國立臺灣大學生物資源暨農學院農藝學系

碩士論文

Graduate Institute of Agronomy
College of Bioresources and Agriculture
National Taiwan University

Master Thesis

光合菌潛力菌株-*Rhodopseudomonas palustris* NTUIOB-PS3 之劑型配方研究

Studies of formulations for the elite phototrophic bacterium, *Rhodopseudomonas palustris* strain NTUIOB-PS3

李淑君

Sook-Kuan Lee

指導教授：盧虎生 博士、劉啟德 博士

Advisor: Huu-Sheng Lur, Ph.D., Chi-Te Liu, Ph.D

中華民國 104 年 5 月

May, 2015

中文摘要

微生物肥料具有改善土壤理化性質，幫助植物吸收養分以及維持健康的特性，可以減少農化產品的施用，被視為一種對環境友善的施肥方式，可兼顧到農作豐產與自然生態平衡。為了將潛力菌株開發成為具商品價值的微生物製劑，如何藉由適當的製劑配方使得產品在儲架期間能保有最高的活菌數遂成為重要課題。*Rhodopseudomonas palustris* NTUIOB-PS3 為分離自台灣水田土壤的光合菌株，它不僅對作物具顯著的生長促進功效，還可提昇植物代謝化學肥料的效率。本研究的目的是要尋找出適合 PS3 菌株的液態/固態劑型配方與儲存條件。 PS3 光合菌分別與六種液態劑型添加物（褐藻酸鈉、聚乙二醇、聚乙烯吡咯烷酮、甘油、葡萄糖、礦物油）以及五種固態劑型載體（泥炭土、蛭石、稻殼、甘蔗渣、菜仔粕）進行加工，並儲存於 4℃、25℃、37℃ 條件下一個月，經由平板菌落計數估算殘存活菌數。實驗結果發現礦物油是最適合做為液態劑型添加劑，泥炭土則是固態劑型的最適載體。經評估兩種劑型之生產加工程序、設備成本、產品儲架壽命、雜菌控制難易度以及對施用環境的影響等要素，液態劑型配方對於 PS3 菌株而言應是較為適合的加工方式。添加 0.5% 的礦物油至 PS3 發酵液即可顯著提昇菌株在儲架期間的殘存活菌數。 在小白菜盆栽試驗中發現經由該方式加工的製劑在不同溫度下儲存一個月後依然能有效促進作物生長， 與未加工的對照組相較其鮮乾重分別增加了 35-70% 與 50-90% 不等程度。由於礦物油本身屬於安全農業資材，而且成本低廉，加工簡易，該製劑技術將有利於 PS3光合菌的商品化與實用性。

關鍵字:促進植物生長根圈微生物、生物性肥料、製劑化、沼澤紅假單胞菌 、儲架壽命

Abstract

Biofertilizers can help to improve soil quality, promote growth of crops, and sustain soil health. Therefore, such approach to farming is regarded as environmental friendly, and can be used to reduce excessive amount of chemical fertilizer application, and ensures a sustainable crop production. To put the embryonic form of elite microbial isolate from bench to practical use, product development and formulation is needed to maintain high quality of the inoculant during storage. A photosynthetic bacterium, *Rhodopseudomonas palustris* strain NTUIOB-PS3 was isolated from Taiwanese paddy soil, which could not only exert beneficial effects on plant growth, but also enhance the efficiency for up taking applied fertilizer nutrients. The aim of this study was to select suitable processing condition and formulation in improving the quality of PS3 inoculant. Six additives (alginate, PEG, PVP, glycerol, glucose, mineral oil) and five carriers (peat, vermiculite, rice husk, sugarcane bagasse, rapeseed meal) were used for liquid and solid-based formulations, respectively. The capacity of these materials for maintaining PS3 cell viability during storage at 4℃, 25℃ and 37℃ was evaluated. On the basis of the survival rate of PS3 cells, mineral oil and peat were chosen to be candidate materials for liquid and solid based formulations, respectively. In consideration of manufacturing process, cost, shelf life, contamination issue, and environmental concerns, etc., liquid-based formulation seems more appropriate for manufacturing PS3 inoculant. Accordingly, mineral oil (0.5%) was chosen as the potential additive candidate for liquid-based formulation according to the survival rate of PS3 cells during storage. In addition, the growth-promoting effects of Chinese cabbage exerted by this formulated inoculant under one-month storage at 4℃, 25℃ and 37℃ were more significant than those of the non-formulated treatments. Shoot fresh and dry weights were significantly increased at 35-70% and 50-90%,

respectively. Due to mineral oil is considered as a safe, low cost, and easy-to-process agricultural material, this formulation technology will facilitate the commercialization and practical use of PS3 inoculant.

Key words: Plant growth promoting rhizobacteria (PGPR), biofertilizer, formulation, *Rhodopseudomonas palustris*, shelf life

誌謝

這份論文的完成，或許不會對世界帶來偉大的影響，但它卻成了我人生中回憶滿滿的旅程。雖然旅程中會遇上困難而感到沮喪與寂寞，其實卻有許多隱藏人物用了屬於他們的方式不斷默默支持與協助我，好讓我能順利走到今天的最後一步。首先非常感激謝盧虎生及劉啟德老師在我碩士生涯裡扮演著重要的角色－人生導師，在學生論文上花上無數的精力與時間，讓學生能夠從無到有的順利完成論文。而且除了論文的指導外，老師平常對我人格上的循循善誘，使我對自己性格上的缺點以及如何去克服有更深刻的認識，老師們不但是我的授業老師，更是成為我內心重要的人生導師。 在論文的修訂及口試上，感謝陳仁治老師、劉嘉睿老師以及謝建元老師提供了寶貴且專業的意見，使得本論文的內容更加完備充實，而對我而言也啟發了許多收穫。

初此之外，我還要感謝實驗室的所有成員，所有曾經幫助過我的學長學弟學姊學妹還有同學們，特別是凱軍學長，不厭其煩在實驗上給予正確的指導與操作，把我訓練成在陌生實驗中也能勇敢的去面對；給我許多研究方面建議的筱涵學姊、婉真姊、書華姊、筑甯姊、曉琳姊、景漢哥、嘉宏哥；常常陪我聊天熬夜做實驗的學弟妹們，球球、孟薇、心玫及爾傑；口試準備隨時給予協助的任祥、泓如、鈺元與澄慧；為我打氣的朋友士弘、仁旭、薪喨以及毓璇；伴我渡過這兩年的研究所生涯。同時也特別感謝在各個方面都盡力給予協助的長輩們－黃文達老師、秀鳳學姊、雅琴姊、崔博士以及志維學長。

從大學到現在，還是要非常感謝與我遠距離的家人與麻吉鍾瑩學妹，感謝您們每份每秒對我的支持與鼓勵，在最困難的時候總是願意當我的忠實聆聽者與默默守在我身旁。

最後，因為有您們，我的研究生活才顯得不乏味，成就今後的我，能夠繼續抱著夢想與熱血勇敢的走進新里程碑，誠實面對自己。謝謝您們。

Contents

Chapter 1
Introduction

Plant growth promoting rhizobacteria (PGPR) was first defined by Kloepper and Schroth (1978) referring to the beneficial soil bacteria that colonize roots and enhance plant growth with various modes of action. PGPR can improve soil fertility, enhance plant nutrition availability and uptake, and support the health of plants (Adesemoye et al., 2009; Berg and Berg, 2009; Lucy et al., 2004; Vessey, 2003) (Figure 1-1). Nowadays, application of the symbiotic or free-living PGPR, such as *Rhizobium* spp., *Azospirillum* spp., *Pseudomonas* spp., *Azotobacter* spp., etc (Bashan et al., 2014; Bhattacharyya and Jha, 2012; Dayamani and Brahmaprakash, 2014) has become a significant component of modern or sustainable agriculture practice in many countries (Bashan, 1998; Das et al., 2013).

Rhodopseudomonas palustris is one of the phototrophic purple non-sulfur bacteria (Imhoff, 2006). This bacterium is widely distributed in various aquatic or terrestrial systems (Gray and Smith, 2005). *R. palustris* can synthesize various ammonium, polysaccharides and vitamins, and is able to grow under different natural environments due to its extraordinary metabolic versatility (Elbadry et al., 1999). *R. palustris* has been extensively used in industry for bioremediation and sewage treatment, furthermore, it is able to act as a biodegradation agent for decomposing the phytotoxin (e.g hydrogen sulfide H_2S) in paddy rhizosphere. In a previous study, we isolated a *R. palustris* strain from Taiwanese paddy soil, which was designated as NTUIOB-PS3 (abbreviated as PS3) (Figure1-2). PS3 could not only exert beneficial effects on plant growth, but also enhance the efficiency of uptake of the applied

fertilizer nutrients in soil cultivation system (Wong et al., 2014) (Figure 1-3). These beneficial traits suggest that the PS3 isolate may serve as a potential PGPR inoculant for agriculture.

Prior to commercialization of potential PGPR inoculant, many hurdles should be crossed over. For example, scale up and production of the microorganism under industrial fermentation conditions, formulation development, quality control, storage and transport processing, etc (Herrmann and Lesueur, 2013). However, some commercial products are inapplicable under field conditions and do not perform equivalent efficacy compared to those under greenhouse or laboratory experiments (Burges, 1998; Stephens and Rask, 2000). In most cases, this gap likely due to inadequate formulations and a poor level of quality, such as poor compatibility and stability of carriers during storage prior to application and appearances of bacterial contaminations (Bashan et al., 2014; Bhattacharyya and Jha, 2012; Dayamani and Brahmaprakash, 2014; Gomez et al., 1997; Hungria et al., 2005; Olsen et al., 1996; Olsen et al., 1995).

To maximize the chances of inoculation success, the formulation of inoculant is highly needed. The aim of formulation is to ensure maximum viability of the microorganisms in the inoculant. The criteria for ideal formulations are as follows (Burges, 1998; Herrmann and Lesueur, 2013): (1) can stabilize the intended microorganisms during production, distribution and extend the shelf-life; (2) protect the intended microorganisms against many environmental stresses at the target sites; and (3) cost-effective and easy to handle and use.

Different PGPR formulations have been developed either use liquid or solid carrier materials (GIFAP, 1989). Liquid inoculant means microbial cultures or suspensions amended with substances that may improve stickiness, stabilization, and surfactant

and dispersal abilities (Singleton, 2002) such as aqueous-, oil-, or polymer-based products (Catroux et al., 2001; Denardin and Freire, 2000; Hynes et al., 1995). Commonly, alginate, glycerol, glucose, PVP, PEG, and various types of oil have been used as additives for microbial inoculant in helping bacteria survive during processing or storage (Dayamani and Brahmaprakash, 2014; Hoben et al., 1991; Rivera, 2014; Singleton, 2002; Tittabutr et al., 2007). On the other hand, solid formulation can be made from inorganic or organic carriers, prepared it into granular and powder form, and are classified on the particle size or application (Adholeya and Das, 2012; Malusá et al., 2012). Among the carriers, peat is considered the most widely used one for solid-based microbial inoculants (Date, 1972; Fernandes Júnior et al., 2009; Sparrow and Ham, 1983). The other carriers, such as lignite, charcoal, coir dust, composts, sugarcane filter mud bagasse, organic amendments, and vermiculite, have also been applied extensively (Albareda et al., 2008; Bashan et al., 2014; Crawford and Berryhill, 1983; Hungria et al., 2005).

In this study, we evaluated a variety of liquid and solid carrier materials for PS3 formulation development. We analyzed the survival of *R. palustris* PS3 in different formulations under various processing conditions and storage temperatures for a period of time, followed by assessment of the beneficial efficacy of these inoculants for plant growth in pot experiments. The schematic diagram of this study is shown in Figure 1-4.

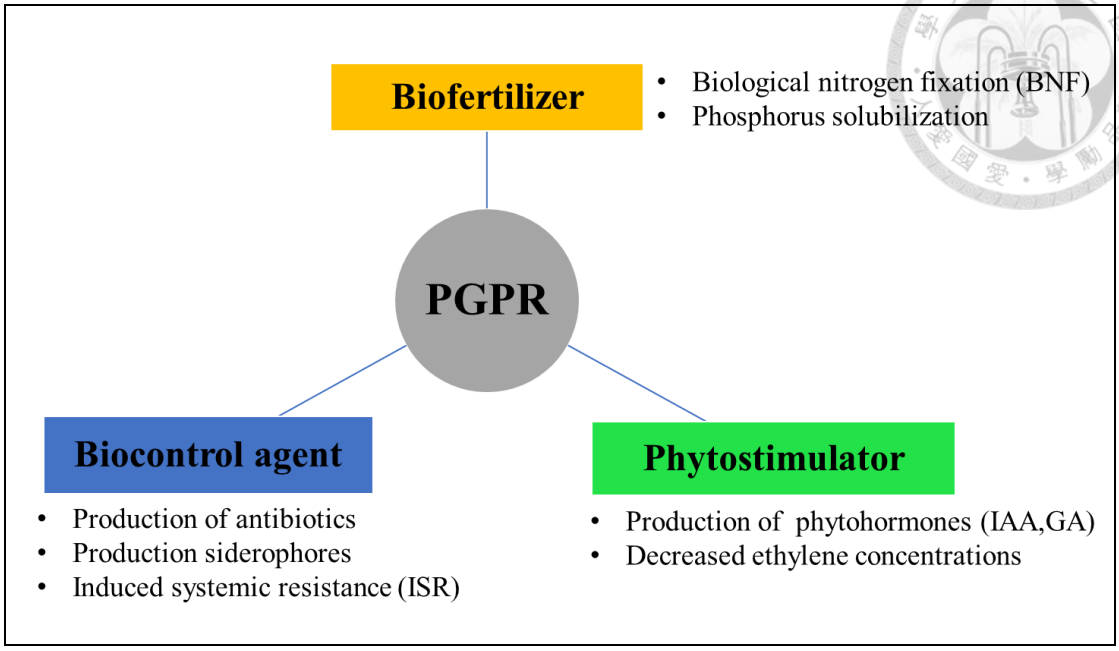


Figure 1-1. Beneficial effects of PGPR (plant growth promoting rhizobacteria) for plant growth and health. PGPR are known to exert the plant growth promotion efficacy through the following modes of action: (1) Biofertilization, PGPR could act as biofertilizer to provide nutrients directly to plants through biological nitrogen fixation (BNF) or solubilisation of phosphorus; (2) Phytostimulation, PGPR could act as phytostimulator by producing phytohormones (such as IAA, GA and so on); (3) Biocontrol, PGPR could act as biocontrol agents against plant pathogens by producing antibiotics, siderophores or triggering induced systemic resistances (ISR).

(This figure was adapted from Martínez Viveros et al. (2010).

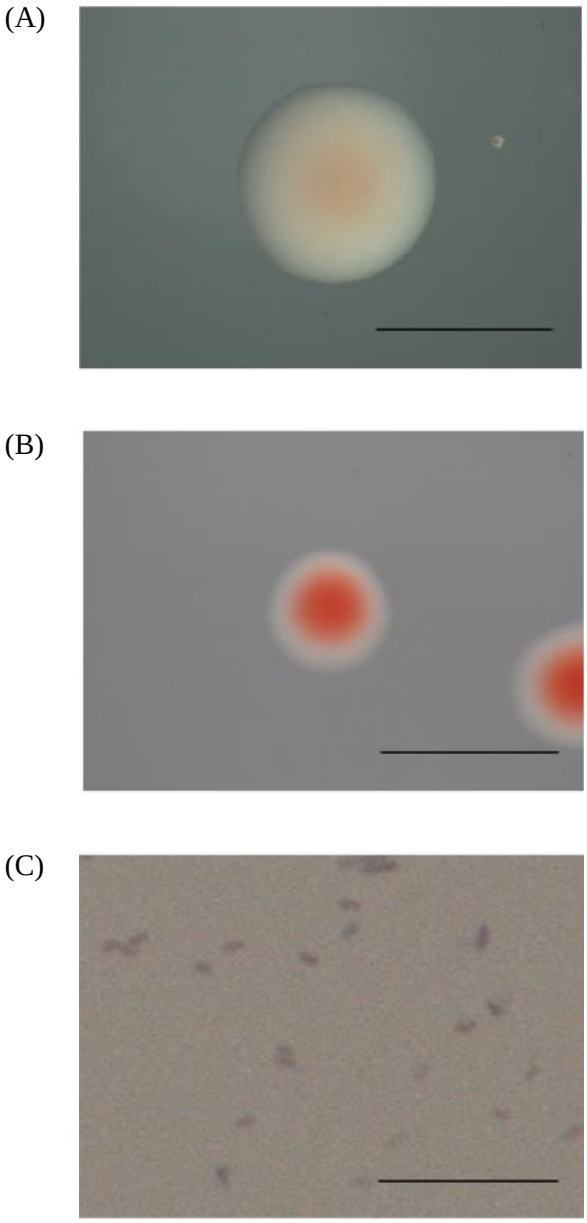


Figure 1-2. Morphological characteristic of *R. palustris* strain PS3. (A) The colonies developed under aerobic growth were pearl white, round, convex, smooth, and shiny, with entire edges. (B) The colonies turned blood red grown anaerobically under illumination (C) Vegetative cells had motile, rod-shaped cells approximately 1.0 μm in length. Scale bars equal 0.5 cm in panels A and B, 10μm in panel C. (Adapted from Wong et al., 2014).

