The Journal of Animal & Plant Sciences, 25 (3 Suppl. 1) 2015 Special Issue Page: 226-231
ISSN: 1018-7081

STUDY ON BIOFILM IN KIWI FRUIT BIOCONTROL

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ABSTRACT

This study aims to develop a biofilm with a biocontrol agent to protect kiwifruit against phytopathogenic bacteria. Strain MHT6 was isolated from kiwifruit orchard soil. In vitro assay results showed that the isolated strain effectively suppressed the growth of plant pathogens, such as*Pseudomonas syringae* pv.*actinida* (PSA) and *Pseudomonas syringae* pv. *Syringae* (PSS), which cause kiwifruit canker and leaf blight. Strain MHT6 was identified as *Bacillus megaterium* through phenotypic, physiological, biochemical, and phylogenetic (16S rDNA) characterization. The strain was mutated under repeated He–Ne laser irradiation to enhance its antagonistic capability. Six mutants were screened, among which mutant MHT67 showed the strongest antagonist capability against PSAand PSS. Mutant MHT67 also demonstrated hereditary stability even after 50 generations. Finally, a biofilm with the biocontrol agent MHT6 was produced and used to protect kiwifruit against phytopathogenic bacteria.

Key words: Kiwifruit, *Bacillus megaterium*, He–Ne laser, Mutation, Biofilm.

INTRODUCTION

Kiwifruit has a distinct flavor and rich nutritional value; many countries worldwide have exerted considerable efforts to develop the kiwifruit industry (Afsharmanesh *et al*., 2014).

Pseudomonas syringae pv. *Actinida* (PSA) and *Pseudomonas syringae* pv. *Syringae* (PSS) are the principal pathogens that cause kiwifruit disease. PSAis reportedly the causal agent of bacterial canker in green-fleshed kiwifruit *(Actinidia deliciosa)* and yellow-fleshed kiwifruit *(A. chinensis)* (Scortichini *et al*., 2012; Carrion *et al*., 2014*)*. PSS is reportedly the causal agent of bacterial blight in kiwifruit(Carrion *et al*., 2014).

Biological control is a non-toxic, safe, effective, and economical process for kiwifruit production system (Ferrante and Scortichini, 2010). *Agrobacterium*, *Pseudomonas*, and *Streptomyces* are bacterial control agent (Shoda, 2000; Marrone, 2002) *Bacillus*is a dominant antagonistic bacterium that has been commonly applied to control fruit and vegetable diseases(Chen *et al*., 2013; Santaella *et al*., 2008) However, reports on the use of *Bacillus megaterium* as a biological control agent of kiwifruit diseases caused by PSA and PSS are lacking.

The breeding of antagonistic bacteria such as laser irradiation technology has attracted increasing attention from researchers (Olano *et al*., 2008; Khaliq *et al*., 2009;Yu *et al*., 2011; Jiang *et al*., 2007) Biofilms which produced by the antagonistic bacteria has caused great attention (Flemming and Wingender, 2010).

The present study isolated and identified strain MHT6 from kiwifruit orchard soil. In order to enhance antagonistic capability of this strain, it was mutated using He–Ne laser irradiation. Finally, a biofilm main produced by biological agents was used in field traits. The results of this study contribute to the discovery of new materials for crop protection.

MATERIALS AND METHODS

Isolation of bacteria: The antagonistic strains were isolated from kiwifruit orchard soil in China. All experiments were performed under sterile conditions as previously described by (Yang and Lee, 2007).

Screening for antagonistic bacteria: The preliminary screening of antagonistic strains were carried out by inhibition zone method (Gnan and Demello, 1999). All isolations were tested for their capability to inhibit PSA and PSS. The strain that showed broad-spectrum and persistent antagonistic capability was selected for further screening. The experiments were conducted in triplicate.

Further screening was carried out by using the Oxford cup method (Vincent *etal*., 1944) to test the antagonistic capability of bacterial fermentation broth. The antagonistic effect was judged on the basis of the inhibition zones surrounding the oxford cups. The experiments were conducted in triplicate.

Morphological and biochemical characteristics: The morphological characteristics of the strains were carried out using standard procedures(Gerhardt *et al*., 1994). The experiments were conducted in triplicate.

