Pertanika J. Trop. Agric. Sci. **31(1)**: 45 - 53 (2008)

ISSN: 1511-3701

Antagonistic Potential of Selected Fungal and Bacterial Biocontrol Agents against *Colletotrichum truncatum* of Soybean Seeds

M.M. Begum[1,*], M. Sariah[1], M.A. Zainal Abidin[1], A.B. Puteh[2] and M.A. Rahman[2]
[1]Department of Plant Protection, [2]Department of Crop Science,
Faculty of Agriculture, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
**E-mail: miss_mahbuba@yahoo.com*

ABSTRACT

Ten fungal and bacterial biocontrol agents (BCAs) were evaluated in vitro for their antagonistic potential against *Colletotrichum truncatum* isolated from soybean seeds. Two fungal BCAs namely *Trichoderma virens* isolate UPM23 and *Trichoderma harzianum* isolate UPM40 and a bacterial BCA namely *Pseudomonas aeruginosa* isolate UPM13B8 strongly inhibited the growth of *C. truncatum* based on high PIRG values in dual culture and culture filtrate tests. Studies on the mechanism of action using mycoparasitism technique and antibiosis observed under light microscope revealed that *T. virens* and *T. harzianum* inhibited the growth of *C. truncatum* by coiling and penetration into the hyphae. Consequently, the hyphae of *C. truncatum* became malformed and swollen. *Pseudomonas aeruginosa* also caused mycelial malformation; the mycelia turned vacuoled and swollen in or at tips of hyphae. *Pseudomonas aeruginosa* gave the highest PIRG value in the culture filtrate test, suggesting that antibiosis could be the main mechanism of antagonism. No inhibitory effect was observed on soybean seeds and seedlings when the seeds were artificially inoculated with *T. virens, T. harzianum* and *P. aeruginosa*. On the contrary, *T. virens* and *T. harzianum* were found to enhance seed germination and seedling establishment, while *P. aeruginosa* enhanced fresh and dry weights of seedlings.

Keywords: Antagonist, seed-borne fungi, *Colletotrichum truncatum*, soybean

INTRODUCTION

Colletotrichum truncatum [(Schw.) Andrus & W. D. Moore] is one of the most important seed-borne fungal pathogens that cause anthracnose of soybean. It reduces seed germination and quality (Manandhar and Hartman, 1999; Ploper and Backman 1992). Fungicidal seed treatment is used mainly to control soybean anthracnose (Hopperly, 1985). However, the growing concern against indiscriminate use of fungicides on health hazard and environmental pollution justify the exploitation of biologically based control strategies (Desai *et al.*, 2002). Recently, numerous fungal and bacterial biological control agents (BCAs) have shown the potential to augment or replace chemical pesticides (Ahmad and Baker, 1988). The most widely used BCAs in the world belong to fungal genus *Trichoderma* and bacteria *Pseudomonas* (Khetan, 2001; Tronsmo and Hjeljord, 1998). *Burkhoderia* spp. and *Serratia* spp. have also been introduced recently in biological control programs. They are mainly patented as seed treatments to provide protection against soil-borne fungi like *Pythium* spp., *Rhizoctonia* spp., *Sclerotium* spp., and *Fusarium* spp. in many economically important crops such as tomato, melon, cotton, wheat and onion (Khetan, 2001; Tronsmo and Hjeljord, 1998; Laha *et al.*, 1996; Ordentlic *et al.*, 1988). It was reported that anthracnose of bean and cucumber caused by *Colletotrichum* spp. could be controlled by non-pathogenic rhizosphere fungi and bacteria (Dean and Kuc, 1986; Kuc, 1981). But, information regarding potential BCAs against *C. truncatum* of soybean is very limited. However, search for the suitable and superior strain of BCAs with greater biocontrol activities are necessary for alternative strategies against *C. truncatum. In vitro* screenings of antagonists have been widely used to select all groups of BCAs

* Corresponding Author

and elucidate its biocontrol mechanisms (Desai *et al.*, 2002). Therefore, an attempt was made to evaluate the biocontrol potential of BCAs against *C. truncatum* of soybean seeds.

