Field monitoring of plant-growth-promoting rhizobacteria by colony immunoblotting

Ganisan Krishnen, Mihály L. Kecskés, Michael T. Rose, Peter Geelan-Small, Khanok-on Amprayn, Lily Pereg, and Ivan R. Kennedy

Abstract: Inoculant plant-growth-promoting bacteria are emerging as an important component of sustainable agriculture. There is a need to develop inexpensive methods for enumerating these organisms after their application in the field, to better understand their survival and impacts on yields. Immunoblotting is one potential method to measure viable cells, but the high cost of the conventionally used nylon membranes makes this method prohibitive. In this study, less expensive alternative materials such as filter papers, glossy photo papers, and transparencies for the purpose of colony immunoblotting were evaluated and the best substance was chosen for further studies. Whatman filter paper No. 541 combined with a 0.01 mol·L^{-1} H_2SO_4 rinsing step gave similar results to nylon membranes but <20% of the overall cost of the original colony immunoblotting assay. The application of the modified immunoblot method was tested on nonsterile clay soil samples that were spiked with high numbers (>10^7 CFU·g^{-1}) of the plant-growth-promoting bacteria *Pseudomonas fluorescens*, *Azospirillum brasilense*, or *Rhizobium leguminosarum*. The modified protocol allowed the identification and recovery of over 50% of the inoculated cells of all three strains, amidst a background of the native soil microflora. Subsequently, the survival of *P. fluorescens* was successfully monitored for several months after application to field-grown rice at Jerilderie, New South Wales, Australia, thus validating the procedure.

Key words: PGPR, inoculants, colony immunoblotting, rice, alternative membrane.

Résumé : Les inoculats de bactéries qui favorisent la croissance des plantes se dégagent actuellement comme composantes importantes de l'agriculture durable. Il existe un besoin de développer des méthodes économiques de numération de ces organismes après leur application dans les champs, afin de mieux comprendre leur survie et leurs impacts sur les récoltes. L'immunobuvardage est une des méthodes éventuelles de mesure des cellules viables, mais le coût élevé des membranes de nylon habituellement utilisées rend cette méthode inabordable. Dans cette étude, du matériel alternatif moins cher comme le papier filtre, le papier photo glacé ou les acétates a été évalué pour l'immunobuvardage des colonies, et le meilleur support a été choisi pour des études subséquentes. Le papier filtre Whatman no. 541 soumis à une étape de rinçage avec du H_2SO_4 à 0,01 mol·L^{-1} donnait des résultats similaires à ceux de la membrane de nylon, mais à un coût inférieur à 20 % du coût total du test d'immunobuvardage des colonies original. L'application modifiée de l'immunobuvardage a été testée sur des échantillons de sols argileux non stériles enrichis d'une quantité importante (>10^7 UFC·g^{-1}) des bactéries qui favorisent la croissance des plantes *Pseudomonas fluorescens*, *Azospirillum brasilense* ou *Rhizobium leguminosarum*. Le protocole modifié a permis d'identifier et de récupérer plus de 50 % des cellules inoculées à partir des trois souches, sur un fond de microflore native du sol. Subséquemment, la survie de *P. fluorescens* a été suivie avec succès pendant plusieurs mois après son application sur du riz en culture à Jerilderie, NSW Australie, validant ainsi le procédé.

Mots-clés : PGPR, inoculats, immunobuvardage de colonies, riz, membrane alternative.

[Traduit par la Rédaction]

Introduction

Monitoring the surviving population of plant-growth-promoting rhizobacteria (PGPR) in inoculants and in rhizosphere soil is a key step for improving the quality control and efficacy of inoculant plant-growth-promoting (PGP) products. However, available counting methods are inefficient because of the difficulties in identifying the inoculant bacteria among the indigenous microbiota of carrier media and soil. Therefore, a specific technique that differentiates inocu-

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G. Krishnen. SUNFix Centre for Nitrogen Fixation, Faculty of Agriculture, Food and Natural Resources, Biomedical Building, 1 Central Avenue, Eveleigh, The University of Sydney, New South Wales 2006, Australia; Strategic Resources Research Centre, MARDI Head Quarters, 43400 Serdang, Selangor, Malaysia.
M.L. Kecskés, M.T. Rose, P. Geelan-Small, K. Amprayn, and I.R. Kennedy. SUNFix Centre for Nitrogen Fixation, Faculty of Agriculture, Food and Natural Resources, Biomedical Building, 1 Central Avenue, Eveleigh, The University of Sydney, New South Wales 2006, Australia.
L. Pereg. School of Science and Technology, University of New England, Armidale New South Wales, Australia.

