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Screening of Phylloplane Bacteria to Control Target Spot Disease of Tomato in Thailand

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The objective of this study was to screen phylloplane bacteria for the control of *Corynespora cassiicola*, a causal agent of target spot disease of tomato. Phylloplane bacteria were isolated from tomato leaves by washing and after dilution they were spread on four media: potato dextrose agar (PDA), nutrient agar (NA), yeast malt agar (YM) and arginine glycerol mineral salt agar (AGMA). Twenty-nine bacterial isolates were obtained and their ability to inhibit the growth of *C. cassiicola* was tested *in vitro*. The results showed that six isolates effectively controlled mycelium growth by 45.01-51.11% inhibition in dual culture on PDA. All effective isolates were tested for their control of target spot disease using the detached leaf technique by spraying a conidial suspension of *C. cassiicola* (10^4 conidia/ml) 24 hr before or after application of the phylloplane bacteria (10^8 cell/ml). We found that antagonistic bacterial isolates BB47, BC29, BC30, BC35, BCP36 and PT4 effectively reduced the disease incidence from 25.00 to 45.00%. Mechanistic studies of cell suspensions of phylloplane showed that the bacteria could inhibit spore germination and mycelial growth of *C. cassiicola,* the culture filtrate gave a similar result as well. Identification of the phylloplane bacteria by morphology and biochemical tests showed that BC29, BC30, BC36 and PT4 were gram-positive and classified into the genus *Bacillus*, while BB47 and BC35 were gram-negative and classified into the genus *Pseudomonas*.

Key words: Antagonistic bacteria, *Bacillus* sp., *Corynespora cassiicola*, *Pseudomonas* sp.

Tomato (*Solanum lycopersicum* L.) is a plant that often has red edible fruit/berries and belongs to the nightshade family Solanaceae. The species originated in South America and it was used as a food first in Mexico, and spread throughout the world following the Spanish colonization of the Americas. In Thailand, tomato is widely cultivated especially in the North, Northeast and Central regions such as Chiang Mai, Lampang, Phetchabun, Nong Khai, Khon Kaen, Nakhon Ratchasima, Ratchaburi, Nakhon Pathom and Kanchanaburi. The demand for tomatoes in Thailand has increased, but the yield is not enough due to problems with diseases and insect pests. A serious disease of tomato is target spot disease, caused by *Corynespora cassiicola* (Berk. & Curt.) Wei[1]

C. cassiicola has spread throughout tropical and subtropical countries and it is a very important plant pathogen in Australia[2], Italy, the United States[3], Argentina[4], Brazil[5], India[6], Sri Lanka[7], Nigeria, China[8], South Asia, South East Asia[9, 10] and Thailand[11]. *C. cassiicola* can damage all parts of the plant including the leaves, stems, flowers and fruits, with more than 700 host varieties such as cucumber[12], rubber[13], tomato[14], soybean[15], tobacco, eggplant[16], sesame[17], bean, cocoa and cotton[18].

Disease control can be achieved via the use of chemicals such as the strobilurin fungicide

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azoxystrobin and a combination product of mancozeb and fumoxate that provide excellent control of target spot[3]. The use of many chemicals wastes money and causes residue on agricultural products and are even dangerous to humans and the environment in the long term[19]. Biological methods are a way to help reduce the use of agricultural chemicals. *Novosphingobium capsulatum* Leifson (UFV-STB6) was reported to have the ability to control diseases of tomatoes on both leaves and fruit[20]. Pernezny[3] reported that *Bacillus subtilis* (Ehrenberg, 1835) Cohn, 1872 (QST713) provided the most effective control of the target spot disease of tomato. Moreover, *B. subtilis* (Ehrenberg, 1835) Cohn, 1872 (BSCBE4), *Pseudomonas chlororaphis* (Guignard & Sauvageau, 1894) Berget et al., 1930 (PA23) and endophytic *P. fluorescens* (ENPF1) inhibited the mycelial growth of *C. cassiicola* under in vitro conditions[21]. The purpose of this study was to select phylloplane bacteria that effectively control target spot disease of tomato.