MATERIALS AND METHODS

Fungal Isolation

The experiment was conducted in 2006 at the Plant Pathology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia (UPM) in Serdang, Selangor. Pathogenic *C. truncatum* was isolated from stored soybean seeds var. palmetto by agar plate method. The fungus was purified by hyphal tip culture method and cultured on potato dextrose agar (PDA) until subsequent studies.

Screening of Fungal and Bacterial BCAs

Seven isolates of *Trichoderma* and three isolates of bacteria obtained from the collection of Plant Pathology laboratory, Faculty of Agriculture, UPM were used in this study. These isolates were screened for their antagonistic activity against *C. truncatum* using dual culture tests based on the percent inhibition of radial growth (PIRG). A 5 mm diameter mycelial agar disc was cut from the margin of 7-day-old culture of *C. truncatum* and placed on one side of a 9 cm Petri dish containing PDA medium and incubated for 48 h. Another 5 mm mycelial agar disc from 7-day-old culture of each *Trichoderma* isolate was placed 3 cm away from the *C. truncatum* disc on the same plate. Plates were incubated at an ambient temperature of 25±1ºC for 15 days. Antagonistic activity of the *Trichoderma* isolates was assessed during incubation period by measuring the radius of the *C. truncatum* colony using the formula

$$\text{Percent inhibition of radial growth (PIRG)} = \frac{R1 - R2}{R1} \times 100$$

where, R1 indicates radial growth of fungal colony in the control plate while R2 indicates radial growth of fungal colony in the dual culture plate. Data regarding the time needed to completely grow over the fungal colony by *Trichoderma* isolates were recorded. The re-growth of the fungus from the inhibition and overgrowth zone was also determined. To test for antagonistic bacteria, a 5 mm diameter of mycelial agar disc from 7-day-old culture was placed in the centre of a 9 cm Petri dish containing nutrient agar (NA) medium. Plates were incubated at an ambient temperature of 25±1°C for 48 h. A loopful of bacteria from 48 h NA culture was then taken and streaked in a circle 3 cm away from the *C. truncatum* disc on the same plate and incubated for 10 days. Data of PIRG and inhibition category were recorded during incubation period. The test was arranged in a completely randomized design with five replications. Based on the highest PIRG values, three promising isolates, namely *Trichoderma virens* isolate UPM23, *Trichoderma harzianum* isolate UPM40 and *Pseudomonas aeruginosa* isolate UPM13B8 were selected to study their mechanism of action.

Mycoparasitism

Hyphal interaction test was used to assess the mycoparasitic activities of *T. virens* isolate UPM23, *T. harzianum* isolate UPM40 and *P. aeruginosa* isolate UPM13B8 against *C. truncatum*. Edges of parasitized fungal hyphae by *T. virens*, *T. harzianum* and *P. aeruginosa* were transferred from the inhibition zone and overgrowth zone from dual culture plate on to clean slides after seven days of incubation. Cover slips were mounted on the mycelia with a drop of lactophenol cotton blue (LCB). Hyphal interaction and morphology were examined under a light microscope.

Antibiosis

Antibiosis test was performed using culture filtrate of UPM40, UPM23 and UPM13B8 on radial growth of the *C. truncatum*. *Trichoderma virens* and *T. harzianum* were grown in potato dextrose broth (PDB) and *P. aeruginosa* in nutrient broth (NB) on an orbital shaker (100 rpm) for 14 days and five days, respectively. Cultures were then centrifuged at 10,000 rpm for 5 min before the supernatant was collected and the pellet discarded. The supernatant was then sterilized and filtered using 0.45 µm and 0.2 µm membrane filters for *Trichoderma* spp. and bacteria, respectively. The sterilized filtrate was then incorporated into sterilized double strength PDA (50ºC) in a ratio of 1:1. Twenty mL of the amended agar was then poured into

each Petri plate and allowed to solidify. A 5 mm diameter mycelial plug of *C. truncatum* was placed centrally in each of the plate and incubated at a room temperature of 25±1°C for 14 days. Non-amended PDA was used as the control. The radial growth of *C. truncatum* was measured and transformed into PIRG in relation to the mycelial growth in the control plate. The hyphal morphology was also examined from the same culture filtrate plates. The edge of fungal mycelia was transferred carefully onto a clean slide after seven days of incubation. A cover slip was placed on the mycelia with a drop of LCB on the slide and the hyphal morphology was observed under a light microscope.

