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Evaluation of the effects of different liquid inoculant formulations on the survival and plant-growth-promoting efficiency of *Rhodopseudomonas palustris* strain PS3

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Abstract Biofertilizers can help improve soil quality, promote crop growth, and sustain soil health. The photosynthetic bacterium *Rhodopseudomonas palustris* strain PS3 (hereafter, PS3), which was isolated from Taiwanese paddy soil, can not only exert beneficial effects on plant growth but also enhance the efficiency of nutrient uptake from applied fertilizer. To produce this elite microbial isolate for practical use, product development and formulation are needed to permit the maintenance of the high quality of the inoculant during storage. The aim of this study was to select a suitable formulation that improves the survival and maintains the beneficial effects of the PS3 inoculant. Six additives (alginate, polyethylene glycol [PEG], polyvinylpyrrolidone-40 [PVP], glycerol, glucose, and horticultural oil) were used in liquid-based formulations, and their capacities for maintaining PS3 cell viability during storage in low, medium, and high temperature ranges were evaluated. Horticultural oil (0.5 %) was chosen as a potential additive because it could maintain a relatively high population and conferred greater microbial vitality under various storage conditions. Furthermore, the growth-promoting effects exerted on Chinese cabbage by the formulated inoculants were significantly greater than those of the unformulated treatments. The fresh and dry weights of the shoots were significantly increased, by 10–27 and 22–40 %, respectively. Horticultural oil is considered a safe, low-cost, and easy-to-process material, and this formulation would facilitate the practical use of strain PS3 in agriculture.

Keywords Plant growth-promoting rhizobacteria (PGPR) · Biofertilizer · Formulation · *Rhodopseudomonas palustris* · Shelf life

Sook-Kuan Lee and Huu-Sheng Lur contributed equally to the work.

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are the beneficial soil bacteria that colonize roots and enhance plant growth through various modes of action (Kloepper and Schroth 1978). PGPR can improve soil fertility, enhance plant nutrition availability and uptake, and support plant health (Adesemoye et al. 2009; Berg 2014; Lucy et al. 2004; Vessey 2003). Currently, the application of symbiotic or free-living PGPR, such as *Rhizobium* spp., *Azospirillum* spp., *Pseudomonas* spp., and *Azotobacter* spp. (Bashan et al. 2013; Bhattacharyya and Jha 2012; Dayamani and Brahmaprakash 2014), has become a significant component of sustainable agriculture practices in many countries (Bashan 1998; Das et al. 2013).

Some commercial PGPR inoculants are not usable under field conditions and do not perform with efficacy equivalent to

those used in greenhouse or laboratory experiments (Burges 1998; Stephens and Rask 2000). In most cases, this gap in performance is likely due to inadequate formulation and poor quality, including poor compatibility and stability of the carriers during storage prior to application and bacterial contamination (Bashan et al. 2013; Bhattacharyya and Jha 2012; Dayamani and Brahmaprakash 2014; Gomez et al. 1997). In general, ideal formulations meet the following criteria (Burges 1998; Herrmann and Lesueur 2013): (1) they can stabilize the microorganisms during production and distribution and extend the shelf life; (2) they protect the intended microorganisms against many environmental stresses at the target sites; and (3) they are cost-effective and easy to handle and use.

Various PGPR formulations have been developed using either liquids or solids as carrier materials. Liquid inoculants are microbial cultures or suspensions modified with water-, oil-, or polymer-based substances (i.e., additives) that may improve stickiness, stabilization, and surfactant and dispersal capacities (Catroux et al. 2001; Denardin and Freire 2000; Hynes et al. 1995; Singleton 2002). Solid formulations, meanwhile, can be made from inorganic or organic carriers, are prepared as granular and powdered forms and are classified based on their particle sizes or applications (Adholeya and Das 2012; Malusá et al. 2012). Because liquid-based formulations are usually more easily processed and much cheaper than the solid-based formulations, they constitute a significant percentage of the inoculant market (Kumaresan and Reetha 2011; Singleton 2002; Tittabutr et al. 2007). However, although liquid inoculants can be packaged and stored for long periods of time, the microorganisms tend to encounter abiotic stresses, such as nutrient depletion, temperature shock, and hypoxia, which result in dramatic declines in the population (Tittabutr et al. 2007). Improving inoculant formulations to maintain the quality of the liquid inoculant has become a major challenge.

Rhodopseudomonas palustris is one of the phototrophic purple non-sulfur bacteria (PNSB) (Imhoff 2006). This non-spore-forming bacterium is widely distributed in various aquatic and terrestrial systems (Gray and Smith 2005). *R. palustris* can synthesize ammonium and various polysaccharides and vitamins and is able to grow in a variety of natural environments due to its extraordinary metabolic versatility (Elbadry et al. 1999). *R. palustris* has been used extensively in industry for bioremediation and sewage treatment; furthermore, it can act as an agent for decomposing phytotoxins (e.g., hydrogen sulfide) in the paddy rhizosphere (Getha et al. 1998; Idi et al. 2014; Kornochalert et al. 2013; Siefert et al. 1978). In a previous study, we isolated *R. palustris* strain PS3 from Taiwanese paddy soil and found that it not only exerted beneficial effects on plant growth but also enhanced the efficiency of nutrient uptake from applied fertilizer in soil cultivation systems (Wong et al. 2014). In addition, this bacterium can be added to hydroponic cultivation systems for better yields with lower nitrate inputs (Hsu et al. 2015). These beneficial traits suggest that the PS3 isolate may serve as an ideal PGPR inoculant for agricultural applications.