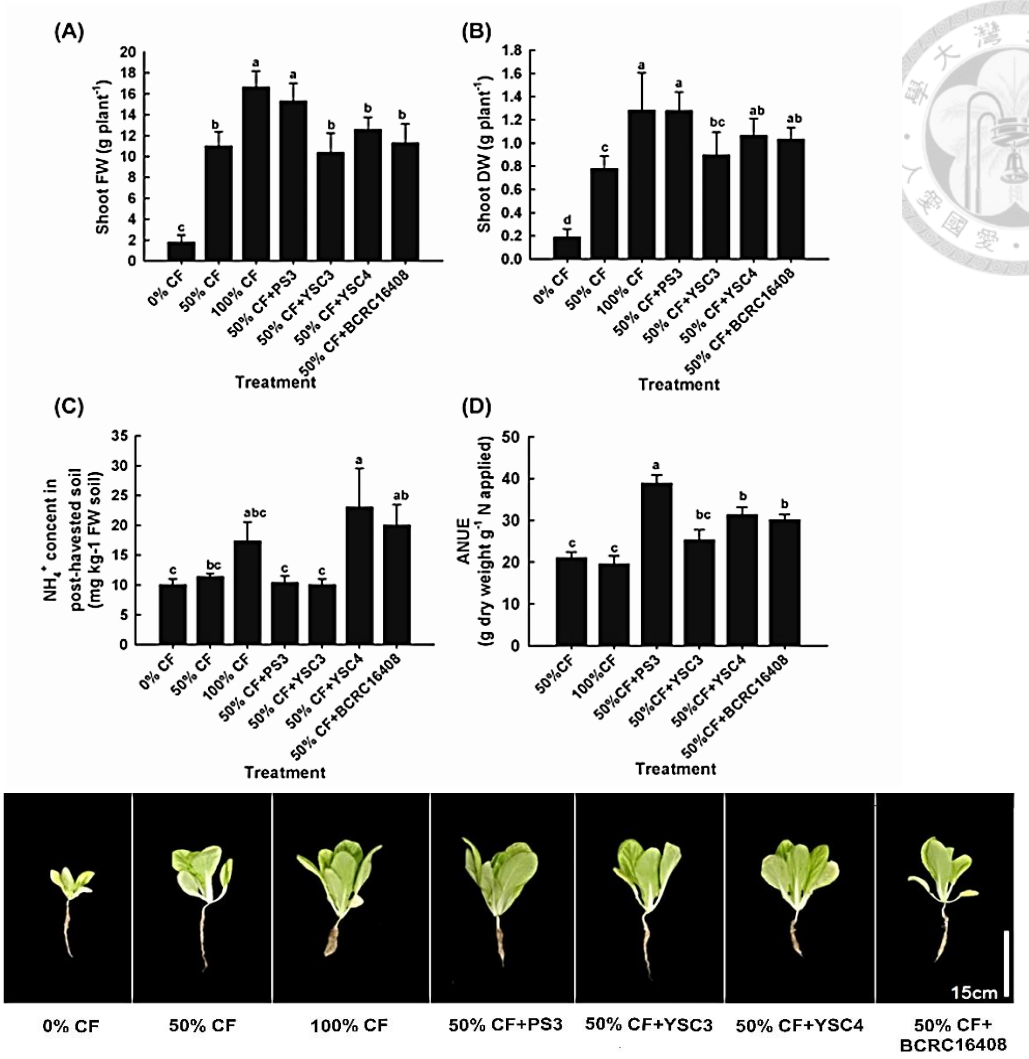


Figure 1-3. Plant growth-promoting effects of bacterial treatments on Chinese cabbage (Adapted from Wong et al., 2014). (A) (B) fresh and dry weights of shoots (C) Agronomic nitrogen use efficiency (ANUE). (D) Appearance of plants under different treatments. The data represent means ± SE, locations marked with the different letters (a to d) are statistically significant ($p < 0.05$). CF: chemical fertilizer, 50% CF+ respective *R. palustris* inoculant, inoculation of the bacterial strain (PS3, YSC3, YSC4, and the type strain BCRC16408, respectively) with half rate of fertilizer. This result is to explain that authors search for promising PGPR inoculant from the *R. palustris* candidates by applied an integrated dosage of fertilizer and results showed that only the PS3 strain had a marked impact on growth in comparison with others (Figure 3A and 3B). Otherwise, the research noted that plant growth response to 50% CF+P3 treatment was significantly greater than 50%CF although the amount

of remaining soil N was at the same level (Figure 3C). Moreover, the agronomic nitrogen use efficiency (ANUE) of the applied fertilizer nutrients was significantly enhanced following the PS3 treatment (Fig. 3D). As reason of that, previous research suggests that increased the plant growth response after inoculated with PS3 strain by enhanced the utilizations of N fertilizer efficiency.

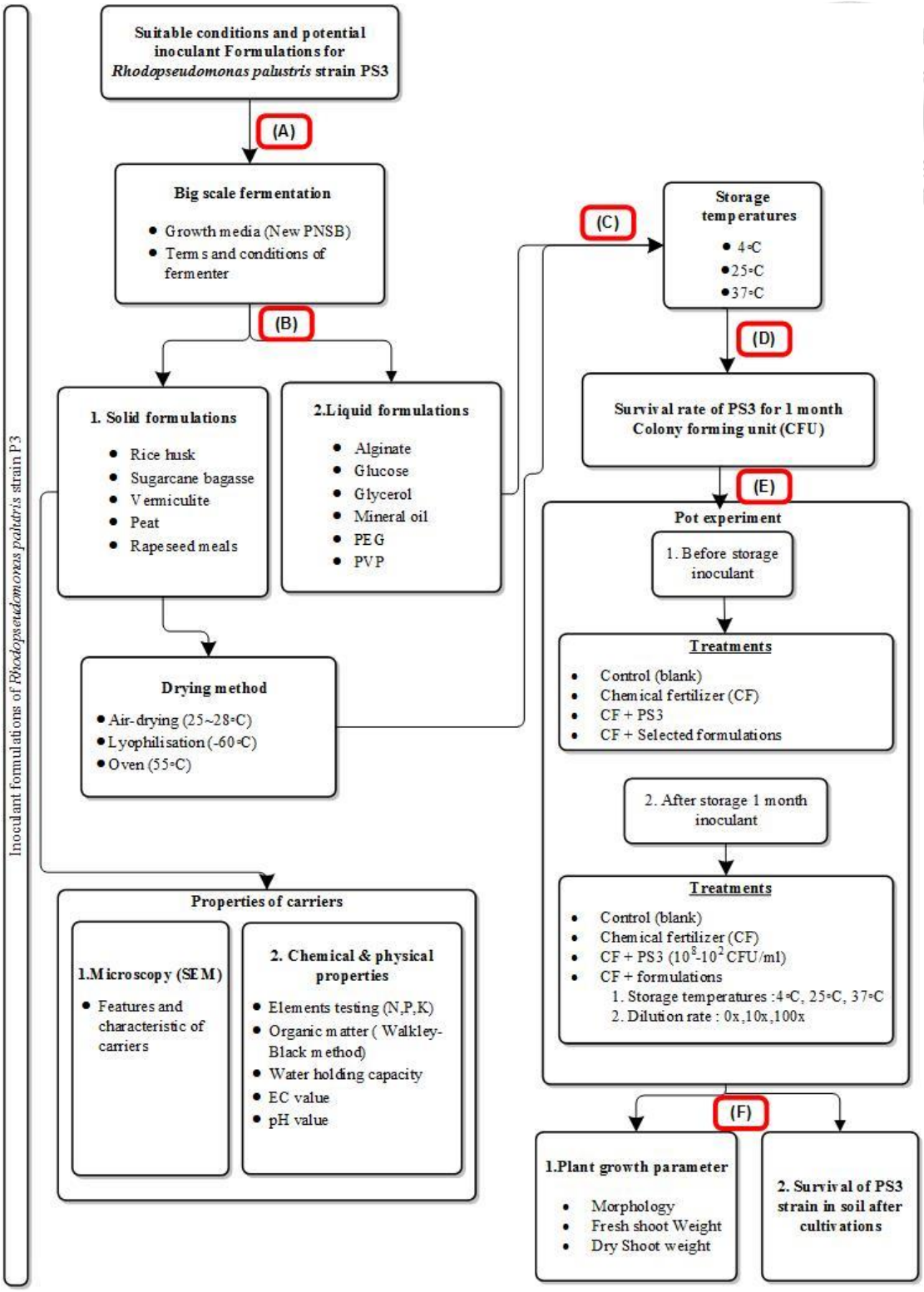


Figure 1-4 Schematic diagram of this study. The aim of this study is to determine the potential formulation for *R. palustris* PS3 strain. Experiments for the study were described as follows: (A) Mass production of PS3 (B) Liquid and solid-based formulations (C) Storage

tests (D) Estimation of survival rate of PS3 (E) Pot experiment (F) Evaluation of plant growth promoting and population of PS3 in rhizosphere soil after 4 weeks of cultivation.

Chapter 2
Materials and methods

2.1 Preparation of inoculant

Rhodopseudomonas palustris strain NTUIOB-PS3 (hereinafter abbreviated as PS3) which was isolated from research paddy field in National Taiwan University (Taipei city, 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 German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany; accession number DSM 29314). It was grown in the purple non-sulfur bacteria (PNSB) medium (KH_2PO_4 1.0 g/L, NH_4Cl 1.0 g/L, $MgSO_4 \cdot 7H_20$ 0.2 g/L, $FeSO_4 \cdot 7H_20$ 0.01 g/L, $CaCl_2$ 0.02 g/L, $MnCl_2 \cdot 4H_2O$ 0.002 g/L, $Na_2MoO_4 \cdot 2H_20$ 0.001 g/L, Yeast extract 0.5 g/L, Malate 10.0 g/L, adjusted pH value to 7.5) at 37℃ (Hansen et al., 1972).

For mass production, PS3 strain was inoculated into 250 ml Erlenmeyer flasks with 50 ml of PNSB broth for 22 hours at 37℃ with shaking (200 rpm). Afterwards, transferred 50 ml of inoculum into fresh PNSB broth (300ml) in 1000 ml Erlenmeyer flasks, cultured for 22 hours at the same condition mentioned above. Finally, feeding 300 ml of above inoculum into the 3 L fresh PNSB broths to carry out the big scale fermentations with suitable growth conditions described previously (Lo et al., published data).

2.2 Liquid formulations

2.2.1 Preparation of liquid-based formulations

Six additives, including polyvinylpyrrolidone-40 (PVP) (Sigma-Aldrich, St Louis, MO, USA), polyethylene glycol (PEG) (Sigma-Aldrich, St Louis, MO, USA), sodium

alginate (Sigma-Aldrich, St Louis, MO, USA), glucose (Amresco, USA), glycerol (Biobasic, Canada), and mineral oil (Yu Tian Di Co.,Ltd, Taiwan) were used in this study (Details refer to Table 2-1 and 2-2). After autoclaving (121℃) for 50 mins, blended the above fermentation broth (1.0-4.5 × 10^9 CFU/ml) with respective sterile additive in various concentrations (Details shown in Table 2-4) as liquid formulations, and 10 ml of each inoculant were then packed and sealed separately into aluminium bags (7 cm × 12 cm). These formulations were stored at different temperatures (4℃, 37℃ and 25℃) to evaluate their shelf life by enumerating the viable cell number by plating methods. Triple replications were conducted.

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

The population dynamics of PS3 cells in respective liquid-based formulation were determined at 2, 4, 6, 12, 18, 24, and 30 days of storage (DAS). Initially, 1.0 ml of inoculant was sampled from each pack. Then, serially diluted by adding 100 µl of the suspension to 900 µl of diluent respectively and dropped 20 µl solutions onto surface of plate. Finally dry it on bench for 15-20 minutes before inversing the plate, and incubated at 37℃ in darkness for 3 days. Calculating the colony forming unit (CFU) per millilitre by using the following formula described by Miles and Misra (1938): average CFU per millilitres = average number of colonies for a dilution × 50 × dilution factor.

2.2.3 Utilization of carbon sources in additives by PS3

The modified API 50CH test was used in this experiment (Wong et al. 2014). The original API 50CH medium (BioMerieux, France) containing phenol red was replaced with an adjusted L2 medium (Suzuki et al., 2007) excluding D-L sodium acetate,

adding 7.6 mM $(NH_4)_2SO_4$ with 0.01% (w/v) resazurin dye (Sigma-Aldrich, St Loius, MO, USA) and 0.1% suspension cell (Optical Density 0.1). When resazurin is reduced to resorfurin by bacterial respiration, a blue to pink color change can be observed. Resofurin is further reduced to hydroresorufin (colourless). Accordingly, resazurin has been used as surrogate indicator of bacterial growth (Sarker et al., 2007). One and half milligram of each additive and 150 µl of above reaction solution were added into each well of 96-well microplate. The utilization reactions were incubated for 24 hours or 48 hours. All the tests had four replicates.

2.3 Solid-based formulations

2.3.1 Characteristics and properties of carriers

Five types of carriers including rapeseeds meal (Grace Fertilizer Co.,Ltd Taichung, Taiwan), rice husk (National Farmers' association Tainan, Taiwan), sugarcane bagasse, peat (Kekkila Co., Ltd Finland), and vermiculite (Ding Lan Co., Ltd Taipei, Taiwan equipment shop) were used in this study for solid-based formulation processing (Table 2-3 and 2-5). pH and electrical conductivity (EC) were determined by pH meter (Eutech pH510, USA) and EC meter (Hanna instrument EC 215, USA) according to the methods described by Mclean (1982) and Janzen (1993) respectively. Total available nitrogen for each carrier was determined by a regular Kjeldahl method and the content of organic matter was analyzed by Walkey-black method (Nelson and Sommers, 1982). Phosphorus was estimated referring to Murphy and Riley (1962) protocols. Potassium contents were estimated by Atomic Absorption Spectroscopy (AAS) (Hitachi ZA3000, Japan) (Knudsen et al., 1982). Calcium, magnesium and other trace elements (Cu、Zn、Ni、Cr、Cd and Pb) were evaluated by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP) (Kontron S-35, Denmark).

2.3.2 Scanning electron microscopy

Surface morphology and characteristics of carriers were determined by scanning electron microscopy as described by Frank's laboratory SEM protocol (2007) http://www4.ncsu.edu/~rgfranks/research/protocols.html. The above five carriers were ground into powder and fixed with 2.5% glutaraldehyde for 24 hours. Then, poured away and added 1% osmium tetroxide solution into each sample. After that, dehydration in a series of ethanol with increasing concentrations (30%-100%), each for 30 min, and stored in refrigerator with 100% alcohol overnight. Samples were dried in freeze drier for 3 days before examination by scanning electron microscope. (Nova Nano SEM 230, Switzerland, Europe).