Effects of Artificial Seed Inoculation with Selected BCAs

Seeds were artificially inoculated with *T. harzianum* isolate UPM40, *T. virens* isolate UPM23 and *P. aeruginosa* isolate UPM13B8. Conidia of UPM40 and UPM23 from 7-day-old cultures were washed off separately with 1.5% sodium alginate solution in sterile distilled water. The conidial suspension obtained was adjusted to a concentration of 3.5×10^7 conidia mL^{-1} using a haemocytometer. Cell suspension of *P. aeruginosa* was adjusted to 1×10^{12} CFU mL^{-1} using a spectrophotometer (Spectronic® 20 Genesys™, USA) following the method of Mortensen (1992). To initiate the treatment, soybean seeds were surface sterilized in 5.25% sodium hypochlorite for 3 min and rinsed thrice with sterilized distilled water, and dried for 1 h in a laminar flow chamber. The Seeds were then soaked in suspensions of the respective biocontrol agents in a ratio of 1: 2 (w/v) separately in 250 mL conical flask for 1 h. The treated seeds were then surface re-dried to remove excess water in the laminar flow chamber. The number of conidia determined by a haemocytometer was 2.2×10^5 conidia $seed^{-1}$ and 2.4×10^5 conidia $seed^{-1}$ for UPM23 and UPM40, respectively. Similarly, *P. aeruginosa* determined by serial plating was 1.1×10^9 CFU $seed^{-1}$. Seeds soaked in only 1.5 % sodium alginate solution for 1 h served as the control.

Fifty seeds of each treatment were sowed at the depth of 2 cm in plastic trays (39 × 28 × 11 cm) containing sterilized soil mixture of top soil, peat grow and sand at the ratio of 3: 2: 1 (v/v/v). Trays were arranged in a completely randomized design with four replications in the glasshouse with each tray being considered as a replication. The daily temperature of the glass house ranged from 25-30ºC with 85±5% relative humidity (RH). Data on germination and seedling establishment were recorded up to 14 days. The mean length, fresh and dry weights of the seedlings was also recorded based on twenty seedlings per replicate. Samples were kept for three days at 60°C before the dry weight was recorded.

Statistical Analysis

Data were subjected to one-way ANOVA (SAS, 1999) and means were subsequently compared using Tukey's Studentized Range test (HSD) of arcsine transformed values at 5% level of probability.

RESULTS

Antagonistic Activity of BCAs

Results from the dual culture test showed that all isolates of *Trichoderma* inhibited mycelial growth of *C. truncatum* however with varying efficiencies (Table 1). The PIRG values ranged from 53.85 to 80.77%, with isolates UPM23 (80.77%) and UPM40 (76.92%) being significantly the best followed by TL1 (71.15%), TV2 (67.23%), TV3 (65.24%), UPM29 (61.54%) and TK1 (53.85%). UPM23 completely overgrew the colony of *C. truncatum* within seven days, while UPM40 overgrew within eight days of co-incubation. The other five isolates did not show strong competitive effect since they were unable to colonize *C. truncatum* after 14 days of co-incubation. No distinct inhibition zone towards *C. truncatum* was discernable in any of the *Trichoderma* isolates. *Colletotrichum truncatum* was lysed and failed to re-grow when parasitized hyphae from the interaction and overgrowth zone was cultured on fresh PDA. Among the three bacterial isolates, UPM13B8 significantly exhibited the strongest antagonism against *C. truncatum* with a high PIRG value of 89.89% followed by UPM14B1 (61.80%) and UPM39B3 (35.77%) (Table 2). A distinct inhibition zone was observed when UPM13B8 and UPM14BI were used towards *C. truncatum*. However, UPM13B8 gave a significantly greater distance inhibition zone than UPM14B1 with the value of 9.25 mm and 4.13 mm after seven days of incubation, respectively. After 10 days of

incubation, the UPM13B8 inhibited *C. truncatum* had significantly the same whereas the fungus had grown to contact with UPM14B1 and overgrown on the colony of UPM39B3. The UPM13B8 parasitized mycelia of the fungal pathogen did not recover when transferred to fresh PDA from the inhibition zone. The isolates UPM23, UPM40 and UPM13B8 clearly exhibited stronger antagonistic potential than the other seven isolates based on the high PIRG values against *C. truncatum*. As such these isolates were selected for further studies on micoparasitism and antibiosis.

