Biocontrol Science and Technology, 2007; 17(5/6): 647–663

Taylor & Francis
Taylor & Francis Group

Biological control of post-harvest late blight of potatoes

PATRICIA J. SLININGER[1], DAVID A. SCHISLER[1], LINDA D. ERICSSON[1], TINA L. BRANDT[2], MARY JO FRAZIER[2], LYNN K. WOODELL[2], NORA L. OLSEN[2], & GALE E. KLEINKOPF[2]

[1]*Crop BioProtection Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA, and* [2]*University of Idaho, Kimberly Research and Extension Center, Kimberly, ID, USA*

(Received 29 December 2006; returned 15 February 2007; accepted 8 March 2007)

Abstract

Introduction of US-8 genotypes of *Phytophthora infestans* has coincided with an increase in severity of potato late blight in North America. As alternatives to chemical fungicides, 18 bacterial strains patented as biological control agents (BCA) of both sprouting and Fusarium dry rot were cultivated in three liquid media and screened in wounded potato bioassays for their ability to suppress late blight incited by *P. infestans* (US-8, mating type A2). Washed or unwashed stationary-phase bacteria were mixed with fungal zoospores to inoculate potato wounds with 5 μL containing $\sim 10^8$ bacterial CFU/mL and 2×10^4 zoospore count/mL. Disease suppressiveness was evaluated after tubers were stored a week at 15°C, 90% relative humidity. One-fifth of the 108 BCA treatments screened, reduced late blight by 25–60%, including among other strains *Pseudomonas fluorescens* S22:T:04 (showing most consistency), P22:Y:05, S11:P:12 and *Enterobacter cloacae S11:T:07*. Small-scale pilot testing of these four strains, alone and in combination, was conducted under conditions simulating a commercial application. Suspensions of 4×10^4 *P. infestans* sporangia/mL were sprayed at a rate of 1.6 mL followed by 0.8 mL of bacteria treatment at $\sim 5 \times 10^9$ CFU/mL per each of 90 unwounded potatoes. Three replicate boxes per treatment (30 tubers per box) were randomized in storage and maintained 4 weeks at 7.2°C, 95% relative humidity. All BCA treatments significantly reduced disease; and unwashed bacteria outperformed those washed free of culture broth. Disease suppression ranged from 35% up to 86% the first test year and from 35 to 91% the second year. Highest overall performance rankings significantly above the control were achieved by the following strains in culture broth: four-strain mix > *P. fluorescens* S22:T:04 > *P. fluorescens* S11:P:12. Combined with previous demonstrations of dry rot and sprout suppression, the consistent late blight control by these strains and strain mixtures suggests the commercial feasibility of a single treatment for broad spectrum suppression of post-harvest potato diseases and sprouting.

Keywords: Solanum tuberosum, Pseudomonas fluorescens, Enterobacter cloacae, *sprout inhibitor,* Phytophthora infestans, *Fusarium dry rot, fungicide*

Correspondence: Patricia J. Slininger, Crop BioProtection Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, 1815 N. University, Peoria, IL 61604, USA. Tel: +1 309 681 6596. Fax: +1 309 681 6693. E-mail: Pat.Slininger@ars.usda.gov

Disclaimer: The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

First published online 11 June 2007

ISSN 0958-3157 print/ISSN 1360-0478 online
DOI: 10.1080/09583150701408881

Introduction

Phytophthora infestans, the causative agent of the potato late blight disease infects tubers primarily via zoospores released by sporangia that are washed down from infected leaves during periods of rain or heavy dew (Lapwood 1977; Sato 1980). In the presence of free moisture, infective zoospores are released from sporangia and form hyphae. Harvested tubers can become infected through eyes or wounds during washing (Fairclough et al. 1997) and during storage and handling (Lambert et al. 1998). *Phytophthora infestans* is considered to be the most significant pathogen of the crop worldwide (Fry et al. 2001) and historically was the cause of the Great Potato Famine of the late 1840s. The introduction of US-8 genotypes of *P. infestans* has coincided with an increase in severity of potato late blight in North America. Members of the Potato Association of America have placed the development of sustainable agricultural practices, involving more effective and efficient use of fungicides in combination with resistant cultivars, as a high priority need for the potato industry to keep late blight disease in check (Platt et al. 2000).

Biological control accomplished by beneficial microorganisms may be a viable alternative approach to reducing late blight disease. Recently, the successful application of rhizobacteria (including strains of *Pseudomonas putida*, *Micrococcus luteus*, and *Flexibacteraceae bacterium*) to suppress late blight was described with respect to disease suppression in greenhouse-grown potato plants (Kim & Jeun 2006). The present work represents the first literature report describing successful post-harvest biological control of late blight on stored potatoes, although research carried out in England during the same time period as these studies has also yielded new isolates showing significant potential to suppress late blight in stored potatoes (Hollywood 2006). A summary of preliminary findings has been given in abstract form (Slininger et al. 2004b).

Eighteen Gram-negative bacteria were originally discovered and developed as biocontrol agents to protect potatoes entering storage from Fusarium dry rot (Schisler & Slininger 1994; Slininger et al. 1994; Slininger et al. 1996a; Schisler et al. 1997, 1998), and they significantly reduced the level of dry rot disease in pilot trials (Schisler et al. 2000b). All of the top six dry rot suppressive strains have also been shown to suppress sprouting (Slininger et al. 2000, 2003a). The present research further explores the breadth of pest control achievable with these beneficial bacteria and the possibility to expand market potential.

As a key feature of this study, the relative late blight suppression of the 18 dry rot antagonistic bacterial strains was ranked after each was grown on three liquid culture media varying in nutritional richness. This ranking system was applied because commercialization of *Pseudomonas fluorescens* strains as biological control agents will require liquid culture production methodologies that foster retention of viability and efficacy when the cells are formulated, dried and stored. The industrial infrastructure for submerged culture fermentation is so well developed that even initial strain selection needs to be based in part on amenability to liquid cultivation. In past studies, we have demonstrated that cultivation conditions can have a major impact on cell yield, metabolite accumulations, and especially, biocontrol agent qualities – such as efficacy, shelf-life, and host compatibility (Slininger et al. 2003b; Zhang et al. 2005, 2006). For example, our process for selecting the most commercially useful dry rot antagonists similarly involved growing the strains on three different liquid media of varying nutritional richness, and then applying them to potato wounds challenged by

the pathogen. The ranking of candidate strains based on the liquid culture growth kinetics and yield and also the disease control efficacy of the product were found to vary widely due to the nutritional environment provided during production of the biological control agent; and these findings showed the critical importance of incorporating the liquid cultivation feature of industrial practice into the strategy used to rank and choose a strain or strains for commercial development (Slininger et al. 1994; Schisler et al. 2000a).

