Biological control of tomato leaf blight disease by high cell density culture of antagonistic *Bacillus subtilis*

Kaltima Phichai[1*]

ABSTRACT: Tomato is one of the important economic vegetable crops which are attacked by several serious diseases such as leaf blight. *Bacillus* genera is the most feasible biocontrol microorganism suppresses several pathogens like *Alternaria* spp. The efficiency of the antagonistic treated plant by strains was evaluated using an in vitro assay. Dual culture examination was performed to investigate the ability of antagonistic bacteria to inhibit the growth of leaf blight caused by *Alternaria* spp. The efficacy of antagonistic *Bacillus subtilis* showed that the bacterium effectively suppressed the development of *Alternaria alternata* and *Alternaria* sp. at 38.67 and 32.89 %, respectively while chemical fungicide agent (mancozeb) could inhibit the pathogen at 38.89 %. The shake flask culture of *B. subtilis* in 7 formulas media was carried at laboratory with 200 rpm for 120 h at room temperature (30°C). The results showed that the high cell density at 96 h has been found for all medium. The highest cell density of ๓,๖ x ๑๐^๘ CFU mL^{-1} was achieved from NGB medium. These findings support the potential use of *B. subtilis* for biological control of *Alternaria* spp. on tomato plants.

Keywords: antagonistic bacteria, biological control, leaf blight, *Bacillus subtilis*, *Alternaria* spp.

Introduction

Tomato (*Lycopersicon esculentum* L.) is considered one of the most important economic vegetable crops in Thailand. Tomato plants are subjected to attack by several soil born fungal pathogens, which cause serious diseases as leaf blight including, early blight and late blight (Montealegre et al., 2005; Srinon et al., 2006). *Alternaria alternata* or tomato early blight alternaria is a fungus that can cause cankers and plant leaf spots on tomato plants. It normally occurs during hot weather when there has been a significant amount of rain and humidity. Plants that have been damaged are particularly susceptible to being infected by tomato early blight alternaria and are responsible for significant economic losses sustained by tomato producers each year (Matthew E.S., and J.E. Alexander. 1999). There is a high incidence of the pathogen especially in Northern and North-Easthern Thailand, where tomato plants are widely cultivated. Currently, few effective fungicides are available to control the pathogen and, when allowed, there are also chemical tools that can have technical, environmental, and toxicological risks (Baker and Cook. 1974). Furthermore, it is difficult to protect the plants by fungicide application when plant foliage is expanded and covers the crown zone where the pathogens start the infection. Recently, the necessity to reduce the energy costs in farming and to develop more environmental friendly and safer methods has encouraged the scientific research to evaluate the efficacy of new control methods against soil-borne pathogens. Among these new methods, there is

[1] Faculty of Science and Technology, Chiangmai Rajabhat University, Chiangmai, 50300, Thailand

* Corresponding author: kaltimap@hotmail.com

the application of antagonistic micro-organisms (yeasts, fungi, bacteria, etc.) alone or combined with fungicides or natural adjuvants (Cao et al., 2011). So far, gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents with regard to the production of antimicrobial metabolites, genetic analysis and regulation of some metabolites, and ecological fitness of soil. The gram-positive bacteria, like *Bacillus* spp., however, has been studied less intensively than the gram-negative bacteria. *Bacillus subtilis* is considered to be a safe biological agent, but evaluation of *B. subtilis* has primarily focused on the degree of disease suppression (Stein et al., 2002; Liu et al., 2006; Grover et al., 2010). The high cell density culture and preferable in medium of the strain has not been as extensively investigated. The main objectives of the current study were to evaluate *Bacillus subtilis* as biocontrol agents against leaf blight disease of tomato and to study shake flask culture in various medium of *B. subtilis* in order to further develop to high cell density culture of the strain.