Mycoparasitism

Microscopic observations revealed that UPM40 and UPM23 hyphae grew initially alongside and coiled compactly around the hyphae of *C. truncatum*. They produced appressorium like structure as attachment to *C. truncatum* hyphae for penetration which led to cell disruption (*Fig. 1B*). Later the hyphae of *C. truncatum* was swollen, malformed and vacuoled (*Fig. 1C*). The parasitized hyphae were unable to regenerate into new colonies when inoculated onto fresh PDA. Parasitized hyphae by UPM13B8 also malformed and swelled (*Fig. 1D*), whereas normal hyphae were smooth and no swelling or vacuolation (*Fig. 1A*).

Antibiosis

Results from the culture filtrate test revealed that UPM23, UPM40 and UPM13B8 strongly inhibited the mycelial growth of *C. truncatum* with different magnitudes of inhibition (Table 3). The isolate UPM13B8 completely inhibited

TABLE 1
Antagonistic effect of *Trichoderma* isolates against *C. truncatum* in dual culture test

Code no.	Species	Antagonism (PIRG)*	Time to over grow
UPM23	*Trichoderma virens*	80.77 a	7 days
UPM40	*T. harzianum*	76.92 ab	8 days
UPM29	*T. harzianum*	61.54 cd	-
TL1	*T. longibrachiatum*	71.15 bc	-
TK1	*T. koningii*	53.85 d	-
TV3	*T. virens*	65.24 c	-
TV2	*T. virens*	67.23 bc	-

- Indicates no overgrowth after 14 days
* indicates percent inhibition of radial growth (PIRG) after five days of incubation
Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.

TABLE 2
Antagonistic effect of bacterial isolates against *C. truncatum* in dual culture test

Code no.	Species	Antagonism (PIRG)*	Inhibition category	
			After 7 days of incubation	After 10 days of incubation
UPM13 B8	*Pseudomonas aeruginosa*	89.89 a	Distance (9.25 a)	Distance (9.25 a)
UPM14 B1	*Burkholderia glumae*	61.80 b	Distance (4.13 b)	Contact (0.0 b)
UPM39 B3	*Serratia marcescens*	35.77 c	Contact (0.0 c)	Fungal Overgrowth (0.0 b)

* indicates percent inhibition of radial growth (PIRG) after 7 days of incubation.
Data in parenthesis indicates the inhibition zone between *C. truncatum* and bacteria in mm
Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.

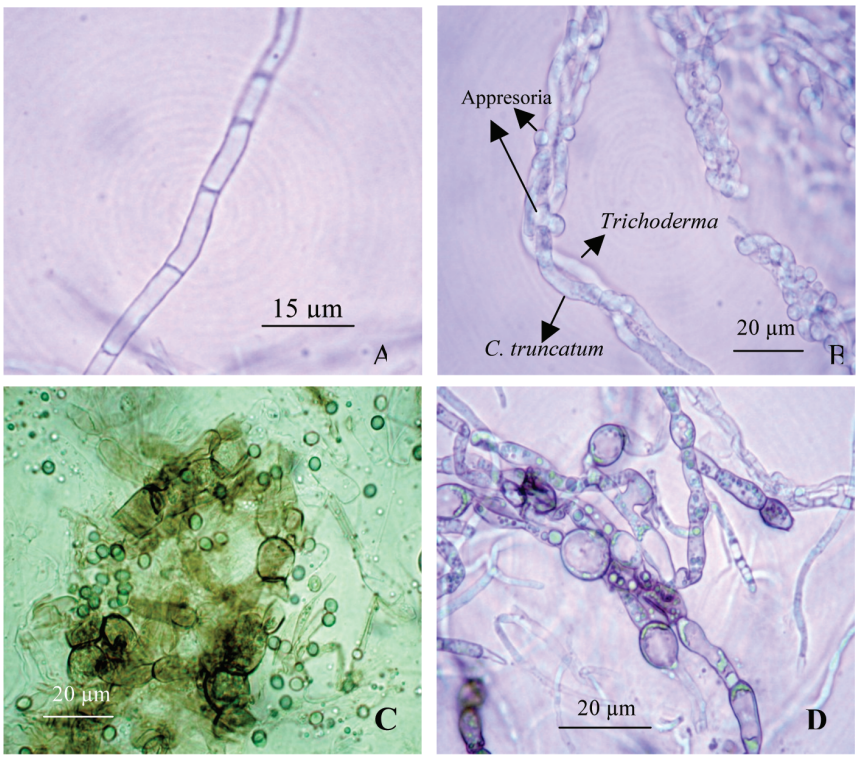

Fig. 1: Photomicrographs showing hyphal morphology of parasitized C. truncatum *by* T. harzianum *(UPM40) and* P. aeruginosa *(UPM13B8)*