2.3.3 Preparation of solid-based formulations

All carriers were finely ground by a SRT-08 pulveriser (S. Shin Co., LTD, Taiwan) and autoclaved (121℃) for 50 mins. After sterilized, respective sterile carrier (20 g) was mixed well with the above fermentation broth to obtain a final moisture at 80% (Table 2-4). The initial population of PS3 in each solid formulation was equivalent to 1.0×10^9 CFU/g carrier (the calculation refer to Table S1).These solid formulations were dried with the following methods: oven-dried (55℃)(Drying oven D 0-6, Deng Yng Co., LTD, Taiwan), air-dried (22 - 25℃, in a fume hood) and lyophilisation (-60℃, 12.8 pa) (Drum type freeze dryer FD6-12P-D, King Mech Co., LTD, Taiwan), respectively. Then they were packed and sealed into aluminium bags (7 cm × 12 cm), and stored at various temperatures (4℃, 25℃ and 37℃, respectively).

2.3.4 Evaluation of viable bacterial cell number in different solid-based formulations

Survival of *R. palustris* strain PS3 with different solid carriers and processing conditions were evaluated at 0, 2, 4, 6, 12, 18, 24, 30 days of storage (DAS). Initially, 1.0 g of respective solid formulation was sampled from each pack and diluted with 9.0 ml of sterile distilled water. Each dilution was shaken vigorously for 20mins by vortex mixer (ELMI Intelli-Mixer RM-2). Then, serially diluted by adding 100 µl of suspension to 900 µl of D. D.W for serial dilution diluent, and dropping 20 µl solutions onto surface of agar in petri dish. Finally, plates were air-dried on the bench for 15 - 20 mins before inversion and incubated at 37℃ in darkness for 3 days. Survival rate of bacterial or colony forming unit (CFU) per gram was calculated by the following formula which described by Miles and Misra (1938): average CFU per gram = average number of colonies for a dilution x 50 x dilution factor. Triple replications were tested.

2.4 Pot experiments

Pot experiments grown with Chinese cabbage were conducted to evaluate (1) effects of fresh inoculants on plant growth (eliminating negative response), and (2) effects of formulated inoculant after storing for one month under various temperatures (4℃, 25℃ and 37℃, respectively). The seeds of Chinese cabbage (*Brassica rapa* var. *chinensis* "Maruba Santoh") were purchased from Taiwan agriculture chemicals and machinery Co., Ltd (Taipei, Taiwan), and sown in Akadama soil-filled pots (containing approximately 300 g of soil per pot) and grown in an incubator (FIRSTEK GC101, Taiwan) with fluorescent light (5000 – 6000x), at 24℃ day/night and 70 (±5) % relative humidity. The pot experiments were conducted in a randomized complete block design (RCBD) with the following treatments: (a) CK, no chemical fertilizer (CF) or inoculant; (b) CF, stands for chemical fertilizer application.

The chemical fertilizer (Sinon Chemical Industry Co., Ltd, Taiwan) used with a N: P : K ratio of 14 : 15 : 10, and 0.05 g of it was applied into each pot (equivalent to N: P_2O_5: K_2O = 44: 48: 31 [kg/ha], comparable to half rate of recommendation fertilizer); (c) CF + respective PS3 formulation, inoculation of each *R. palustris* PS3 formulation with chemical fertilizer; Each treatment had at least 10 replicates. The plants were fertilized once a week, except the CK group. In the first part, 2 ml of the fresh liquid PS3 formulation (10^8 CFU/ml) was applied weekly on the surface soil of each pot (i.e the total amount of the inoculant for 4 weeks was 8.0×10^8 CFU/ml, equivalent to 3.0×10^6 CFU/g soil). In the second part, 2 ml of the liquid formulations for one month of storage was applied weekly on the surface soil of each pot. The plants were harvested after planting for 4 weeks, and the growth parameters, such as fresh and dry weight of shoot, were recorded.

Table 2-1. Sources and cost for various formulations.

Additives for liquid based formulation	Cost (NT)/100g(ml)	Sources/ Company
PVP	2480.00	Sigma Co., Ltd, USA
PEG	814.00	Sigma Co., Ltd, USA
Alginate	1356.00	Sigma Co., Ltd, Norway
Glucose	98.00	Amresco Co., Ltd, USA
Glycerol	144.00	Biobasic Co., Ltd, Canada
Mineral oil	80.00*	Yu Tian Di Co., Ltd, Taiwan
Rice husk	2.80	Xin Gan Kun Co. ,Ltd, Taiwan
Peat	1.90	Kekkila Co., Ltd, Finland
Vermiculite	5.70	Ding Lan Co., Ltd, Taiwan
Sugarcane bagasse	0.00*	Taipei fresh market, Taiwan
Rapeseed meal	1.80	Grace fertilizer Co. Ltd

Asterisks (*) means cheapest material compared with others.

Table 2-2. Special characteristic and references for liquid-based formulations.

Additives f	Characteristics	References
PVP	Large molecular weight (Av. Mol. Wt. 40000), water soluble compound with stabilization and adhesive properties, high water binding capacity, high phenolic compound binding capacity, useful in reducing toxic substances	(Deaker, 2004; Singleton, 2002; Vincent et al., 1962)
PEG	Small molecular weight (Av. Mol. Wt. 3000) water soluble compound with adhesive properties	(McAneney et al., 1982; Temprano et al., 2002)
Alginate	Large molecular weight, nontoxic compound with adhesive property, limit heat transfer, high water activity, useful in supporting long term survival of inoculant	(Bashan, 1998; Bashan et al., 1999; Jung et al., 1982)
Glucose	Protects cell by enhances the storage product glycogen and exopolysaccharide production, no toxic effect on cell survival	Singleton (2002)
Glycerol	Carbon source, high water binding capacity, protect cells from desiccation	Singleton (2002)
Mineral oil	Prevent from desiccation, heat stress and water evaporation, bio-control agents	(Fernandez et al., 2006; Helmy, 2012; Hoben et al., 1991)

Table 2-3. Special characteristic and references for solid-based formulations.

Carriers	Characteristics	References
Rice husk	Agricultural wastes, environmental friendly, readily available, low cost, nutrient for plant (High silica content), high water holding capacity	(Hafeez et al., 1989; Kaljeet, 2011; Varela Milla et al., 2013)
Peat	Common and commercial materials, finely powdered inexpensive, high water holding capacity, high organic matter, nutritive environment for microbial, biodegradable, strong buffering capacity, easy to apply	(Bashan, 1998; Gupta et al., 2013; Herrmann and Lesueur, 2013)
Vermiculite	Inorganic material, greater bulk density, provide elements and mineral nutrients for microbes, protect microbes by creating an envelope, inexpensive and readily available.	(Bashan, 1998; Graham Weiss et al., 1987; Müller and Défago, 2006)
Sugarcane bagasse	By products of sugar industry, high organic matter and nutrient value, non-toxic, localized sources	(Anyango, 1984; Balume, 2013; Mohamed and Abdel-Moniem, 2010)
Rapeseed meal	Organic fertilizer, provide high nutrient for plant. 2014 experiment result showed that plant growth was increased when apply together with fresh PS3 strain (refer to Figure S5-S6).	(Zhang et al., 2014; Zheng et al., 2009)

Table 2-4. Different additives and their concentrations for liquid formulations of *R. palustris* strain PS3.

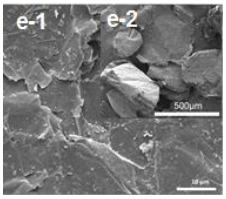
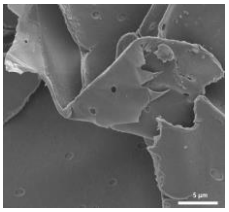
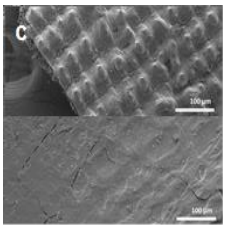
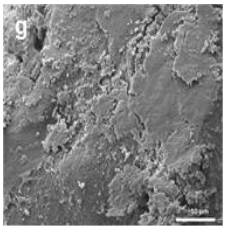
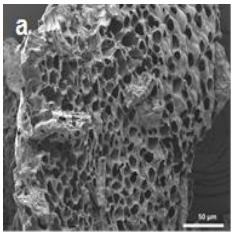
Additives	Concentrations (%w/v)[a]				
PVP	1.0	2.0	3.0	5.0	10.0
PEG	0.1	0.5	1.0	5.0	10.0
Sodium alginate	0.5	1.0	1.5	2.0	2.5
Glucose	0.1	0.5	1.0	3.0	5.0
Glycerol	0.5	1.0	2.0	3.0	5.0
Mineral oil	0.5	1.0	2.0	3.0	5.0

‘ [a]’ Each additive was mixed with the bacterial suspension in the ratio shown.

Table 2-5. Volume of PS3 fermentation broth contained in various carriers.

Carriers	Volumes of broth added (ml)[a]	CFU/ml	CFU/g carrier
Vermiculite	38±2	5.29×10^{8}	1.0×10^{9}
Sugarcane bagasse	68±2	2.91×10^{8}	1.0×10^{9}
Rice husk	44±2	4.52×10^{8}	1.0×10^{9}
Rapeseed meals	30±2	4.27×10^{8}	1.0×10^{9}
Peat	53±2	3.76×10^{8}	1.0×10^{9}

[a] indicates each carrier was prepared about 20g with 80% (w/w) water content.

Carriers for solid based formulation	Morphology	SEM image [a]
Vermiculite		
Sugarcane bagasse		
Rice husk		
Rapeseed meal		
Peat		

'a' surface and texture of carriers observed under scanning electron microscope (SEM).

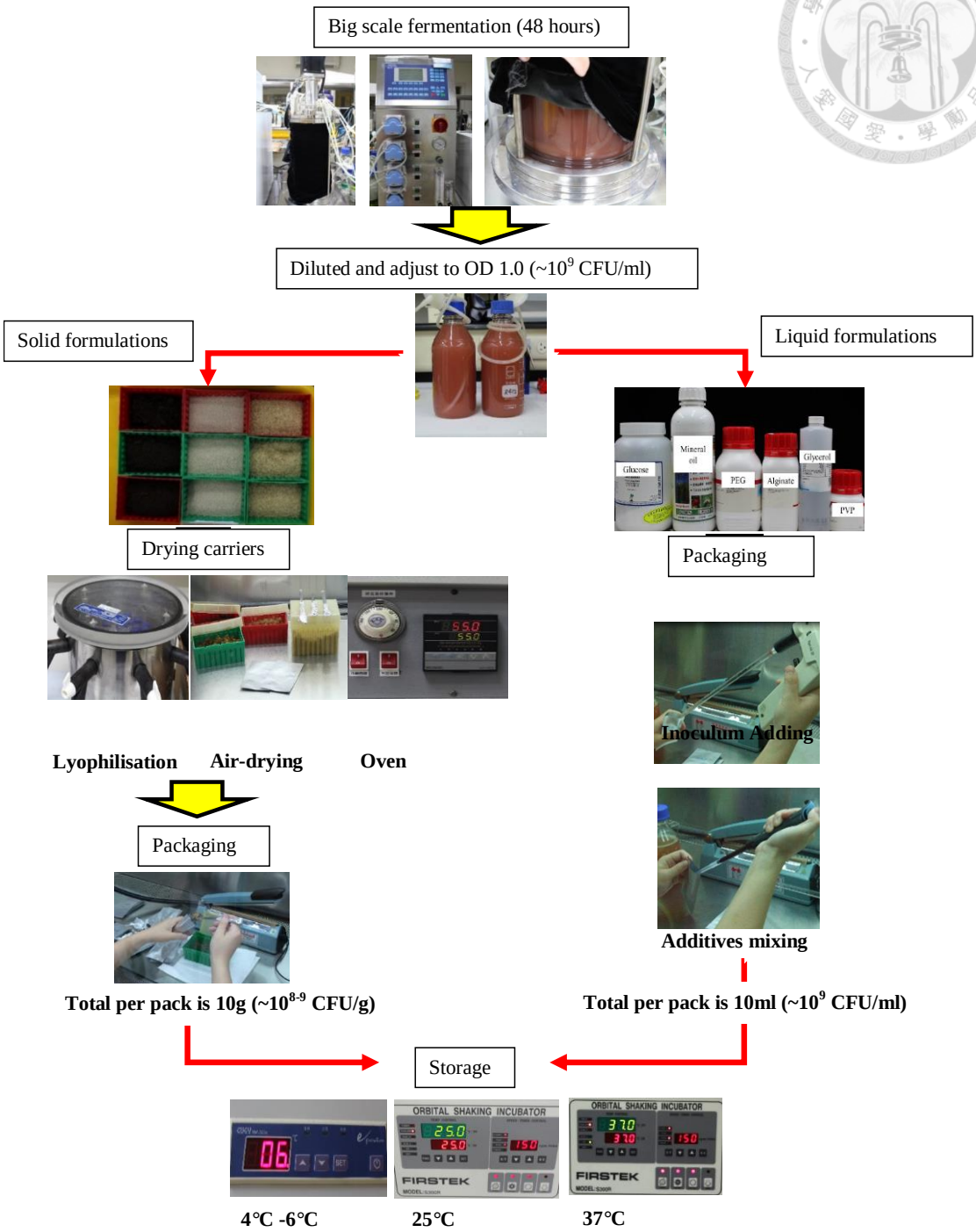


Figure 2-1 Morphology of solid-based formulations (carriers).

Chapter 3
Results

3.1 Liquid-based formulations

3.1.1 Utilization of additives by PS3 strain

To confirm whether the additives (alginate, PEG, PVP, glucose, glycerol, and mineral oil) can be assimilated by *R. palustris* strain PS3, we conducted carbohydrate utilization assay by API 50CH + LR method. As shown in Table 3-1, the colours for the sample solutions have been turned to light purple or pink after incubation, suggesting all additives could be assimilated by PS3. More than that, efficiency rate of utilization by PS3 correlated with the intensity reaction levels. Our experiment revealed that mineral oil and alginate have the higher intensity value (4 and 3, respectively). Other treatments get an intermediate intensity value (2 and 1), including glycerol, glucose and followed by PVP and PEG.

3.1.2 Viability of *R. palustris* PS3 strain under different liquid-based formulation process

To select potential additives that is able to increase the stability of PS3 liquid inoculant, the effects of carrier types, concentration of additive and storage temperature on the survival of this bacterium were evaluated. As shown in Table 3-2 and Figures S1-S3, the PS3 cell numbers in different formulations declined dramatically with increasing storage temperatures after one month of storage. The storage temperature was the most important factor to maintain the quality and survive ability of various inoculant formulations (Table 3-3). The final bacterial concentrations were equivalent to 10^3-10^5 CFU/ml when stored under 37℃, and they

were declined slowly (final bacterial concentrations: 10^6 - 10^8 CFU/ml) when stored at 4℃ (Table 3-2).

Under 4℃ storage condition, the highest viable count of the PS3 cells (8.7 Log CFU/ml) were found in the 5.0% mineral oil treatment (Table 3-2). The other formulations, such as 0.5 - 3.0% mineral oil, 0.1%, 3.0%, and 5.0% glucose, 3.0% glycerol, 1.0% and 5.0% PVP could also confer higher population stability during storage than that of control treatment (determined by the letter grades of "a" to "c" on statistical analyses, Table 3-2) . When the inoculants were stored at room temperature (25℃), the inoculant formulations with the following carriers: 1.0% glucose, 0.5% alginate, 2.0% PVP, 0.5% - 2.0% mineral oil were found to give higher viable count in the superior range (5.9 - 6.5 Log CFU/ml, Table 3-2). While the inoculants were preserved under high temperature (37℃), the survival of PS3 cells in most of the liquid formulations were declined dramatically (Table 3-2 and Figure S3). The formulations with 1.0% glycerol, 0.5% and 2% mineral oil, and 3.0% glucose, or 0.5% PEG could seem protect the PS3 cells from high temperature stress (viable count 4.7 - 5.3 Log CFU/ml, Table 3-2).

Among the additives, only glucose (0.1%, 3.0%), glycerol (1.0%, 3.0%) and mineral oil (0.5% - 5.0%) could support higher viable cell of PS3 under all three temperature grades (4, 25, and 37℃) than those in control treatments (Table 3-2). According to the survival rate of PS3 cells (Table 3-2) as well as the purchase cost for respective material (Table 2-2), we finally chose 0.5% mineral oil, 1.0% glucose, and 1.0% glycerol as the potential additive candidates.

