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Contributed Paper

Growth Inhibition of *Ralstonia solanacearum* PT1J by Antagonistic Bacteria Isolated from Soils in the Northern Part of Thailand

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ABSTRACT

The most serious infectious disease found in Siam Tulip or Patumma (*Curcuma alismatifolia* Gagnep.) was a bacterial wilt caused by *Ralstonia solanacearum*. Among ninety-four isolates of the bacteria isolated from infected Patumma's rhizomes, the isolate PT1J, which was identified as *R. solanacearum*, was selected for further study due to its high virulence against Patumma rhizomes upon re-infection. Concurrently, five of seventy-six bacterial isolates, screened for their ability to inhibit growth of *R. solanacearum* PT 1J, showed high potential for minimizing growth of the pathogen; namely *Bacillus* sp. Ba4, N9C8 and N11IV, and enterobacteria N9A and Ba3. Optimal conditions, for production of inhibitory substances from these antagonistic bacteria, were 30°C in a medium pH of 7, containing 0.25% (w/v) glucose and 2.0% (w/v) peptone. The promising time for introducing mixed cultures of these five antagonistic strains to Patummas grown in pots was at the beginning of Patumma cultivation, in order to prevent and protect the plants from *R. solanacearum* infection.

Keywords: agar disc diffusion, bacterial wilt, *Bacillus*, *Curcuma alismatifolia* Gagnep., Patumma, *Ralstonia solanacearum*, enterobacteria.

1. INTRODUCTION

Patumma (*Curcuma alismatifolia* Gagnep.) is annual member of the family *Zingiberaceae*, which has been named "Siam tulip" due to its exotic flowering shapes and colors [1]. It is a native Thai flower, found in the northern, north eastern and central parts of Thailand. The growing season of Patumma is March through June with peak flowering occurring during June through September. After flowering, the plants lie dormant from September to February, when rhizome harvesting usually takes place. The domestic market for Patumma is of low value, compared with foreign markets. Beginning from private trading, between Thai producers and foreign customers, nowadays the trade has developed into a government concern with export of Patummas rhizomes becoming more important than exports of flowers. The annual exported value of rhizomes is 15-30 million baths [2, 3]. For more than a decade the best markets have been European countries, particularly the Netherlands and the United States of America and Japan.

Prior to cultivation of Patummas, soils must be treated with sunlight and chemicals such as urea and lime to minimize the amount of some soil-borne pathogens for around a month [4]. During cultivation, Patummas must be frequently inspected for any phenotypic abnormalities that indicate incidence of diseases. Some Patumma diseases are caused by microbes and nematodes. For example, bacterial wilt is caused by *Ralstonia solanacearum*, leaf spot is caused by *Acremonium* sp.; and algal disease is caused by *Sphaceloma* sp. All mentioned diseases can effectively spread from farm to farm, especially bacterial wilt. If a plant control department in a foreign country detects *Ralstonia solanacearum* in Patumma rhizomes, all rhizomes will be rejected and destroyed [4]. Therefore, once infected plants are observed in fields, they must be discarded immediately, otherwise it will bring down the whole growing Patummas in that farm and hence affect the overall economics of the country.

Ralstonia solanacearum, formerly *Pseudomonas solanacearum*, is an aerobe, Gram-negative rod and motile. It is an heterogeneous, species indigenous in soils, and displays phenotypic and genetic variability [5, 6]. The bacterium had been reported to be a wilt-causing culprit in *Zingiberaceae* plants such as gingers, and tomatoes. The pathogenic mechanism is not well understood, but it has been suggested that the bacteria enters the plant vascular system, rapidly multiplies and blocks xylem vessels. Several virulence factors, produced by *R. solanacearum*, have been reported, including extracellular polysaccharides and a consortium of cell wall-degrading enzymes [7]. Infected rhizomes are water-soaked, unable to shoot and eventually die. If infected rhizomes germinate, after the onset of wilt, the leaves turn brown and dry out from top to bottom and down to the pseudostem of the plant. Ultimately the plants die, while the bacterial culprit still remains in the soil, if it is not removed. Nowadays, farmers use many chemicals to control *R. solanacearum* and they change the cultivating area each year, but this results in soil pollution.