To develop this microbial isolate for practical use, product development and formulation are needed. The aim of this study was to select suitable processing conditions and formulations to improve the survival and beneficial effects of the PS3 inoculant. We evaluated six additives for PS3 formulation development: the water-soluble additives glucose and alginate, the oil-based additives glycerol and horticultural oil, and the polymer-based substances polyethylene glycol (PEG), and polyvinylpyrrolidone-40 (PVP). We analyzed the survival of *R. palustris* PS3 in various formulations and at different storage temperatures for a set period of time and assessed the beneficial effects of these inoculants on plants grown in pots.

Materials and methods

Preparation of the inoculant

R. palustris strain PS3 (hereafter abbreviated as PS3), which was isolated from a research paddy at the National Taiwan University (Taipei, Taiwan), was used in this study (Wong et al. 2014). This strain was deposited at the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan; accession number BCRC910564) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany; accession number DSM 29314). It was grown in PNSB medium (KH_2PO_4 1.0 g/L, NH_4Cl 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L, $CaCl_2$ 0.02 g/L, $MnCl_2 \cdot 4H_2O$ 0.002 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.001 g/L, yeast extract 0.5 g/L, malate 10.0 g/L, pH adjusted to 7.5) at 37 °C (Hansen and van Gemerden 1972).

For large-scale production, 50 mL of PNSB broth was inoculated with the PS3 strain in 250-mL Erlenmeyer flasks and incubated for 22 h at 37 °C with shaking (200 rpm). To prevent contamination during dispensation, we deliberately transferred the whole amount of culture broth as a starter for the next inoculation step. Therefore, 50 mL of inoculum was subsequently transferred into fresh PNSB broth (300 mL) in 1000-mL Erlenmeyer flasks and was cultured for 22 h under the same conditions described above. Finally, 300 mL of the inoculum was transferred into 3 L of fresh PNSB broth to produce large-scale cultures until the cell density reached approximately $OD_{600} = 2.0$ (~2×10^9 colony-forming units [CFU]/mL).

Preparation of liquid-based formulations

Six additives, PVP (Sigma-Aldrich, St. Louis, MO, USA), PEG (Sigma-Aldrich, St. Louis, MO, USA), sodium alginate (Sigma-Aldrich, St. Louis, MO, USA), glucose (Amresco,

USA), glycerol (Biobasic, Canada), and horticultural oil (SK-Enspray N, Yu Tian Di Co., Ltd., Taiwan), were used in this study. We prepared a highly concentrated stock solution of each additive and blended the appropriate amount of the stock with PS3 inoculant to produce a liquid formulation with the desired concentration. For example, to prepare 0.5 % (*w*/*v*) horticultural-oil-based formulation, PS3 broth was adjusted to a suspension containing 1.0–1.2 × 10^9 CFU/mL. Then, 9.95 mL of that PS3 suspension and 0.05 mL of 100 % horticultural oil were mixed thoroughly and packed into an aluminum bag (7 cm × 12 cm). Accordingly, the bacterial population for each formulated inoculant is equivalent to ~1.0 × 10^9 CFU/mL (~9 log CFU/mL), and the additive (horticultural oil) accounts for 0.5 % of the liquid product. These formulations were stored at low (4 ± 2 °C), medium (25 ± 2 °C), and high temperatures (40 ± 3 °C) for evaluation of their shelf life by enumerating the viable cell numbers using plating methods. Each of these evaluations was performed in triplicate.

Evaluation of PS3 population dynamics in the different liquid-based formulations

The population dynamics of PS3 cells in each of the liquid-based formulations were evaluated at 30 days after storage (DAS). Initially, 1.0 mL of inoculant was sampled from each pack. The inoculant was then serially diluted by adding 100 μL of the suspension to 900 μL of diluent, and 20 μL of this suspension was spread onto the surface of a plate. Finally, the plate was allowed to dry on the bench for 15–20 min before being inverted and then incubated at 37 °C in darkness for 3 days. The number of CFUs per milliliter was calculated using the following formula described by Miles et al. (1938): average CFU per milliliters = average number of colonies for a dilution × 50 × dilution factor.

Utilization of carbon sources in additives by PS3

A modified API 50 CH test (Wong et al. 2014) was applied for determining the utilization of carbon sources from each of the additives by PS3. The original API 50 CH medium (BioMerieux, Marcy-l'Étoile, France) containing phenol red was replaced with a modified L2 medium (Suzuki et al. 2007) excluding the carbon source DL sodium acetate but including 7.6 mM $(NH_4)_2SO_4$ (nitrogen source) with 0.01 % (*w*/*v*) resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) dye (Sigma-Aldrich, St. Louis, MO, USA) in a 96-well microplate. Resazurin is used as an oxidation-reduction indicator in bacterial cell viability and aerobic respiration assays (Borra et al., 2009; GonzálezPinzón et al., 2012; Twigg, 1945). Because this indicator dye undergoes rapid photochemical decay, the following reaction was conducted under light-shielded conditions by covering the plate with aluminum foil. We put 1.5 mg of each additive and 150 μL of the above reaction solution containing a 1.0 % (*v*/*v*), i.e., 1.5 μl, suspension of PS3 cells (initial OD_{600} = 0.1, equivalent to 10^8 CFU/mL) in each well (96-well microplate). The utilization reactions were incubated at 37 °C for 48 h. Each test was repeated four times.

Quantitative estimation of polysaccharides in the liquid formulations

Quantities of exopolysaccharides (EPS) were determined using the phenol-sulfuric acid method (DuBois et al. 1956). In a preliminary test, crude EPS of PS3 was extracted by centrifugation, followed with or without cold ethanol precipitation treatment before determining the concentration. Because the regression statistics showed that the two methods (i.e., purified EPS vs. crude EPS) were in good agreement (r^2 = 0.98, data not shown), we applied the simplified protocol (i.e., without cold ethanol precipitation treatment) in further assays.

