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Effect of different concentrations of bacterial suspensions on biocontrol efficacy and storage stability of *Bacillus cereus* AR156, *Pantoea ananatis* YT11 and *Pseudomonas fluorescens* ABc9

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The influence of bacterial suspension concentration on biocontrol efficacies and storage stabilities of three biocontrol agents, including *Bacillus cereus* AR156, *Pantoea ananatis* YT11 and *Pseudomonas fluorescens* ABc9, were studied. The results indicated that biocontrol efficacy and storage stability did not increase along with the enhancement of the concentration of biocontrol agents, and 10^9 CFU ml⁻¹ of the strain *P. fluorescens* ABc9, 10^9 CFU ml⁻¹ of *B. cereus* AR156 and 10^8 CFU ml⁻¹ of *P. ananatis* YT11 provided the best biocontrol efficacy as 63.06, 49.55 and 52.25% respectively, and the most suitable concentration of the biocontrol agents for preservation ranged from 10^8 to 10^9 CFU ml⁻¹.

Key words: Biological control, strain concentration, biocontrol efficacy, storage viability.

INTRODUCTION

Biological control using microbial antagonists has attracted much interest as an alternative to chemical methods of controlling pre- and post-harvest plant pathogens and pests of agricultural and horticultural crops, which can give a good environmental compatibility, non-toxic harmless advantage (Janisiewicz, 1988; Wilson and Chalutz, 1989).

For practical use, microbial agents must be formulated as products capable of storage, distribution and application in agricultural marketplace, requiring different approaches from traditional agrochemical product design (Rodham et al., 1999). The main problem in formulation studies is how to maintain biocontrol efficacy and livingness of the products which is influenced by many factors. One of the most important factors is the strain concentration used for biological control and conserved for storage. A useful microbial formulation should be easy to distribute to the intended environment, inexpensive to produce, and has a long shelf-life (Melin et al., 2007).

Few studies have been carried out to evaluate the impact of the strain concentration on the biocontrol efficacies and storage stabilities of biocontrol agents. *Bacillus cereus* AR156, *Pantoea ananatis* YT11 and *Pseudomonas fluorescens* ABc9 were biocontrol agents selected to control root-knot nematode caused by *Meloidogyne incognita*, bacterial wilt caused by *Ralstonia solanacearum* on tomato, cucumber and some other plants in our previous work and unpublished data. In order to obtain well-formulated products of the three biocontrol agents for commercial application, we screened the suitable strain concentration of them for the best biocontrol efficiency and the storage.

MATERIALS AND METHODS

Bacterial strains and cell preparation

The *B. cereus* AR156, *P. ananatis* YT11 and *P. fluorescens* ABc9 used in this study were all isolated from the rhizosphere of tomato plants in Jiangsu Province of China. And they were all identified by 16S rRNA sequencing. Stock cultures of three biocontrol agents were stored at -70°C. The three biocontrol agents were all cultured in a 1 L conical flask at 30°C. A working volume of 500 ml of LB

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Table 1. Biocontrol efficacies of *B. cereus* AR156, *P. ananatis* YT11 and *P. fluorescens* ABc9 with a range of concentrations on tomato bacterial wilt caused by *Ralstonia solanacearum*.

Species	Treatment(CFU ml ⁻¹)	Disease severity (%)	Biocontrol efficacy (%)
<i>B. cereus</i> AR156	2.47×10 ⁹	45.83±2.08 ^{*gh†}	40.54±2.70 ^{cd}
	1.26×10 ⁸	28.47±3.18 ^k	63.06±4.13 ^a
	2.17×10 ⁷	39.58±3.61 ⁱ	48.65±4.68 ^b
	5.84×10 ^{6c}	58.33±2.08 ^d	24.32±2.70 ^{fg}
<i>P. ananatis</i> YT11	2.69×10 ¹⁰	50.00±2.08 ^f	35.13±2.70 ^d ^e
	3.62×10 ⁹	45.83±2.08 ^{gh}	40.54±2.70 ^{cd}
	4.39×10 ⁸	38.89±1.20 ^{ij}	49.55±1.56 ^b
	4.64×10 ⁷	45.14±1.20 ^{gh}	41.44±1.56 ^c
	4.00×10 ⁶	61.11±3.18 ^{cd}	20.72±4.13 ^g
<i>P. fluorescens</i> ABc9	4.25×10 ¹⁰	47.22±3.18 ^{fgh}	38.74±4.13 ^{cd}
	1.04×10 ⁹	36.81±2.41 ^{ij}	52.25±3.12 ^b
	1.06×10 ⁸	44.44±3.18 ^h	42.34±4.13 ^c
	1.76×10 ⁷	54.17±2.08 ^e	29.73±2.70 ^{ef}
	6.11×10 ⁶	67.36±2.41 ^b	12.61±3.12 ^h
Control		77.08±2.08 ^a	—

Note:*Means standard deviation; †Means followed by the same letter within a column are not significantly different as determined by the LSD test ($P=0.05$). Data are means for four replications.