Methods

Alternaria spp. was isolated from leaf blighted tomato plants cultivated in green house of Chiangmai Rajabhat University. It was microscopically identified on the basis of cultural and microscopic characteristics as *Alternaria alternata* and *Alternaria* sp. The isolate was maintained on PDA medium at 4°C. The antagonistic bacterium, *Bacillus subtilis*, was isolated from the rhizospheric area of tomato plants grown from a farm in Chiangmai, Thailand and identified by Microbiology center, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The bacterium was maintained on Nutrient agar (NA) at 4°C before experimental use. The antagonistic effect of the tested biocontrol agents against *Alternaria* spp. was examined using the technique of dual culture analysis (Morton and Stroube, 1955). *Alternaria alternata* and *Alternaria* sp. were cultured on PDA medium for 7 days at 30°C. Then, a plug (0.5 cm diameter) of the pathogen fungal colony was cut from periphery of active growing culture using a sterile cork borer and aseptically placed on to a PDA plate, approximately 2.5 cm from the center of the plate. Single bacterial colonies were patched along the perimeters of the plates at a distance of 5 cm from the fungal block. PDA plates singly inoculated with *Alternaria* spp. and Mancozeb (4 g/L) were prepared as controls. Five replicates were prepared in each experiment. Inoculated plates were incubated at 30°C until the fungal radial growth of the control plates reached 4.5 cm, the inhibition zone in between the bacterial and fungal colonies was recorded (see Figure 1). The percent inhibition of radial growth; PIRG was calculated according to following equation (Tronsmo, 1992).

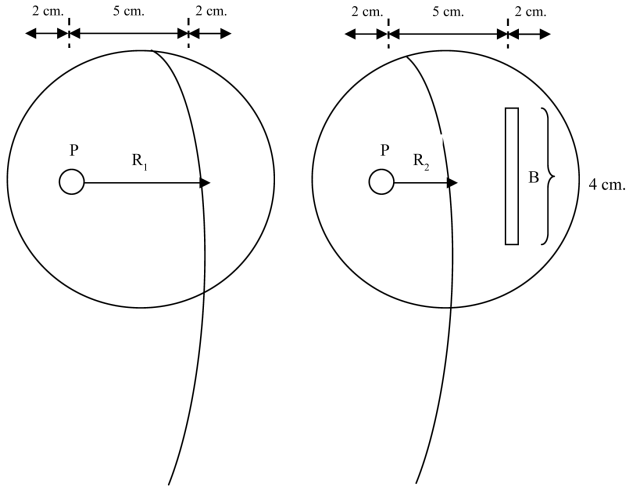

Figure 1 The placement of pathogen and antagonistic bacterium in bioassay plates (in vitro) and the measurement of inhibition zone. (P: Pathogen, B: Antagonistic bacteria, R: Radial growth of pathogenic fungi)

$$PIRG = \frac{R_1 - R_2 \times 100}{R_1}$$

Where, R1 = Radial growth of *Alternaria* spp. in control plate

R2 = Radial growth of *Alternaria* spp. interacting with antagonistic bacteria

- Shake flask culture of *Bacillus subtilis*

For inoculum preparation, the organism was grown on NA at 30 °C for 24 h. One loop of organism was transferred to 250 mL flask with 100 mL Nutrient broth (NB) working volume and incubated at 30 °C, 200 rpm for 16 h. Then, cells were centrifuged at 10,000 g for 15 mins. and supernatant was discarded. Cells were washed with distilled water for 3 times. Number of cells was determined by spectrophotometer at 600 nm and inoculum was adjusted to absorbance of A_{600} 0.2 unit.

Seven media (Table 1) were used for comparative purpose including, Malt-yeast extract medium (MY), Yeast peptone medium (YP), Glucose-malt extract-peptone medium (GMP), NTG, Nutrient glucose broth (NGB), Nutrient broth (NB) and Molasses soybean meal medium (MS). Five mL of seed culture were inoculated into 95 mL of the respective medium in a 250 mL Erlenmeyer flask and incubated at 30 °C, 200 rpm, for 120 h. The samples were collected at interval time of 24, 48, 72, 96 and 120 h. measurement of optical density at 600 nm (OD600), and determination of viable cell count by 10-folds serial dilution spread plate technique on NA.

Table 1 Composition of media used for shake flask cultur

Media	Composition (g/L)
MY	Malt extract, 3; Yeast extract, 3; Peptone, 5; Glucose, 10
YP	Yeast extract, 5; Peptone, 10
GMP	Glucose, 15; Peptone, 6; Malt extract, 3; Yeast extract, 3; NaCl, 5; $MgSO_4.7H_2O$, 0.25
NTG	Glucose, 20; KH_2PO_4, 0.4; NaCl, 1; $MgSO_4.7H_2O$, 0.2
NGB	Beef extract, 3; Peptone, 5; Glucose, 2.5
NB	Peptone, 5; Beef extract, 3
MS	Soy bean meal, 10; Molasses, 5