A = Normal appearance of *C. truncatum* hyphae
B = Parasitized hyphae of *C. truncatum* coiled by *T. harzianum* and *T. virens*
C = Malformed and swolled hyphae of *C. truncatum* parasitized by *T. harzianum* and *T. virens*
D = Malformed and swolled hyphae of *C. truncatum* parasitized by *P. aeruginosa*

fungal growth and gave the highest PIRG value of 100% compared to UPM23 (82.47%) and UPM40 (69.23%) after seven days of incubation. After 14 days of co-incubation, the PIRG value of UPM23 and UPM40 had increased to 83.50% and 76.99%, respectively. On microscopic observation, the parasitized hyphae of *C. truncatum* by BCAs became malformed, thickened and vacuoled (*Figs. 1C, D*). Many swellings were observed in the hyphae, whereas the normal hyphal walls remained smooth (*Fig. 1A*).

Effect of BCAs on Soybean Seed Germination, Seedling Vigor and Establishment

All of the promising isolates tested did not inhibit seed germination, seedling growth and establishment based on seedling length, fresh weight and dry weight (Table 4). The highest germination was recorded in seeds treated with UPM23 (98%) and UPM40 (97%), while UPM13B8 treated seeds recorded 94% germination which was statistically similar with that of water-treated control seeds (93%). Seedling establishment was significantly higher in UPM40 (98%) treated seeds followed by UPM23 (96%), UPM13B8 (92%) and the control (93%). The maximum seedling length was recorded from seeds treated with UPM40 (50.85%) followed by UPM13B8 (46.83%), UPM23 (45.13%) and water (43.43%) treated seeds. Regarding the fresh and dry weights of seedling, the highest effect was obtained in UPM13B8 treated seeds with the values of 1.66 and 0.20 g seedling^{-1}, respectively. Statistically, similar fresh and dry weights were recorded from treated seeds with UPM23, UPM40 and the control.

TABLE 3
Antagonistic effect of UPM23, UPM40 and UPM13B8 against *C. truncatum* in culture filtrate test

Code no.	Species	Antagonism (PIRG) After 7 days of incubation	Antagonism (PIRG) After 14 days of incubation
UPM23	*T. virens*	82.47 b	83.50 b
UPM40	*T. harzianum*	69.23 c	76.99 c
UPM13 B8	*P. aeruginosa*	100.00 a	100.00 a

Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.

TABLE 4
Effect of UPM23, UPM40 and UPM13B8 on the seed germination, seedling vigor and establishment of soybean under glass house conditions

Treatments	Germination (%)	Seedling establishment (%)	Length seedling^{-1} (cm)	Fresh weight seedling^{-1} (g)	Dry weight seedling^{-1} (g)
UPM23	97.00 a	96.00 b	45.13 bc	1.54 b	0.19 b
UPM40	98.00 a	98.00 a	50.85 a	1.58 b	0.19 b
UPM13 B8	94.00 b	92.00 c	46.83 b	1.66 a	0.20 a
Control	93.00 b	93.00 c	43.43 c	1.50 b	0.19 b

Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of four replications with fifty seeds per replication.

DISCUSSION

In the present study, seven isolates of *Trichoderma* and three isolates of bacteria were tested *in vitro* for the preliminary screening to look for potential biocontrol agents against the pathogenic fungus *C. truncatum*. A considerable variation was observed between, as well as within, the fungal and bacterial isolates with regard to the hyphal interaction and subsequent events to the inhibition in pathogen growth. Of these isolates, two isolates of *Trichoderma* namely *T. virens* isolate UPM23, *T. harzianum* isolate UPM40 and a bacterium namely *P. aeruginosa* isolate UPM13B8 demonstrated stronger antagonistic activities to inhibit the radial growth of *C. truncatum* using dual culture and culture filtrate tests. The time needed for colony overgrowth is an important parameter in the assessment of antagonistic ability to compete against the pathogen for limited nutrient resources and space (Ibrahim, 2005). The isolates UPM23 and UPM40 were able to overgrow fully the colony of *C. truncatum* within seven and eight days in the dual culture test, respectively.