3.1.3 Efficiency of selected inoculant formulations under greenhouse conditions

We evaluated the plant growth promoting effectiveness of the PS3 inoculants that were formulated with 0.5% mineral oil, 1.0% glucose, or 1.0% glycerol. The morphology of Chinese cabbage (*B. rapa chinesis*) plants treated by respective inoculant was shown in Figure 3-1. There was no inhibition effect of the additives on normal plant growth of Chinese cabbage while comparing the growth of CF group and those with the additives (CF - MO 0.5, CF - Glu 1.0, and CF - Gly 1.0) (Figure 3-2). While supplementation with the PS3 inoculant without any formulation (i.e CF + PS3), the plant growth was greater than that of CF alone, and there were 40% and 60% increment in fresh and dry shoot weight, respectively, in comparison with those of the CF control (Figure 3-2). We inoculated the formulated PS3 inoculants (CF - MO 0.5 - PS3, CF - Glu 1.0 - PS3, or CF - Gly 1.0 - PS3) respectively into the pots, and found that all the treatments could promote plant growth effectively. In addition, there was no statistical difference between the formulated and the unformulated (i.e CF + PS3) samples.

3.1.4 Survival of *R. palustris* inoculants in rhizospheres

We analysed the populations of PS3 (with or without formulation) in rhizospheres after 4 weeks of cultivation using the Miles and Misra (1938) method. The final bacteria concentrations showed that no significant differences between the treatments ($p > 0.05$) by Tukey's HSD test, which were equivalent to 5.1 Log CFU/g for the CF soils with inoculants PS3, and 5.1 - 5.4 Log CFU/g soil for the CF + PS3 soils with mineral oil (Figure 3-3).

Taken together, based on the results mentioned above, including shelf life (i.e. the survival rate of PS3 cells under different storage temperatures, Table 3-2), the

purchase cost for respective material (Table 2-2) and the plant growth promoting effectiveness (Figure 3-2), we finally chose mineral oil 0.5% as the potential additive candidate for liquid-based formulation.

3.1.5 Efficiency of mineral oil inoculant formulations (after one month of storage) under greenhouse conditions

We evaluated the effectiveness of the selected liquid formulation for PS3, ie. 0.5% mineral oil, after storing under various conditions (4℃, 25℃ and 37℃) for one month on the growth of Chinese cabbage in pot experiments (Figure 3-4). As shown in Figure 3-4A, there was no statistical significant differences in fresh shoot weight between the non-formulated treatments (CF - PS3, stored at 4℃, 25℃ and 37℃, respectively) and the CF alone treatment. While supplementation with 0.5% mineral oil in the PS3 suspension (CF - MO O.5-PS3), the plant growth of Chinese cabbage was greater than those of non-formulated treatments at any storage condition (4℃. 25℃ and 37℃). Comparing with the growth of non-formulated treatments, 35~70% and 50-90% increased for fresh and dry yields were observed in the formulated ones. Furthermore, we noticed that the formulated treatments stored at 4℃ and 25℃ had similar efficacy of plant growth promotion, which were shown to be superior to that of 37℃ ($p < 0.05$).

3.1.6 Survival of *R. palustris* inoculants in rhizosphere

The survival of formulated or non-formulated PS3 cells in the post-harvested Akadama soils after 4 weeks of cultivation were determined by the Miles and Misra

method (1938). As shown in Figure 3-5, there were significant differences between the populations of 0.5% mineral oil formulated samples (CF - MO 0.5 - PS3) and those of non-formulated ones (CF - PS3) (Student's t test, $p < 0.05$). The PS3 populations were approximately 5.8 Log CFU/g soil when the formulated inoculants were stored under 4℃ or 25℃, whereas that dropped dramatically to ~4.0 Log CFU/g soil while it was stored under 37℃ (Figure 3-5).

3.2 Solid formulation

3.2.1 Physiochemical properties of carrier materials

The physiochemical characteristics of the carriers used were analysed. As shown in Table 3-4, all solid carriers had different pH values in the range of 3.5 to 7.5. Sugarcane bagasse had the lowest value (pH 3.5) and vermiculite was considered as a neutral carrier (pH 7.2). The latter gained less value in electrical conductivity (EC) and the others were in the range of 2.5-3.5%. The maximum amount of bacterial culture that can be added to a carrier was determined by the water holding capacity (WHC). Except vermiculite (30%) and rapeseed (40%), the other carriers had exceed 60% of WHC, and peat had the highest value of it (80%). Most of the carriers had substantial proportion of organic matters (>50%), and rapeseed had the highest contents (75%). We also found that the available N and P of rapeseed were superior to the others (5.3% and 2.1%, respectively). Intriguingly, although vermiculite contained the least amounts of available nutrient (N, P, and K) and organic matter content (3.8%), it has abundant trace elements, such as copper (72 ppm), nickel (240 ppm), chromium (898 ppm) and lead (41 ppm), etc.

3.2.2 Carriers morphology and surface characteristics

The SEM images of surface characteristics for respective carrier were shown in Figure 2-1. In peat (P), there were a great amount of pores interlinked, and predominant fibre like structures with varying sizes and thickness. Rice husk (R) has smooth, flat, cracks and compact surfaces. Sugarcane bagasse (SB) possessed rigid fibril and swirl shape owing to cut blades effects or milling process. Sometimes, fragmented structure of SB contained some piths and small holes. Crystal, flakes and

smooth surface, multilayer effects were observed in vermiculite (V). Rapeseed meal (Ra) had large particles of uneven sizes, granulate and compact structures in comparison with other materials.

3.2.3 Viability of *R. palustris* PS3 strain under different solid-based formulation process

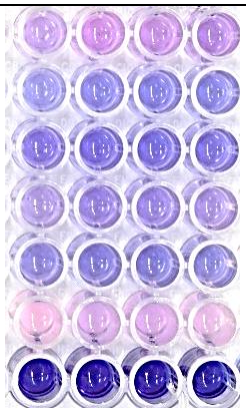
Among the formulation processes for solid-based inoculant, drying carrier is an important step. As shown in Table 3-5, there was no viable cell in the carriers while drying by oven at high temperature (55℃). On the other hand, while the carriers were dried by lyophilisation (freeze drying), the population of PS3 declined from ~ 9.0 Log CFU/g carrier to ~7.5-5.0 Log CFU/g carrier. In the case of air-drying, those were maintained at relative high level (8.0 ± 0.7 Log CFU/g carrier) in most carriers.

We further assessed the survival rate of PS3 in each carrier under different drying methods (55℃ oven, lyophilisation, and air–drying, respectively) in combination with the effects of storage temperatures (4℃, 25℃, and 37℃, respectively) (Table 3-6 and Fig S4). The effective variances were analysed and found that the storage temperature was the most critical factor to maintain the quality and survive ability of various inoculant formulations (Table 3-7). The PS3 cell numbers in different carriers declined dramatically with increasing storage temperatures after one month of storage. As shown in Fig S4, the viable PS3 cells greatly reduced when they were stored under 37℃, and they were remained much more stable under 4℃ storage condition.

Among the carriers, the air-dried peat and rice husk formulations could confer higher population of PS3 (7.7 Log CFU/carrier and 7.4 Log CFU/carrier, respectively than those in the control treatments (7.1 Log CFU/carrier for air-drying treatment)

after one month of storage at 4℃ (Table 3-6) . When the inoculants were stored at room temperature (25℃), the air-dried rice husk and vermiculite formulations were found to give higher viable counts (7.5 Log CFU/g carrier and 7.8 Log CFU/g carrier, respectively) than those of control treatments (6.1 Log CFU/carrier for air-drying treatment). While the inoculants were preserved under relative high temperature (37℃), the survival of PS3 cells in most of the solid based formulations were declined dramatically (Table 3-6 and Figure S4). Only the air-dried peat and vermiculite formulations could protect the PS3 cells from high temperature stress (6.3 Log CFU/g carrier). However, none of the carriers could support higher viable cell of PS3 than those in control treatments under all three temperature grades (4, 25, and 37℃).

Table 3-1. Utilizations in respective additive by *R. palustris* strain PS3.

Additives	Reactions		Replications			
	Before	After 48 hours	R1	R2	R3	R4
Alginate			3	3	3	3
PVP			1	1	1	1
PEG			1	1	1	1
Glycerol			2	2	2	2
Glucose			1	1	1	1
Mineral oil			4	4	4	4
L2-N medium[b]			0	0	0	0

'a' indicates that resazurin test values ranging from 0–4: 0 corresponds to a negative reaction, 4 to a reaction of maximum intensity and values 1, 2, or 3 are intermediate reactions depending on the level of intensity. 'b' indicates that L2-N medium serves as a negative control, there is no carbon source in the solution.

Table 3-2. Effect of additives on populations of *R. palustris* PS3 strain stored under different temperatures.

Additives	Concentrations (%w/v)[a]	Survival rate of PS3 strain Log(CFU/ml)					
		4℃		25℃		37℃	
Control		7.5	de	5.0	nm	4.6	cdefg
Alginate	0.5	6.3	i	6.2	b	3.2	p
	1.0	5.2	l	5.6	f	4.2	jkl
	1.5	4.1	m	5.3	ghi	4.3	hijkl
	2.0	6.4	j	4.6	p	3.6	o
	2.5	6.5	ij	4.9	no	2.7	q
Glucose	0.1	7.8	c	5.2	jkl	4.6	cdefg
	0.5	7.4	def	5.3	igh	4.3	ijkl
	1.0	5.5	k	6.5	a	3.8	no
	3.0	8.2	b	5.1	lm	4.8	bc
	5.0	7.8	c	5.1	lm	4.4	ghijkl
Glycerol	0.5	7.1	g	4.9	no	4.7	cdefg
	1.0	7.6	d	5.1	klm	5.3	a
	2.0	7.4	def	5.4	gh	4.1	lmn
	3.0	8.1	b	5.6	fe	4.5	cdefghij
	5.0	7.3	efg	4.9	no	4.7	bcd
Mineral Oil	0.5	8.0	b	5.9	c	4.8	b
	1.0	8.1	b	6.0	c	4.7	bcdef
	2.0	7.9	c	5.8	cd	4.7	bcde
	3.0	8.3	b	5.6	fe	4.5	cdefghi
	5.0	8.7	a	5.2	ijk	4.6	cdefg
PEG	0.1	7.1	g	5.3	ghi	4.4	fghijkl
	0.5	7.2	fg	5.3	hij	4.7	bcde
	1.0	6.7	h	5.2	jkl	4.5	efghijk
	5.0	7.2	fg	5.3	ghi	4.4	efghijk
	10.0	7.2	fg	5.1	klm	4.2	lm
PVP	1.0	7.3	c	5.4	g	4.5	efghijk
	2.0	6.6	hi	6.2	b	4.3	hijkl
	3.0	7.4	ef	5.7	de	4.3	ijkl
	5.0	8.1	b	4.9	o	3.9	ijkl
	10.0	5.6	k	4.7	p	4.2	kl

[a]indicates the concentrations of additives (% w/v); [b]the values are the mean of three replications. Different letters in a column indicate significant differences at the 5% level among the treatments at

different temperatures as determined by Tukey's HSD (Honest significant differences) test. Treatments that performed well than control treatment are highlighted in color of gray.

Table 3-3. Analysis of variances showing the effect of different additives (A), concentrations (C), storage temperatures (T) and their interactions on the viability of PS3 strain in the inoculants.

Source	Sum of squares[a]	df[b]	Mean square[c]	F value[d]	P-value[e]
Additives (A)	13.45	5	2.69	588.27	**
Concentrations (C)	8.43	8	1.05	230.25	**
Temperatures (T)	271.60	2	135.80	29688.56	**
A × C × T	40.44	32	1.26	276.25	**
A × C	10.46	16	0.65	142.85	**
A × T	6.79	10	0.68	148.45	**
C × T	27.53	16	1.72	376.19	**

** indicates significant at 1% level.

'a' In analysis of variance (ANOVA), the total sum of squares is calculated as a summation of the squares of the differences from the mean. It represents the variation attributed to various factors.

'b' degrees of freedom (n-1), to describe the number of values in the final calculation of a statistic that are free to vary. n: the size of samples.

'c' is an estimate of the population variance based on the sum of squares multiplied by n (the size of samples). The great the numerical values, the higher the total variations.

'd' is the ratio of the variance between groups to the variance within groups. If the variables are large, the result is more significant.

'e' derived from F value that is computed from ANOVA. P-value test means the probability of obtaining the observed sample results (or a more extreme result) when the null hypothesis is actually true. If the *p* -value is smaller than 0.01, 0.05, 0.1, the result is significant.

Table 3-4. Selected physiochemical properties of different carrier materials.

Carriers	pH	EC	WHC	OM	N	P_2O_5	K_2O	CaO	MgO	Cu	Zn	Cd	Ni	Cr	Pb
	Percentage (%)									ppm (kg/ha)					
Vermiculite	7.2	0.4	30.0±2	3.8	0.1	0.4	3.6	2.4	19.3	72.4	123.4	0.5	239.5	898.0	40.6
Sugarcane	3.5	3.6	60.0±2	68.2	0.6	0.3	0.9	0.2	0.2	18.4	139.0	0.3	5.3	9.9	14.4
Rice husk	5.9	2.4	60.0±2	59.4	0.7	0.3	0.8	0.2	0.1	5.1	36.5	0.3	2.0	0.8	4.5
Rapeseed	6.5	2.6	40.0±2	75.0	5.3	2.1	1.0	7.5	0.5	10.3	547.0	0.6	5.9	10.0	13.0
Peat	5.2	2.7	80.0±2	71.0	1.0	0.6	0.3	3.2	1.0	16.8	32.9	0.1	5.3	4.6	19.8

EC: Electric conductivity; OM: Organic matter; WHC: water holding capacity N: available nitrogen; P_2O_5: available Diphosphorus pentoxide; K_2O: Potassium oxide.

Table 3-5. Survival of *R. palustris* PS3 strain under various drying methods

Carriers	Drying methods (Log CFU/g carrier)[a]			
	Before drying	Air-drying	Lyophilisation	Oven[b]
Control	9.7±0.3 a	8.0±0.5 a	5.1±0.5 b	0
Rapeseed	8.9±1.0 a	8.7±0.9 a	7.7±0.9 a	0
Peat	8.9±0.9 a	7.6±0.9 b	6.5±0.7 b	0
Vermiculite	9.0±0.7 a	8.1±0.7 a	5.2±0.8 a	0
Sugarcane	9.9±1.0 a	8.4±0.7 a	7.7±0.7 a	0
Rice husk	9.2±0.9 a	8.0±0.6 a	7.6±1.0 a	0

'a' indicated that average of three replications ± standard error (SE). Means with different letters differ significantly within column (a to b in each group) by Duncan's New Multiple Range Test (DMRT), $p < 0.05$.

'b' represented that no viable cell of PS3 strain after oven treatment.

Table 3-6. Survival of *R. palustris* strain PS3 in various carriers for solid-based formulations under three different storage temperatures after lyophilisation and air drying.

Carriers	Drying	Survival rate Log CFU/ g carrier		
		4℃	25℃	37℃
Control	Lyophilisation	6.2e	0.0g	0.0f
	Air-drying	7.1c	6.1c	4.7b
Rapeseed	Lyophilisation	5.5h	0.0g	0.0d
	Air-drying	5.8f	3.4f	0.0d
Peat	Lyophilisation	5.2j	0.0g	0.0d
	Air-drying	7.7a	5.4d	6.3a
Vermiculite	Lyophilisation	5.1j	0.0g	0.0f
	Air-drying	6.9d	7.5b	6.3a
Sugarcane	Lyophilisation	5.8g	0.0g	0.0d
	Air-drying	5.6h	3.6e	3.4c
Rice husk	Lyophilisation	5.3i	0.0g	0.0d
	Air-drying	7.4b	7.8a	0.0d

Data showed in decimal logarithm of viable cells g^{-1} of inoculant. Data are means of three replications. Different letters in a column indicate significant differences at the 5% level among the treatments at different temperatures as determined by Tukey's HSD (Honest significant differences) test. Treatments that performed well than control treatment are highlighted in color of gray.

Table 3-7.Analysis of variances showing the effect of different carriers (C), drying methods (D), storage temperatures (T) and their interactions on the viability of PS3 strain in the inoculants.