The EPS standards were calibrated using 5 different glucose standard solutions: 100, 200, 400, 800, and 1600 ppm. Bacterial cells were removed by centrifugation for 15 min at 14,000 rpm. The cell-free supernatants were collected, and 1.0 mL of each sample was added to 1.0 mL of 5.0 % phenol; 5.0 mL of 96 % sulfuric acid was then added to all of the tubes. All of the solutions were measured at a wavelength of 490 nm using a Victor3 multilabel plate reader (PerkinElmer, MA, USA). The concentration of EPS produced in each sample was determined using a graph of the 490-nm absorbance versus the EPS concentration (ppm) and the calibration standards.

Quantifying the major nutrients in the different liquid inoculants after 1 month of storage

The major nutrients (N, P, and K) in the formulated and unformulated inoculants after 30 days of storage at different temperatures were determined using the following methods. Total available nitrogen for each inoculant was determined using the standard Kjeldahl method (Bremner and Mulvaney 1982) and the phosphorus content was estimated using the protocol proposed by Murphy and Riley (1962). Potassium content was estimated via atomic absorption spectroscopy (AAS) (Hitachi ZA3000, Tokyo, Japan) (Knudsen et al. 1982).

Recovery activities of PS3 cells in liquid formulations after storage

A simple resazurin-based assay was performed to evaluate the effects of temperature on the speed of recovery of PS3 cells after storage. The contents in the reaction solution were slightly different from those in the modified API 50 CH test described above. This solution contained both carbon and nitrogen sources, including complete L2 medium (Suzuki et al. 2007) and 7.6 mM ammonium sulfate with 0.01 % (*w*/*v*) resazurin dye (Sigma-Aldrich, St. Louis, MO, USA). The formulated (PS3–

0.5 % horticultural oil (H.o.)) or unformulated PS3 inoculants (with an initial cell concentration of 1.0×10^9 CFU/mL) were stored at low, medium, or high temperatures for 3 days. The survival of PS3 in each sample was determined using the plating method after storage. We added 15 μL of each sample to 135 μL of the above reaction solution. The reactions were conducted under light-shielded conditions by covering the plate with aluminum foil and were incubated at 37 °C for 48 h. Cell vitality (i.e., metabolic activity) was confirmed through the changes in the color of resazurin. These color changes were from blue (oxidized, resazurin) to purple/pink (reduced, resorufin) and then to colorless (hydroresorufin). The optical density or absorbance at 600 nm was determined as described by Oliveira et al. (2010) using a microplate reader (PerkinElmer, MO, USA), and the numerical values were converted into 7 levels in response to the extent of color changes. For this assay, "0" represents no change (dark blue), and "1–6" represent positive reactions of increasing color intensities. All of the tests were repeated three times.

Root colonization assay

Chinese cabbage seeds (*Brassica rapa* var. *chinensis* "Maruba Santoh") were purchased from the Taiwan Agriculture Chemicals and Machinery Co., Ltd. (Taipei, Taiwan). For surface sterilization, seeds were immersed in 70 % alcohol for 2 min and then in 3 % hydrogen peroxide solution for 5 min, followed by thorough washing with sterile distilled water. These seeds were germinated on soft water-agar (0.6 %) plate and grown at 25 °C and 75 ± 5 % relative humidity in a light-emitting diode (LED) plant growth chamber with an alternating light (7000 Lux, 12 h) and dark (12 h) regimen. After 4 days of germination, the radicles (embryonic roots) were dipped in the inoculant samples described in the recovery assay section (1.0×10^9 CFU/mL). The seedlings were subsequently transferred to agar plates with half-strength MS medium (Murashige and Skoog 1962). After 3 days of incubation, the whole seedlings were carefully removed from the plates, rinsed several times with sterile distilled water to remove the loosely bound bacteria, and then placed in vials containing 5 mL phosphate-buffered saline (PBS). The whole seedlings were then subjected to sonication (Ultrasonic cleaner DC 400H) (40 KHz) for 10–20 min in a vial to remove the tightly bound bacteria, and the populations of the colonized PS3 cells were determined using the plating method as described by Miles and Misra (1938). The morphology of the colonized roots was observed by stereomicroscopy (VEM-100, Optima, Taipei, Taiwan).

Pot experiments for the evaluation of plant-growth-promoting effects

Pot experiments were conducted to evaluate the growth-promoting effects of unformulated and formulated inoculants after 1 month of storage at each temperature (4 ± 2, 25 ± 2, and 40 ± 3 °C for low, medium, and high temperatures, respectively). Chinese cabbage seeds were sown in Akadama soil-filled pots (approximately 300 g of soil per pot). Plants were grown at 25 °C and 75 ± 5 % relative humidity in a light-emitting diode (LED) plant growth chamber with an alternating light (7000 Lux, 12 h) and dark (12 h) regimen. The pot experiments were conducted in a randomized complete block design (RCBD). The amount of chemical fertilizer (Sinon Chemical Industry Co., Ltd., Taiwan, with an N:P:K ratio of 14:15:10) applied in this study was 0.05 g/pot (equivalent to $N:P_2O_5:K_2O$ = 44:48:31 [kg/ha]). Each week, 2 mL of the formulated or unformulated liquid culture that had been stored for 30 days at the indicated temperature was poured around the roots of the plant in each pot by micro-dispenser. Fresh PS3 culture adjusted to 10^8 CFU/mL was applied as a reference. Each treatment was performed at least 10 times. The plants were harvested 4 weeks after planting, and the growth parameters (fresh and dry weights) were determined.

Results

Assimilation of additives by the PS3 strain

To confirm whether the six additives (glucose, sodium alginate, glycerol, horticultural oil, PVP, and PEG) used in this study could be assimilated by *R. palustris* strain PS3, we conducted a modified API 50 CH assay (Wong et al. 2014). As shown in Fig. 1, the sample solutions turned light purple or pink after 48 h of incubation with resazurin. These results suggest that all of the additives used in this study can be assimilated by PS3. The utilization rate of PS3 for each of the additives was determined based on the extent of the color change. We found that the media containing horticultural oil or alginate produced colors of higher intensities (values of 4 and 3, respectively) than the other treatments (glycerol, glucose, PVP, and PEG, which produced values between 1 and 2) (Fig. 1).