medium was used as a growth medium after inoculation with 1% (v/v) of an inoculum which was cultured 24 h to exponential phase before use. Cells were harvested at the beginning of the stationary phase (24 h) by centrifugation at 5,000 g for 10 min at 20°C in an Avanti-TM J-25I centrifuge (Beckman, Palo Alto, CA, USA). The highest concentrations of three biocontrol agents cultured on LB medium were 3.0-5.0×10⁸ CFU ml⁻¹ for *B. cereus* AR156, 1.0-3.0×10⁹ CFU ml⁻¹ for *P. ananatis* YT11 and 4.0-6.0×10⁹ CFU ml⁻¹ for *P. fluorescens* ABc9. The cell paste was resuspended in sterile distilled water at the concentrations of 10⁹, 10⁸, 10⁷ and 10⁶ CFU ml⁻¹ on *B. cereus* AR156, 10¹⁰, 10⁹, 10⁸, 10⁷ and 10⁶ CFU ml⁻¹ on *P. ananatis* YT11, and 10¹⁰, 10⁹, 10⁸, 10⁷ and 10⁶ CFU ml⁻¹ on *P. fluorescens* ABc9.

The *R. solanacearum* was cultured in a 1 L conical flask at 30°C. A working volume of 500 ml of YGPA medium was used as a growth medium after inoculation with 1% (v/v) of an inoculum which was cultured 24 h to exponential phase before use. Cells were harvested at the beginning of the stationary phase (24 h) by centrifugation at 5,000 g for 10 min at 20°C and the cell paste was resuspended in sterile distilled water to 10⁷ CFU ml⁻¹.

Biological control ability of biocontrol strains in different concentrations in assay

Tomato seedlings (cv. Shanghai 903) at the age of 30 days were treated with antagonistic strains in one of the following methods. In the drenching method, 20 ml suspension of antagonistic strains with different concentrations was poured into each pot. Plants treated with sterile water served as control. The pots were placed in a greenhouse maintained at 28°C with relative humidity of 30%, and a 12 h/12 h photoperiod. In the greenhouse experiments, there were 12 plants in each replication and three replications for each treatment. Thirty days after treatment with antagonists (28 days after pathogen inoculation), the disease index was recorded based on a scale of 0-4 as described by Kempe and Sequeira (1983).

Disease incidence and biocontrol efficiency were calculated as

follows:

Disease incidence = $[\sum (\text{The number of diseased plants in this index} \times \text{Disease index}) / (\text{Total number of plants investigated} \times \text{The highest disease index})] \times 100\%$. Biocontrol efficacy = $[(\text{Disease incidence of control} - \text{Disease incidence of antagonist-treated group}) / \text{Disease incidence of control}] \times 100\%$.

Test of storage viability of biocontrol strains in different concentrations

The impact of the strain concentrations of biocontrol agents on durable storage in liquid formulation was also detected. Based on the results of biocontrol effect of *B. cereus* AR156, *P. ananatis* YT11 and *P. fluorescens* ABc9 with different concentrations on tomato bacterial wilt, 250 ml suspension of biocontrol agents with the different concentrations of 10⁹, 10⁸ and 10⁷ CFU ml⁻¹ on *B. cereus* AR156, 10¹⁰, 10⁹, 10⁸ and 10⁷ CFU ml⁻¹ on *P. ananatis* YT11, and 10¹⁰, 10⁹, 10⁸ and 10⁸ CFU ml⁻¹ on *P. fluorescens* ABc9 stored in 300 ml glass bottles at room temperature (25°C). The viabilities of the cells after 1, 2, 4, 8, 12, 24 and 36 months post-storage were observed, and the samples with appropriate dilutions plated in LB agar by plate dilution methods (Hoben and Somasegaran, 1982).

The plates were incubated at 30°C for 24 h and the resulting colonies were determined as colony forming units per milliliter (CFU ml⁻¹).

RESULTS AND DISCUSSION

When the strain concentrations of *B. cereus* AR156 and *P. ananatis* YT11 were 10⁶-10⁸ CFU ml⁻¹, and *P. fluorescens* ABc9 was 10⁶-10⁹ CFU ml⁻¹, the biological efficacies of three biocontrol agents had a positive trend with the increasing concentration (Table 1). But when the