Corresponding author: Ganisan Krishnen (e-mail: ganisan@mardi.gov.my).

lant PGP bacteria from the soil microbiota is needed. Among the simplest techniques available, the combination of dilution plating and antibody based identification method, also known as colony immunoblotting (CIB), has been shown to meet this challenge (Kecskés et al. 2009). Once specific antibodies are available for a certain PGP bacterium, CIB has the potential to evaluate hundreds of samples from large-scale crop field trials in a relatively simple manner. In this technique, bacterial colonies grown on standard dilution plates are transferred onto a membrane and subjected to CIB assay. Three types of membranes are commonly used for enumeration of bacteria from various samples by the CIB assay, including nitrocellulose (Singh et al. 1997; Duez et al. 2000; Grimm et al. 2007), polyvinylidene fluoride (Bhunia and Johnson 1992; Carroll et al. 2000), and nylon (Rodrigue and Lavoie 1990; Kecskés et al. 2007). Nevertheless, the widespread use of current protocols is prohibited by expensive membranes that may represent up to 87% of the cost involved in CIB.

A number of earlier attempts sought to evaluate economical membranes using various blotting techniques. Cost efficient filter papers Whatman No. 1, 40, and 5 were used in dot immunoblotting (Littlewood and Munkres 1972; Tan et al. 1983; Fletcher 1987), colony blot radioimmunoassay (Gulig et al. 1983), and CIB (Rodrigue et al. 1989), respectively. In our preliminary work, with limited success, various economical materials such as glossy photo paper and transparency and filter papers were tested as a nylon membrane (NM) substitute for CIB (Moutouvirin 2007). The aim of this detailed study was to find the best practical membrane replacement material and CIB conditions and validate its use for routine monitoring (quality control) of inoculant PGP bacteria in soil.

Materials and methods

Strains and media

Azospirillum brasilense Sp 245 was kindly provided by Lily Pereg from the University of New England, Armidale, New South Wales, Australia. *Pseudomonas fluorescens* 1N and *Rhizobium leguminosarum* bv. *trifolii* R4 were obtained from the SUNFix Centre, the University of Sydney culture collection. *Azospirillum brasilense* Sp 245 and *P. fluorescens* 1N were grown on modified nutrient (MN) agar or in liquid media with agitation (120 r·min^{-1}) for 48 h at 28 °C, and *R. leguminosarum* bv. *trifolii* R4 was grown on yeast mannitol (YM) agar or in liquid media with agitation (120 r·min^{-1}) for 48 h at 28 °C.

Antisera production, immunoglobulin purification, and immunoglobulin-horseradish peroxidase conjugation

Polyclonal antibodies against whole cells of *A. brasilense* Sp 245, *P. fluorescens* 1N, and *R. leguminosarum* bv. *trifolii* R4 were prepared and supplied by N. Trang from Southern Sub-Institute of Agricultural Engineering and Postharvest Technology (SIAEP), Ho Chi Minh City, Vietnam (Rose et al. 2011). Rabbit immunization, serum collection, and IgG purification by protein A – Sepharose (Sigma) affinity chromatography were conducted according to Kecskés et al. (2009). IgG was conjugated to horseradish peroxidase (HRP) (Sigma) using a sodium periodate method modified from Tsang et al. (1995).