Viability of the PS3 strain in different liquid-based formulations

To select potential additives that can increase the stability of PS3 liquid inoculant during storage, the survival rates of this bacterium in different additives at various concentrations and at different temperatures (low 4 ± 2 °C, medium 25 ± 2 °C, and high 40 ± 3 °C) were determined using the plate counting method. As shown in Table 1, the storage temperature was the most important factor in the maintenance of the quality and survival rate of the various inoculant formulations. PS3 cell numbers declined dramatically with increasing temperatures after one month of storage. The living bacteria concentrations were 2.7–5.3 log CFU/mL (equivalent to 10^3–10^5 CFU/mL)

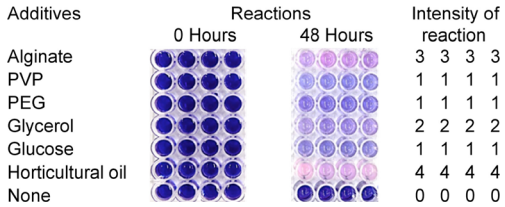

Fig. 1 Assimilation of additives by *R. palustris* strain PS3. Each sample was incubated at 37 °C for 48 h. Resazurin is reduced by bacterial respirations. Color changes from *dark blue* to *purple* or *pink* were taken as positive reactions. For numerical interpretation in all cases, "0" designated negative reactions, and "1–4" designated positive reactions with different intensity. "None" indicates that L2 medium without supplementation additive serves as a negative control because there is no carbon source in the solution

when stored at the high temperature (40 ± 3 °C), and they declined relatively slowly (final bacterial concentrations of 4.1–8.7 log CFU/mL, equivalent to 10^4–10^9 CFU/mL) when stored at the low temperature (4 ± 2 °C).

Under the low-temperature storage condition, the highest count of viable PS3 cells (~8.7 log CFU/mL) was measured in the 5.0 % horticultural oil (H.o) formulation (Table 1). The 0.5, 1.0, and 3.0 % horticultural oil, 3.0 % glucose, 3.0 % glycerol, and 5.0 % PVP formulations could also confer high population stability (> 8.0 log CFU/mL) during storage. When the inoculants were stored at the medium temperature, the inoculant formulations containing 1.0 % glucose, 0.5 % alginate, 2.0 % PVP, and 0.5 % or 1.0 % horticultural oil resulted in greater viability (5.9–6.5 log CFU/mL, Table 1). Although the inoculants were preserved at the higher temperature, the survival of PS3 cells was dramatically decreased in most of the liquid formulations (Table 1). The formulations containing 1.0 % glycerol, 0.5 and 2.0 % horticultural oil, 3.0 % glucose, and 0.5 % PEG appeared to protect the PS3 cells from high-temperature stress (viabilities of 4.7–5.3 log CFU/mL, Table 1).

Among the additives, we observed that only 0.5 % horticultural oil could support higher levels of viable PS3 cells (marked with italic letters in Table 1) than the control treatments at multiple storage temperatures. Accordingly, we chose 0.5 % horticultural oil as the candidate additive for further experiments in this study. We also compared the growth curves of PS3 with and without 0.5 % horticultural oil in the formulation and found that these cultures grew at almost the same rate (Fig. 2), indicating that this additive does not affect the normal growth of PS3 cells.

Exopolysaccharides synthesized in PS3 cells after storage

When liquid inoculants are packaged and stored, bacteria may be subjected to abiotic stresses, such as nutrient depletion, temperature shock, and hypoxia (Tittabutr et al. 2007). EPS synthesized by bacteria are thought to be adaptations to environmental stresses (Lloret et al. 1998). We determined the amounts of EPS produced by PS3 cells in the unformulated and formulated (with 0.5 % horticultural oil) inoculants that had been stored at different temperatures for 30 days. In the unformulated inoculants, PS3 cells produced more EPS in the low- and high-temperature storage conditions than in the medium temperature (Fig. 3). However, all of the formulated PS3 (PS3–0.5 % H.o.) inoculants exhibited higher EPS production than the unformulated inoculants at any of the storage temperatures.

Recovery activities and root-colonization abilities of PS3 cells after storage at different temperatures

We conducted a resazurin-based assay to evaluate the recovery activities of PS3 cells in the formulated (PS3–0.5 % H.o.) and unformulated inoculants after storage at different temperatures. The population of PS3 in each sample after 3 days of storage was almost the same as that before storage (approximately ~10^9 CFU/mL, data not shown). However, all of the formulated PS3 inoculants exhibited higher metabolic activities than the unformulated inoculants at any temperature (Fig. 4 and Fig. S1 in the Supplementary Material). The PS3 cells derived from PS3 + 0.5 % H.o. (M) recovered the fastest, followed by the cells derived from PS3 + 0.5 % H.o. (L).

The ability of inoculated bacteria to colonize the rhizosphere is generally considered an essential step in the application of PGPR for beneficial purposes. To directly evaluate the root-colonization abilities of the PS3 cells derived from the above samples, the radicles of Chinese cabbage were dipped in respective inoculant solution (10^9 CFU/mL) and transplanted onto 1/2 strength MS agar plates. As shown in Fig. 5, we observed the formation of light red colonies of PS3 cells on the root surface of Chinese cabbage seedlings, suggesting that PS3 cells can interact directly with host plants in the rhizosphere. We also determined the populations of the colonized PS3 cells using the plating method. The total viable cell numbers of PS3 were approximately 10^7–10^8 CFU (Fig. 5), and there was no significant difference among the treatments.