Results and Discussion

Bacillus subtilis strain was evaluated for antagonistic effect against *Alternaria alternata* and *Alternaria* sp. on Petri dishes containing PDA medium. Table 2 shows that the bioagent strain succeeded in reducing the radial growth of *A. alternata* and *Alternaria* sp. *B. subtilis* was more active on *A. alternata* than for reducing the radial growth of *Alternaria* sp. being 2.76 and 3.02 cm, respectively. The percent inhibition of radial growth of *A. alternata* and *Alternaria* sp. by *B. subtilis* strain was 38.67 and 32.89 %, respectively (Table 3). Comparing with the control Mancozeb, non-systemic fungicide treatment, the result gained from *B. subtilis* against *A. alternata* was not significantly different ($P \leq 0.05$). The mycelial growth reduction, noted in the present study, was mainly due to the important competitive potential of the antagonistic used (Weller, 1988). This behavior represents an important approach for controlling a leaf blight disease of tomato plants. The potentiality of the used strain could be also attributed to its effect to secrete hydrolytic enzymes or antifungal metabolites (Berg and Ballin, 1994). Also, Peng and Mustafa (2012) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens. However, the successful use of bio-control may be needed the further study as Hashem and Abo-Elyousr (2011) suggested to use the strain with combinations of different biocontrol organisms.

Table 2 Effect of *B. subtilis* on the radial growth of *Alternaria* spp.

Treatment	Radial growth (cm.)	
	Alternaria sp.	*Alternaria alternata*
Mancozeb	2.75 ± 0.110[a]	2.75± 0.059[a]
B.subtilis	3.02 ± 0.131[b]	2.76 ± 0.364[a]
Untreated control	4.50 ± 0.152[c]	4.50 ± 0.152[b]

Means of radial growth in any column with different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 3 In vitro efficacies of the strain *B. subtilis* against the two strains of *Alternaria*

Treatment	% inhibition of radial growth (n = 5)	
	Alternaria sp.	*Alternaria alternata*
Mancozeb	38.89	38.89
B.subtilis	32.89	38.67
Untreated control	0	0

Shake flask culture for a comparison of the growth of *B. subtilis* on the seven different media was performed. Average profiles of total viable cell count at 96 h of *B. subtilis* in various media are presented in Table 4. The highest number of cells gained from NGB medium was 3.6 x 108 CFU mL^{-1} and followed by NB and MS with the cells of 2.3 x 10^8 and 2.1 x 10^8 CFU mL^{-1}, respectively. This may be due to glucose was included in NGB medium while glucose did not appear in NB medium. This implied that glucose is needed in medium formulations when high cell density culture is required (Phichai, 2008). This is not surprising as the organism uses glucose as major carbon source to obtain energy for assimilation. This experiment suggested that glucose was the essential carbon source required for high cell yield of *B. subtilis.*

Table 4 Number of cells *B. subtilis* at 96 h of cultivation (CFU mL^{-1})

Media	Viable cells of *Babillus subtilis* (CFU mL^{-1})
MY	1.9 x $10^{6\ e}$*
YP	2.1 x $10^{6\ e}$
GMP	8.2 x $10^{6\ d}$
NTG	7.3 x $10^{7\ c}$
NGB	3.6 x 108 ^{a}
NB	2.3 x $10^{8\ b}$
MS	2.1 x $10^{8\ b}$

*Means of viable cells in column with different letters are significantly different (P≤ 0.05) according to Duncan's multiple range test.

However, there are some issues needed to be considered when glucose is included in medium for high cell density. Firstly, the solubility and sterilization of high concentration of glucose in feed solution (50 – 70 % of glucose), it may not be convenient to prepare, especially for large scale production compared with other carbon sources which can be used in liquid form such as glycerol or fructose and maltose syrup (Dahod S.K. 1999). It is known that a high glucose concentration in medium of related bacteria (e.g. *E. coli*) can lead to the accumulation of acetate. Riesenberg (1991) has reported that the growth of *E. coli* was inhibited when glucose concentration exceeded 50g/L. Interestingly, MS medium gave the similar result to NB with no significantly

different ($P \leq 0.05$). Due to molasses in MS composed of simple sugar such as glucose and other nutrients are essential for the growth of microorganism. Thus, molasses is beneficial substrate for the strain (Lazaridou et al., 2002). Soy bean meal is flour made by grinding the solid residue of soybean oil production which can be used as nitrogen and carbon sources (Phichai, 2008). Both molasses and soy bean meal are agricultural, therefore, they are free from toxic and available for using as substrate to grow the desired microorganisms (Dahod, 1999).