In agriculture, numerous studies have been reported on the antagonistic activity against mainly soil-borne plant pathogens, but only a few studies have investigated the antagonistic activity against *Colletotrichum* spp. of different crops. *Trichoderma harzianum* was found to decrease significantly the incidence of *Glomerella* (*Colletotrichum*) *glycines* on soybean (Fernandez, 1992) and anthracnose caused by *C. lindemuthianum* on beans was controlled by *P. aeruginosa* (De Meyer *et al.*, 1999). Severity of foliar anthracnose of soybean caused by *C. dematium* was reduced significantly by fluorescent pseudomonad as stated by Tripathi *et al.* (2006).

Beside competition for resources and space, the invasive mechanism of Trichoderma includes lysis, mycoparasitism, antibiosis and local or systemic induced resistance (Harman, 2005; Howell, 2003). Similarly, in this study it was observed that the mechanisms of antagonism for UPM23 and UPM40 were through competition, lysis, mycoparasitism and antibiosis. In the dual culture test both UPM23 and UPM40 were able to compete and inhibit the mycelial growth of *C. truncatum*. They parasitized and lysed the hyphae of *C. truncatum* through coiling and subsequent penetration. They produced appressorium like structures which aided in the penetration of the host cell wall (Goldman *et al.*, 1994). A similar observation was reported on parasitized hyphae of *Sclerotium rolfsii* by *T. harzianum* (Widyastuti *et al.*, 2003), *Botryodiplodia theobromae* by *T. harzianum* (Gupta *et al.*, 1999) and *Rhizoctonia solani* by *T. virens* (Howell, 2003). Subsequent degradation of the fungal cell wall might be due to the actions of different lytic enzymes. Strong support has been given by Lorito *et al.* (1993) on the importance of cell-wall degrading enzymes secreted by *T. harzianum* and *T. virens* (syn. *G. virens*) in controlling fungal diseases. This finding strongly suggested that the inhibition of mycelial growth of *C. truncatum* in the presence of *T. harzianum* and *T. virens* were due to the effect of antifungal substances as proven by the culture filtrate test through the mechanism of antibiosis. Secreted enzymes mainly chitinase, _-1, 3 glucanase and _-1, 3 glucosidase were reported to be responsible for the degradation of the host cell wall by *T. harzianum* and *T. virens* (Howell, 2003; Khetan, 2001; Tronsmo and Hjeljord, 1998).

Pseudomonas aeruginosa inhibited the radial growth by establishing a clear inhibition zone in a dual culture test; no further growth of *C. truncatum* was observed when the PDA was amended in a culture filtrate from this strain. Moreover, mycelial malformation and vacuolation occurred frequently with parasitized mycelia as revealed in both tests. Several mechanisms are responsible for suppression of pathogens by bacteria, including competition, antibiotic and metabolite production and also induction of systemic resistance (Compant *et al.*, 2005; Whipps, 2001). The inhibition of radial growth by forming inhibition zone against fungal pathogen is considered as antibiosis, whereby the antibiotic metabolites may penetrate the cell and inhibit its activity by chemical toxicity. The mycelial malformation observed was probably due to the toxic effect of antibiotic substances interfering with normal growth processes (Sariah, 1994). *Pseudomonas aeruginosa* is known to produce metabolites such as pyoverdin, pyochelin and salicylic acid which are effective against various pathogens (De Meyer and Hofte, 1997; Buysens, 1996). The vacuolar appearance of the mycelium might be due to the antibiotic metabolites produced by the bacterium, which may penetrate and cause protoplasmic dissolution and disintegration (Rahman *et al.*, 2007). *Pseudomonas aeruginosa* produced a higher PIRG value than *T. harzianum* and *T. virens* in the cultural filtrate test thus indicating that more antibiotic substances were produced by *P. aeruginosa*.