Plant growth-promoting effects of formulated and unformulated PS3 inoculants after storage

We determined the availability of N-P-K nutrients in the formulated (PS3–0.5% H.o.) and unformulated inoculants that had been stored for 30 days at different temperatures. As shown in Table 2, there were no significant differences between the nutrient compositions after the different treatments. We further evaluated their effectiveness at promoting plant growth by conducting pot experiments with Chinese cabbage. We had already confirmed that horticultural oil did not influence the growth of Chinese cabbage (Fig. S2 in the

Table 1 Effects of additives on populations of *R. palustris* PS3 strain stored at different temperatures

Additives	Concentrations (% *w*/*v*)[a]	Survival rate of PS3 strain log(CFU/mL)[b]					
		4 ± 2 °C		25 ± 2 °C		40 ± 3 °C	
Control (w/o additives)		7.50 ± 0.03	de	5.02 ± 0.05	mn	4.60 ± 0.00	cdefg
Alginate	0.5	6.32 ± 0.03	i	*6.24 ± 0.01*	*b*	3.21 ± 0.05	p
	1.0	5.22 ± 0.01	l	*5.59 ± 0.02*	*f*	4.23 ± 0.06	jkl
	1.5	4.11 ± 0.04	m	*5.30 ± 0.01*	*ghi*	4.31 ± 0.00	hijkl
	2.0	6.38 ± 0.00	j	4.64 ± 0.03	p	3.64 ± 0.01	o
	2.5	6.47 ± 0.01	ij	4.94 ± 0.01	no	2.68 ± 0.05	q
Glucose	0.1	*7.84 ± 0.02*	*c*	*5.16 ± 0.04*	*jkl*	4.62 ± 0.00	cdefg
	0.5	7.42 ± 0.04	def	*5.34 ± 0.02*	*igh*	4.28 ± 0.00	ijkl
	1.0	5.46 ± 0.02	k	*6.47 ± 0.02*	*a*	3.84 ± 0.01	no
	3.0	*8.21 ± 0.04*	*b*	5.09 ± 0.03	lm	4.77 ± 0.03	bc
	5.0	*7.84 ± 0.01*	*c*	5.09 ± 0.02	lm	4.35 ± 0.00	ghijkl
Glycerol	0.5	7.14 ± 0.07	g	4.92 ± 0.01	no	4.63 ± 0.03	cdefg
	1.0	7.60 ± 0.03	d	5.12 ± 0.03	klm	*5.30 ± 0.00*	*a*
	2.0	7.42 ± 0.01	def	*5.40 ± 0.02*	*gh*	4.13 ± 0.00	lmn
	3.0	*8.10 ± 0.01*	*b*	*5.63 ± 0.01*	*fe*	4.51 ± 0.05	cdefghij
	5.0	7.34 ± 0.05	efg	4.94 ± 0.03	no	4.73 ± 0.02	bcd
Horticultural oil	0.5	*8.00 ± 0.02*	*b*	*5.94 ± 0.01*	*c*	*4.82 ± 0.01*	*b*
	1.0	*8.12 ± 0.00*	*b*	*6.00 ± 0.03*	*c*	4.66 ± 0.01	bcdef
	2.0	*7.88 ± 0.01*	*c*	*5.83 ± 0.02*	*d*	4.53 ± 0.08	bcde
	3.0	*8.29 ± 0.06*	*b*	*5.64 ± 0.02*	*fe*	4.60 ± 0.01	cdefghi
	5.0	*8.68 ± 0.07*	*a*	*5.23 ± 0.01*	*ijk*	4.37 ± 0.03	cdefg
PEG	0.1	7.14 ± 0.04	g	*5.34 ± 0.06*	*ghi*	4.70 ± 0.01	fghijkl
	0.5	7.23 ± 0.03	fg	*5.26 ± 0.01*	*hij*	4.70 ± 0.04	bcde
	1.0	6.72 ± 0.03	h	*5.16 ± 0.02*	*jkl*	4.45 ± 0.02	efghijk
	5.0	7.23 ± 0.01	fg	*5.30 ± 0.01*	*ghi*	4.41 ± 0.01	efghijk
	10.0	7.23 ± 0.01	fg	*5.11 ± 0.00*	klm	4.15 ± 0.02	lm
PVP	1.0	*7.83 ± 0.08*	*c*	*5.44 ± 0.01*	*g*	4.46 ± 0.02	efghijk
	2.0	6.64 ± 0.02	hi	*6.15 ± 0.02*	*b*	4.30 ± 0.01	hijkl
	3.0	7.35 ± 0.02	ef	*5.72 ± 0.03*	*de*	4.29 ± 0.24	ijkl
	5.0	*8.09 ± 0.02*	*b*	4.88 ± 0.02	o	3.88 ± 0.01	ijkl
	10.0	5.58 ± 0.06	k	4.72 ± 0.01	p	4.18 ± 0.00	kl

Different letters in a column indicate significant differences at the 5 % level among the treatments at the different temperatures, as determined by Tukey's HSD (Honest Significant Differences) test. Treatments that performed better than the control treatment are italicized

[a] The concentrations of additives (% *w*/*v*)

[b] The values are the means of three replications

Supplementary Material). As shown in Fig. 6, with the application of either the 0.5 % H.o.-formulated or the unformulated PS3 inoculants that were stored under high temperature for 30 days, the yields (fresh and dry shoot weights) were lower than the yields of the inoculants stored under low or medium temperatures. Regardless of the storage temperature, all of the 0.5 % H.o.-formulated PS3 inoculants produced better yields than the unformulated inoculants; the average increases in fresh and dry shoot yields were 10–27 and 22–40 %, respectively.

