodiplodia theobromae* or sterile distilled water one month after cassava stake treatment and planting. [2]Diseases severity was evaluated 7 days after challenge. Each value represents a mean of three replicate plants with three leaves per plant. Means in the column followed by the same letter are not significantly different according to the LSD test (a = 0.05).

investigated and studied (Tronsmo and Dennis, 1977; Zhihe et al., 1998; Thrane et al., 2000; Yedidia et al., 2000; Spadaro and Gullino, 2005; Ubalua and Oti, 2007; Buensanteai et al., 2010; Akinbode and Ikotun, 2011; Buensanteai, 2011). The beneficial filamentous fungi inoculants are able to improve seedling emergence and vigor, responses to biotic/abiotic stress factors and protect plants from phytopathogens infection (Harman et al., 2004; Buensanteai et al., 2010).

This current study confirms the earlier experiments which revealed that under the laboratory and greenhouse conditions, cassava stake treatment with *T. virens* strain TvSUT10 showed the effective control mechanisms against the aggressive strain of *L. theobromae* causal agent stem rot disease on cassava in Thailand. Similar improvement of biological control mode of action by *Trichoderma* sp. has been reported in other plant as radish and peas (Harman, 1981), yam (Okigbo and Ikediagwu, 2000) maize (Sobowale et al., 2005; Sobowale et al., 2007) and strawberry (Tronsmo and Dennis, 1977). Moreover, Smith et al. (1990) had reported that antagonistic interaction of some soil fungi as *Phytophtora* sp.

The β-1,3-glucanase enzyme produced by *Trichoderma* strain might be interacted with the antagonism mode of action of antagonistic fungi. Mishra (2010) reported that the *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3-

glucanases and proteases. In the current study, *Trichoderma* sp. TVSUT10 was investigated to be the highly producing of β-1,3-glucanase approximately 25.7 U/ml. These results have confirmed that the mode of action might be involved in hydrolysis of fungal pathogen cell wall during antagonism of its biocontrol ability, as the cell wall of plant pathogenic fungi are composed of β-1, 3-glucan (El-Katatny et al., 2000; Monteiro and Ulhoa, 2006; Mishra, 2010.

Conclusion

In conclusion, the results of this experiment suggest that simultaneous *Trichoderma* for stem rot disease reduction under laboratory and pot experiment is a valuable tool to select effective beneficial filamentous fungi strain for our microbial biofertilizer in the near future.

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