Biocontrol has been proposed to prevent growth of *R. solanacearum* in some *Zingiberaceae* plants, although few microbial biocontrol products has been fully commercialized. *Trichoderma harzianum* T39 is an example of commercial biocontrol for control of *Botrytis cinerea* in vineyards [8]. However, until now, there has not been any reported biocontrol method for Patumma. The objective of this research was to isolate and screen for antagonistic bacteria, capable of inhibiting growth of the wilt-causing bacteria, *R. solanacearum*, isolated from infected Patumma rhizomes.

2. MATERIALS AND METHODS

2.1 Isolation of Wilt Causing Bacteria

Water-soaked and badly smelling Patumma rhizomes were aseptically cut to scrape out the mucus-like contents which were streak plated onto the TZC agar [9], containing 0.05% glucose, 0.1% peptone, 0.01% pancreatic digest of casein, 0.05% 2, 3, 5 – triphenyltetrazoliumchloride and 1.5% agar. The culture plates were incubated at 30°C for 24-48 hours. Selected colonies were tested for their ability to cause wilt disease by stabbing them into Patumma rhizomes, which were then cultivated and observed for the occurrence of wilt disease. A selected isolated culture, PT1J, was then kept on TZC agar slants at 4°C for further study.

2.2 Isolation and Screening of Antagonistic Bacteria

Samples of approximately 100 grams of soil from Patumma beds and from around the roots of *Zingiberaceae* plants were collected and used to pour plates of tryptic soy agar

(TSA, Merck®), pH 7.3±0.2, containing 0.25% glucose, 0.3% peptone from soy meal, 1.7% peptone from casein, 0.5% NaCl, 0.25% K_2HPO_4 and 1.5% agar. Each pure isolate obtained was kept at 4°C for further evaluation.

An agar disc diffusion method was performed to determine the ability of each bacterial isolate to inhibit growth of the wilt-causing bacterium, PT1J. Each bacterial isolate was cultivated in tryptic soy broth (TSB) at 30°C for 48 hours. The culture broth was centrifuged to obtain a supernatant. Concomitantly, the isolate PT1J was grown in TZC broth at 30°C for 24 hours prior swabbing it onto TZC agar plates. A paper disc, 6 mm in diameter, was dipped into each supernatant obtained before placing it onto the surfaces of the PT1J agar plates. The plates were incubated at 30°C for 24 hours, prior to observing the appearance of clear inhibition zones.

2.3 Production Optimization

Five antagonistic bacterial isolates; N9C8, N11IV, N9A, Ba3 and Ba4 were selected for further production optimization. All experiments were conducted three times.

2.3.1 Carbon and Nitrogen Sources

Each isolate was grown at 30°C in 25 ml of TSB and modified TSB in which glucose had been substituted with fructose, maltose, mannitol or sucrose for 48 hours. The cultures were centrifuged at 6,000 rpm for 10 minutes to collect supernatants for further agar disc diffusion method. The carbon source with the highest activity was selected to determine the optimal concentration. Each antagonistic bacterial isolate was grown at 30°C in 25 ml of modified TSB, containing various concentrations of the carbon source; 0.1, 0.15, 0.2, 0.25, 0.5, 1.0, 1.5, 2.0 or 2.5 % (w/v), for 48 hours before conducting the agar disc diffusion method against the pathogenic isolate PT1J.

The effects of nitrogen was evaluated by growing each isolate at 30°C in 25 ml of TSB and modified TSB in which peptone had been substituted by meat extract, yeast extract, $NH_4H_2PO_4$ or ammonium tartrate, for 48 hours before conducting the agar disc diffusion method. The nitrogen source with the highest activity was chosen to determine the optimal concentration, by growing each isolate in the modified TSB, containing various concentrations of peptone; 0.25, 0.5, 1.0, 1.5, 2.0 or 2.5% (w/v) for 48 hours. Cultures were centrifuged before conducting the agar disc diffusion test against the PT1J.

2.3.2 Temperature

Each antagonistic isolate was grown in 25 ml of modified TSB (obtained from the experiment 2.3.1) at 25, 30, 35 or 37°C for 48 hours. The supernatants were then tested for their ability to inhibit growth of PT1J.