Discussion

The successful development of a PGPR formulation is mainly dependent on the use of suitable materials and application methods (Bashan et al. 2013; Tittabutr et al. 2007; Trivedi and Pandey 2008). In this study, we chose to test horticultural oil as a potential additive for liquid-based formulations of *R. palustris* PS3. Horticultural oil is commonly used as a safe and effective insecticide for crop protection (Fernandez et al. 2006; Helmy et al. 2012; Opande

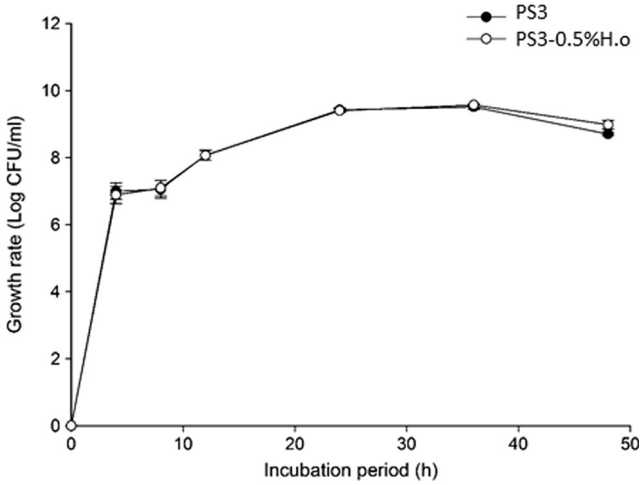

Fig. 2 Growth curves of *R. palustris* strain PS3 with or without addition of 0.5 % horticultural oil. In the PS3–0.5 % H.o. inoculant, PS3 cells were cultivated at 37 °C in purple non-sulfur bacteria (PNSB) broth, which was supplemented with horticultural oil to a final concentration of 0.5 % (*w/v*). Data are presented as the means from four experiments, with *bars* showing standard error (SE)

et al. 2013; Shaw et al. 2000; Stansly and Connor 2005). To our knowledge, this case is the first in which horticultural oil has been used in PGPR formulation development. According to our results, use of this additive could support greater viability of PS3 cells than formulations without horticultural oil for storage at low, medium or high temperatures (Table 1). This oil can be catabolized by PS3

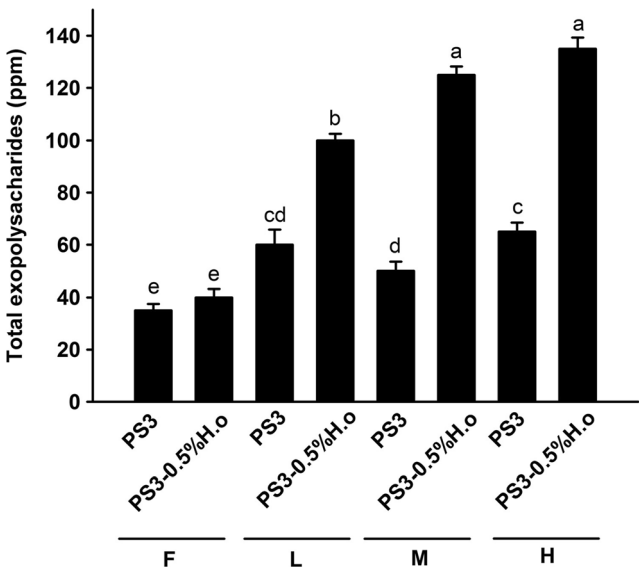

Fig. 3 Exopolysaccharides synthesized by PS3 after storage. The different storage temperatures are labeled as L (low temperature, 4 ± 2 °C), M (medium temperature, 25 ± 2 °C), and H (high temperature, 40 ± 3 °C). The data are presented as the mean values ± SE from 4 biological replicates, and *different letters* represent significantly different mean values ($P < 0.05$, Tukey's HSD test). "F" indicates fresh PS3 sample without storage. PS3: *R. palustris* strain PS3 inoculant; PS3–0.5 % H.o.: PS3 inoculant supplemented with 0.5 % horticultural oil

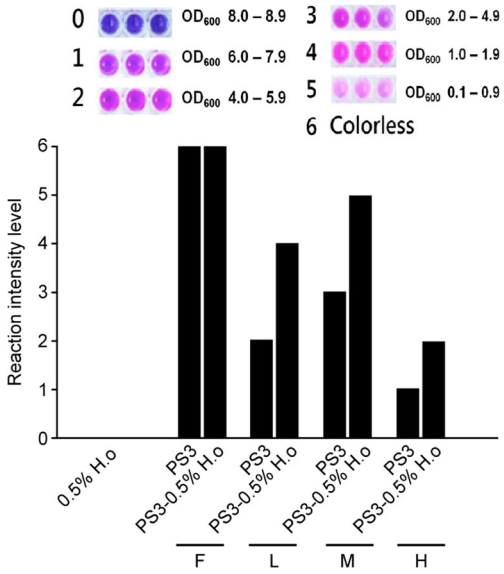

Fig. 4 Recovery activities of PS3 inoculants after 3 days of storage. Each sample was stored at the indicated temperature range for 3 days and then incubated with resazurin-containing reagent at 37 °C for 48 h. Resazurin is reduced by bacterial respirations, and the optical density or absorbance at 600 nm was determined. The numerical values were shown in Fig. S1 in the Supplementary Material, and converted into 7 levels in response to the extent of color changes. Each *bar* represents the extent of resazurin color change, from *blue* (oxidized, resazurin) to *purple/pink* (reduced, resorufin), and then to *colorless* (hydroresorufin). As shown in the upper illustration, "0" designates negative reactions, and "1–6" designate positive reactions with different intensities. "0.5 % H.o" indicates the use of L2 medium with 0.5 % horticultural oil as a negative control, to confirm that the resazurin test is not influenced by horticultural oil supplementation. "F" indicates the use of a fresh PS3 sample without storage. "L", "M", and "H" indicate the storage temperatures low (4 ± 2 °C), medium (25 ± 2° C), and high (40 ± 3 °C), respectively. This experiment was repeated three times