The second key feature of our studies was to test the consistency of late blight reduction by top-ranked single strains and strain mixtures in assays incorporating application and storage conditions that simulate commercial use of the biological control agent. The consistent achievement of disease control efficacy has been one of the major hurdles to overcome in the commercial development of biological control agents. Several researchers have reported that mixtures of strains can enhance and/or improve the consistency of biological control (Pierson & Weller 1994; Duffy & Weller 1995; Duffy et al. 1996; Janisiewicz 1996; Leeman et al. 1996; de Boer et al. 1999; Guetsky et al. 2001; Krauss & Soberanis 2001; Hwang & Benson 2002; Cruz et al. 2006). Our preliminary research has shown that formulations containing multiple strains of our dry rot antagonists performed more consistently than individual strains did when subjected to 32 storage environments varying in potato cultivar, harvest year, potato washing procedure (microflora exposure), temperature, and storage time (Slininger et al. 2001). Therefore, the performances of top individual biocontrol strains were compared with the mixture of strains in our evaluations simulating commercial use.

Experiments were designed with these key features to assess the ability of dry rot suppressive strains grown on industrially relevant liquid culture media to reduce late blight infection of stored potatoes in laboratory wounded potato bioassays and in trials conducted under commercial application and storage conditions. The ability to control late blight in addition to dry rot and sprouting by spraying a single treatment to harvested potatoes entering storage would further expand the commercial market and feasibility of this biocontrol product.

Materials and methods

Bacterial antagonists

Dry rot suppressive strains isolated by Schisler and Slininger (1994) were stored lyophilized in the ARS Patent Culture Collection (NCAUR, USDA, Peoria, IL) (Table I). Stock cultures of bacteria in 10% glycerol were stored at -80°C. Glycerol stocks were streaked to one-fifth strength trypticase soy broth agar plates (1/5 TSA; Difco Laboratories, Detroit, MI) which were incubated 2–3 days at 25°C and refrigerated up to one week as a source for preculture inoculation.

Cultivation media of varying nutritional richness

The disease control efficacies of the eighteen bacteria were compared for cells produced on three different liquid media which varied in nutritional richness. Minimal defined liquid (MDL) medium was prepared with 2 g/L each K_2HPO_4 and KH_2PO_4, 1.26 g/L urea, 0.1 g/L $MgSO_4(7H_2O)$, 10 mg/L NaCl, 10 mg/L $FeSO_4(7H_2O)$, 4.4 mg/L $ZnSO_4(7H_2O)$, 11 mg/L $CaCl_2(2H_2O)$, 10 mg/L $MnCl_2(4H_2O)$, 2 mg/L

Table I. Dry rot suppressive bacterial antagonists assayed for late blight suppressiveness.

	NRRL	
Strain	Accession number	Identification
P22:Y:05	B-21053	*Psuedomonas fluorescens biovar V*
S09:P:06	B-21049	*Pseudomonas corrugata*
S09:P:14	B-21105	*Pseudomonas corrugata*
S09:T:12	B-21104	*Pantoea* sp.
S09:T:14	B-21051	*Pseudomonas corrugata*
S09:Y:08	B-21128	*Pseudomonas fluorescens biovar I*
S09:P:08	B-21129	*Pseudomonas corrugata*
S09:T:04	B-21103	*Enterobacter* sp.
S09:T:10	B-21101	*Enterobacter* sp.
S11:P:08	B-21132	*Enterobacter* sp.
S11:P:12	B-21133	*Pseudomonas fluorescens biovar V*
S11:P:14	B-21134	*Pseudomonas fluorescens biovar V*
S11:T:06	B-21135	*Pseudomonas* sp.
S11:P:02	B-21136	*Pseudomonas corrugata*
S11:T:04	B-21048	*Pantoea agglomerans*
S11:T:07	B-21050	*Enterobacter cloacae*
S22:T:04	B-21102	*Pseudomonas fluorescens biovar I*
S22:T:10	B-21137	*Pseudomonas* sp.

$(NH_4)_6MO_7O_{24}(4H_2O)$, 2.4 mg/L H_3BO_3, 50 mg/L EDTA, and 35 g/L glucose (initial pH 6.8–7.0). Semi-defined complete liquid (SDCL) medium contained MDL minerals plus the same C:N ratio and C loading via combination of only 15 g/L glucose with amino acids (instead of urea), i.e. 15 g/L Difco vitamin-free casamino acids, 0.15 g/L tryptophan, and 0.6 g/L cysteine. SDCL also contained growth factors: 0.01 g/L each of adenine, cytosine, guanine, uracil, thymine; 0.5 mg/L each of vitamins thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine, thioctic acid; and 0.05 mg/L each of vitamins folic acid, biotin, B_{12}. Undefined liquid (UDL) medium was prepared by enriching SDCL medium with 5 g/L each Difco Bacto-tryptone, -peptone, and -yeast extract. All ingredients were autoclaved, except vitamins, urea and minerals <1 g/L, which were filter sterilized.

Shake-flask cultivations of bacteria

Fifty-mL precultures were the source of inocula for 200-mL experiment cultures (0.1 initial absorbance, 620 nm). Pre-cultures and experiment cultures contained like media in 125- and 500-mL flasks, respectively, and were shaken at 250 rpm (2.5 cm eccentricity) and 25°C in a New Brunswick Psychrotherm incubator. Cultures harvested after 72 h incubation were used to supply bacteria for bioassays. Typical cell accumulations reached $\sim 0.5-1 \times 10^{10}$ per mL.