16S rDNA amplification and sequence analysis: Genomic DNA was extracted using a commercial kit (DP302, Tiangen Biotech CO., LED, China). A pair of universal primers was prepared according to previously described methods (Fani *et al*., 1995).The PCR reaction

mixtures (30 µL) contained 1 µL of template, 1 µL of each primer, 13 µL of deionized water, and 15 µL of 2 ×Pfu PCR MasterMix (KP201-01, Tiangen Biotech CO. Ltd., China). The 2 ×Pfu PCR MasterMix was composed of 0.1 U Pfu Polymerase, 500 µM dNTP, 50 mM Tris-HCl (pH 8.7), 20 mM KCl, and 4 mM $MgCl_2$.The PCR was run in a Peltier PTC-100TM Thermal Cycler (MJ Research TM, Inc., Massachusetts, USA) under the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 50 s, 52 °C for 50 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min.

The PCR productwas performed by electrophoresis analysis. The target fragments in the gel were purified, recovered and sequenced by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (China).

A phylogenetic tree was constructed using the neighbor-joining method with MAGE3.1 software (Han *et al*., 2005).

Screening of enhanced antagonistic mutant using He–Ne laser irradiation: The strain was cultured and irradiated as described methods (Liao *et al*., 2014).The suspension was irradiated for 15 min using the He–Ne laser generator (Northwest University Photo electricity Factory, China). The suspension without irradiation was used as a negative control. The experiments were performed in triplicate.

After irradiation, the suspension was serially diluted (10^{-5}, 10^{-6}, and 10^{-7}). Then, each diluted suspension was spread onto NA plates. After incubating at 37 °C for 20 h, the mutants were observed and the survival rate and the positive mutation rate were calculated.

Test for antagonistic capability of the mutant cell and fermentation broth: The antagonistic capability of the mutant cell was test by the inhibition zone method (Gnan and Demello, 1999).

The antagonistic capability offermentation broth was tested by Oxford cup method (Vincent *etal*., 1944).

All selected mutants were tested for their capability to inhibit PSA and PSS.

Hereditary stability of the mutants: The selected mutant was activated, inoculated onto NA plates, and then incubated at 30°C for 24 h as the first generation. The mutant was incubated up to the 50th generation using the same method. Using inhibition zone method to test the antagonistic capability of the mutants in every ten generations.

Biological control of field trials by biofilm Formation: The fermentation broth of the mutants, with CFU of 10^9/mL, was respectively diluted to 1% and 0.2%. Then each concentration of the broth was combined with some membrane-forming agent such as sodium alginate, for the biofilm formation. The biofilm with different concentration of broth, was separately sprayed onto the leaves of kiwifruit, taking the fresh water as control. The spray were done for 5 times, with the frequency of 7 days per one time. Control efficacy was calculated as following equation :

Control efficacy=(Infection rate of control-Infection rate of treatment)/ Infection rate of control×100%

RESULTS AND DISCUSSION

Isolation and screening of antagonistic bacteria: Six strains isolated from kiwifruit orchard soil were all bacteria. The antagonistic capability of cell and fermentation broth against PSA and PSS was shown in Table 1. From the table, Strain MHT6 exhibited strongest antagonistic effects than the other strains. Therefore, strain MHT6 was selected as the biocontrol agent for further experiments.

Phenotypicand Physiological characterization of strain MHT6: The cells of MHT6 were rod-shaped, G^+, 1.1 µm × 0.9 µm in size, They formed mid-borne oval spores with enlarged cysts. The colonies were dry and opaque, with irregular diffused edge. It was strictly aerobic and can ferment various carbohydrate compounds, including glucose (only acid produced) and mannose, but not indole and H_2S.

Table 1 The antagonistic effect of the six strains' cell and fermentation broth

Inhibition zone(mm) / bacteria	cell		fermentation broth	
	PSA	PSS	PSA	PSS
MHT1	3.8±0.11	1.1±0.23	3.1±0.13	1.0±0.12
MHT2	2.8±0.31	2.6±0.05	2.2±0.16	2.1±0.07
MHT3	4.3±0.11	4.1±0.21	2.6±0.10	2.3±0.21
MHT4	2.4±0.38	2.1±0.17	1.9±0.15	2.0±0.00
MHT5	1.8±0.33	1.7±0.24	1.6±0.15	1.5±0.14
MHT6	4.5±0.00	4.3±0.00	4.3±0.11	3.1±0.00

Each data is an average of three replicates.

It was tested positive for the following reactions: starch and casein hydrolysis; gelatin liquefaction; VP test; citrate utilization; and catalase, oxidase, and nitrite reductase tests.

16S rDNA sequence and phylogenetic analysis: Approximately 1500 bp of the 16S rDNA product was amplified by PCR (Fig.1). The complete 16S rDNA of strain MHT6 (1491 bp) was submitted to GenBank under accession number JX402906 and was verified by DNA sequence analysis. Based on the sequence similarity analyzed by BLAST, strain MHT6 showed 100% homology with *B. megaterium* 108 (GenBank accession number AB334764). A phylogenetic tree (Fig. 2) was constructed based on the alignment of 1320 bp of 16S rDNA sequences using the neighbor-joining method.