All selected BCAs did not show any adverse effect on seed germination and seedling growth performance under glass house conditions. *Trichoderma virens* and *T. harzianum* seemed to enhance seed germination, seedling stand and length, but did not provide any positive effect on the fresh and dry weights of seedlings. However, *P. aeruginosa* improved fresh and dry weights of seedlings from treated seeds. Enhancement of plant growth is well documented by *Trichoderma* spp. (Harman, 2000; Yedidia *et al.*, 1999) and *P. aeruginosa* (Hofte *et al.*, 1991). But, the effect on seed germination and seedling growth promotion seemed to be inconsistent among antagonists. This contradictory effect in growth promotion may be dependent on the antagonistic performance to survive and develop actively in the rhizosphere (Devliegher *et al.*, 1995; Kleifeld and Chet, 1992). Thus, activities of *T.* virens, *T. harzianum* and *P. aeruginosa* in this study suggested that all the three antagonists could be utilized as BCAs against C. *truncatum*. The use of these biocontrol agents could be an economically feasible alternative to chemical biocides and environmental friendly in suppressing the anthracnose disease in biological control programs of soybean.

ACKNOWLEDGEMENTS

The authors are sincerely grateful to the Third World Organization for Women in Science (TWOWS) for the financial support to conduct this research at Universiti Putra Malaysia.

REFERENCES

AHMAD, J.S. and BAKER, R. (1988). Implications of rhizosphere competence of *Trichoderma harzianum*. *Can. J. Microbiol.*, *34,* 229-234.

BUYSENS, S., HEUNGENS, K., POPPE, J. and HOFTE, M. (1996). Involvement of pyochelin and pyoverdin in suppression of *Pythium*-induced damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Appl. Environ. Microbiol.*, *62,* 865–871.

COMPANT, S., DUFFY, B., NOWAK, J., CLEMENT, C. and BARKA, E.A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospects. *Appl. Environ. Microbiol.*, *71,* 4951 - 4959.

DE MEYER, G. and HOFTE, M. (1997). Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinera* on bean. *Phytopathol.*, *87,* 588-593.

DE MEYER, G., CAPIAU, K., AUDENAERT, K., BUCHALA, A., METRAUX, J.P. and HOFTE, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Molecular Plant-Microbe Interactions*, *12,* 450-458.

DEAN, R.A. and KUC, J. (1986). Induced systemic protection in cucumber: time of production and movement of the signal. *Phytopathol.*, *76,* 966-970.

DESAI, S., REDDY, M.R. and KLOEPPER, J.W. (2002). Comprehensive testing of biocontrol agents. In S.S. Gnanamanickam (Ed.), *Biological control of crop diseases* (p. 387-420). New York: Marcel Dekker Inc.

DEVLIEGHER, W., ARIF, M.A.S. and VERSTRAETE, W. (1995). Survival and plant growth promotion of detergent-adapted *Pseudomonas fluorescens* ANP15 and *Pseudomonas aeruginosa* 7NSK2. *Appl. Environ. Microbiol.*, *61,* 3865-3871.

FERNANDEZ, M.R. (1992). The effect of *Trichoderma harzianum* on fungal pathogens infesting soybean residues. *Soil Biol. Biochem.*, *24,* 1027-1029.

GOLDMAN, G.H., HAYES, C. and HARMAN, G.E. (1994). Molecular and cellular biology of biocontrol by *Trichoderma* spp. *Tibtech*, *12,* 478-482.

GUPTA, V.P., TEWARI, S.K., GOVINDAIAH and BAJPAI, A.K. (1999). Ultrastructure of mycoparasitism of *Trichoderma*, *Gliocladium* and *Laetisaria* species on *Botryodiplodia theobromae*. *J. Phytopathol.*, *147,* 19-24.

HARMAN, G.E. (2005). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathol.*, *96,* 190-194.

HARMAN, G.E. (2000). Myth and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Dis.*, *84,* 377-393.

HOFTE, M., SEONG, K.Y., JURKRVITCH, E. and VERSTRAETE, W. (1991). Pyoverdin production by plant growth beneficial *Pseudomonas* strain 7NSK2: ecological significance in soil. *Plant and Soil*, *130,* 249-258.

HOPPERLY, P.R. (1985). Soybean anthracnose. In R. Shibles (Ed.), *World Soybean Research Conference III* (p. 547-554). Boulder and London: Westview Press.