(Fig. 1), suggesting that it may act as an additional nutrient source for sustaining bacterial growth during storage. Moreover, when the horticultural oil-formulated PS3 inoculant was applied to the rhizosphere, the growth of Chinese cabbage plants was enhanced (Fig. 6). When liquid inoculants are packaged and stored for a period of time, bacteria may be subjected to abiotic stresses, such as nutrient depletion, temperature shock, and hypoxia (Tittabutr et al. 2007). As shown in Table 1, PS3 cell numbers declined dramatically after 30 days of storage. Such reduced cell viability under unfavorable conditions may result from the accumulation of microbial wastes or toxins (Bazilah et al. 2011; Šantek and Marić 1995). When in harsh environments, microorganisms will activate stress responses and produce EPS to protect themselves from damage (Hartel and Alexandar 1986; Öztürk and Aslim 2010). Accordingly, microbial EPS production has been considered an indicator for survival. We observed that the formulated PS3 (PS3–0.5 % H.o.) inoculants produced higher levels of EPS than those without formulation at any storage temperature (Fig. 3), and there were more viable cells in

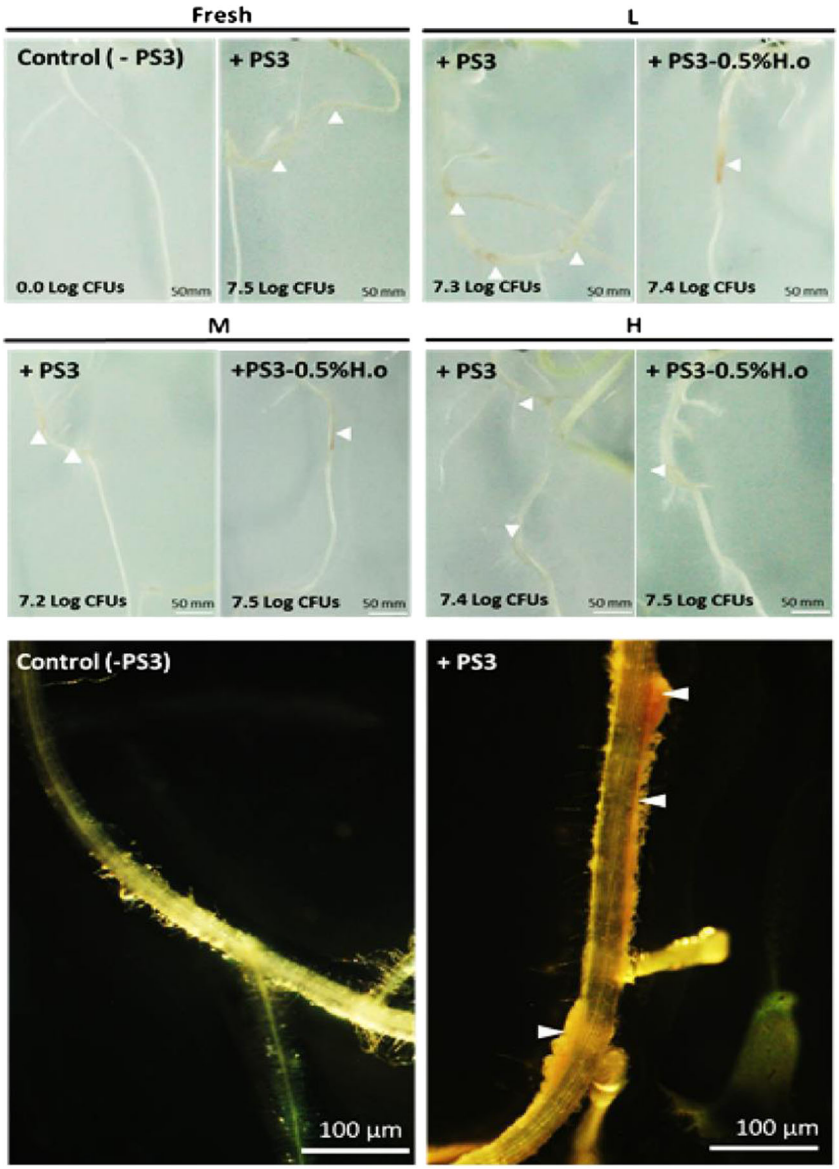

Fig. 5 Survival and root colonization of *R. palustris* PS3 on the root surface of Chinese cabbage seedlings. The Chinese cabbage seedlings grew on 1/2 strength MS agar (0.6 %), and the light red colonies of the PS3 cells formed along the emerging radicles (indicated by triangles). The value shown in each image corresponds to the average population of the colonized PS3 cells. The bottommost panels are close-up images of the untreated control (*left*) and PS3 inoculated (*right*) root surfaces. "Fresh" indicates the use of fresh PS3 sample without storage. Different storage temperatures are labeled as *L* (low temperature, 4 ± 2 °C), M (medium temperature, 25 ± 2 °C), and H (high temperature, 40 ± 3 °C). PS3: *R. palustris* strain PS3; 0.5 % H.o: supplementation with 0.5 % horticultural oil

Table 2 Major nutrients (N-P-K) for plant growth in the formulated and unformulated inoculants after 30 days of storage at different temperatures

Treatment	*N* Percentage (%)	P_2O_5	K_2O
PS3 (L)	0.08	0.02	0.06
PS3–0.5 % H.o. (L)	0.07	0.01	0.04
PS3 (M)	0.07	0.02	0.09
PS3–0.5 % H.o. (M)	0.07	0.01	0.07
PS3 (H)	0.07	0.01	0.07
PS3–0.5 % H.o. (H)	0.07	0.02	0.09

This experiment was repeated 3 times. *N* available nitrogen, P_2O_5 available diphosphorus pentoxide, K_2O potassium oxide

the former than in the latter (Table 1). This observation suggests that the EPS synthesized by PS3 cells can alleviate stress during storage. EPS synthesis is highly affected by the availability of carbon substrates (Sutherland 2001). The horticultural oil used in this study is a highly refined mineral oil mainly composed of alkanes, cycloalkanes, and aromatic hydrocarbons. This additive was well assimilated by PS3 (Fig. 1) and did not affect bacterial growth (Fig. 2). Accordingly, we deduced that in addition to the stress responses (i.e., nutrient deficiency, temperature shock, and reduced oxygen content, etc), the elevated EPS productivity by PS3 may be due to the presence of excess available carbon substrates in horticultural oil.