2.3.3 Initial Media pH

Each selected antagonistic bacterial isolate was grown in 25 ml of modified TSB (obtained from experiment 2.3.1) with the pH adjusted to 4, 5, 6, 7, 8, 9, 10 or 11, and incubated at 30°C for 48 hours prior to conducting the agar disc diffusion method.

2.4 The Effects of Mixed Cultures of the Antagonistic Bacteria on Patummas

The five antagonistic bacterial isolates; N9C8, N11IV, N9A, Ba3 and Ba4; were mixed cultivated at 30°C in 25 ml of the modified TSB for 24 hours. The quantity of the bacteria was determined by using the viable cell count method. Concurrently, the wilt-causing bacterium, PT1J, was cultivated in 25 ml of TZC broth at 30°C for 24 hours. Three treatments were performed. First, both antagonistic and pathogenic bacteria were co-applied to Patumma rhizomes and to soil in pots before cultivation. Secondly, both antagonistic and pathogenic bacteria were

co-applied to shooting Patummas. Finally pathogenic bacteria PT1J were applied to rhizomes and to soil before Patumma cultivation, while the mixed culture of five antagonistic bacteria was added to the plant pots after shooting. All treatments were examined amounts of both antagonistic and wilt-causing bacteria, including physiologically change of the plants every 15 days for 6 months.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of Wilt-Causing Bacteria

Ninety-six bacterial isolates with reddish or pinky colonies, surrounded by a white slime layer, were obtained from water-soaked and badly smelling of Patumma rhizomes (Figure 1). They were Gram negative, short rods, aerobic and motile. According to their similar microscopic and gross characteristics, only the isolate PT1J was chosen for further study. The PT1J was classified as *Ralstonia solanacearum* (data not shown) due to its physical and biochemical characteristics (such as carbohydrate fermentation scheme, motility test, growth temperature, growth at various pHs and pigment formation). After the bacterial isolate PT1J was re-infected into Patumma rhizomes, the plants wilted and died, indicating successful isolation of the virulent strain from infected rhizomes with TZC medium [10].

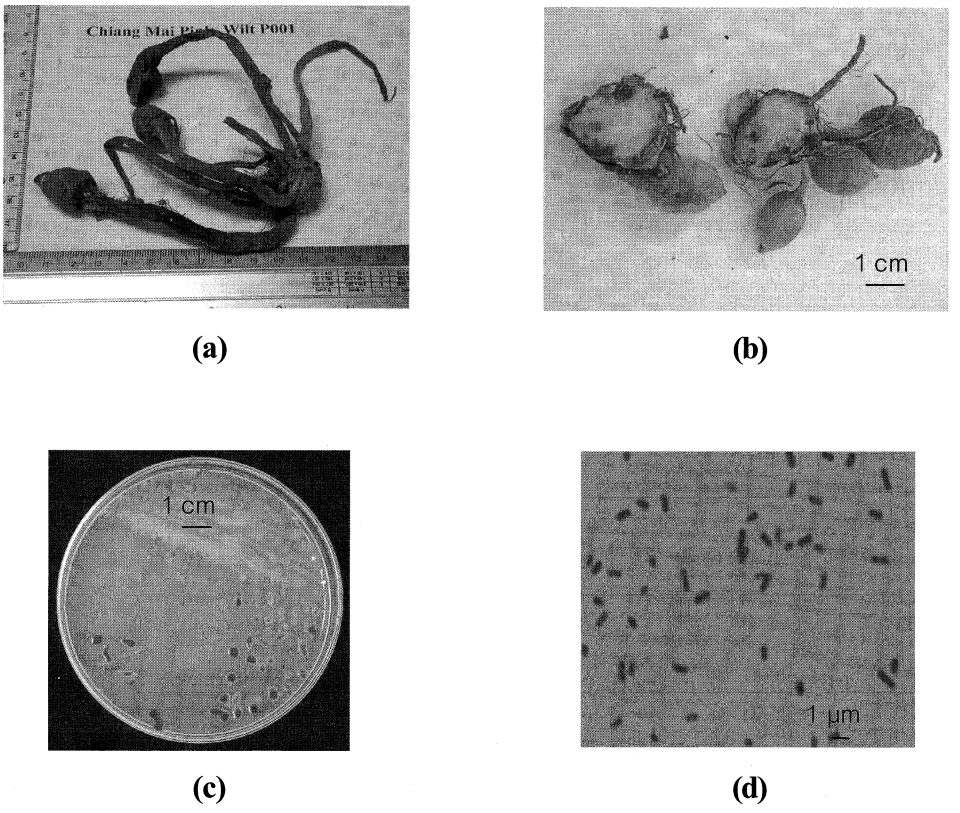


Figure 1. (a) and (b) The infected Patumma's rhizomes used for bacterial isolation. (c) The isolate PT1J colonies on TZC agar after 24 hours of incubation at 30°C. Notice the reddish colonies surrounded by white slime layer. (d) Gram stain of the isolate PT1J.