Fermentor cultivation of bacteria

To supply inocula for larger scale efficacy studies in a commercial storage environment, bacteria were cultivated in 2-L B. Braun Biostat E or ED fermentors charged with 1.6 L of the SDCL medium above enriched to contain 40 g/L glucose, 60 g/L casamino acids, 0.6 g/L tryptophan, and 2.4 g/L cysteine. Fermentors were controlled

at 25°C, pH 7 (with 6 N NaOH or 3 N HCl additives), 1 L/min aeration, and variable stirring 300–1500 rpm to maintain dissolved oxygen at 30% of saturation. To control foaming, a 20% solution of Cognis FBA 3107 was dosed as needed. Bacteria were harvested after growth 72 h; at which time, viable cell accumulations were typically $\sim 2-3 \times 10^{10}$ per mL.

Laboratory wounded potato bioassay of late blight suppression by bacteria (Peoria)

Phytophthora infestans strain 693-3 (US-8, mating type A2) was obtained from Dr Gary Secor and Viviana Rivera, North Dakota State University, Fargo, ND for laboratory wounded potato bioassays in Peoria. Stock plates were initiated by placing 7-mm diameter plugs from the periphery of the source fungal matt in the center of CV8/RGA plates that were prepared as described below. Plates were sealed with Parafilm™ and incubated in darkness at 15°C for about 10 days until two-thirds of the surface was covered by fungal growth. Stock plates were then stored inverted in a box to maintain darkness in the refrigerator for at least 3 months. Stock cultures used to initiate inoculum production for experiments were no more than three transfers from the source plate.

For bioassays, plugs from stock culture plates were transferred to several CV8-RGA plates for incubation 14 days as described above. Maximum sporangia and zoospore yield occurred in a 12–17 day time window. Sporangia were harvested by adding 10 mL of sterile, cold distilled water to each plate, gently scraping with a loop, transferring the suspension to a second plate to harvest that plate, and finally transferring the sporangia suspension to a sterile 15-mL conical tube which is refrigerated for 2 h. After refrigeration, tubes were warmed for an hour in a 28°C incubator to liberate zoospores before counting in a hemacytometer.

Clarified V8 juice rye grain agar (CV8/RGA) was prepared by mixing and autoclaving (20 min, 121°C) 50 mL CV8 stock, 1 L rye broth, 20 g sucrose, 50 mg β-sitosterol (Sigma S-5753), and 15 g Bacto agar (Becton-Dickinson). The CV8 stock was prepared by autoclaving centrifuged V8 supernate that had been treated with 14.4 g $CaCO_3$ per 480 mL of supernate. The rye broth was prepared by diluting to 1 L and autoclaving the mixture of a first supernate poured from 60 g organic rye grain soaked overnight in 200 mL distilled water with a second supernate prepared by boiling the remaining grain 2 h in 1 L of distilled water followed with straining out the grain in four layers of cheese cloth.

Pathogen inoculum was diluted to 4×10^4 zoospores/mL in sterile distilled water, and each bacterial antagonist culture was diluted to 1.0 optical density at 620 nm ($\sim 10^9$ viable cells/mL) in cold sterile pH 7.2 phosphate buffer (0.004% phosphate buffer with 0.019% $MgCl_2$; Fisher Aid-Pack, Gloucester, MA). 'Washed' bacteria were twice centrifuged (7000 rpm, 7 min) and resuspended in cold sterile phosphate buffer to remove culture ingredients and products prior to use as inocula. Pathogen and bacterial inocula (or buffer only in place of bacteria for the disease controls) were then mixed 1:1 just prior to coinoculation of potato wounds with 5 μL per wound. Each bacteria treatment was repeated on six Russet Burbank potatoes that had been washed and dried a day ahead at room temperature (Wisconsin Seed Potato Certification Program, University of Wisconsin Madison, Antigo, WI). Each potato had four wounds equally spaced around the middle – three wounds receiving bacteria and pathogen and one control wound receiving only pathogen with either water or

buffer controls. Each potato was placed in a plastic weigh boat, and boats were held in trays that were supplied 80 mL water in the bottom, plastic bagged, and stored one week at 90% relative humidity and 15°C.

After 1 week storage each potato was quartered, slicing through the center of each of the four wounds. The extent of disease in each wound was rated by adding the greatest depth and width measurements (mm) of discolored necrotic tissue extending below and to the sides of the wound. Relative disease (%) was calculated as 100 × (wound disease rating/average disease rating of wounds receiving pathogen only).

For each bacterium, a relative performance index (RPI) was calculated based on its disease suppressiveness within each of six inoculum experiments, i.e. six combinations derived from three cultivation media (MDL, SDCL, or UDL) × two cell wash conditions (washed or unwashed). Given disease ratings normally distributed across the group of bacteria stains tested, the value of $F = (X - X_{avg})/s$ ranges from -2 to $+2$. Here, X designates a disease rating observed per bacterium, and X_{avg} and s are the average and standard deviation, respectively, of all values observed for the group of bacteria strains within the applied inoculum type. Since F decreases as disease suppressiveness improves, then $RPI = (2 - F) \times 100/4$, such that the value of RPI ranges from 0 to 100 percentile from least to most suppressive, respectively.

Late blight suppression by bacteria under commercial storage conditions (Kimberly)

Phytophthora infestans JMUIK-2002 (US-8, mating type A2) was obtained from Dr Jeff Miller (University of Idaho, Aberdeen, ID) for small pilot testing of disease suppression under commercial storage conditions at the University of Idaho Kimberly Research and Extension Center, Kimberly, ID. Kimberly rye agar plates were grown in darkness for 2 weeks at 18°C, where rye agar was prepared as follows: 60 g rye seed were soaked over night; seeds were then ground in blender 3 min and strained out using four layers cheesecloth; deionized water was poured through pulp until 1 L total volume suspension was collected; 10 g glucose and 15 g agar were added just prior to autoclaving. Plates were harvested by adding 10 mL of ~4°C water per plate and scraping with a glass hockey stick to recover about 9 mL containing 2×10^5 sporangia/mL. For tuber inoculation, 2.5 L of 4×10^4 sporangia/mL were chilled 1.5 h and then warmed to room temperature ~15 − 18°C for 45 min to liberate zoospores.