In general, the titer value of PGPR is determined by measuring the number of viable cells using the plate

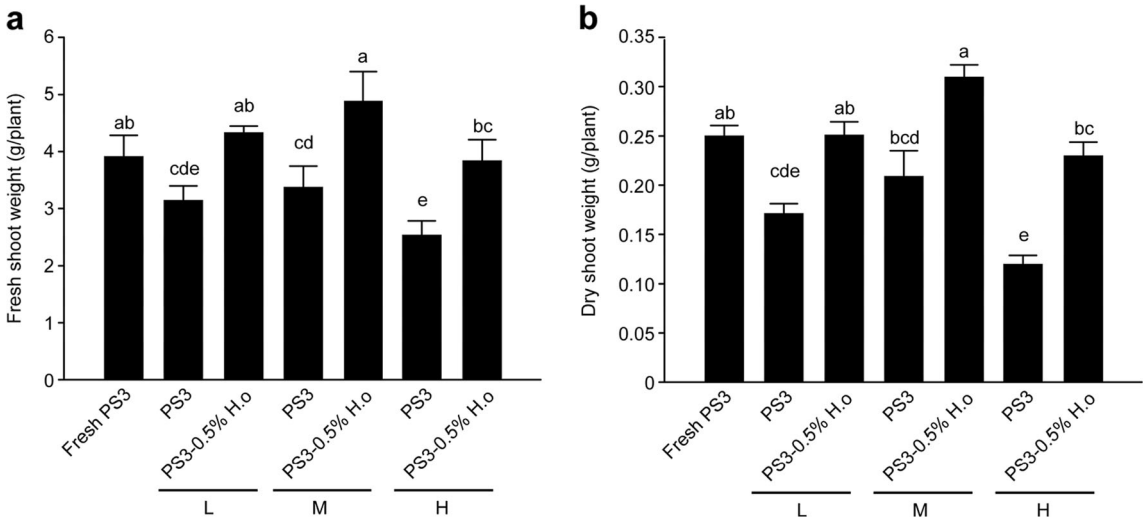

Fig. 6 Plant-growth-promoting efficiency of the PS3 inoculants that were stored at different testing temperatures after 30 days in storage. Biomass of Chinese cabbage was determined after 30 days of cultivation. **a** Fresh weights of shoots. **b** Dry weights of shoots. Bars represent mean values ± SE from 10 biological replicates, and different letters represent significantly different mean values ($P < 0.05$, Tukey's HSD test). Different storage temperatures are labeled as *L* (low temperature, 4 ± 2 °C), M (medium temperature, 25 ± 2 °C), and H (high temperature, 40 ± 3 °C). PS3: supplementation with *R. palustris* strain PS3; 0.5 % H.o: supplementation with 0.5% horticultural oil

counting method (CFU) or the most probable number (MPN). This value is considered to be an index of inoculant quality in relation to plant growth-promoting effectiveness (Pindi and Satyanarayana 2012), and several studies have demonstrated that the decline of populations over time in inoculants results in a lower inoculation efficiency (Biederbeck and Geissler 1993; Trivedi and Pandey 2008). On the other hand, many studies have indicated that even high titer values in the inoculants after storage do not always result in a demonstrable increase in crop yield under field conditions (Vessey 2003). In this study, we noticed that although the titer value of the PS3 + 0.5 % H.o. (M) (log 5.9 CFU/mL) treatment was much lower than that of the fresh PS3 inoculant treatment (log 8.0 CFU/mL) (Table 1), their beneficial effects on crop yields were nearly identical (Fig. 6). This result suggests that formulation with horticultural oil can mitigate the negative effects of population decline of PS3 cells to some extent. In a preliminary test, we confirmed that horticultural oil did not influence the growth of Chinese cabbage (Fig. S2 in the Supplementary Material). Consequently, we deduced that the distinct benefit of enhanced plant growth promotion is largely due to the action of the PS3 cells. Because the titer value reflects the viability (i.e., culturability) but not the vitality (i.e., metabolic activity) of bacterial cells (Kell 2000), we further assessed the vitality of PS3 cells in their respective inoculants after storage. We found that although the survival and root-colonization ability of PS3 cells in each inoculant sample was almost identical (Fig. 5), the formulated PS3 (PS3–0.5 % H.o.) inoculants exhibited higher recovery activities from stress than the unformulated PS3 cells at any temperature treatment (Fig. 4). Accordingly, we assumed that the 0.5 % H.o.-formulated PS3 cells were able to interact more vigorously with host plants than the unformulated PS3 cells in the rhizosphere. Taken together, to address the gaps between the expected and actual performance of bacterial inoculants, we propose that not only the viability but also the vitality of bacteria should be regarded as quality control indices.

We have shown that formulation with horticultural oil can provide PS3 cells with either protection or robustness during storage for up to 30 days at any of the temperatures examined. In general, the expiration dates of commercial inoculants are set at least 6 months after their production (Herridge 2008). For practical application in agriculture, we should extend the shelf life of the PS3 + 0.5 % H.o.-formulated inoculant to retain its biological traits intact. Because our selected additive is a safe, low-cost material and the production of the inoculant is easy, we expect that this formulation technology will facilitate the integration of the PS3 inoculant into the agricultural distribution system.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animals participants.

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