Source	Sum of squares[a]	df[b]	Mean square[c]	F value[d]	P-value[e]
Carriers (C)	25.03	5	5.01	2.84	0.02*
Temperatures (T)	431.11	2	215.56	122.47	0.00*
Drying (D)	210.66	1	210.66	119.69	0.00*
T × D	51.96	2	25.98	14.76	0.00*
Error	170.73	97	1.76		

* indicates significant at 1% level.

[a] In analysis of variance (ANOVA), the total sum of squares is calculated as a summation of the squares of the differences from the mean. It represents the variation attributed to various factors.

[b] degrees of freedom (n-1), to describe the number of values in the final calculation of a statistic that are free to vary. n: the size of samples.

[c] is an estimate of the population variance based on the sum of squares multiplied by n (the size of samples). The great the numerical values, the higher the total variations.

[d] is the ratio of the variance between groups to the variance within groups. If the variables are large, the result is more significant.

[e] derived from F value that is computed from ANOVA. P-value test means the probability of obtaining the observed sample results (or a more extreme result) when the null hypothesis is actually true. If the P-value is smaller than 0.01, 0.05, 0.1, the result is significant.

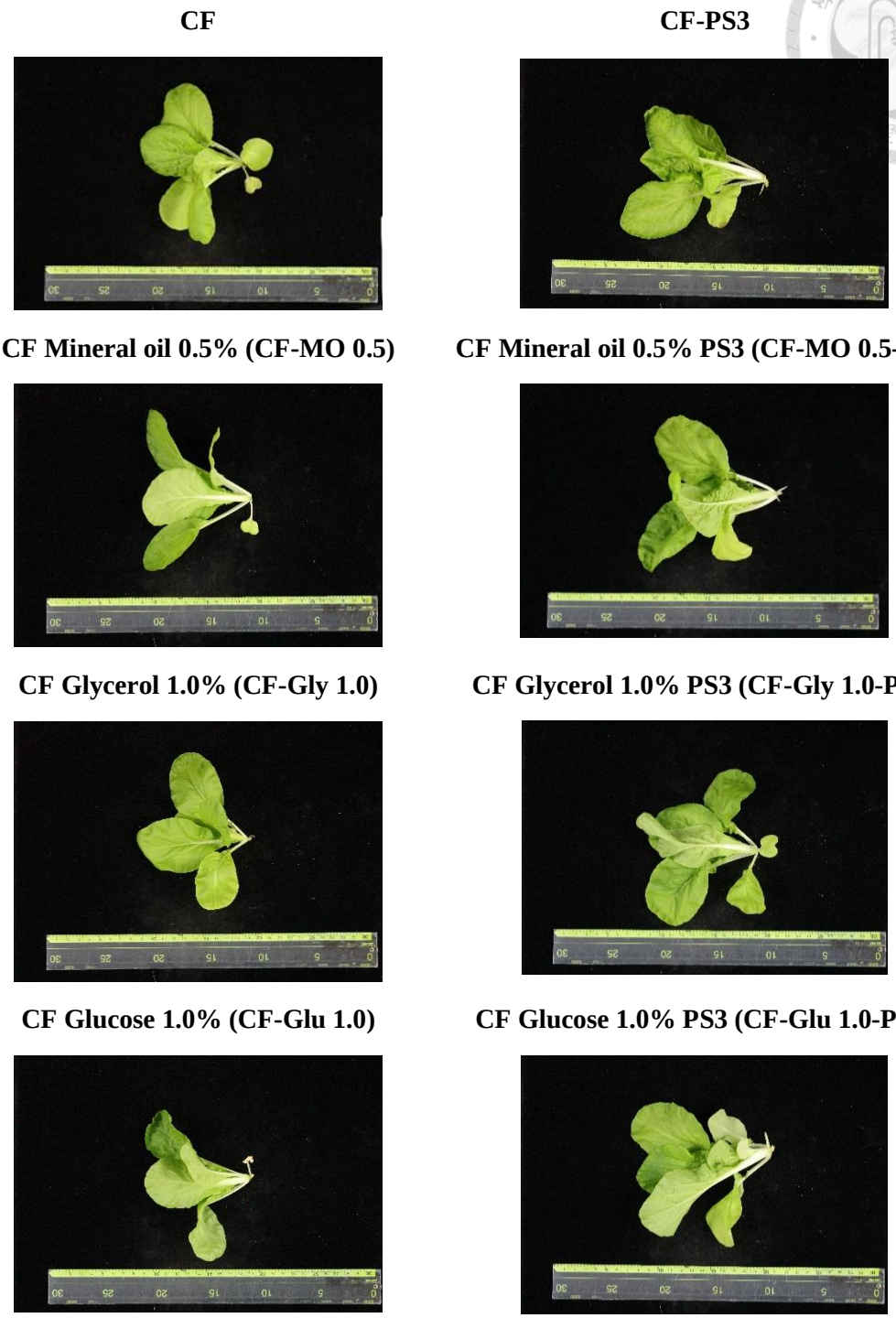
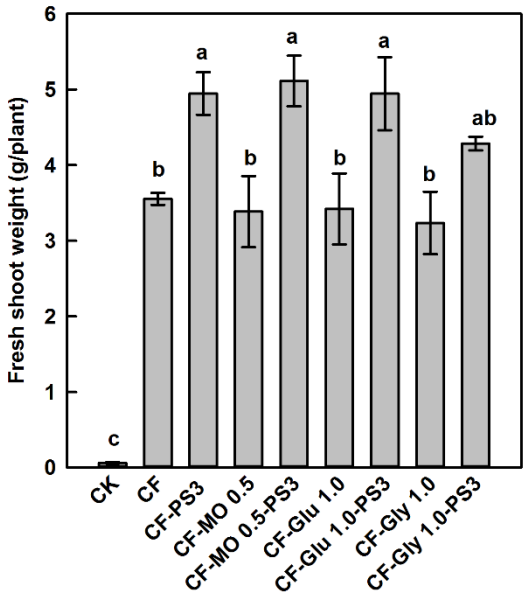


Figure 3-1. Morphology of *B.rapa chinesis* plants grown in sterile Akadama soil after 4 weeks. CF: chemical fertilizer; MO 0.5: Mineral oil 0.5%; Glu 1.0: Glucose 1.0%; Gly 1.0: Glycerol 1.0%. PS3: *R. palustris* strain PS3.

(A)



(B)

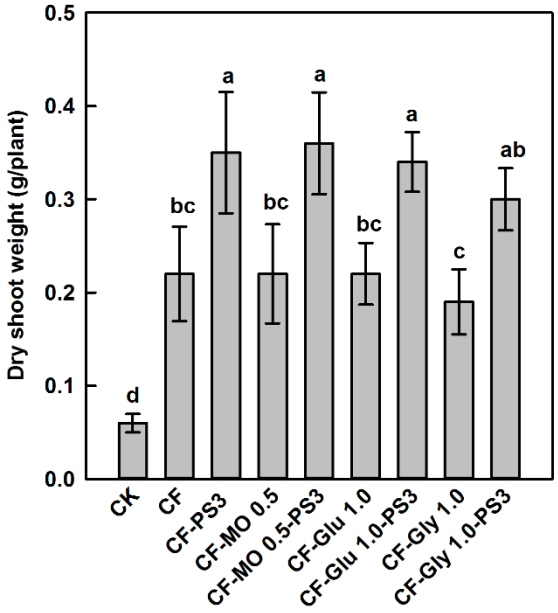


Figure 3-2. Effects of (fresh) inoculant formulations of *R. palustris* PS3 strain on (A) shoot fresh weight (g/plant) and (B) shoot dry weight (g/plant) under pot experiment. The growth responses were analysed at the end of 4 weeks. Bars represent mean values ±SE from ten replicates and labelled with different letter represent significantly different mean values, according Tukey's HSD test. CF: chemical fertilizer; MO 0.5: Mineral oil 0.5%; Glu 1.0: Glucose 1.0%; Gly 1.0%: Glycerol 1.0%.

.

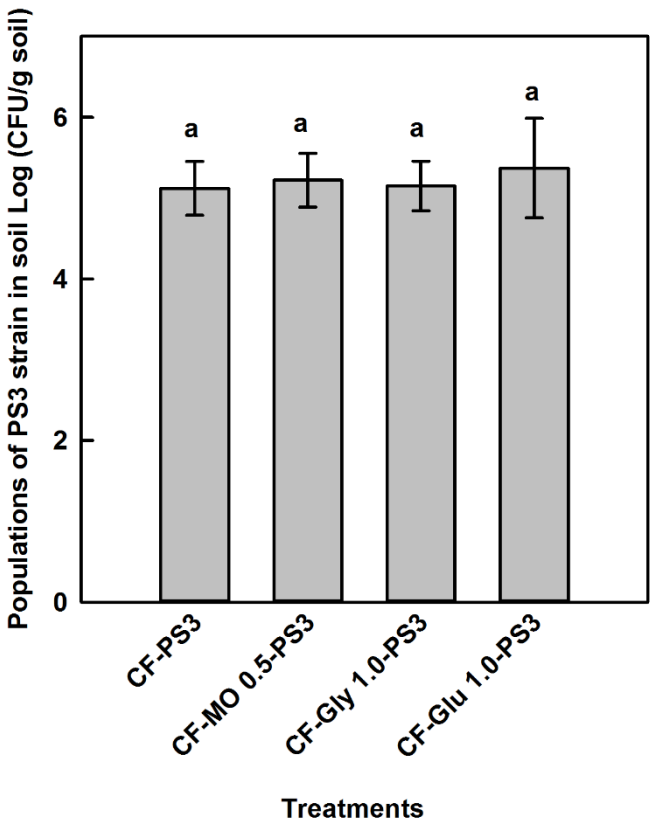
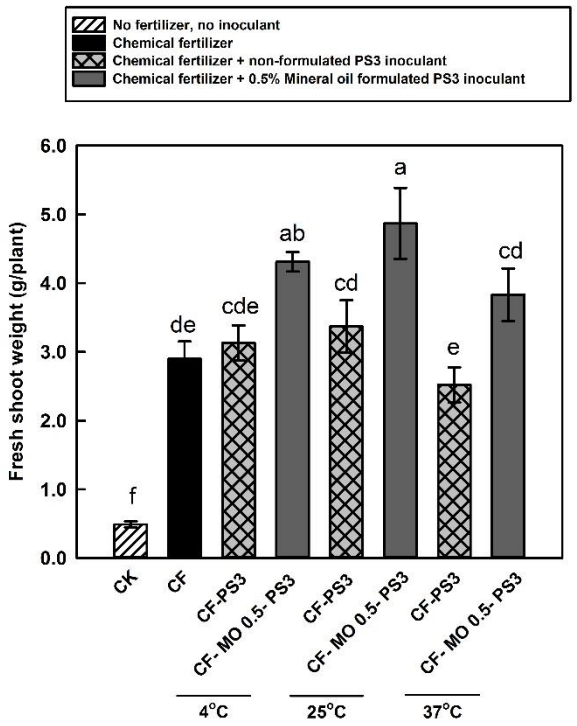


Figure 3-3. Survival of *R. palustris* PS3 strain inoculant to Akadama soils after 4 weeks of cultivations. Bars represent mean values ±SE from three biological repeats and labelled with same letter represent non-significantly different mean values, according Tukey's HSD test. CF: chemical fertilizer; MO 0.5: Mineral oil 0.5%; Gly 1.0: Glycerol 1.0%; Glu: Glucose 1.0%; PS3: *R. palustris* strain PS3.

(A)



(B)

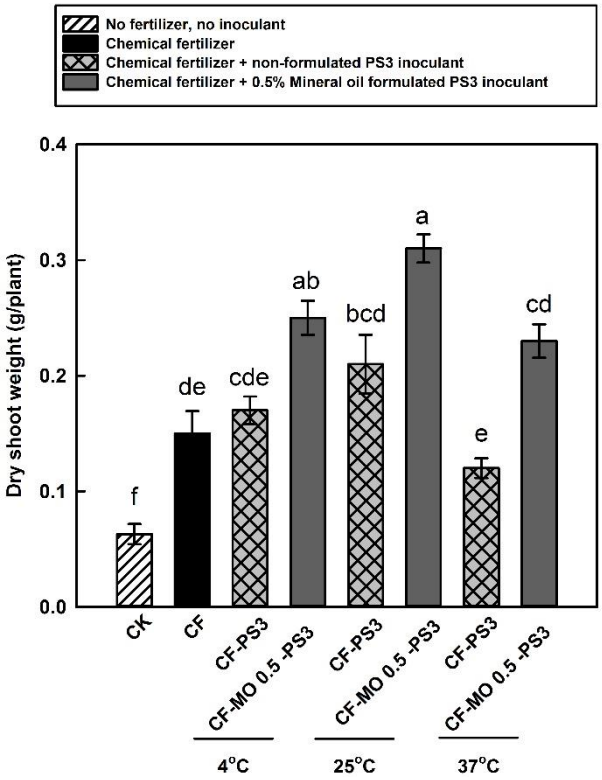


Figure 3-4. Effects of one-month-storage *R. palustris* PS3 inoculants on (A) shoot fresh weight (g/plant) and (B) shoot dry weight (g/plant) under pot experiments. The

growth responses were analysed at the end of 4 weeks. Bars represent mean values ±SE from ten replicates and labelled with different letter represent significantly different mean values, according Tukey's HSD test. 4°C, 25°C, 37°C: Storage temperatures; CF: chemical fertilizer; MO 0.5: 0.5% mineral oil; PS3: *R. palustris* strain PS3.

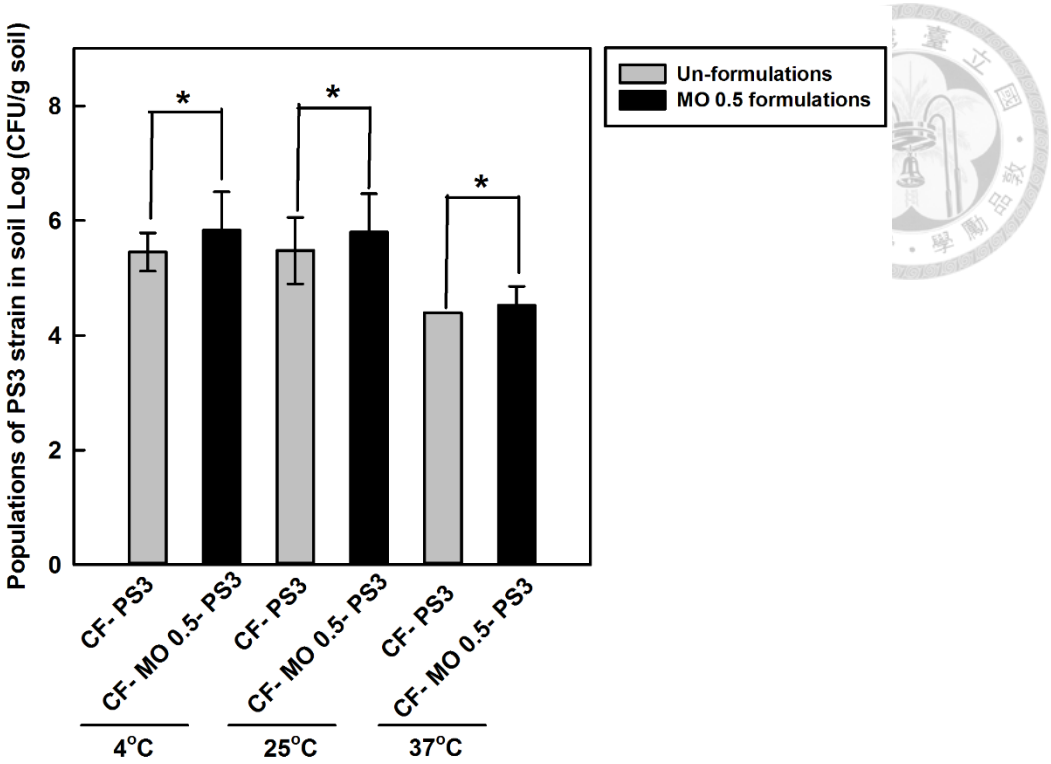


Figure 3-5. Survival of *R. palustris* PS3 inoculants in Akadama soils after 4 weeks of cultivation with and without mineral oil 0.5% formulation. Bars represent mean values ±SE from three biological repeats. The asterisks (*) indicate there are two pairs of groups whose means differ significantly ($p < 0.05$) from each other. 4℃, 25℃, 37℃: Storage temperatures; CF: chemical fertilizer; MO 0.5: 0.5% Mineral oil; PS3: *R. palustris* strain PS3.