Conclusion

The study suggested to use *B. subtilis* as a common biocontrol practice against *A. alternata* or tomato early blight alternaria on tomato plants. Development of medium optimization from agricultural residual is needed to be further investigated in order to make the possibility of value added and cost effective in agricultural production.

References

Baker, K.F., and R.J. Cook. 1974. Biological Control of Plant Pathogen. W.H. Freeman, San Francisco.

Berg, G., and G. Ballin. 1994. Bacterial antagonists to Verticillium dahliae Kleb. J. Phytopathol. 141: 99-110.

Cao, S., Z. Yang, Z. Hua, and Y. Zheng. 2011. The effects of the combination of Pichia membranefaciens and BTH on controlling of blue mould decay caused by Penicillium expansum in peach fruit. Food Chem. 124: 991-996.

Dahod, S.K. 1999. Raw materials selection and medium development for industrial fermentation processes. p. 213-220. In: A.L. Demain, J. E. Davies, editors. Manual of Industrial Microbiology and Biotechnology. 2nd ed. Washington, D.C, American Society for Microbiology.

Grover, M., L.Nain, S.B. Singh, and A.K. Saxena. 2010. Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent Bacillus subtilis RP24. Curr. Microbiol. 60: 99-106.

Hashem, M., and K.A. Abo-Elyousr. 2011. Management of the root-knot nematode Meloidogyne incognita on tomato with combinations of different biocontrol organisms. Crop Prot. 30: 285-292.

Lazaridou, A., T. Roukas, C.G. Biliaderis, and H. Vaikousi. 2002. Characterization of pullulan produced from beet molasses by Aureobasidium pullulans in a stirred tank reactor under varying agitation. Enzyme. Microbiol. Tech. 31: 122–132

Liu, Y.F., Z.Y. Chen, T.B. Ng, J. Zhang, M.G. Zhou, F.P. Song, and Y.Z. Liu. 2006. Bacisubin, an antifungal protein with ribonuclease and hemagglutinating activities from Bacillus subtilis strain B-916. Peptides 28: 553-559.

Matthew, E.S., and J.E. Alexander. 1999. Salicylic Acid Induces Resistance to Alternaria solani in Hydroponically Grown Tomato. Phytopathology. 89: 722-727.

Montealegre, J.R., R. Herrera, J.C. Velasquez, P. Silva, X. Besoain, and L.M. Perez. 2005. Biocontrol of root and crown rot in tomatoes under greenhouse conditions using Trichoderma harzianum and Paenibacillus lentimorbus. Additional effect of solarisation. Electronic Biotech. 8: 249-257.

Morton, D.J., and W.H. Stroube. 1955. Antagonistic and stimulatory effects of soil microorganisms upon Sclerotium rolfsii. Phytopathology. 45:417-420.

Peng, L.P., and M. Mustafa. 2012. Production of non-volatile antifungal from Bacillus subtilis on selected solid medium. Available: http://www.fsas.upm- edu.my/~muska/Thesis%20AH%20 Peng.pdf. Accessed Apr. 20, 2012.

Phichai, K. 2008. Fermentation Technology. Chiangmai, Chiangmai Rajabhat University.

Riesenberg, D. 1991. High-cell-density cultivation of Escherichia coli. Biotechnol. 2: 380-384.

Srinon W., K. Chuncheen, K. Jirattiwarutkul, K. Soytong, and S. Kanokmedhakul. 2006. Efficacies of antagonistic fungi against Fusarium wilt disease of cucumber and tomato and the assay of its enzyme activity. J. Agric. Technol. 2: 191-201.

Stein T., S. Borchert, B. Conrad, J. Feesche, B. Hofemeister, J. Hofemeister, and K.D. Entian. 2002. Two different antibiotic-like peptides originate from the ericin gene cluster of Bacillus subtilis A1/3. J. Bacteriol. 184: 1703-1711.

Tronsmo, A. 1992. Leaf and blossom epiphytes and endophytes as biological agents of plant diseases. p. 43 – 54. In: E.C. Tjamos, G.C. Papavizas, R.J. Cook Editors. Biological Control of Plant Disease; Progress and Challenges for the Future, NATO ASI series. A life sciences Vol 230, Plenum Press, New York.