MATERIALS AND METHODS

Fungal pathogen

The causal agent of target spot disease of tomato (*C. cassiicola*) was obtained from the Branch of Plant Pathology, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University.

Isolation of phylloplane bacteria

Phylloplane bacteria were isolated from healthy tomato leaves (5 leaves/plant and 10 plants/farm) obtained from three farmers in Mahasarakham Province. The leaves were washed with sterile distilled water and cut into small pieces with a size of about 1x1 cm, and then they were put into a test tube containing sterile distilled water. These were shaken with a vortex mixer to expose the leaf surface to water and the dilution from 10^{-1} to 10^{-3} were spread on potato dextrose agar (PDA), nutrient agar (NA), arginine glycerol mineral salt agar (AGMA) and yeast malt medium (YM) incubated at 28 ± 2°C for 24-72 hours. The isolates were purified and stored at 4°C until use.

Efficacy test of phylloplane bacteria by dual culture

The dual culture technique was used to test the ability of the phylloplane bacteria to inhibit the growth of *C. cassiicola*. A 7 mm diameter plug of *C. cassiicola* was taken from the edge of a 7-day-old pure culture using a cork borer and placed at the centre of a new PDA plate for 4 days, and after that the stab method was used with phylloplane bacteria from 48 hour old cultures at four points in a cross pattern and incubated at 28 ± 2°C. There were five replications per treatment and daily measurements of the diameter of *C. cassiicola* were taken for 10 days. The effective phylloplane bacteria were selected for use in the next experiment.

Efficacy test of phylloplane bacteria by detached leaf technique

Healthy tomato leaves were taken from plants grown in pots that had not had any chemicals used. These were then washed with water for five minutes and surface sterilized with 70% alcohol. The leaf stalks were covered with sterile cotton and dipped into sterile distilled water before covering with aluminium foil. Then a cell suspension of the effective phylloplane bacteria after culture on NA for 48 hr was prepared and adjusted to 10^8 cells/ml[22]. Spore suspensions of *C. cassiicola* were prepared after culture on PDA for 10 days. The spores were swept into sterile distilled water and the concentration was adjusted to 10^4 spores / ml[22]. Two experiments were conducted: 1) spray 10 ml of the spore suspension of *C. cassiicola* on tomato leaves 24 hours before spraying 10 ml of the suspension of the effective phylloplane bacteria and 2) spray 10 ml of the spore suspension of *C. cassiicola* on tomato leaves 24 hours after spraying 10 ml of the suspension of the effective phylloplane bacteria. There were five replications per treatment with spraying sterile distilled water as the control and experiments were repeated twice. Disease severity was scored using the method adapted by Dixon[23]: 0 = no symptoms, 1 = 1-10% disease area on leaf, 2 = 11-25% disease area on leaf, 3 = 26-50% disease area on leaf, 4 = 51-75% disease area on leaf and 5 = 76-100% disease area on leaf.

The effective phylloplane bacteria that inhibited the target spot severity on leaves were further observed microscopically to determine their antagonistic effects on spore germination and hyphal growth of *C. cassiicola.* Then identification by morphology characteristics consisting of morphology, colour and size of colony was

performed. Biochemical tests such as motility, catalase and oxidase tests were also done.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) and treatment mean comparisons were performed using the least significant differences at P=0.05 by (LSD).

RESULTS

Isolation of phylloplane bacteria

Twenty-nine isolates of phylloplane bacteria were obtained from the four media: PDA, NA, AGMA and YM. The result showed that more bacteria could be isolated on the NA and PDA media than the AGMA and YM. From the NA nine isolates were obtained (BB44, BB46, BC37, PT1, PT3, PT4, PT5, PT7 and PT15). While from the PDA 10 isolates were also obtained (BB41, BB58, BC29, BC35, BC36, PT2, PT9, PT18, PT19 and PT20). From the AGMA and YM, five isolates were obtained from each (BB47, BB53, BC30, BC38 and PT28 and BB42, BB51, PT12, PT13 and PT22, respectively).

Table 1. Ability of phylloplane bacteria isolated from tomato leaves to inhibit *Corynespora cassiicola* as determined by dual culture technique.