Chapter 4
Discussion and conclusion

In this study, we evaluated a variety of materials for development of liquid and solid based formulations of *R. palustris* PS3 inoculant. We analysed the survival of PS3 in different formulations under various processing conditions and storage temperatures for a period of time, followed by assessment of the beneficial efficacy of these inoculants for plant growth in pot experiments.

In the case of liquid-based formulations, each of the additive has its own merits, and most of them perform better than the control treatment in viable cell counts. Based on the results mentioned, we found that glucose, glycerol and mineral oil could support higher viable cells of PS3 under 4℃, 25℃, 37℃ for one month of storage (Table 3-2), and also possess the plant growth promoting effectiveness (Figures 3-1 and 3-2). The advantages of these materials have been reported in many studies (summarized in Table 2-1). For example, mineral oil can prevent the microbes from stress by contributing the oil envelope and good adhesive for seed inoculations (Fernandez et al., 2006; Helmy, 2012; Hoben et al., 1991); glucose can protect microbes by increasing the storage products (such as glycogen or polysaccharides), or act as osmoprotectant (Singleton, 2002); and glycerol is one of the nutrient compounds or carbon sources for microbes, and it can protect microbes from desiccation by providing high water binding capacity (Singleton, 2002).

In the carbon source utilization assay, mineral oil, glucose and glycerol were able to be catabolized by PS3 (intensity level is 4, 3, 2, respectively, Table 3-1). We suggest that these carbohydrate compounds could act as additional nutrient sources to sustain the growth of PS3 during storage. Unexpectedly, although alginate was well

utilized by PS3 efficiently (with maximum intensity level 4 in resazurin test, Table 3-1), it was not a suitable additive in this study since it had poor performances in survival rate of PS3 strain cell in most of the treatments (Table 3-2). The criteria for successful development of PGPR formulation mainly depends on suitable materials and application methods (Bashan et al., 2014; Tittabutr et al., 2007; Trivedi et al., 2008). In general, alginate is used for encapsulation or macro-alginate beads for PGPR formulation (Bashan et al., 2014). Therefore, we speculate that merely mixed alginate with PS3 broth may not make good use of this material.

As shown in Figure 3-2, we noted that although the same amount of chemical fertilizer (CF) was applied in the treatments, plant growth responses were boosted and performed well only in the groups supplemented with PS3 inoculant. Furthermore, the remaining population of PS3 after 4 weeks of cultivations retain in a high level (more than 5 Log CFU/g in soil) (Figure 3-3). Since the beneficial effects were comparable between the fresh/ non-formulated PS3 inoculant (CF-P3) and the formulated ones (CF-MO 0.5-P3, CF-Glu 1.0-P3, and CF-Gly 1.0-P3) (Figure 3-2), we deduced that the formulated PS3 inoculants do not give adverse effect to either bacterium itself or plants.

We chose mineral oil as the potential additive candidate for liquid-based formulation according to the survival rate of PS3 cells (Table 3-2), purchase cost (Table 2-1) and the plant growth promoting effectiveness (Figure 3-2). To our knowledge, mineral oil has not been applied as additive for biofertilizer formulation. It is typically used as a biocontrol agent in crop protection (Fernandez et al., 2006; Helmy, 2012; Opande and Arama, 2013; Shaw et al., 2000; Stansly.P.A, 2005), and has been certified as nature and safe material in agriculture application (refer to Taiwan Organic Information Portal website:

http://info.organic.org.tw/supergood/front/bin/ptdetail.phtml?Part=sick-41&). Other than that, Hoben (1991) also indicated that the oil type additives, such as mineral oil, peanut oil and soybean oil, have a lot of advantages. For example, they can provide reasonably sticky, are inexpensive and readily available, and are non-toxic to seeds or plants and microbes, etc.

In the case of solid-based formulations, drying is a critical manufacturing procedure. As shown in Table 3-5, the populations of PS3 were maintained at relative high level (~10^7 CFU/g) in most carriers while using aseptic air-drying to desiccate the carriers that were amended with PS3 suspension. By contrast, there was no viable PS3 cell in the carriers while drying by heating oven (55℃) (Table 3-5). Generally, the fast drying approaches, such as using heating and drying ovens, have no applicability to the non-spore-forming bacteria. This result is comparable to that reported by Chao and Alexander (1984) indicating that the survival rate of *Rhizobium* sp. dropped dramatically under fast drying process. Lyophilisation (freeze drying) is another extensively applied drying method for either spore-forming PGPR (e.g. *Bacillus* sp. (Wacek, 1997), or non-spore-forming ones (e.g. *Azospirillum* sp. or *Pseudomonase* sp.) (Mputu Kanyinda, 2012; Okon, 1993; Wainwright, 1999). However, the populations of PS3 declined dramatically while the carriers were dried by lyophilisation, indicating such drying process somehow damaged the viability of PS3 cells. Deaker et al (2012) have pointed out that osmotic stress occurs during freeze-drying will result in excessive cell death. Therefore, the moderate air-drying method is considered more appropriate for processing solid-based formulation of PS3.

As shown in Table 3-6, none of the carriers could support higher viable cells of PS3 than those in control treatments under all the three testing temperature grades (4, 25, and 37℃). Peat, vermiculite, and rice husk are relative good candidates for they

showed better survival rate than the other ones under two of the three storage conditions. For example, the air-dried peat formulation could confer higher population of PS3 (7.7 Log CFU/carrier and 6.3 Log CFU/carrier) at 4 and 37℃, respectively (Table 3-6). Peat has been considered as a potential carrier for solid-based inoculant formulation (Date, 1974; Roughley, 1970; Thompson, 1984). As shown in Table 3-4, peat was superior to the other carriers in some physiochemical features, such as it was rich in organic matter and nutrients, furthermore, and possessing high water holding capacity (WHC), etc. However, the effectiveness of peat for PS3 survival at 25℃ storage conditions was inferior to that of vermiculite or rice husk in this study. The PS3 cell viability (5.4 Log CFU/carrier) under this condition was even less than that in control treatment (6.1 Log CFU/carrier). Although peat can sustain high numbers of rhizobia (>10^8 per g) when incubated at 3 to 28℃ (Kremer, et al. 1983), the reason for the population decline of PS3 at room temperature remains to be elucidated.

Microbial contamination has become a troublesome sort, and severely affects the reliability and quality of PGPR inoculants (Arora, 2008; Sparrow and Ham, 1983). Among the carriers, we noted that rapeseed meal and rice husk treatments were easily contaminated with fungi or other microbes during storage under processing or storage (data not shown). The contaminants frequently outcompeted the growth of the indigenous PS3 cells, resulted in the populations of this bacterium declined rapidly (Table 3-6, Figure S4). Accordingly, these two carriers are not appropriate candidates for solid-based formulation of PS3 inoculant, although rice husk could support higher viable cells of PS3 than those in control treatments under 4 and 25℃ storage condition.

According to the physiochemical characteristics of carriers, we observed considerable amounts of heavy metals (Ni, Cu) were existed in vermiculite (Table 3-

4). It may damage microbial viability for long-term storage, and pose potential hazards for environments when applied into fields (Lowe and Evans, 1962; Wilson and Reisenauer, 1970). Now, therefore, although both peat and vermiculite had better performance in shelf life (Table 3-6), we would rather select peat as a candidate carrier for solid-based formulation of PS3.

In this study, mineral oil and peat were chosen to be candidate materials for liquid and solid based formulations, respectively. However, in comprehensive consideration of shelf life, manufacturing process [including composting, drying, milling, grinding, and sterilization, etc (Singleton.P et al., 2002; Kumaresan and Reetha, 2011; Girisha et al., 2006; Sridhar et al., 2004)], cost, contamination issue, storing space and environmental concerns, etc., liquid-based formulation seems more appropriate for manufacturing PS3 inoculant. Accordingly, we finally adopt mineral oil (0.5%) as the potential formulation for preparing PS3 inoculant, although the survival rate of PS3 in mineral oil (0.5%) at 37℃ (4.8 Log CFU/ml) (Table 3-2) was inferior to that in peat formulation (air-dried) (6.3 Log CFU/carrier) (Table 3-6). According to a preliminary test, we have evaluated the effective dosage of PS3 inoculant that was able to exert beneficial effects on plant growth was within the range of 6~8 Log CFU/ml (equivalent to 4~6 Log CFU/g soil) (data not shown). Therefore, to retain high-quality of PS3 inoculant, the formulated product should keep in a cool place during shipping and storage.

Mineral oils is a complex mixture of hydrocarbons containing traces of nitrogen and sulphur-linked compounds (Paranjape et al., 2015). According to the results of Table 3-1, we deduced that PS3 cells are able to use mineral oil as an additional nutrient to sustain their metabolism during storage. Besides, due to mineral oil is considered as a safe, low cost, and easy-to-process agricultural material, this

formulation technology will facilitate the commercialization and practical use of PS3 inoculant. We refer to current regulations or standards for microbial inoculants (mainly *Rhizobium* sp. inoculant) proposed in some developed and developing countries, and found that the viable cell number should retain 10^7-10^9 cells g^{-1} or ml^{-1} for shelf life and minimum standard are given for the date expirations, at least 6 months. Furthermore, there must be no contaminant in the inoculants or in compliance with the standards with respect to contaminations (Table S2). To meet the regulation of biofertilizer in Taiwan and other countries (Table S2) (Herrmann and Lesueur, 2013; Young et al., 2006), further investigation of the factors such as the metabolically active resting (i.e., nongrowing) bacterial cells, or packaging techniques may improve the survival rate and storage condition of PS3.

References

Adesemoye A.O., Adesemoye H.A., Torbert J.W., Kloepper. (2009) Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology 58:921-929.

Adholeya A., Das M. (2012) Biofertilizers: Potential for crop improvement under stressed conditions, Improving crop productivity in sustainable agriculture, Wiley-VCH Verlag GmbH & Co. KGaA. pp. 183-200.

Albareda M., Albareda D., Rodríguez Navarro M., Camacho F., Temprano. (2008) Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. Soil Biology & Biochemistry 40:2771-2779.

Anyango B. (1984) Assessment of filtermud as a carrier for legume seed inoculants, Department of Botany, University of Nairobi, Kenya. pp. 129.

Arora N.K. (2008) Sawdust as a superior carrier for production of multipurpose bioinoculant using plant growth promoting rhizobial and pseudomonad strains and their impact on productivity of *trifolium repense*. Current Science 95:90.

Balume I. (2013) Assessment of quality control of inoculants used on bean and soybean in Eastern and central Africa, Land resource management and agricultural technology faculty of agriculture, University of Nairobi, Kenya. pp. 78.

Bashan Y. (1986) Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. Applied and environmental microbiology 51:1089-98.

Bashan Y. (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnology Advances 16:729-770.

Bashan Y., de-Bashan L., Prabhu S.R., Hernandez J.-P. (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant and Soil 378:1-33.

Berg G., Berg. (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11-18.

Bhattacharyya P.N., Jha D.K. (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology 28:1327-1350.

Boonkerd N. (2002) Development of inoculant production and utilisation in Thailand, in: D. Herridge (Ed.), Inoculants and nitrogen fixation of legumes in Vietnam. pp. 95-104.

Burges H.D. (1998) Formulation of microbial biopesticides: beneficial microorganisms, nematodes, and seed treatments Springer Netherlands.

Catroux G., Catroux A., Hartmann C., Revellin. (2001) Trends in rhizobial inoculant production and use. Plant and Soil 230:21-30.

Chao W.L., Alexander M. (1984) Mineral soils as carriers for *Rhizobium* inoculants. Applied and environmental microbiology 47:94-7.

Crawford S.L., Berryhill D.L. (1983) Survival of *Rhizobium phaseoli* in coal-based legume inoculants applied to seeds. Applied and Environmental Microbiology 45:703-5.

Das A.J., Kumar M., Kumar.R. (2013) Plant growth promoting rhizobacteria (PGPR): An alternative of chemical fertilizer for sustainable, environment friendly agriculture. Research Journal of Agriculture and Forestry Sciences 1:21-23.

Date R.A. (1974) Legume inoculant production, Proceedings of the Indian National Science Academy, Section B, Bangalore. pp. 667-686.

Date R.A. (1972) Sources and quantities of yeast extract for growth of rhizobia. Journal of Applied Bacteriology 35:379-387.

Dayamani K.J., Brahmaprakash G.P. (2014) Influence of form and concentration of the osmolytes in liquid inoculants formulations of plant growth promoting bacteria. International Journal of Scientific and Research Publications (IJSRP) 4.

Deaker R., Hartley E., Gemell G. (2012) Conditions affecting shelf-life of inoculated legume seed. Agriculture 2:38-51.

Denardin N.D., Freire J.R.J. (2000) Assessment of polymers for the formulation of legume inoculants. World Journal of Microbiology and Biotechnology 16:215-217.

Elbadry M., Elbadry A., El Bassel K., Elbanna. (1999) Occurrence and dynamics of phototrophic purple non-sulphur bacteria compared with other asymbiotic

nitrogen fixers in rice fields of Egypt. World Journal of Microbiology & Biotechnology 15:359-362.

Fernandes Júnior P.I., Rohr T.G., Oliveira P.J.d., Xavier G.R., Rumjanek N.G. (2009) Polymers as carriers for rhizobial inoculant formulations. Pesquisa Agropecuária Brasileira 44:1184-1190.

Fernandez D., Fernandez E.H., Beers J.F., Brunner M.D., Doerr J.E., Dunley. (2006) Horticultural mineral oil applications for apple powdery mildew and codling moth, *Cydia pomonella* (L.). Crop Protection 25:585-591.

GIFAP (1989) *Catalogue of Pesticide Formulation Types and International Coding System*, GIFAP, Brussels.

Girisha H.C., Brahmaprakash G.P., Mallesha B.C. (2006) Effect of osmoprotectant (PVP-40) on survival of *Rhizobium* in different inoculants formulation and nitrogen fixation in Cowpea. Geobios (Jodhpur) 33:151-156.

Gomez M., Gomez N., Silva A., Hartmann M., Sagardoy G., Catroux. (1997) Evaluation of commercial soybean inoculants from Argentina. World Journal of Microbiology & Biotechnology 13:167-173.

Graham Weiss L., Bennett M.L., Paau A.S. (1987) Production of bacterial inoculants by direct fermentation on nutrient-supplemented vermiculite. Applied and Environmental Microbiology 53:2138-41.

Gray E.J., Smith D.L. (2005) Intracellular and extracellular PGPR: Commonalities and distinctions in the plant–bacterium signaling processes. Soil Biology and Biochemistry 37:395-412.

Gupta V.K., Schmoll M., Maki M., Tuohy M., Mazutti M.A. (2013) Applications of microbial engineering CRC Press.

Hafeez F.Y., Hafeez M., Idris K.A., Malik. (1989) Growth and survival of cowpea bradyrhizobia in various carrier materials. Biology and Fertility of Soils 7:279-282.

Hansen T., Van Gemerden H., Gemerden H. (1972) Sulfide utilization by purple nonsulfur bacteria. Archives of Microbiology 86:49-56.

Helmy E.I, Kwaiz.F. A. and El-Sahn.O. M. N. (2012) The usage of mineral oils to control insects. Egyptian Academic Journal of Biological Sciences 5:167-174.

Herrmann L., Lesueur D. (2013) Challenges of formulation and quality of biofertilizers for successful inoculation. Applied Microbiology and Biotechnology 97:8859-8873.

Hoben H.J., Aung N., Somasegaran P., Kang U.G. (1991) Oils as adhesives for seed inoculation and their influence on the survival of *Rhizobium* spp. and *Bradyrhizobium* spp. on inoculated seeds. World Journal of Microbiology and Biotechnology 7:324-330.