Membrane materials for CIB

Eight types of economical material were screened for their suitability as a replacement for commercial NM in the CIB assay. We tested four types of Whatman filter papers, namely Nos. 1, 115, 5, and 541, three types of transparencies (two of them were surface modified) (Highland brand), namely transparency (T), painted and blotted on unpainted side (TUS), and painted and blotted on painted side (TPS), and a glossy photo paper (GP) (GBC brand). The performance of all economical membranes were compared with a commercial NM (diameter = 82 mm) (Roche). The alternative materials were prepared by using a disk cutter with same size as the NM.

Performance of alternative membrane in CIB

The CIB was carried out according to Duez et al. (2000). Briefly, the pure culture of *A. brasilense* Sp 245 was grown at 28 °C for 48 h in MN broth using 0.5 g of sucrose and 0.5 g of glucose with agitation (120 r·min^{-1}). Two-day-old liquid culture was serially diluted and spread on MN agar and incubated at 28 °C for 48 h. Plates with 30–200 colonies were selected for the CIB assay. Initially the membranes were placed on the plate and pressed with forceps until the whole membrane was completely in contact with the agar surface, followed by blotting for 30 min. Bubble formation in between the membrane and agar surface was avoided by pressing the area with forceps until the air was released out via the edge of membrane. After 30 min, the membranes were lifted using forceps and heat fixed (colony blotted side up) for 30 min at 80 °C. After cooling, membranes were incubated in freshly prepared blocking solution (phosphate-buffered saline (PBS) with 5% skim milk and 0.2% Tween 20 (Sigma), pH 7.4) for 1 h. The membranes were then submerged for 1 h in blocking solution with polyclonal antibody (1:4000) against *A. brasilense* Sp 245 and subsequently washed three times for 5 min with blocking solution. The membranes were incubated with commercial HRP–conjugate (Dako) amended blocking solution (0.75 μL·mL^{-1} blocking solution) for 1.5 h followed by washing three times, twice with washing solution (PBS with 0.2% Tween 20, pH 7.4) and once with PBS alone. All the blocking, antibody reactions, and washing steps were conducted on a rotary shaker (85 r·min^{-1}). Colour was developed by incubation with HRP-substrate (urea peroxide (Sigma) and cyclodextrin (Sigma) solution, pH 5) and chromogen (10 mg·mL^{-1} TMB (3,3′,5,5′-tetramethylbenzidine) (Sigma) in dimethyl sulfoxide (DMSO) (Sigma). Finally, the reaction was stopped by spraying with 1 mol·L^{-1} H_2SO_4 (Univar) on the membrane, and the membrane was rinsed with distilled water and air dried. Each treatment consisted of 3 replicates.

The colony recovery rate was calculated based on the number of purple colony imprints recovered on the membrane, relative to the number of colonies on the original plate. The scoring of colony imprint quality and membrane background stains was scored by 5 respondents. The colony imprints were ranked from poorest quality (1) to best (4), and the membrane background stains from highest (1) to lowest (4), respectively.

Optimization of membrane destaining

The best performing economical membrane in colony recovery and imprint quality, FP541, was subjected to numer-

ous treatments to remove its intense green background. The tests included increasing the amount of blocking solution (8 mL instead of 4 mL), the blocking time (2 h instead of 1 h), the concentration of Tween 20 in blocking and washing solution (0.5% and 1% instead of 0.2%), and decreasing the concentration of H_2SO_4 as background decolourizing agent (0.05, 0.1, and 0.5 mol·L^{-1} instead of 1 mol·L^{-1}), the concentration of urea peroxide in substrate solution (22.5 mg·mL^{-1} substrate instead of 45 mg·mL^{-1} substrate), and the duration of the substrate-chromogen reaction (0.5, 1, and 3 min instead of 5 min).

After rinsing the FP541 membrane with distilled water, an extra rinse step with household bleach (0.01%, 0.1%, 0.5%, and 1%), household detergent (Laundrite Laundry Powder) (0.05%, 0.1%, 0.5%, and 1%), or diluted H_2SO_4 (0.001, 0.01, 0.1, and 0.5 mol·L^{-1}) was also completed. For all these treatments, the control was not subjected to a second rinse with any of the material tested above. Each treatment consisted of three replicates.