HOWELL, C.R. (2003). Mechanisms employed by *Trichoderma* species for the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.*, *87,* 1-10.

IBRAHIM, M.D. (2005). *Delivery system of Trichoderma inoculant for the control of Rhizoctonia diseases in Brassica rapa* (Ph. D. thesis. Universiti Putra Malaysia, Malaysia, 2005).

KHETAN, S.K. (2001). *Microbial Pest Control.* New York: Markel Dekker Inc.

KLEIFELD, O. and CHET, I. (1992). *Trichoderma* – Plant interaction and its effect on increased growth response. *Plant and Soil*, *144,* 267-272.

KUC, J. (1981). Multiple mechanisms, reaction rates and induced resistance in plants. In R.C. Staples and G.H. Toenniessen (Eds.), *Plant disease control* (p. 259-272). New York: John Wiley and Sons.

LAHA, G.S., SINGH, R.P. and VERMA, J.P. (1996). Role of growth promoting rhizobacteria in plant disease management. In V.P. Agnihorti, O. Prakash, R. Kishun and A.K. Misra (Eds.), *Disease scenario in crop plants, Vol. II-Cereals, Pulses, oilseeds and cash crops* (p. 233-241). Delhi, India: International Books and Periodicals.

LORITO, M., HAYES, C.K., PETERBAUER, C., TRONSMO, A., KLEMSDAL, S. and HARMAN, G.E. (1993). Antifungal chitinolytic enzymes from *Trichoderma harzianum* and *Gliocladium virens*: purification, characterization, biological activity and molecular cloning. In R.A.A. Muzzarelli (Ed.), *Chitin enzymology* (p. 383-392). Lyon and Ancona: European Chitin Society.

MANANDHAR, J.B. and HARTMAN, G.L. (1999). Anthracnose. In G.L. Hartman, J.B. Sinclair and J.C. Rupe (Eds.), *Compendium of soybean diseases* (4th edn., p. 13-14). APS Press: St. Paul.

MORTENSEN, C.N. (1992). Seed bacteriology laboratory guide: Danish Government Institute of seed pathology for developing countries. Copenhagen, Denmark.

ORDENTLICH, A.Y., ELAD, Y. and CHET, I. (1988). The role of chitinase of *Serratia macescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathol.*, *78,* 84-88.

PLOPER, L.D. and BACKMAN, P.A. (1992). Nature and management of fungal diseases affecting soybean stems, pods and seeds. In L.G. Copping, M.B. Green and R.T. Rees (Eds.), *Pest management in soybean* (p. 174-184). London and New York: SCI: Elsevier Applied Science.

RAHMAN, M.A., KADIR, J., MAHMUD, T.M.M., RAHMAN, R.A. and BEGUM, M.M. (2007). Screening of antagonistic bacteria for biocontrol activities on *Colletotrichum gloeosporioides* in papaya. *Asian J. Plant Sci.*, *6,* 12-20.

SARIAH, M. (1994). Potential of *Bacillus* spp. as a biocontrol agent for anthracnose fruit rot of chilli. *Mal. Appl. Biol.*, *23,* 53-60.

SAS. (1999). SAS/STAT Guide to Personal Computer. Version 8.1. SAS Institute Inc., Cary, North Carolina, USA.

TRIPATHI, M., JOHRI, B.N. and SHARMA, A. (2006). Plant growth-promoting *Pseudomonas* sp. strains reduce natural occurrence of anthracnose in soybean (*Glycine max* L.) in central himalayan region. *Curr. Microbiol.*, *52,* 390-394.

TRONSMO, A. and HJELJORD, L.G.. (1998). Biological control with *Trichoderma* species. In G.S. Boland and L.D. Kuykendall (Eds.), *Plant-microbe interactions and biological control* (p. 111-124). New York: Marcel Dekker Inc.

WHIPPS, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Experi. Botany*, *52,* 487-511.

WIDYASTUTI, S.M., HARJONO, SUMARDI and YUNIARTI, D. (2003). Biological control of *Scleorotium rolfsii* damping-off of tropical pine (*Pinus merkusii*) with three isolates of *Trichoderma* spp. *Online Journal of Biological Sciences*, *3,* 95-102.

YEDIDIA, I., BENHAMOU, N. and CHET, I. (1999). Induction of defense response in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.*, *65,* 1061-1070.