Hungria M., Loureiro M.F., Mendes I.C., Campo R.J., Graham P.H. (2005) Inoculant preparation, production and application, in: D. Werner and W. Newton (Eds.), Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment, Springer Netherlands. pp. 223-253.

Hynes R.K., Craig K.A., Covert D., Rennie R.J., Smith R.S. (1995) Liquid rhizobial inoculants for lentil and field pea. Journal of Production Agriculture 8:547-552.

Imhoff J. (2006) The phototrophic alpha-proteobacteria, in: M. Dworkin, et al. (Eds.), The Prokaryotes, Springer New York. pp. 41-64.

Janzen, H.H. (1993) Soluble salts, in: M.R. Carter (Ed.), Soil sampling and methods of analysis. Lewis Publishers, Boca Raton, Fla. pp. 161-166.

Jung G., Mugnier J., Diem H.G., Dommergues Y.R. (1982) Polymer-entrapped *rhizobium* as an inoculant for legumes. Plant and Soil 65:219-231.

Kaljeet S. (2011) Temperature on survivability of rhizobial inoculant. Asian Journal of Plant Sciences 10:331.

Kloepper J.W., Schroth M.N. (1978) Plant growth promoting rhizobacteria on radishes, Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Angers, France : Station de Pathologie Végétale et Phytobactériologie, INRA. pp. 879-882.

Knudsen D., Peterson G.A., Pratt P.F. (1982) Lithium, sodium, and potassium, in: A. L. Page (Ed.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties, American Society of Agronomy, Soil Science Society of America, Madison. pp. 225-246.

Kremer R.J., Peterson H.L. (1983) Effects of carrier and temperature on survival of *Rhizobium* spp. in Legume Inocula: Development of an improved type of inoculant. Applied and Environmental Microbiology 45:1790-1794.

Kumaresan G., Reetha D. (2011) Survival of *Azospirillum brasilense* in liquid formulation amended with different chemical additives. Journal of Phytology 3.

Lowe R.H., Evans H.J. (1962) Cobalt requirement for the growth of rhizobia. Journal of Bacteriology 83:210-211.

Lucy M., Reed E., Glick B. (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86:1-25.

Maheshwari D. (2008) Potential Microorganisms For sustainable agriculture: A Techno-commercial perspective. IK International Pvt Ltd.

Malusá E., Malusá L., Sas Paszt J., Ciesielska. (2012) Technologies for beneficial microorganisms inocula used as biofertilizers. The Scientific World Journal 2012:1-12.

Martínez Viveros O., Martínez Viveros M.A., Jorquera D.E., Crowley G., Gajardo M.L., Mora. (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. Journal of Soil Science and Plant Nutrition 10:293.

Marufu L., Marufu N., Karanja M., Ryder. (1995) Legume inoculant production and use in East and Southern Africa. Soil biology & biochemistry 27:735-738.

McAneney K.J., Harris R.F., Gardner W.R. (1982) Bacterial water relations using polyethylene glycol 40001. Soil Sci. Soc. Am. J. 46:542-547.

McLean, E. O. (1982) Soil pH and lime requirement, in: A. L., R. H. Miller and D. R. Keeney (Eds.) Methods of soil analysis. Part 2 - Chemical and microbiological properties. (2nd Ed.). Agronomy 9:199-223.

Miles A.A., Miles S.S., Misra J.O., Irwin. (1938) The estimation of the bactericidal power of the blood. The Journal of hygiene 38:732.

Mohamed H.M., Abdel-Moniem A.-b. (2010) Evaluation of water hyacinth and sugarcane bagasse composts as a carrier for *Rhizobium* Inoculants and their effects on faba bean. Research Journal of Agriculture & Biological Sciences 6.

Mputu Kanyinda J.-N. (2012) Accelerated storage testing of freeze-drying Pseudomonas fluorescens BTP1, BB2 and PI9 strains. African Journal of Biotechnology 11.

Müller B., Défago G. (2006) Interaction between the bacterium *Pseudomonas fluorescens* and vermiculite: Effects on chemical, mineralogical, and mechanical properties of vermiculite. Journal of Geophysical Research: Biogeosciences 111:G02017.

Murphy J., Riley J.P. (1962) A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta 27:31-36.

Nelson D.W., Sommers L.E. (1982) Total carbon, organic carbon, and organic matter, in: A. L. Page (Ed.), Methods of soil analysis. part 2. chemical and microbiological properties, American Society of Agronomy, Soil Science Society of America. pp. 539-579.

Okon Y. (1993) Azospirillum / plant associations CRC Press, United state of America.

Olsen P., Olsen W., Rice L., Bordeleau A.H., Demidoff M., Collins. (1996) Levels and identities of nonrhizobial microorganisms found in commercial legume inoculant made with nonsterile peat carrier. Canadian Journal of Microbiology 42:72-75.

Olsen P.E., Olsen W.A., Rice M.M., Collins. (1995) Biological contaminants in North American legume inoculants. Soil Biology & Biochemistry 27:699-701.

Olsen P.E., Rice W.A., Bordeleau L.M., Biederbeck V.O. (1994) Analysis and regulation of legume inoculants in Canada: The need for an increase in standards. Plant and Soil 161:127-134.

Opande G.O., Arama C.M.P.F. (2013) Innundative biocontrol of water hyacinth (*Eichhornia crassipes* (Mart.) Solms. Laubach) using zonate leaf spot (*Acremonium zonatum* Sawada Gams) fungal agent. IOSR Journal of Agriculture and Veterinary Science 6:69-71.

Paranjape K., Gowariker V., Krishnamurthy V.N., Gowariker S. (2015) The pesticide encyclopedia CAB International.

Rivera D. (2014) Evaluation of polymers for the liquid rhizobial formulation and their influence in the Rhizobium-Cowpea interaction. Universitas scientiarum 19:265.

Roughley R. (1970) The preparation and use of legume seed inoculants. Plant and Soil 32:675-701.

Sarker S., Sarker L., Nahar Y., Kumarasamy. (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42:321-324.

Scaglia J.A. (1991) Production and quality control of inoculants in Rwanda FAO, Italy.

Shaw P., Bradley S., Walker J. (2000) Efficacy and timing of insecticides for the control of San Jose scale on apple, Procceddings of the New Zealand Plant

Protection Conferences, New Zealand Plant Protection Society; 1998. pp. 13-17.

Singleton P. (2002) Development and evaluation of liquid inoculants. Inoculants and Nitrogen Fixation of Legumes in Vietnam:52-66.

Sridhar V., Brahmaprakash G., Hegde S. (2010) Development of a liquid inoculant using osmoprotectants for phosphate solubilizing bacterium (Bacillus megaterium). Karnataka Journal of Agricultural Sciences 17.

Smith R. (1992) Legume inoculant formulation and application. Canadian journal of microbiology 38:485-492.

Sparrow S.D., Ham G.E. (1983) Survival of *Rhizobium phaseoli* in six carrier materials. Agronomy Journal 75:181-184.

Stansly.P.A. (2005) Crop and insect response to horticultural mineral oil on tomato and pepper, in: F. S. H. Society (Ed.), Annual meeting; 118th, Florida State Horticultural Society, Florida. pp. 132-141.

Stephens J.H.G., Rask H.M. (2000) Inoculant production and formulation. Field Crops Research 65:249-258.

Suzuki S., Aono T., Lee K.B., Suzuki T., Liu C.T., Miwa H., Wakao S., Iki T., Oyaizu H. (2007) Rhizobial factors required for stem nodule maturation and maintenance in Sesbania rostrata-*Azorhizobium caulinodans* ORS571 symbiosis. Applied and Environmental Microbiology 73:6650-9.

Temprano F., Temprano M., Albareda M., Camacho A., Daza C., Santamaría N., Rodríguez N. (2002) Survival of several *Rhizobium/Bradyrhizobium* strains on different inoculant formulations and inoculated seeds. International microbiology 5:81-86.

Thompson J.A. (1984) Production and quality control of carrier-based legume inoculants ICRISAT Andhra Pradesh, India.

Tittabutr P., Payakapong W., Teaumroong N., Singleton P.W., Boonkerd N. (2007) Growth, survival and field performance of bradyrhizobial liquid inoculant formulations with polymeric additives. Science Asia 33:69-77.

Trivedi P., Trivedi A., Pandey. (2008) Recovery of plant growth-promoting rhizobacteria from sodium alginate beads after 3 years following storage at 4℃. Journal of Industrial Microbiology & Biotechnology 35:205-209.

Varela Milla O., Rivera E.B., Huang W.-J., Chien C., Wang Y.-M. (2013) Agronomic properties and characterization of rice husk and wood biochars

and their effect on the growth of water spinach in a field test. Journal of Soil Science and Plant Nutrition 13:251-266.

Vessey J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.

Vincent J., Thompson J., Donovan K. (1962) Death of root-nodule bacteria on drying. Australian Journal of Agricultural Research 13:258-270.

Wacek T.J. (1997) Liquid formulations of non-Spore forming, in: G. R. G. M. J. H. H. M. Collins (Ed.), Pesticide formulations and application systems, ASTM International. pp. 94-97.

Wainwright M. (1999) Environmental biotechnology in agriculture, An introduction to environmental biotechnology, Springer US. pp. 127-141.

Wilson D.O., Reisenauer H.M. (1970) Effect of manganese and zinc ions on the growth of *Rhizobium*. Journal of Bacteriology 102:729-32.

Wong W.T., Tseng C.H., Hsu S.H., Lur H.S., Mo C.W., Huang C.N., Hsu S.C., Lee K.T., Liu C.T. (2014) Promoting effects of a single *Rhodopseudomonas palustris* inoculant on plant growth by *Brassica rapa chinensis* under low fertilizer input. Microbes and Environments 29:303-313.

Young C.C., Young P.D., Rekha W.A., Lai A.B., Arun. (2006) Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnology and Bioengineering 95:76-83.

Zhang S., Winestrand S., Guo X., Chen L., Hong F., Jansson L. (2014) Effects of aromatic compounds on the production of bacterial nanocellulose by *Gluconacetobacter xylinus*. Microbial Cell Factories 13:62.

Zheng H., Shi J., Fan L., Huang J. (2009) Effects of combined application of rapeseed cake and chemical fertilizer on rhizosphere microbes of flue-cured tobacco. Chinese Tobacco Science 6:13.

Supplementary

Table S1. Calculation for initial viable cell of PS3 strain in solid-based formulations.

Carriers	Broths added（ml）	CFU/ml	CFU/g carrier
Vermiculite	37.84	5.29×10^{8}	1.0×10^{9}
Sugarcane	68.61	2.91×10^{8}	1.0×10^{9}
Rice husk	44.28	4.52×10^{8}	1.0×10^{9}
Rapeseed	30.53	4.27×10^{8}	1.0×10^{9}
Peat	53.17	3.76×10^{8}	1.0×10^{9}

*Each carrier was prepared about 20g with 80%（w/w） water content.

Known:

OD 0.1 considered as 1.0×10^{8} CFU/ml; OD1.0 was equivalent to 1.0×10^{9} CFU/ml

1.0×10^{9} CFU/ml = 1.0×10^{9} CFU in 1 millilitre (ml)

Then, 20ml contained 20.0×10^{9} CFU/ml

Changed millilitre (ml) into gram (g) unit (Elimination methods 消除法)

Hence, 20.0×10^{9} CFU in 20 gram (g)

Ask: How many CFU/ml if each carrier was prepared about 20g with 80% (w/w) water content?

Vermiculite 20.0×10^{9} cfu / 37.48 ml = 5.33×10^{8} cfu/ml

Sugarcane 20.0×10^{9} cfu / 68.61 ml = 2.91×10^{8} cfu/ml

Rice husk 20.0×10^{9} cfu / 44.28 ml = 4.52×10^{8} cfu/ml

Rapeseed 20.0×10^{9} cfu / 30.53 ml = 4.27×10^{8} cfu/ml

Peat 20.0×10^{9} cfu / 53.17 ml = 3.76×10^{8} cfu/ml

* Broths volume according the result of water holding capacity of carrier (Data not shown).

Table S2. Descriptions of inoculant quality standard in various countries.

Country	Rhizobium standard		Contaminations standard	References and Sources
	cells g^{-1}	cells per $seed^{-1}$		
Taiwan	10^7 cells g^{-1}	10^6	Solid formulations: less than 15%	Young et al. (2006) AFA*
	10^8 cells m^{-1}	-	Liquid formulations: less than 5%	
France	10^9	$> 10^5$	None, even during storage	(Bashan, 1998; Catroux et al.,1991)
Australia	10^9	-	0.1% of the total microbial population	(Bashan, 1998; Thompson, 1984)
Rwanda	10^9	-	less than 0.001% of viable rhizobia	(Scaglia, 1991)
Zimbabwe	10^9	-	Frequently of use of unsterile carriers	(Marufu et al., 1995)
Kenya	10^9	-	Frequently of use of unsterile carriers	(Marufu et al., 1995)
South Africa	10^8	-	Frequently of use of unsterile carriers	(Hungria et al., 2005; Smith, 1992)
New Zealand	10^8	-	None	(Smith, 1992)
Netherland	4 to 25×10^9	-	None	(Maheshwari, 2008)
Thailand	5×10^7	-	No regulation	Boonkerd (1991)
Canada	10^{11} $hectare^{-1}$	$10^3 – 10^5$	No standard	(Olsen et al., 1994)
USA	-	-	Manufactures dissertations	(Date, 2000)
UK	-	-	Manufactures dissertations	(Date, 2000)
India	10^8 (Manufactured)) 10^7 (Expired)	-	None	(Maheshwari, 2008)

(*) means Agriculture and Food Agency, Council of Agriculture, Executive Yuan. http://www.afa.gov.tw/laws_index.asp?CatID=228

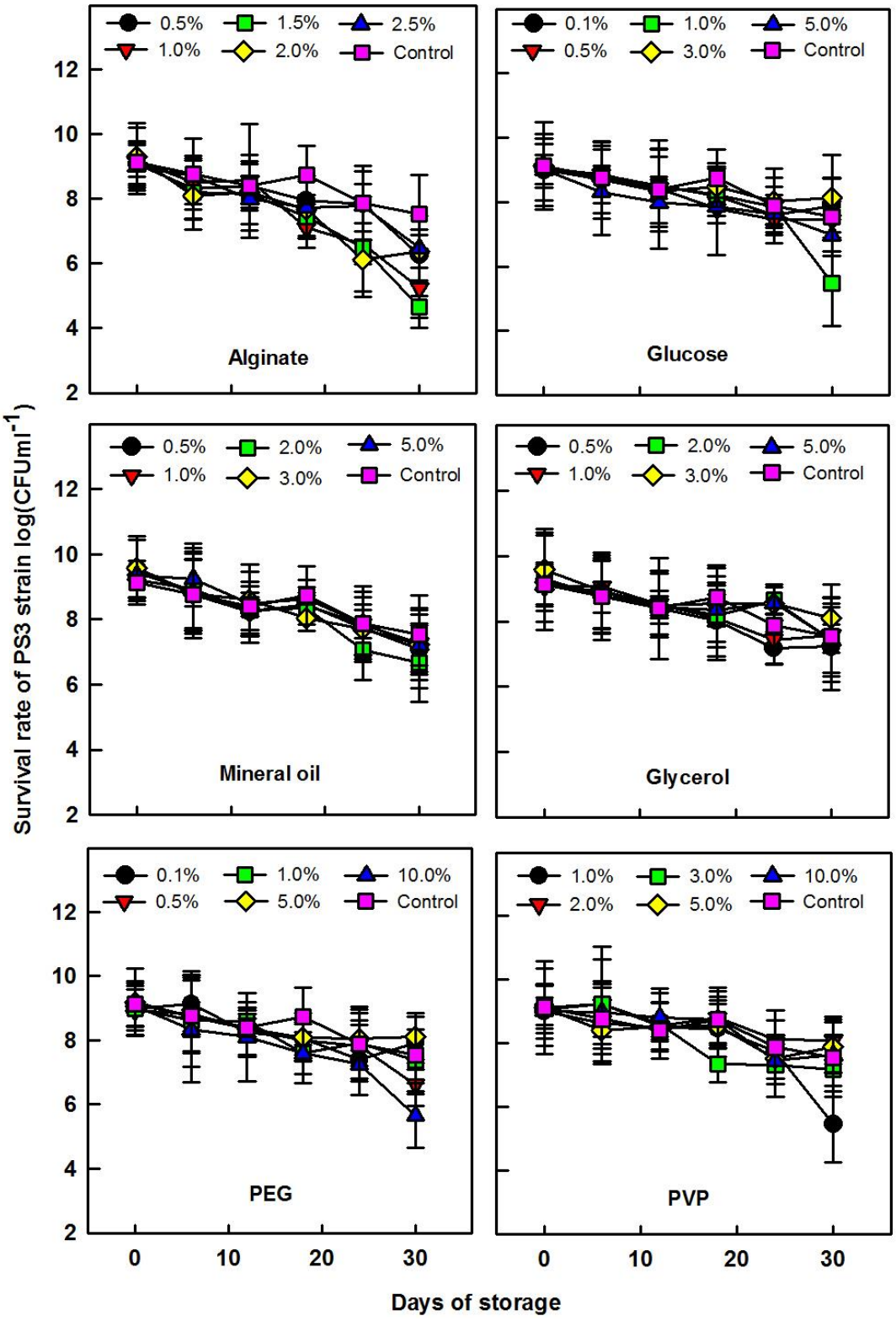


Figure S1. Shelf life of *Rhodopsedomonas palustris* PS3 strain with various additives in different concentrations under 4℃ for one month of storage. Data represents as mean values ±SE from three replicates.