Isolates	% inhibition of *C. cassiicola* [1]
BC29	51.11 ± 0.19 a
BC30	45.01 ± 5.76 a
BC35	48.88 ± 0.48 a
BC36	47.23 ± 3.88 a
BC37	20.09 ± 1.59 defg
BC38	29.63 ± 1.28 cde
BB41	29.92 ± 0.00 bcd
BB42	27.28 ± 0.89 cdef
BB44	37.88 ± 2.45 abc
BB46	10.60 ± 1.23 g
BB47	46.97 ± 2.14 a
BB51	15.91 ± 3.45 efg
BB53	43.94 ± 4.64 ab
BB58	28.41 ± 1.13 cdef
PT1	17.79 ± 0.78 defg
PT2	14.99 ± 4.63 fg
PT3	16.10 ± 3.39 defg
PT4	51.11 ± 4.45 a
PT5	10.45 ± 1.36 g
PT7	15.67± 4.55 efg
PT9	9.38 ± 0.61 g
PT12	9.30 ± 3.54 g
PT13	15.56 ± 2.18 efg
PT15	10.97 ± 2.30 g
PT18	8.15 ± 2.79 g
PT19	10.81 ± 1.66 g
PT20	14.80 ± 6.52 fg
PT22	9.26 ± 1.69 g
PT28	9.26 ± 1.08 g
CV (%)	11.52

[1]Means (n=5) followed by different letters within a column are not significantly different at P=0.05 by LSD.

Efficacy test of phylloplane bacteria by dual culture

The dual culture method showed that six isolates of phylloplane bacteria could inhibit the mycelial growth of *C. cassiicola* (BB47, BC29, BC30, BC35, BC36 and PT4) and they showed 45.01- 51.11% inhibition. However, BB44 and BB53 (37.88 and 43.99% inhibition) showed a not significantly different inhibit of mycelial growth of *C. cassiicola* when compared with all of six isolates above (Table 1).

Efficacy test of phylloplane bacteria by detached leaf technique

The results of the phylloplane bacteria to control *C. cassiicola* using the detached leaf

Table 2. Ability of phylloplane bacteria isolated from tomato leaves to inhibit *Corynespora cassiicola* as determined by the detached leaf technique four days after inoculation.

Treatment	Disease index	Disease severity [1](%)
Control (dH_2O)	0	0.00 ± 0.00 d
C. cassiicola + dH_2O	5	76.67 ± 15.28 a
dH_20 + *C. cassiicola*	4	55.00 ± 5.00 ab
BC29+*C. cassiicola*	3	26.67 ± 3.82 c
BC30+*C. cassiicola*	3	35.00 ± 8.66 bc
BC35+*C. cassiicola*	3	38.33± 10.41 bc
BC36+*C. cassiicola*	2	25.00± 5.00 c
BB47+*C. cassiicola*	3	26.67± 5.77 c
PT4+*C. cassiicola*	3	26.67± 8.93 c
C. cassiicola+BC29	3	28.33± 2.89 c
C. cassiicola+BC30	3	26.67± 5.28 c
C. cassiicola+BC35	3	45.00± 5.00 bc
C. cassiicola+BC36	2	25.00± 5.00 c
C. cassiicola+BB47	3	28.33± 2.89 c
C. cassiicola+PT4	3	30.00± 13.23 c
CV (%)		34.81

[1] Means (n=5) followed by different letters within a column are not significantly different at P=0.05 by LSD.

technique showed that all treatments significantly reduced disease severity when compared to the control. They showed 25.00 - 38.33 and 25.00 – 45.00% disease severity reduction when spraying phylloplane bacteria 24 hr before or after spraying *C. cassiicola*, respectively (Table 2).

The results of the effective phylloplane bacteria to inhibit spore germination and hypha growth of *C. cassiicola* showed that the cell suspension and culture filtrates of the effective phylloplane bacteria did not affect spore germination, but inhibited the development of hypha and resulted in hyphal malformation.

The identification of the effective phylloplane bacteria via morphological study and biochemical properties could classify the effective phylloplane bacteria into two groups that were gram-positive and gram-negative bacteria. BC29, BC30, BC36 and PT4 were *Bacillus* sp., while BB47 and BC35 were *Pseudomonas* sp. (Table 3).