The suspension of *P. infestans* was sprayed onto all of the tubers for the trial at a rate of 1.6 mL per 8 oz. tuber (0.8 mL/tuber first to one side plus 0.8 mL/tuber to the other side). An air-assist syringe sprayer was used with a Delevan brass nozzle (#4, 80°LF). The sprayer and nozzle were sterilized between treatments by rinsing once with 70% ethanol and then three times with distilled water. Potatoes were sprayed in groups of about 100 spread out on plastic sheeting which could be drawn up to cover the potatoes to retain moisture until the bacterial treatments or water controls were applied. Pathogen-inoculated potatoes were collected into three replicate groups, and allowed to set no more than 45 min prior to treatment with biocontrol agents. Bacterial cultures were harvested from fermentors and stored refrigerated or in chilled shipping coolers 2–5 days before the trial. On the day of the trial, cultures were diluted in half with cold distilled water and returned to the refrigerator until application. Each bacterial treatment or water control treatment was then similarly sprayed at a rate of 0.8 mL per tuber to 30 potatoes from each of the three pathogen-treated replicate groups.

Each treatment replicate of 30 tubers was placed in an unventilated plastic box with lid and positioned randomly on shelves in the storage bay which was maintained at 7.2°C (45°F) and 95% relative humidity. After 4 weeks storage, potatoes were peeled and rated based on the percentage of surface area showing the discoloration typical of late blight infection.

Results and discussion

Strain efficacy influenced by culture media/broth in lab wounded potato bioassays

One-fifth of the 108 biocontrol agent treatments screened, reduced late blight by 25–60%. Table II(A)–(C) shows the results of six inocula experiments corresponding to the six production-formulation conditions applied to the 18 bacterial strains – three different culture media (MDL, SDCL, and UDL) and the application of washed versus unwashed bacteria. Results were normalized between experiments by converting wound disease ratings (mm) to relative disease ratings (%), which is disease development relative to the control wounds receiving only the *P. infestans* 693-3 pathogen and no bacteria.

In Table II strains are listed in order of decreasing suppressiveness (increasing relative disease rating), and it is notable that the ranking of strains varied sometimes considerably across the six different inocula experiments. This variation may have arisen because of specific differences in nutritional requirements of each strain for bioefficacy or because of the presence or absence of bioactive metabolites or culture broth nutrients when bacteria are applied unwashed or washed. However, a two-way analysis of variance across strains showed that neither washing or media type had significant impact on overall disease development. Relative disease rating means for MDL, SDCL, and UDL were 88.6, 84.7, and 90.2%, respectively; whereas means for washed and unwashed bacteria were 85 and 89%, respectively.

With an overall average relative performance index of 86.2, strain S22:T:04 most consistently suppressed disease regardless of growth nutrition and culture broth inclusion (Table III). This strain is of most interest for late blight suppression compared with other strains in the group because it would be expected to perform more consistently and at lower cost due to fewer production/formulation requirements in commercial applications. Other strains having RPI >50 included S09:T:14, S09:T:12, S09:P:14, S11:T:07, S09:Y:08, and P22:Y:05. In order to assess the overall best-performing strain, relative performance indices were calculated for each strain in each of the six inocula experiments and then averaged to obtain the overall average strain RPI's as shown in Table III. The RPI value within an experiment indicates the percentile ranking of a given strain relative to the performance of the others tested. Assuming a perfectly normally distributed data set, the best-performing strain would potentially have an RPI of 100 and the worst, 0. The overall average relative disease rating is also shown in Table III for each strain to indicate what level of disease suppression it achieved.

In earlier studies, the following strains were top-performers (overall RPI >50) when considering both dry rot suppressiveness and cultivation kinetics of strains produced on MDL, SDCL and UDL media: *E. cloacae* S11:T:07^{kdsl}, *P. fluorescens* S09:Y:08^{kdl}, *P. fluorescens* S11:P:12^{kds}, *P. fluorescens* S11:P:14^{kd}, *Enterobacter* sp. S11:P:08^{kds}, *P. fluorescens* S22:T:04^{kdl}, *P. corrugata* S11:P:02^{kd}, *Enterobacter* sp. S09:T:10^{kd}, *Enterobacter* sp. S09:T:04^{kd} and *P. fluorescens* P22:Y:05^{kdl} (Slininger et al. 1994). The

Table II. Late blight suppression by bacterial antagonists cultivated on three different media and applied with or without washing to potato wounds co-inoculated with *P. infestans* in a laboratory bioassay.

	Unwashed cells				Washed cells		
Treatment	Disease rating (mm)	Relative disease rating[a,b,c] (%)		Treatment	Disease rating (mm)	Relative disease rating[a,b,c] (%)	
A. MDL medium							
S11T07	4.3	43	A	**S22T04**	**8.0**	**39.6**	**A**
S11P08	4.7	39	A	S09T12	9.5	49.5	AB
S09T14	4.8	52	A	S09P06	13.5	75.8	AB
S09Y08	5.0	55	A	S09T14	13.7	76.9	AB
S22T04	5.2	58	A	S09P14	13.8	78.0	AB
P22Y05	6.3	79	A	S11T07	14.5	82.4	AB
S09P14	6.3	79	A	S09Y08	15.2	86.8	AB
S11T06	6.5	82	A	S11T04	15.2	86.8	AB
S09T12	6.5	82	A	P22Y05	15.5	89.0	AB
S22T10	6.8	89	A	S22T10	16.0	92.3	AB
S11P02	7.0	92	A	S11T06	16.0	92.3	AB
S11T04	7.2	95	A	**Buffer**	**17.2**	**100**	**B**
Buffer	**7.5**	**100**	**A**	S11P02	17.7	103.3	B
S09T10	7.7	104	A	S09T10	19.5	115.4	B
S09T04	8.0	110	A	S11P14	19.5	115.4	B
S09P06	8.3	116	A	S11P12	20.7	123.1	B
S11P14	8.7	122	A	S09P08	21.5	128.6	B
S11P12	9.7	141	A	S09T04	21.7	129.7	B
S09P08	9.8	145	A	S11P08	22.2	133.0	B

Table II (*Continued*)