3.2 Isolation and Screening of Antagonistic Bacteria

Seventy-six bacterial isolates were obtained from soils surrounding *Zingiberaceae* plants. Among these, 28 isolates were cocci, 16 were short rods and 32 were rods. After testing their ability to inhibit growth of the pathogenic bacteria PT1J, only five, namely N9C8, N9A, N11IV, Ba3 and Ba4, displayed a high ability, with inhibition zones ranging from 15-22 mm (Table 1 and Figure 2).

The isolates N9C8, N11IV and Ba4 were Gram-positive rods with cell lengths greater than 1 μm. Their ellipsoidal endospores were located at the cell terminal. These three aerobic bacteria were motile and able to grow at 45°C but not at pH 4.0 and 11.0. Catalase enzyme was produced. They fermented fructose, galactose, glucose, maltose, mannitol, raffinose, rhamnose, sorbitol, sorbose and sucrose. Fumarate was fermented only by N9C8. Combinations of both biochemical and physical properties of these bacteria indicated that isolate Ba4, N9C8 and N11IV were *Bacillus*.

Isolates N9A and Ba3 were Gram-negative shot rods, with cell lengths less than 1 μm and they did not produce any endospores. These facultative bacteria were able to grow at pH 4.0 but not 11.0. Ba3 grew well at 45°C, while N9A could not. Both isolates were non-motile and did not produce oxidase enzyme, but catalase. They successfully fermented fructose, galactose, glucose, mannitol, rhamnose, sorbitol, sorbose and sucrose. N9A could also ferment maltose and raffinose. Both isolates were placed in the family *Enterobacteriaceae*, in accordance with the report of Weller (1998) about the ability of these bacteria to inhibit growth of soil borne pathogens [11].

Table 1. Inhibition of five antagonistic bacteria against growth of the PT1J pathogen.

Bacterial isolate	Inhibition zone diameter (mm)
N9C8	20 ± 2
N9A	15 ± 0.8
N11IV	18 ± 1
Ba3	19 ± 0
Ba4	22 ± 1

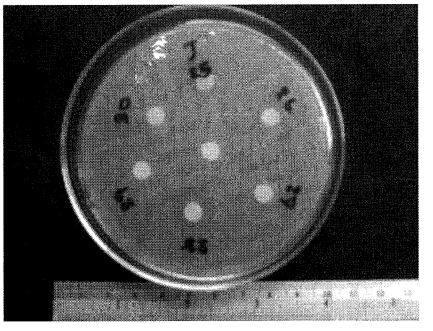


Figure 2. Clear zone of PT1J growth inhibition by the isolate N9C8.

3.3 Optimization of the N9C8, N9A, N11IV, Ba3 and Ba4 Isolates

3.3.1 Carbon Sources

Among five types of carbon sources evaluated, glucose showed the highest activity to inhibit growth of the PT1J with inhibitory zones of 1.8, 1.6, 1.5, 1.6, and 2.1 cm for isolates N9C8, N9A, N11IV, Ba3 and Ba4, respectively (Figure 3). Glucose was the best carbon source due to its readiness to enter many major metabolic pathways in the cells. The role of glucose for production of inhibiting substances from these antagonistic bacteria is under investigation. Nonetheless, this simply requirement for carbon source of these antagonistic bacteria could be the advantage for a bio-control preparation.