According to results mentioned above, the antagonistic strain MHT6 was identified as *B. megaterium*

He–Ne laser irradiation: After the irradiation, six mutants (MHT61, MHT63, MHT65, MHT66, MHT67, and MHT69) were selected on the basis of the inhibition zone method. Taking wild strain MHT6 as control, the inhibition zone of MHT67 against PSA and PSS were tested using SPSS19.0software(SPSS, Inc., USA) with P=0.05. The result was shown in table 2. From the table, we can see the cell of mutant MHT67 have a significant inhibition effect on PSS and PSA. After testing the antagonistic capabilityof the fermentation broth of mutant MHT67 and MHT6 (Table3), we can find that the fermentation broth of mutant MHT67 significantly inhibited PSS growth. Thus, the MHT67 was selected out for the further study.

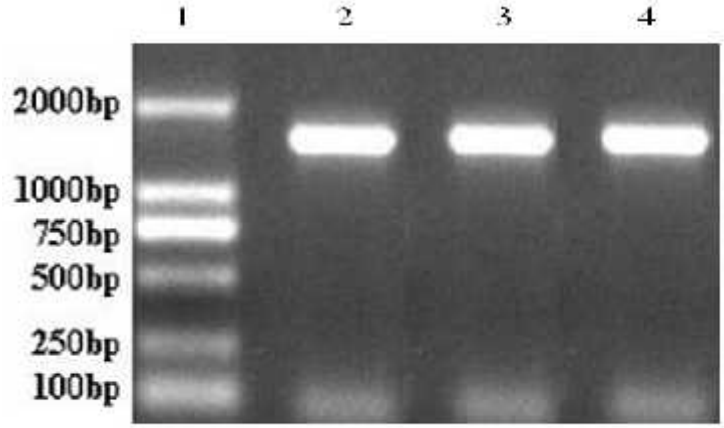

Figure 1.Gel electrophoresis of PCR amplification for 16SrDNA of strain MHT6 with a pair of universal primer F27 and R 1492.
Lanes: 1, DNA Marker (Qiagen) in base pairs of D2000; from 2to 4, Isolate MHT6 (three replicates)

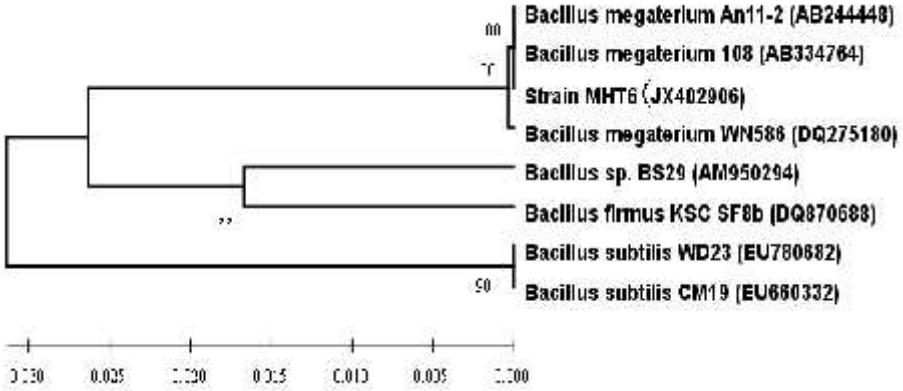

Figure 2. Neighbor-joining tree based on the 16SrDNA sequences.

The number at each branch points is the percentage supported by bootstrap. The bootstrap analysis was based on 1000 replicates. The scale bar indicates the 0.005 evolutionary distance unit.

Hereditary stability of mutant MHT67: The selected mutant MHT67was subcultured to 50th generation. The inhibition zone method was used totest the antagonistic capability of the mutant in every ten generations. The

result (Table4) indicated that mutant MHT67 exhibits hereditary stability.

Table 2.The antagonistic distance against PSA and PSS and descriptive statistics using One-sample t-test

Strains	*Test Value (control parent) = 4.5(mm)*			
	against PSA		against PSS	
	Inhibition zone (mm)	Sig.	Inhibition zone (mm)	Sig.
MHT61	4.90±0.1002	0.020	4.63±0.1528	0.063
MHT63	4.61±0.1900	0.422	4.53±0.1528	0.118
MHT65	4.62±0.1305	0.243	4.40±0.3000	0.622
MHT66	5.10±0.1000	0.009	4.47±0.2082	0.300
MHT67	5.41±0.2000	0.016	5.07±0.2082	0.024
MHT69	4.73±0.1528	0.118	4.83±0.0577	0.004

Each data is an average of three replicates

Table 3. The antagonistic effect of the fermentation broth of the mutant and parent strain against the two pathogens in vitro

Strain \ Pathogen	PSA	PSS
MHT6	4.3±0.11	3.1±0.00
MHT67	5.1±0.13	4.8±0.07

Experiments were performed on agar plates and replicated six times.