	Unwashed cells				Washed cells		
Treatment	Disease rating (mm)	Relative disease rating[a,b,c] (%)		Treatment	Disease rating (mm)	Relative disease rating[a,b,c] (%)	
B. SDCL medium							
S11P12	16.7	73.9	A	**S22T04**	**7.5**	**38.7**	**A**
S11P14	18.3	82.3	A	S09T04	8.8	48.0	AB
S22T04	19.0	85.6	A	P22Y05	9.5	52.7	AB
S22T10	19.5	88.2	A	S09T14	10.5	59.8	AB
S09P14	19.8	89.8	A	S09Y08	10.5	59.8	AB
S11T06	20.2	91.5	A	S11P02	11.0	63.3	AB
S09T12	20.3	92.3	A	S09P08	11.2	64.5	AB
S11P02	20.5	93.2	A	S09P06	12.7	75.0	AB
S11T07	21.2	96.5	A	S11P14	13.0	77.3	AB
P22Y05	21.7	99.0	A	S11T07	13.2	78.5	AB
Buffer	**21.9**	**100.0**	**A**	S09T12	13.2	78.5	AB
S09T14	22.7	104.1	A	S09T10	13.3	79.7	AB
S11P08	23.0	105.8	A	S11P12	13.5	80.9	AB
S11T04	23.8	110.0	A	S11T06	14.8	90.2	AB
S09T10	24.7	114.2	A	S11P08	15.0	91.4	AB
S09Y08	24.7	114.2	A	S11T04	16.2	99.6	AB
S09P06	25.3	117.5	A	**Buffer**	**16.2**	**100.0**	**B**
S09P08	26.0	120.9	A	S22T10	17.7	110.2	B
S09T04	28.3	123.9	A	S09P14	17.8	111.3	B

Table II (*Continued*)

Treatment	Unwashed cells: Disease rating (mm)	Unwashed cells: Relative disease rating[a,b,c] (%)		Treatment	Washed cells: Disease rating (mm)	Washed cells: Relative disease rating[a,b,c] (%)	
C. UDL medium							
S09T14	**12.5**	**53.2**	**A**	**S22T04**	**13.0**	**58.5**	**A**
S09P14	16.0	71.0	AB	S11P12	15.5	71.8	AB
P22Y05	18.3	82.8	AB	S09P14	17.3	81.6	AB
S09T12	18.7	84.5	AB	S09T14	17.8	84.3	AB
S09T10	19.8	90.4	AB	S09Y08	18.7	88.7	AB
S11T04	19.8	90.4	AB	S11T06	18.7	88.7	AB
S22T04	20.2	92.1	AB	S09T12	19.2	91.4	AB
S09T04	21.0	96.3	AB	S09P08	19.2	91.4	AB
S11P14	21.3	98.0	AB	S11T04	19.3	92.2	AB
Buffer	**21.7**	**100.0**	**B**	S11T07	20.0	95.8	AB
S09Y08	21.8	100.5	B	S11P08	20.2	96.6	AB
S11P08	21.8	100.5	B	S11P02	20.3	97.6	AB
S09P06	21.8	100.5	B	S09T10	20.3	97.6	AB
S11T07	22.0	101.4	B	**Buffer**	**20.8**	**100.0**	**B**
S22T10	22.3	103.1	B	S09T04	21.7	104.7	B
S09P08	22.8	105.6	B	S11P14	21.8	105.5	B
S11T06	22.8	105.6	B	S09P06	24.3	118.8	B
S11P02	24.5	114.0	B	S22T10	25.7	125.9	B
S11P12	25.0	116.6	B	P22Y05	26.2	128.6	B

[a]Within the relative disease rating columns, values with no letters in common are significantly different ($P < 0.05$) based on results of the Student–Newman–Keuls pairwise comparison method. Each value represents the mean of disease ratings from six replicate wounds, each on a different potato; since one control and three treatments were applied per potato, buffer control means are the average of 36 wound ratings. [b]Embolding highlights buffer controls (containing no biological control agent) and biocontrol treatments that show significant disease suppression relative to controls. [c]In this screening experiment, each bacteria was evaluated once on each of the six culture medium-cell washing conditions, and then statistical analyses were applied to rank overall strain performance as shown in Table III.

Table III. Superior late blight suppression achieved by *Pseudomonas* sp. strain S22:T:04 that was significantly different from controls as shown by overall relative disease and relative performance index (RPI) analyses.

					RPI					
					Unwashed cells			Washed cells		
Treatment	Overall average relative disease rating (%)[a]		Overall average RPI[a]		MDL	SDCL	UDL	MDL	SDCL	UDL
S22T04	**62.1**	**A**	**86.2**	**A**	**76.9**	**75.9**	**55.2**	**104.9**	**97.8**	**106.5**
S09T14	71.7	AB	75.9	AB	82.2	44.0	122.9	67.6	71.4	67.5
S09T12	79.8	AB	64.7	AB	55.7	64.3	68.4	95.0	47.9	56.7
S09P14	85.2	AB	60.6	AB	58.3	68.6	92.0	66.6	6.8	71.5
S11T07	82.9	AB	57.7	AB	90.1	57.0	39.0	62.2	47.9	50.0
S09Y08	84.2	AB	56.1	AB	79.5	26.6	40.5	57.8	71.4	60.7
P22Y05	88.6	AB	53.1	AB	58.3	52.7	71.4	55.6	80.2	0.2
S11T06	91.8	AB	49.9	AB	55.7	65.7	31.7	52.3	33.2	60.7
S11P02	93.8	AB	47.2	AB	47.7	62.8	17.0	41.4	67.0	47.3
S11T04	95.6	B	45.3	AB	45.1	33.9	58.2	57.8	21.4	55.4
S11P08	96.0	B	43.1	AB	84.8	41.1	40.5	11.8	31.7	48.6
S11P14	100.1	B	43.6	AB	21.2	81.6	44.9	29.3	49.3	35.2
S11P12	101.1	B	44.5	AB	5.4	96.1	12.6	21.6	44.9	86.3
S09T10	100.2	B	40.8	B	37.1	26.6	58.2	29.3	46.4	47.3
Buffer	**100.0**	**B**	**40.3**	**B**	**40.5**	**51.1**	**41.4**	**44.6**	**20.9**	**43.6**
S09P06	100.6	B	37.3	B	26.6	20.8	40.5	68.7	52.3	15.0
S22T10	101.4	B	37.1	B	50.4	71.5	36.1	52.3	8.2	4.3
S09T04	102.1	B	35.4	B	31.8	−5.3	47.9	15.1	86.0	36.5
S09P08	109.1	B	31.3	B	2.7	15.0	31.7	16.2	65.5	56.7

[a]Within columns, values with no letters in common are significantly different ($P < 0.05$) based on results of the Student–Newman–Keuls pairwise comparison method.
[b]Embolding highlights buffer controls (containing no biological control agent) and biocontrol treatments that show significant disease suppression relative to controls.