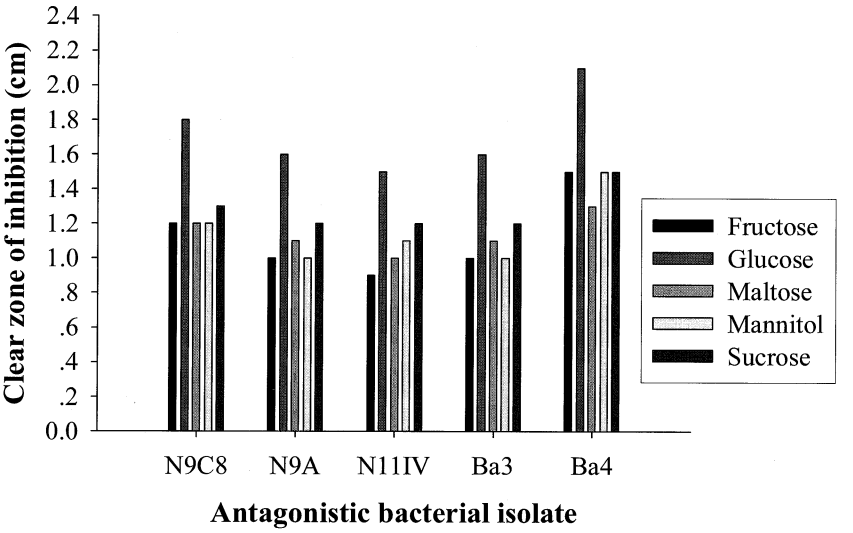


Figure 3. Effect of carbon sources on production of inhibiting substances by the antagonistic bacteria to inhibit growth of the PT1J. The experiments were conducted three times at 30°C using 0.25% (w/v) carbon source.

3.3.2 Nitrogen Sources

Peptone yielded the highest activity for all five antagonistic bacteria, due to its high content of trace elements and ready-to-use amino acids. Two other organic nitrogen sources, meat extract and yeast extract, displayed lower activity (Figure 4).

Peptone is commercially prepared by hydrolysis of proteins. The ratio of amino nitrogen (AN) and total nitrogen (TN) was 18-24%. Yeast extract was produced by baker's and brewer's yeast. It had an AN/TN ratio of between 40-50%, depending on the enzymes used in autolysis [12]. Hence, AN in yeast extract should enhance production of inhibitory substances better than peptone.

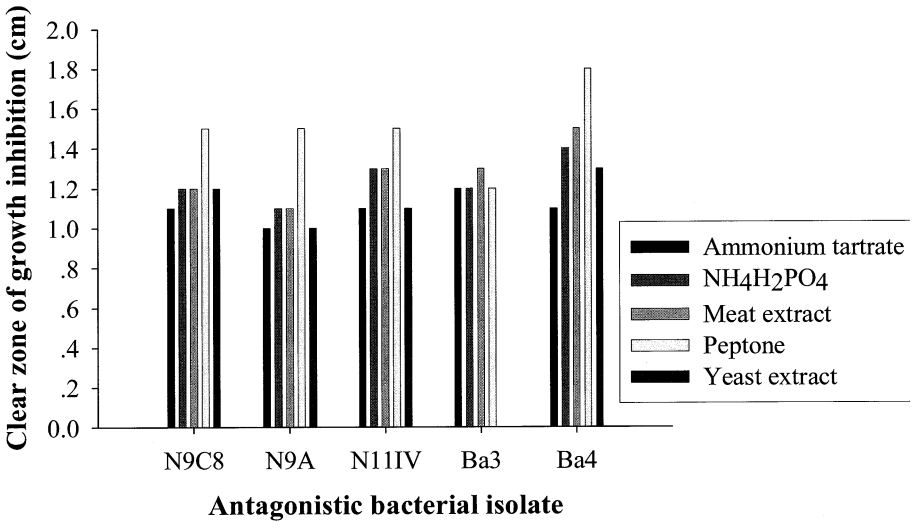


Figure 4. Effect of type of nitrogen sources on growth inhibition of the wilt causing bacterium, PT1J, by the five antagonistic bacteria. The cultures were grown at 30°C for 48 hours in the basal media containing 2.0% (w/v) nitrogen source.

3.3.3 Temperature

All tested temperatures supported the production of antagonistic substances by all five antagonistic bacteria. Antagonistic substances were best produced at 30°C for all bacteria but except Ba3 (Figure 5). The pathogen PT1J grew best at this temperature as well. Therefore, antagonistic bacteria were promising as bio-control agents against Patumma wilt.