Table 3. Properties of effective phylloplane bacteria

Properties	Gram positive bacteria					Gram negative bacteria		
	BC29	BC30	BC36	PT4	*Bacillus* sp.	BB47	BC35	*Pseudomonas* sp.
Colony size	0.9 cm	0.7 cm	0.5 cm	0.7	Large	0.5 cm	0.5 cm	Medium
Colour	Turbid white	White	Turbid white	Turbid white	White dull	Turbid white	Turbid white	Diffusible green
Form	Circular	Irregular	Circular	Irregular	Irregular	Irregular	Circular	Circular
Margin	Entire	Undulate	Entire	Undulate	Undulate	Undulate	Wavy	Wavy
Elevation	Convex	Flat	Raised	Raised	Umbonate	Convex	Convex	Umbonate
Surface	Smooth	Dry	Smooth	Smooth	Dry	Mucoid	Mucoid	Mucoid
Optical properties	Opaque	Opaque	Translucent	Opaque	Opaque	Opaque	Opaque	Opaque
Cell shape	Rod	Rod	Rod	Rod	Rod	Short rod	Short rod	Rod
Endospore position	C[1]	C	C	C	C	None	None	None
Endospore shape	E[2]	E	E	E	E	None	None	None
Cell motility	+[3]	-[4]	+	+	+	+	+	+
Catalase	+	-	+	+	+	+	+	+
Oxidase	-	+	+	-	D[5]	+	+	+

[1] C = Central, [2] E = Ellipsoidal, [3] + = positive for 90 – 100%,
[4] - = negative for 90 – 100% and [5] D = Rare aerotolerant[34]

DISCUSSION

Twenty-nine isolates of phylloplane bacteria were obtained from tomato leaves. PDA and NA could isolate more bacteria than AGMA and YM as NA is suitable for the cultivation of a wide variety of microorganisms. The American Public Health Association[24] suggested the formula of NA as a standard culture medium to be used in water testing. NA continues to be a widely used general purpose medium for growing non-fastidious microorganisms. PDA is a non-selective medium for the cultivation and enumeration of yeasts and moulds. PDA is composed of dehydrated potato infusion and dextrose that encourages luxuriant fungal growth. Agar is added as the solidifying agent. Many standard procedures use a specified amount of sterile tartaric acid (10%) to lower the pH of this medium to 3.5 that inhibits bacterial growth, but in this study we did not add tartaric acid and as a result bacterial growth was possible[25,26]. YM is a selective growth medium with low pH that is useful for cultivating yeasts, moulds or other acid-tolerant or acidophilic organisms, while deterring the growth of most bacteria and other acid intolerant organisms[27]. AGMA was found to be the most suitable medium for the rapidly increase of *Streptomyces*. Yaeram et al.[28] selected

Streptomyces and cultured it on three types of solid media. They found that all isolates of *Streptomyces* grew rapidly on yeast extract malt extract agar (YEMA) and AGMA. Six isolates (BB47, BC29, BC30, BC35, BC36 and PT4) could inhibit the growth of the mycelium of *C. cassiicola* as determined by the dual culture technique with 45.01- 51.11% inhibition. The detached leaf technique was performed by spraying 10^8 cells/ml of the effective phylloplane bacteria 24 hr before or after spraying 10^4 spores/ml of *C. cassiicola* on to tomato leaves. The result showed that all isolated could reduce the disease severity incidence when compared with the control. When spraying the effective phylloplane bacteria before or after spraying *C. cassiicola*, there was a significant reduction.

The effective phylloplane bacteria inhibited the spore germination, germ tube development and hypha growth of *C. cassiicola*. This was similar to Wang and Qing[12] who isolated an actinomycete strain XN-1 from a cucumber phyllosphere and then tested its potential application as an antagonist against *C. cassiicola*. The result showed that the spore germination and hyphal growth inhibition rate of the XN-1 fermentation filtrate against *C. cassiicola* could reach 96.50% and 51.17%, respectively, as well as give 63.54% control on the cucumber leaves. The experiment indicated that the exogenous actinomycete XN-1 has the potential to act as an antagonistic agent in controlling the occurrence and development of cucumber target leaf spot in the greenhouse.