superscript 'k' designates top kinetic performers in liquid culture, and the 'd' indicates top dry rot suppressive strains. The three strains marked with a superscript 's' were also found to be top-performers with respect to sprout suppression and demonstrated overall average RPI's >50 (Slininger et al. 2003a). The four strains marked with the superscript 'l' are common to the set of top late blight suppressive strains above. Only strain *E. cloacae* S11:T:07 was among the top performers with overall RPI >50 in all three assays for dry rot, sprout, and late blight suppression. The multiple bioactivities of S11:T:07 as well as its consistency in controlling each of three potato storage maladies may arise at least in part because of its ability to produce multiple bioactive metabolites. Phenylacetic acid, indoleacetic acid, and tyrosol were isolated from *Enterobacter cloacae* strain S11:T:07 and shown to have not only antifungal activity against *G. pulicaris* but also sprout inhibitory activity (Burkhead et al. 1998; Slininger et al. 2004a), with greatest disease suppression occurring when all three bioactive metabolites were present. Consistent with our observations of indoleacetic acid (IAA) as a major antifungal product, Martiniez Noel et al. (2001) showed that IAA also attenuates disease severity in potato-*Phytopthora infestans* interactions and inhibits the pathogen growth *in vitro*.

Previous studies indicated that a variety of metabolites, including antifungal compounds, are produced by our 18 patented dry rot antagonistic bacteria, and that each of the strains produced at least one and some produced several antifungal compounds (Burkhead et al. 1995). Thus, current and previously reported findings suggest the potential utility of applying one or a combination of strains to accomplish multiple problem solutions in a single biological control treatment. In addition, the availability of multiple bioactive components accomplishing control may have the added advantages of improving biocontrol consistency and reducing the likelihood of the target pathogen becoming resistant.

Best strain efficacy enhanced by culture broth in lab and commercial storage simulations

With the idea of developing a single multi-use biocontrol agent mixture, four strains of varying biocontrol abilities were chosen for further small-scale pilot testing under conditions simulating commercial application: *E. cloacae* S11:T:07^{kdsl}, *P. fluorescens* S11:P:12^{kds}, *P. fluorescens* S22:T:04^{kdl}, and *P. fluorescens* P22:Y:05^{kdl}. To accommodate the larger-scale testing, all strains were grown similarly in controlled fermentors on SDCL medium enriched in amino acids and aerated to enhance viable cell accumulation. Disease reduction results from trials conducted at Peoria using the wounded potato bioassay and at Kimberly under simulated commercial storage conditions are compared in Table IV. Significant disease reduction was observed in all trials, but the largest reductions were observed in the Kimberly trials where disease suppression ranged from 35% (worst treatment) to 86% (best treatment) the first year and from 35 to 91% the second year. Two-way analysis of variance of disease rating with strain and suspending medium showed that for all trials, except Peoria Year 1, unwashed bacterial treatments containing culture broth allowed significantly less disease development than did treatments composed of washed cells suspended in buffer ($P \leq 0.01$). The comparison of broth: buffer relative disease rating (%) means is as follows: Peoria Year 1 (98:78), Peoria Year 2 (41:70), Kimberly Year 1 (21:55), and Kimberly Year 2 (19:42). Significant variation in relative disease ratings among strains was also observed in Kimberly trials (though not Peoria trials), but the relative

Table IV. Comparative treatment efficacies in laboratory wounded potato bioassays (Peoria) and small-pilot commercial simulations (Kimberly).

	Kimberly						Peoria					
	Year 1			Year 2			Year 1			Year 2		
Treatment[b,c]	Disease rating (mm)	Relative disease (%)[a]		Disease rating (mm)	Relative disease (%)[a]		Diseased surface (%)	Relative disease (%)[a]		Diseased surface (%)	Relative disease (%)[a]	
Mix+buffer	16.8	71.0	AB	17.8	77.3	ABCD	7.3	34.3	CD	17.7	50.4	BC
S11T07+buffer	16.0	67.0	B	14.3	62.2	BCDEF	10.4	48.8	BC	15.4	43.9	CD
P22Y05+buffer	18.3	78.2	AB	19.8	86.0	ABC	13.8	64.7	B	9.4	26.8	DEF
S11P12+buffer	16.3	68.6	B	18.5	80.2	ABC	13.9	65.3	B	7.8	22.2	DEF
S22T04+buffer	23.0	100.5	AB	10.0	43.4	DEFG	13.1	61.5	B	22.8	65.0	B
Mix+broth	18.0	76.6	AB	5.8	25.3	GHI	2.9	13.6	DE	11.2	31.9	CDE
S11T07+broth	25.2	110.8	AB	9.5	41.2	EFGH	5.7	26.7	CD	3.2	9.1	F
P22Y05+broth	20.2	86.9	AB	15.3	66.5	BCDE	3.8	17.8	D	9.1	25.9	DEF
S11P12+broth	23.0	100.5	AB	9.5	41.2	EFGH	5.4	25.3	CD	4.2	11.9	EF
S22T04+broth	24.5	107.7	AB	6.7	28.9	FGHI	4.0	18.7	D	5.0	14.2	EF
Broth of mix	27.3	121.2	A	17.7	77.0	ABCD	9.3	43.7	BC	5.3	15.1	EF
Buffer (Control)	–	–		–	–		12.3	57.7	B	28.3	80.6	A
Water (Control)	22.9	100.0	AB	23.1	100	A	21.3	100.0	A	35.1	100.0	A

[a]Within relative disease columns, values followed with no letters in common are significantly different ($P < 0.05$) based on results of the Student–Newman–Keuls pairwise comparison method. [b]'Mix' refers to a mixture of all four bacterial strains S11:T:07, P22:Y:05, S11:P:12, and S22:T:04; the designations '+ buffer' or '+ broth' indicate that the strain or mix were either washed free of culture broth and suspended in buffer or applied suspended in culture broth, respectively; 'broth of mix' refers to the broth that was separated from the four-strain mix and cleared of cells; and 'buffer' and 'water' controls were applied in place of BCA or broth treatments. [c]This experiment design was repeated twice in Peoria laboratory bioassays (shown in years 1 and 2) and twice in Kimberly small-pilot commercial simulations (also shown in years 1 and 2). In each Peoria experiment, each treatment was applied to six replicate potato wounds, each on a separate potato. In each Kimberly experiment, each treatment was sprayed to three replicates of 30 whole potatoes per replicate.