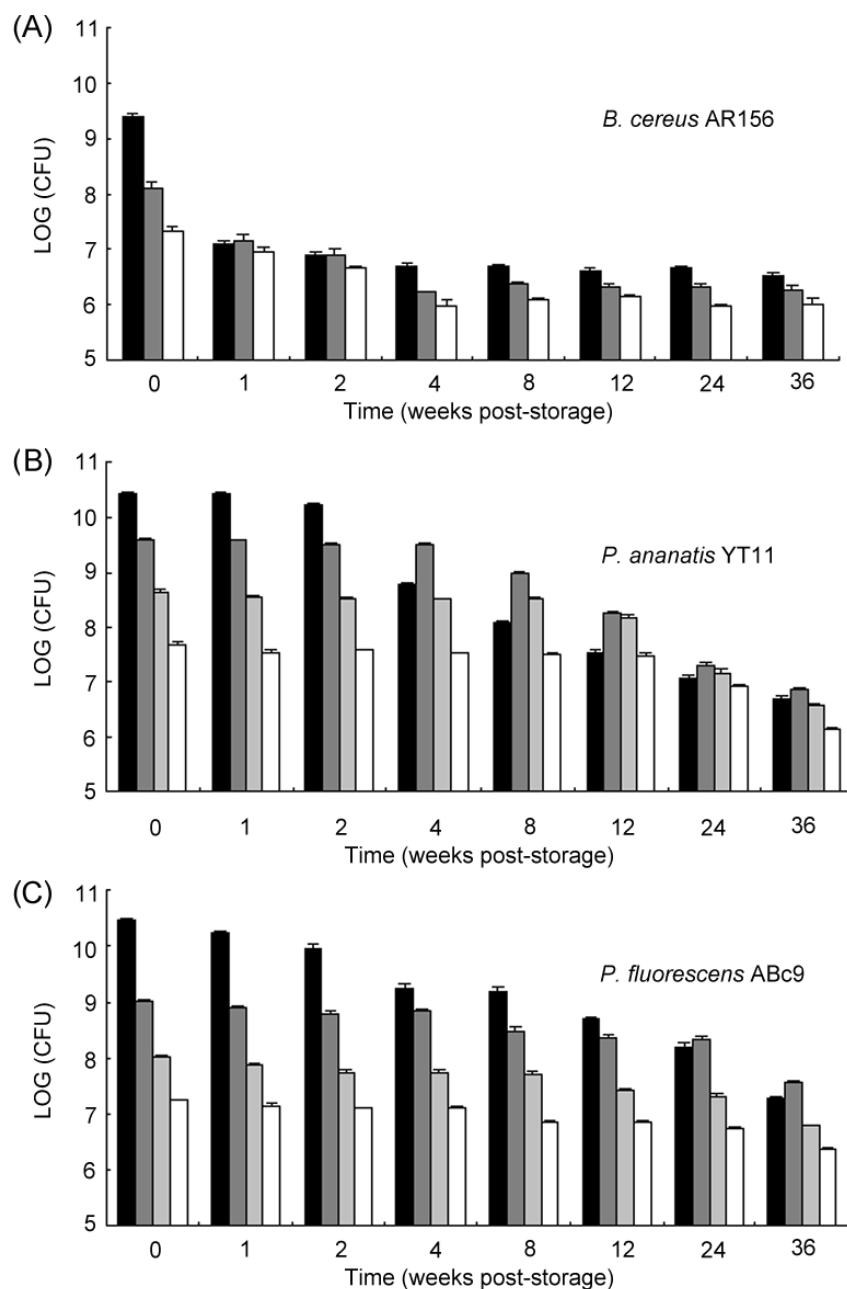


Figure 1. Comparison of the viability performance among (A) *B. cereus* AR156, (B) *P. ananatis* YT11, (C) *P. fluorescens* ABc9 at a range of initial concentrations 10^7 (□), 10^8 (■), 10^9 (■) and 10^{10} (■) CFU ml⁻¹ during storage.

strain concentrations exceed a certain level, such as the concentration of *B. cereus* AR156 and *P. ananatis* YT11 were higher than 10^8 CFU ml⁻¹, the one of *P. fluorescens* ABc9 was higher than 10^9 CFU ml⁻¹, the biological efficacies of the biocontrol agents were weakened. This indicated that increasing the strain concentration of the three biocontrol agents used were not always good for biological control against tomato bacterial wilt. In actual application, the concentration of the three biocontrol

agents should be 10^8 - 10^9 CFU ml⁻¹.

Significant differences in change of the viability on different storing concentrations of *B. cereus* AR156, *P. ananatis* YT11 and *P. fluorescens* Abc9 were observed (Figure 1). The three biocontrol agents showed a similar trend in storage, and the higher the initial concentration, the greater the survival rate. It exhibits that the initial concentration with 10^{10} CFU ml⁻¹ of *P. ananatis* YT11 and *P. fluorescens* Abc9 had lower viabilities than the ones

with 10^9 CFU ml⁻¹, indicating that the higher the concentration of the three biocontrol agents stored, the more it is harmful to their preservation. Considering the costs of products, the most suitable stored initial concentrations of the three biocontrol agents were 10^8 - 10^9 CFU ml⁻¹.

In conclusion, the strain concentration showed an important role on the biological efficacy and preservation of biocontrol agents. Some studies showed the higher the strain concentration, the better effect of biocontrol agents got (Bonaterra et al., 2003; Patino-Vera et al., 2005). But some other reports detected there was no positive trend on the strain concentration to the biological efficacy (Wilson and Chalutz, 1989; Nam, 2009), which were similar to our study. It is possible that too much foreign biocontrol agents in the micro-ecological environment in soil creates unsuitable condition for indigenous bacteria and host plant, which might make it easier for the infection of pathogens. There are several mechanisms for different biocontrol agents, and the ultimate aim of these biocontrol bacteria is to regulate ecological balance for controlling pathogen and promoting host plant growth, which also needs to cooperate with the indigenous bacteria in soil and plant. Therefore, the strain concentration of biocontrol agents is very important in practical application.

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