The identification of the antagonistic bacteria by biochemical characters showed that BC29, BC30, BC36 and PT4 were gram-positive bacteria classified into the genus *Bacillus*, while BB47 and BC35 were gram-negative bacteria classified into the genus *Pseudomonas*. Bacterial biocontrol agents (BCAs) have been widely employed to minimize the incidence and severity of several economically important crop diseases. Studies on the interactions between BCAs and the target microbial plant pathogens have revealed the involvement of different mechanisms for their biocontrol activities. The bacterial antagonism may be due to the production of toxic metabolites (enzymes, antibiotics and volatile organic compounds), competition for nutrients and space, prevention of pathogen colonization of host tissues and induction of resistance in plants to crop diseases. Bacterial species from the genera *Pseudomonas*, *Bacillus*, *Burkholderia*, *Lysobacter*, *Serratia* and *Pantoea* have been reported to be effective against several plant pathogens by acting through one or more mechanisms[29]. (Narayanasamy, 2013). For instance, *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 (*P. aureofaciens*) were effective biocontrol agents against *Pythium aphanidermatum*, the causal agent of damping-off of hot pepper (*Capsicum annum* L.) in greenhouse vegetable production systems. The two bacterial strains induced the development of plant defence-related enzymes including phenylalanne ammonialyase, peroxidase, polyphenol oxidase and phenol content, while suppressing the incidence of damping-off and increasing the growth of hot pepper seedlings[30]. Mathiyazhagan et al.[21] reported that *B. subtilis* (BSCBE4), *P. chlororaphis* (PA23) and endophytic *P. fluorescen*s ($ENPF_1$) inhibited the mycelial growth of *C. cassiicola* (Berk.&Curt.)Wei *in vitro*. All these bacterial isolates produced hydroxamate and carboxylate type siderophores. Delivering the talc based formulation of BSCBE4 through seedling dip and foliar application effectively reduced stem blight disease incidence and increased the dry matter production under pot culture and field conditions. The application of BSCBE4, PA23 and ENPF1 increased defence related enzymes such as peroxidase, polyphenol oxidase, chitinase and ^{2}-1,3 glucanase in *P. amarus* up to ten days after the challenge inoculation with *C. cassiicola*. Therefore, Ji et al.[31] reported that *B. licheniformis* (Weigmann 1898) Chester 1901 strain SDYT-79 isolated from soil had the highest antagonistic activity against *C. cassiicola*. The strain SDYT-79 greatly inhibited the spore germination of *C.cassiicola*. The spores of *C.cassiicola* could germinate in the fermentation filtrate liquor, but the germination tube was abnormal. Inhibition spectrum tests showed that the strain SDYT-79 could significantly inhibit the 11 tested fungal phytopathogens. *P. aeruginosa* (Schroeter, 1872) Migula, 1900 was strongly antagonistic to the mycelial growth of *C. cassiicola*, with a bacterium-inhibiting width of 10 mm. In addition, the inhibitory effects of six kinds of biological medicaments

on *Pogostemon cablin* (Blanco) Benth. were tested in the laboratory. The results showed that by applying $3x10^{10}$ spores/gram *B. cereus* WP, $2x10^{9}$ spores/ml Bituo, 4% Pyamidine nucleotide bacteriophages WG, 1.1% d-catectin WP and 10% Polynactins WP there were good inhibitory effects on the mycelial growth, while applying 3% Zhongshengmycin WP had no control effect on the mycelial growth[32]. While, *B. amyloliquefaciens* CNU114001 showed significant antagonistic activity against pathogenic fungi and showed broad spectrum activity against 12 phytopathogenic fungi using the dual culture method. A semi purified compound from CNU114001 significantly inhibited the mycelial growth of *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea* and *Sclerotinia sclerotiorum* at a concentration of 200 ppm. Spore germ tube elongation of *B. cinerea* was inhibited by the culture filtrate of the isolate[33]. In this study we found that the effective phylloplane bacteria that controlled *C. cassiicola* including *Bacillus* spp. and a *Pseudomonas* sp. However, studies on the other properties of these bacterial are needed in the future.

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