Table V. Significant disease suppression by all biocontrol treatments based on relative disease analysis across both locations and years; and superior ranking of strain mix, S22:T:04 and S11:P:12 treatments in culture broth that significantly differed from controls based on relative performance index (RPI) analysis.

					RPI			
					Peoria		Kimberly	
Treatment[b]	Overall average relative disease (%)[a]		Overall average RPI[a]		Year 1	Year 2	Year 1	Year 2
Mix+broth	36.9	A	71.8	A	69.2	86.7	78.8	52.6
S22T04+broth	42.4	A	63.2	A	27.0	82.7	73.8	69.2
S11P12+broth	44.7	A	61.4	A	36.7	70.1	67.5	71.4
S11T07+broth	47.0	A	58.2	AB	22.4	70.1	66.1	74.0
P22Y05+broth	49.3	A	58.0	AB	54.9	44.2	74.7	58.3
S11T07+buffer	55.5	A	54.3	AB	82.2	48.7	44.7	41.4
Mix+buffer	58.2	A	51.0	AB	77.0	33.0	58.8	35.2
S11P12+buffer	59.1	A	50.2	AB	80.3	29.9	28.8	61.7
P22Y05+buffer	63.9	A	44.5	AB	67.3	24.1	29.3	57.5
Broth of mix	64.3	A	40.1	AB	8.8	33.5	49.7	68.4
S22T04+buffer	67.6	A	39.7	AB	36.7	67.9	32.5	21.6
Water (Control)	100.0	B	7.6	B	37.4	9.3	−4.8	−11.3

[a]Within columns, values followed with no letters in common are significantly different ($P < 0.05$) based on results of the Student–Newman–Keuls pairwise comparison method. [b]'Mix' refers to a mixture of all four bacterial strains S11:T:07, P22:Y:05, S11:P:12, and S22:T:04; and the designations '+ buffer' or '+ broth' indicate that the strain or mix were either washed free of culture broth and suspended in buffer or applied suspended in culture broth, respectively; 'broth of mix' refers to the broth from the four-strain mix cleared of cells; and 'water' controls were applied in place of BCA or broth treatments.

performance of strains depended on whether they were applied in broth or buffer. A two-way analysis of variance of either relative disease rating (%) or RPI with treatment and trial (location-year) indicated no significant treatment × trial interaction.

In order to rank overall treatment performance, relative performance indices were calculated for each treatment in each of the trials at Peoria and Kimberly and then averaged to obtain the overall average RPI's of treatments as shown in Table V. Using this analysis, all strains in broth ranked higher in ability to suppress late blight than strains in buffer. With overall average RPI = 71.8, the four-strain mix in broth was top ranked and significantly different from the water control. The S22:T:04 and S11:P:12 in broth treatments followed in order and were also significantly different from the water control. By itself, the broth of the mix (prepared by centrifuging culture to remove the cells) usually reduced disease, and exhibited an overall average relative disease rating of 64.3%, which was significantly less than that of the water control. This result further suggests the importance of the culture broth in enhancing biological control of late blight. In previous studies, bioactivity of the broth played a role in sprout suppression by strain S11:P:12 (Slininger et al. 2003a). The activity of the broth may arise because of bioactive metabolites produced by strains (as noted above) or because of other media components present, and further studies to elucidate this are necessary.

In summary, ranking of strains relative to ability to reduce late blight infection varied with the culture medium they were produced on and also depended on whether the culture broth was retained or washed away from cells applied in bioefficacy assays. For example, the strain most suppressive to late blight, *Pseudomonas* sp. S22:T:04, significantly reduced disease by 14–60% (37% on average), depending on the cultivation medium and washing condition. Disease suppression by treatments was greater under test conditions simulating a commercial setting where pathogen entry to the potato was mainly through eyes compared to the laboratory bioassay where pathogen entry was via wounds. Under conditions simulating commercial application and storage processes, single strains and strain mixtures were shown to significantly reduce late blight by 35–91% in unwounded tubers; and the presence of culture broth significantly enhanced the bioefficacy of all four of the strains selected for study. The mixture of four strains in broth had an RPI of 71.8, which was higher than that of any of the individual strains in broth or buffer, but not significantly different from the top late blight suppressive single strains S22:T:04 and S11:P:12 in broth. These findings suggest the utility of applying a mix of strains along with their culture broth in achieving consistent late blight control under commercial application and storage conditions. The ability to control late blight in addition to dry rot and sprouting in a single treatment further expands the commercial market and feasibility of this biocontrol product. This technology offers an approach to disease suppression where no effective chemicals are registered for food-grade potatoes and where chemical use is undesirable or prohibited – such as for organic potatoes, fresh-pack potatoes, some foreign export markets, and seed potatoes.

Acknowledgements

We thank Ms Maureen Shea-Wilbur and Ms Jennifer Sloan for their excellent assistance in the laboratory phases of these experiments.

References

Burkhead KD, Schisler DA, Slininger PJ. 1995. Bioautography shows antibiotic production by soil bacterial isolates antagonistic to fungal dry rot of potatoes. Soil Biology and Biochemistry 27:1611–1616.

Burkhead KD, Slininger PJ, Schisler DA. 1998. Biological control bacterium *Enterobacter cloacae* S11:T:07 (NRRL B-21050) produces antifungal compound phenylacetic acid in Sabouraud maltose broth culture. Soil Biology and Biochemistry 30:665–667.

Cruz LP, Gaitan AL, Gongora CE. 2006. Exploiting the genetic diversity of *Beauveria bassiana* for improving the biological control of the coffee berry borer through the use of strain mixtures. Applied Microbiology and Biotechnology 71:916–926.

de Boer M, van der Sluis I, van Loon LC, Bakker PAHM. 1999. Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of Fusarium wilt of radish. European Journal of Plant Pathology 105:201–210.

Duffy BK, Weller DM. 1995. Use of *Gaeumannomyces graminis* var. *graminis* alone and in combination with fluorescent *Pseudomonas* spp. to suppress take-all of wheat. Plant Disease 79:907–911.

Duffy BK, Simon A, Weller DM. 1996. Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. Phytopathology 86:188–194.