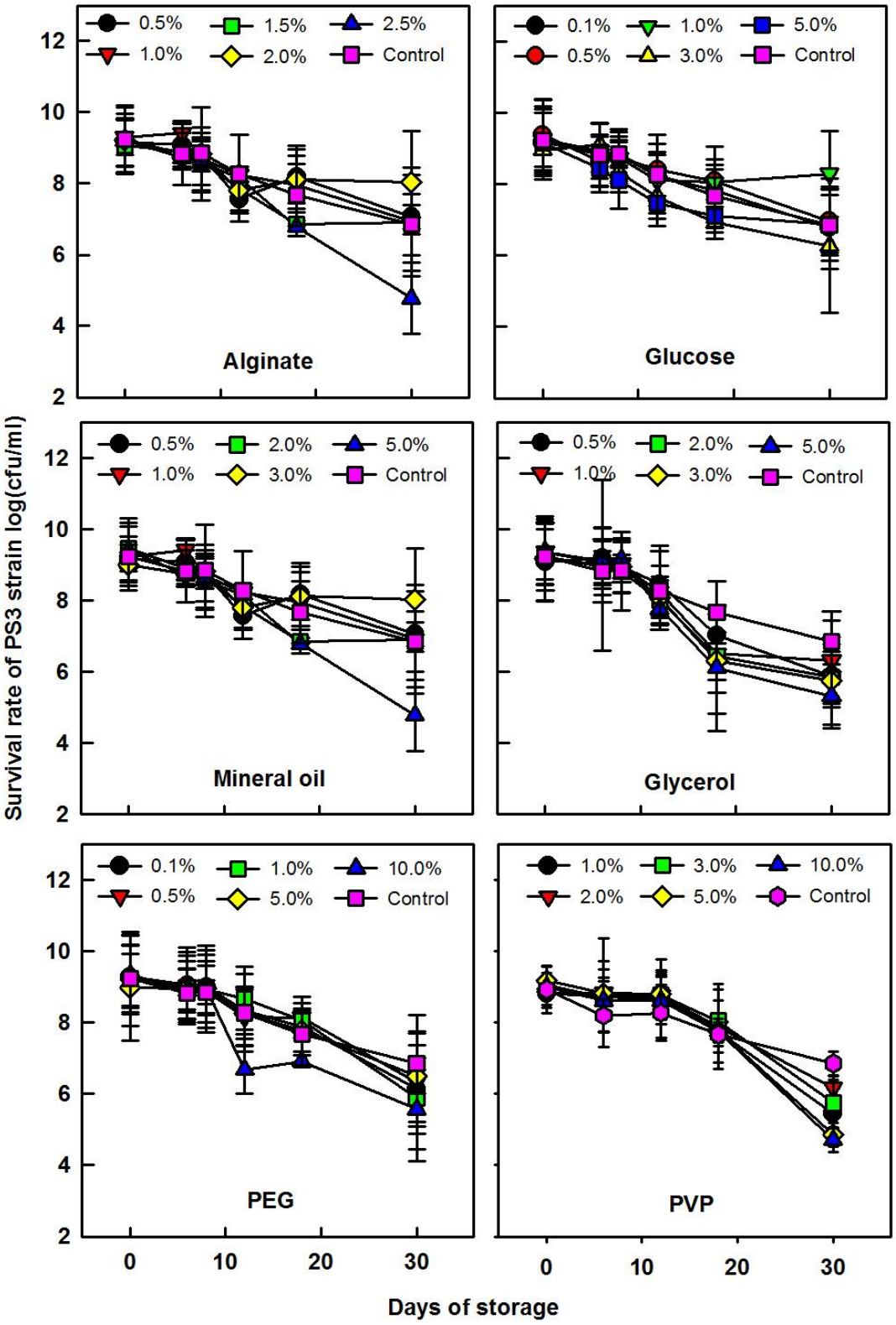


Figure S2. Shelf life of *Rhodopsedomonas palustris* PS3 strain with various additives in different concentrations under 25℃ for one month of storage. Data represents as mean values ±SE from three replicates.

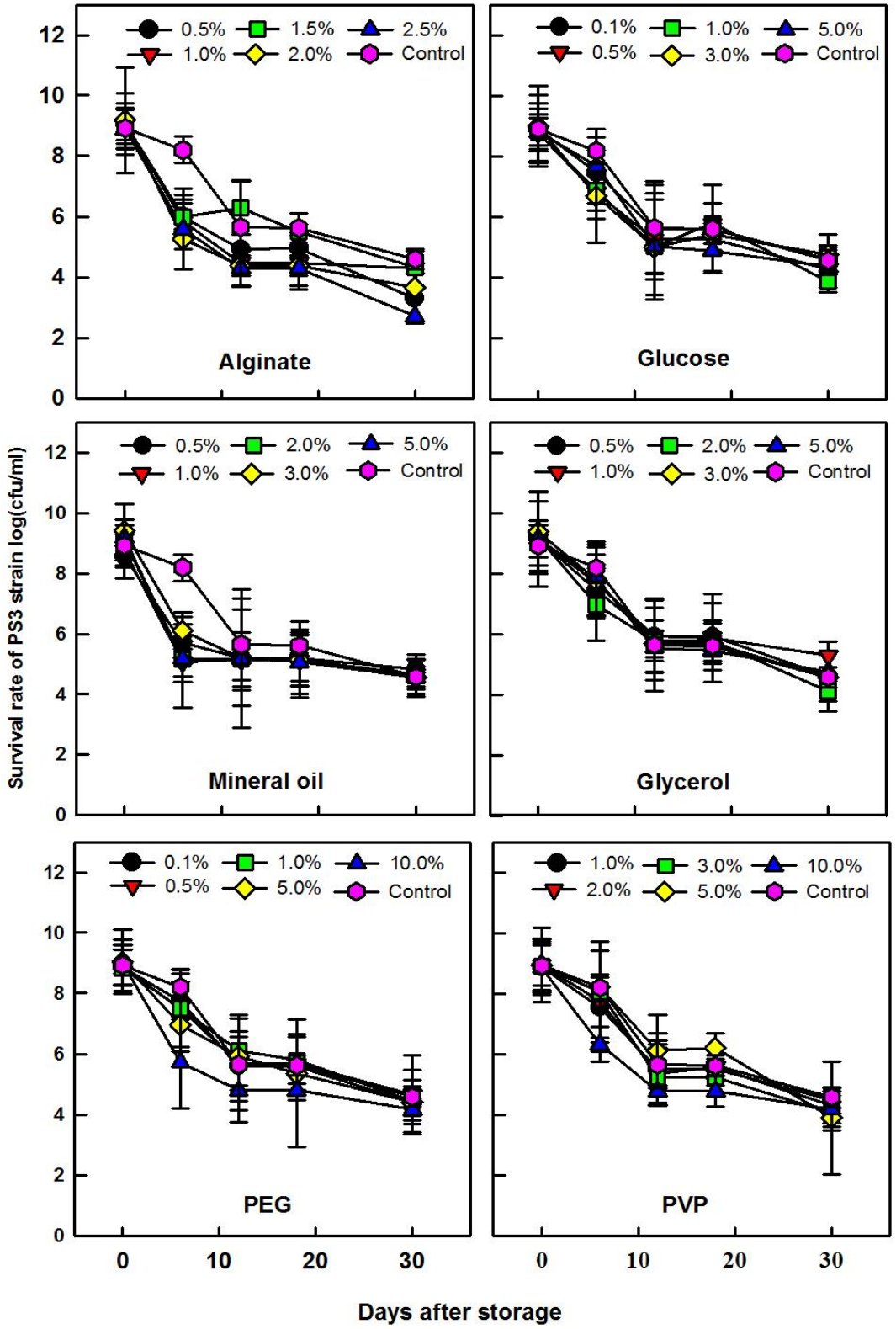


Figure S3. Shelf life of *Rhodopsedomonas palustris* PS3 strain with various additives in different concentrations under 37℃ for one month of storage. Data represents as mean values ±SE from three replicates.

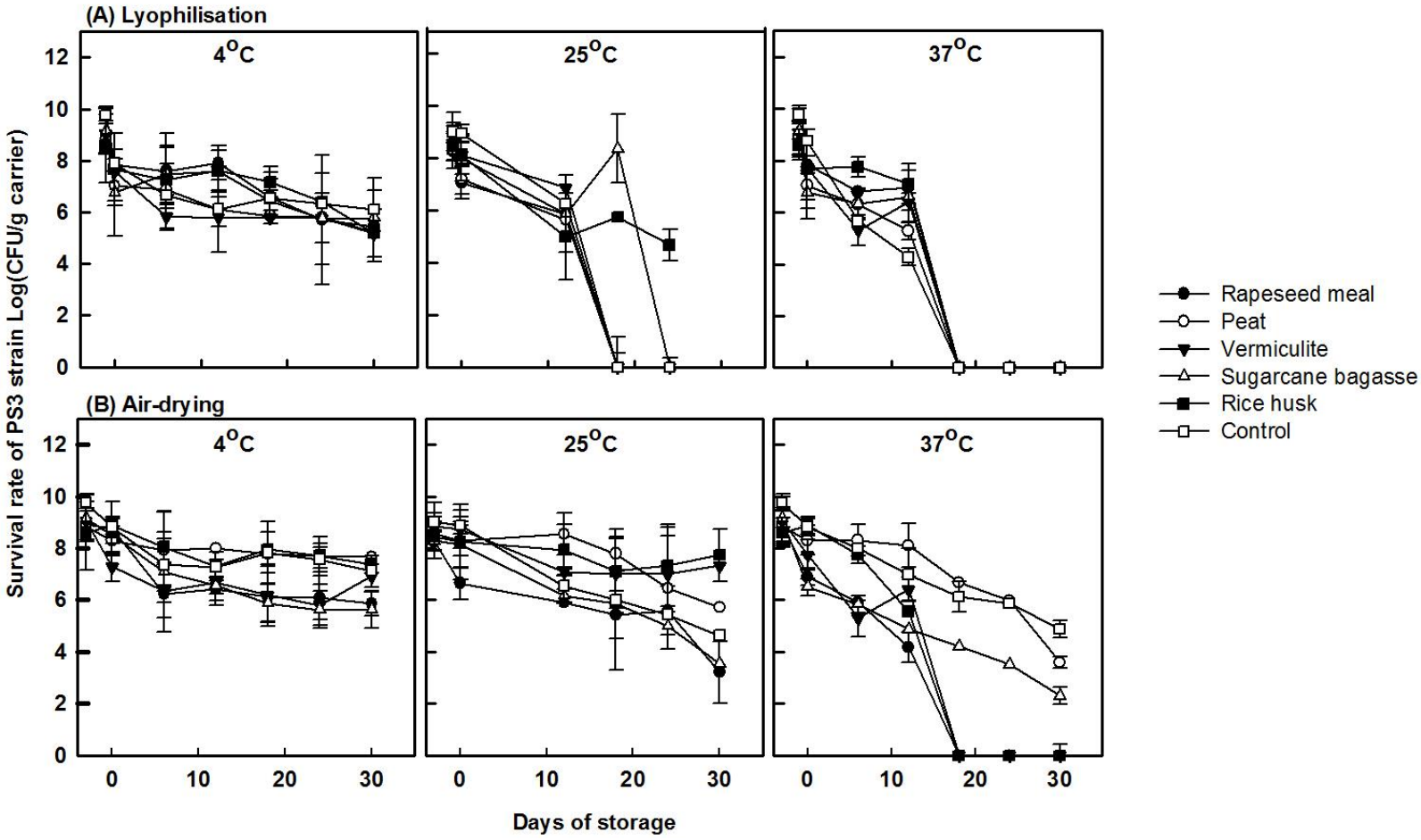


Figure S4. Shelf-life of *Rhodopseudomonas.palustris* PS3 strain in different carriers and storage temperatures. Values are averages of three replications. Error bars of treatments indicate standard error of the mean; where error bars are not visible, they are smaller than the symbol.

(A) Comparison of inoculated PS3 with chemical and organic fertilizer under pot experiment.

(B) Comparison of inoculated PS3 with various organic fertilizer.

Figure S5. Morphology of *B.rapa chinesis* plants grown in sterile Akadama soil after 4 weeks.

Abbreviations:

CK: Control 50% and 100%: Dosage of chemical fertilizer; P3: *R. palustris* strain PS3

SC: Sugarcane bagasse; Bio: Commercial bio-fertilizer; R: Rapeseed meals.

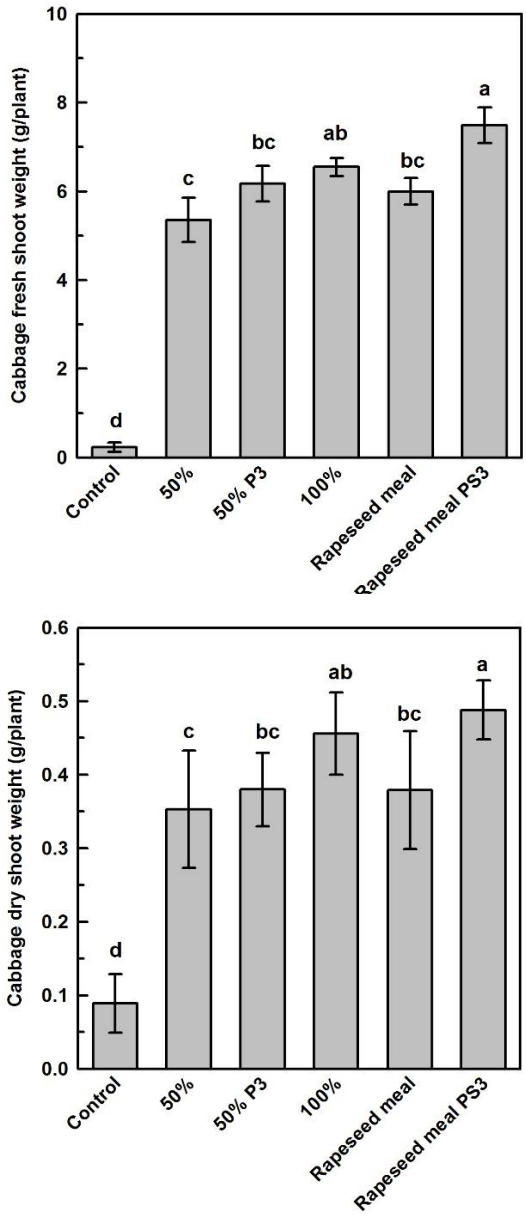


Figure S6. Effects of organic fertilizer (rapeseed meal) inoculated with *R. palustris* PS3 strain on shoot fresh weight (g/plant) under pot experiment. The growth responses were analysed at the end of 4 weeks. Bars represent mean values ±SE from ten replicates and labelled with different letter represent significantly different mean values, according Fisher's least significant different (LSD) test after ANOVA. CF: chemical fertilizer (refer to material and methods). Experimental date and place: October to November, horticulture planting room.