Table 4. Hereditary stability of mutant MHT67

Generation	Inhibition zone(mm) against PSA	Inhibition zone(mm) against PSS
1th	5.3±0.11	5.00±0.05
11th	5.3±0.10	5.00±0.03
21th	5.3±0.09	4.97±0.11
31th	5.3±0.07	4.95±0.10
41th	5.3±0.03	4.95±0.08
51th	5.3±0.00	4.95±0.05

Application of biofilm with MHT67: The biofilm with the different concentration of biological control agent MHT6 and MHT67 were used to protect kiwifruit. As was shown in the table5, the biofilm formed by mutant MHT67showed the significant biological effect compared to its wild strain MHT6.The results of this study will contribute to the discovery of new materials for kiwifruit protection.

Conclusion: Six strains that exhibitedantagonistic effects on kiwifruit pathogens were isolated in this study. After primary and further screening,

Table 5.Control effect of biofilm formed by MHT6 and MHT67 on bacterial leaf blight and canker of kiwifruit against PSS and PSA

Treatment	Control effect on leaf blight against PSS		Control effect on canker against PSA	
	Concentration%	Control effect%	Concentration%	Control effect%
MHT67	100	91.4a	100	90.2a
	1	86.8b	1	86.6b
	0.2	80.5c	0.2	81.1c
MHT6	100	80.1bc	100	85.8b
	1	74.2cd	1	77.6c
	0.2	68.5d	0.2	70.3d
CK	-	-	-	-

strain MHT6 exhibited the strongest antagonistic effect against PSA and PSS among all strains. Based on the morphological, physiological and molecular characterization, MHT6 was identified as *B. megaterium*.

The inhibition zone induced by the fermentation broth of antagonistic bacteria was used to determine the antagonistic activity of the metabolite produced by strain MHT6 and mutant MHT67 against PSA and PSS. The inhibition zone indicates that strain MHT6 and mutant MHT67 exert an antibiotic activity similar to that of iturins synthesized by *B. subtilis*(Ongena and Jacques, 2008) The in vitro agar plates showed that both the cells and the fermentation broth of the antagonistic bacteria inhibited the kiwifruit pathogens. We also found that the cells of strain MHT6 produced larger inhibition zones than its fermentation broth. This result suggests that other interactions such as special competition and nutrition competition exist between the antagonistic bacteria and the pathogens.

To enhance the antagonistic capability of this biological control agent, strain MHT6 was mutated using He–Ne laser irradiation. As a new efficient mutation breeding technique, He–Ne laser irradiation has the advantage of high mutation rate and mutant capacity These characteristics explain why He–Ne laser irradiation is widely used in microbial breeding(Gao *et al*., 2014).

However, the application of He–Ne laser irradiation in breeding highly efficient antagonistic bacteria is still limited. This study initiated helpful explorations. After screening, mutant MHT67 whose antagonistic capability was significantly enhanced was subcultured to the 50th generation with a good genetic stability. This mutant was used for further studies.

As far as we know, this study is the first time to use He–Ne laser irradiation to screen antagonistic bacteria against PSA and PSS. The results of this study may be used as a reference to improve the antagonistic capability of kiwifruit against plant disease and pests.

The biofilm, which take antagonistic bacterial fermentation broth as main effecter, is a new research direction in biological control. It is the combination of biological membrane technology and modern microbial engineering technique. After breeding high-efficient antagonistic strains, the fermentation broth was combined with some membrane-forming agent such as sodium alginate sprayed on the fruit surface. Then a protective film, with good film-forming and extensible property, can be formed on the fruit surface rapidly. This biofilm can effectively protect the fruit from the damage of pesticide and pathogen. In this paper, a biofilm formed by different concentration of MHT6 and MHT67showed significant effect to control the canker and leaf blight diseases in kiwifruit.

In conclusion, a biofilm formed by biological agent MHT67 was developed and proven to exert a strong antagonistic activity against pathogen. The biofilm with MHT67 could be served as a potential biocontrol agent for kiwifruit protection.

Acknowledgements: This work was financially supported by Agricultural Science and Technology Achievements Transform Fund Project (2012GB2G000451)

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