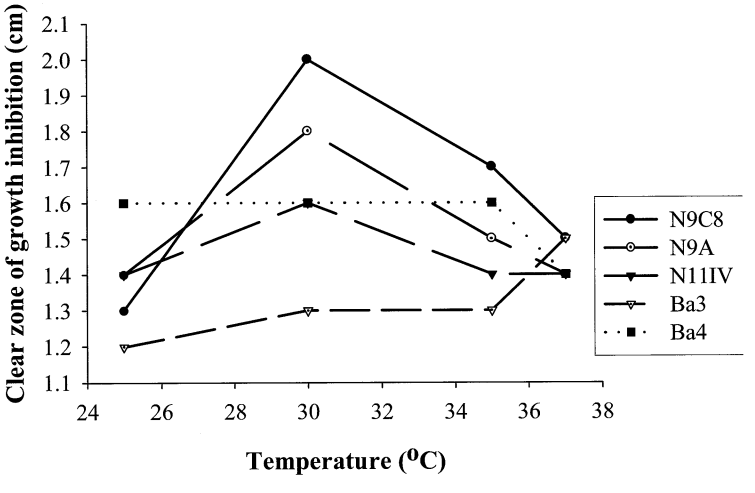


Figure 5. Effect of temperature on production of growth inhibitory substances from the antagonistic bacteria. The cultivations were performed at various temperatures for 48 hours prior to conduct the agar disc diffusion test.

3.3.4 Media pH

Normally bacterial strains stabilize intracellular pH at a certain value, despite changes in extracellular pH, allowing all enzymes in metabolic pathways to work properly. Large differences between extracellular and intracellular pHs cause cells to malfunction and die. Neutral pH was most suitable for all five antagonistic isolates to produce substances that inhibit growth of the wilt-causing bacterium PT1J, with the clear zones of inhibition ranging from 15 to 18 mm (Figure 6). Several reports indicated that most bacteria were capable of synthesizing an antibiotic in media with pHs ranging from 5.5 through 8.5 [13]. Meanwhile, *R. solanacearum* PT1J preferred an acidic environment, hence, a bio-control prepared at this neutral pH would effectively impede growth of the wilt pathogen.

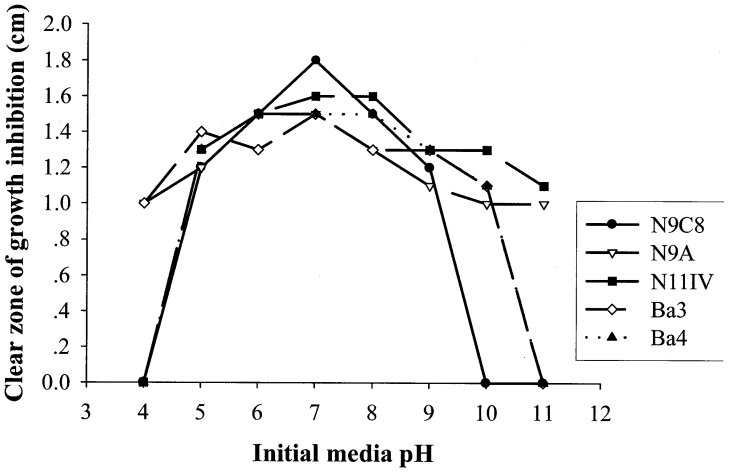
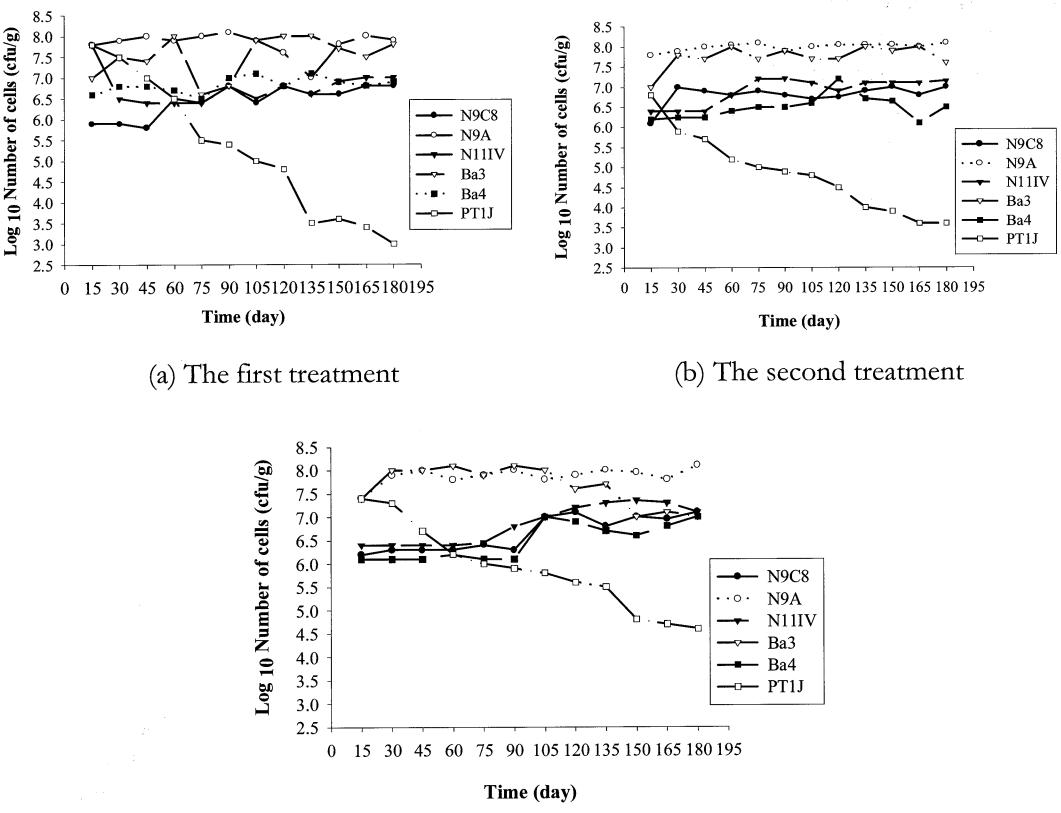