Fairclough RW, Bain RA, Holmes SJ. 1997. The infection of potatoes by *Phytophthora infestans* during simulated washing and its control using a hot water treatment. Potato Research 40:91–99.

Fry WE, Thurston HD, Stevenson WR. 2001. Late blight. In: Stevenson WR, Loria R, Franc GD, Weingartner DP, editors. Compendium of potato diseases. St. Paul, MN: APS Press. pp 28–30.

Guetsky R, Shtienberg D, Elad Y, Dinoor A. 2001. Combining biocontrol agents to reduce the variability of biological control. Phytopathology 91:621–627.

Hollywood JC. 2006. Biological control of late blight of potatoes: *in vivo* and *in vitro* evaluation of microbial antagonists against tuber blight [PhD Thesis, advised by Richard Strange]. London (England): University of London. 220 p.

Hwang J, Benson DM. 2002. Biocontrol of Rhizoctonia stem and root rot of poinsettia with *Burkholderia cepacia* and binucleate *Rhizoctonia*. Plant Disease 86:47–53.

Janisiewicz W. 1996. Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of post-harvest diseases of apples. Phytopathology 86:473–479.

Kim HJ, Jeun YC. 2006. Resistance induction and enhanced tuber production by pre-inoculation with bacterial strains in potato plants against *Phytophthora infestans*. Mycobiology 34:67–72.

Krauss U, Soberanis W. 2001. Biocontrol of cocoa pod diseases with mycoparasite mixtures. Biological Control 22:149–158.

Lambert DH, Currier AI, Olanya MO. 1998. Transmission of *Phytophthora infestans* in cut potato seed. American Journal of Potato Research 75:257–263.

Lapwood DH. 1977. Factors affecting the field infection of potato tubers of different cultivars by blight (*Phytophthora infestans*). Annals of Applied Biology 85:23–42.

Leeman M, Den Ouden FM, Van Pelt JA, Cornelissen C, Matamala-Garros A, Bakker PAHM, Schippers B. 1996. Suppression of Fusarium wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. European Journal of Plant Pathology 102:21–31.

Martiniez Noel GMA, Madrid EA, Bottini R, Lamattina L. 2001. Indole acetic acid attenuates disease severity in potato-*Phytopthora infestans* interaction and inhibits the pathogen growth *in vitro*. Plant Physiology and Biochemistry 39:815–823.

Pierson EA, Weller DM. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology 84:940–947.

Platt HW, Plissey ES, MacKay GR, Adams M, Hinkle MK, MacDonald E. 2000. Symposium: Sustaining potato crop, land and industry health beyond the year 2000: Current issues and future needs. American Journal of Potato Research 77:333–352.

Sato N. 1980. Sources of inoculum and sites of infection of potato tubers by *Phytophthora infestans* in soil. Annals of the Phytopathological Society of Japan 46:231–240.

Schisler DA, Slininger PJ. 1994. Selection and performance of bacterial strains for biologically controlling Fusarium dry rot of potatoes incited by *Gibberella pulicaris*. Plant Disease 78:251–255.

Schisler DA, Slininger PJ, Bothast RJ. 1997. Effects of antagonist cell concentration and two-strain mixtures on biological control of Fusarium dry rot of potatoes. Phytopathology 87:177–183.

Schisler DA, Bothast RJ, Slininger PJ. 1998. Bacterial control of Fusarium dry rot of potatoes (method of selection). US Patent 5, 783, 411.

Schisler DA, Slininger PJ, Hanson LE, Loria R. 2000a. Potato cultivar, pathogen isolate, and antagonist cultivation medium influence the efficacy and ranking of biological control strains of Fusarium dry rot. Biocontrol Science and Technology 10:267–279.

Schisler DA, Slininger PJ, Kleinkopf G, Bothast RJ, Ostrowski RC. 2000b. Biological control of Fusarium dry rot of potato tubers under commercial storage conditions. Amercian Journal of Potato Research 77:29–40.

Slininger PJ, Schisler DA, Bothast RJ. 1994. Two-dimensional liquid culture focusing: A method of selecting commercially promising microbial isolates with demonstrated biological control capability. In: Ryder MH, Stephens PM, Bowen GD editors. Improving plant productivity with rhizosphere bacteria. The 3rd international workshop on plant growth-promoting rhizobacteria, 7–11 March 1994, Adelaide, S. Australia. Glen Osmond, South Australia: CSIRO Division of Soils. pp 29–32.

Slininger PJ, Schisler DA, Bothast RJ. 1996. Bacteria for the control of Fusarium dry rot of potatoes (method of use and strains). US Patent 5, 552, 315.

Slininger PJ, Burkhead KD, Schisler DA, Bothast RJ. 2000. Biological control of sprouting in potatoes. US Patent 6, 107, 247.

Slininger PJ, Schisler DA, Kleinkopf G. 2001. Combinations of dry rot antagonistic bacteria enhance biological control consistency in stored potatoes. Phytopathology 91:S83.

Slininger PJ, Schisler DA, Burkhead KD, Bothast RJ. 2003a. Postharvest biological control of potato sprouting by Fusarium dry rot suppressive bacteria. Biocontrol Science and Technology 13:477–494.

Slininger PJ, Behle RW, Jackson MA, Schisler DA. 2003b. Discovery and development of biological agents to control crop pests. Neotropical Entomology 32:183–195.

Slininger PJ, Burkhead KD, Schisler DA. 2004a. Antifungal and sprout regulatory bioactivities of phenylacetic acid, indole-3-acetic acid, and tyrosol isolated from the potato dry rot suppressive bacterium *Enterobacter cloacae* S11:T:07. Journal of Industrial Microbiology and Biotechnology 31:517–524.

Slininger PJ, Schisler DA, Kleinkopf G. 2004b. Biological control of postharvest late blight of potatoes in storage. Phytopathology 94:S96.

Zhang S, Schisler DA, Boehm MJ, Slininger PJ. 2005. Carbon-to-nitrogen ratio and carbon loading of production media influence freeze-drying survival and biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9. Phytopathology 95:626–632.

Zhang S, Schisler DA, Jackson MA, Boehm MJ, Slininger PJ, Liu Z. 2006. Cold shock during liquid production increases storage shelf-life of *Cryptococcus nodaensis* OH 182.9 after air drying. Biocontrol Science and Technology 16:281–293.