Figure 6. Effect of initial media pH on production of growth inhibitory substances of the antagonistic bacteria. The cultivations were conducted at 30°C for 48 hours.

3.4 Evaluation of the Inhibition Potential of the Five Antagonistic Bacteria on Patummas in Pots

When the mixed cultures of the antagonistic bacteria were introduced into Patummas' rhizomes and soil in pots upon cultivation as well as the pathogen PT1J, the plants grew well and displayed no signs of wilt. Quantities of antagonistic bacteria increased by 2-10% while those of PT1J declined by 65%, after 6 months of observation. Similarly, when antagonistic bacteria were co-applied with PT1J on shoots of Patummas in pots, the antagonistic bacteria increased by 2-10% while PT1J declined by 60% and the plants showed no sign of wilt. When PT1J was applied to cultivated Patummas, while the antagonistic bacteria were introduced to the shooting plants; the amounts of all antagonistic bacteria increased by 2% while PT1J declined by only 35% (Figure 7). Thirty-three percent of tested Patumma in this group had wilt, indicating partial prevention against *R. solanacearum* PT1J.



(a) The first treatment

(b) The second treatment

(c) The third treatment

Figure 7. Three treatments were applied to Patummas in pots. Soil samples were collected every 15 days to perform viable cell counts.

Hence, the proper period of time to inoculate the antagonistic bacteria as a bio-control agent is prior to crop cultivation, in order to allow the antagonistic bacteria to multiply and cover all cultivated areas, resulting in prevention of the wilt pathogen growth on the sites. The preventing role is based on nutrient competition and/or the action of substances produced by antagonistic bacteria. Patumma rhizomes are then protected against the wilt-causing organism and so are the cultivating fields. Control of *R. solanacearum* by antagonistic bacteria minimizes use of chemicals and brings about a cleaner environment as well.

4. CONCLUSION

This is the first report of using an antagonistic bacterial culture for minimizing growth of the wilt-causing bacteria, *Ralstonia solanacearum*, isolated from Patummas. The antagonistic bacteria are now under investigation for suitable formulation for use in Patumma fields, including a delivery system and shelf-life of the antagonistic bacteria in formulations. In order to encourage farmers to be more interested in using a bio-control, preparations should be simple, low cost as well as prevention and protection of the crops against *R. solanacearum* infection.

5. ACKNOWLEDGEMENT

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