Recovery of spiked inoculant-PGP bacteria from a rice field soil using FP541-based CIB

Inoculant-PGP strains *A. brasilense* Sp 245, *P. fluorescens* 1N, and *R. leguminosarum* bv. *trifolii* R4 were grown as described above. The soil for this study was collected from a rice field at Rice Research Australia Pty. Ltd., Jerilderie (RRAPL), New South Wales, Australia, and was ground using a mortar and pestle (0.5–1.5 mm in size). In a sterile Petri dish, 10 g of soil was spiked with 3 mL of 48 h grown liquid inoculant PGP strains, mixed thoroughly, sealed with parafilm, and left to stand for 1 h. Subsequently, 2 g of the soil–inoculant mixture was suspended with sterile PBS with a final volume of 20 mL. Serial dilutions were plated on MN agar for *A. brasilense* Sp 245 and *P. fluorescens* 1N and YM agar for *R. leguminosarum* bv. *trifolii* R4. All the plates were incubated at 28 °C for 48 h, and the colonies grown on the plates were counted and blotted using FP541. The CIB was conducted on a rotary shaker (85 r·min^{-1}) as mentioned above by using IgG–HRP conjugate diluted in blocking solution (1:200 for *A. brasilense* Sp 245 and *P. fluorescens* 1N; and 1:1000 for *R. leguminosarum* bv. *trifolii* R4) with omission of commercial HRP conjugate reaction. The background stains were eliminated by rinsing the membranes in 0.01 mol·L^{-1} H_2SO_4. Each treatment consisted of three replicates.

Monitoring of inoculated inoculant PGP strain *P. fluorescens* 1N from field-grown rice

Rice cultivation

A field experiment was carried out at the RRAPL to study the effect of inoculant PGP strains (among others, *P. fluorescens* 1N) on rice to partially replace chemical fertilizer. This trial consisted of 6 inoculant PGP treatments and a control.

The size of each plot was 37.5 m^2 (2.5 m × 15 m). As common practice, the plot was wet-up 1 week after sowing and subsequently the soil moisture maintained until flooding. Chemical fertilizers (150 kg N·ha^{-1} as urea (270 kg urea·ha^{-1}) (Incitic/Pivot) and 60 kg P·ha^{-1} (91 kg·ha^{-1} of P as monoammonium phosphate) (N10:P22:K0:S1.5; Incitic/Pivot)) were applied just before flooding. Once the field had been flooded, the water level was increased according to plant size to ensure that the root regions were submerged in water. Three weeks before harvesting, the water was drained off and the crop was left to dry.

Each plot was sown with 563 g (150 kg·ha^{-1}) rice variety Jarrah using a cone seeder. At this time, biofertilizer inoculants were applied to all treatments plots with (10^{13} CFU·ha^{-1}) except for all the controls during sowing via a sprayer tubing system attached to the machine. Subsequently, the plots were wet-up 1 week after sowing for all the treatments and control except treatment 2. For treatment 2, the plot was wet-up immediately after sowing.

A second application of biofertilizer inoculant was conducted 15 days after sowing (DAS). The second application of biofertilizer was conducted using an irrigation nozzle and booster pump in the plot. Just before the plot was flooded (day 18), the chemical fertilizer monoammonium phosphate (N10:P22:K0:S1.5) was applied for all the treatments and controls (341 g·plot^{-1}). The N-fertilizer urea was applied to all the treatments at a rate of 25% of the recommended rate (334 g·plot^{-1}). For the controls, various rates ranging from 0% to 100% of recommended rate of urea were applied. The rates of urea used were 0, 334, 668, and 1336 g·plot^{-1} for controls 1, 2, 3, and 4, respectively. All the treatments and controls contained six replicates.

Sampling of rice plants for PGPR enumeration

Three rice plants were carefully extracted from the soil of each plot and the root portion was excised from the bulk soil. The roots were frozen using liquid nitrogen and transferred to the University of Sydney in a portable freezer.

Isolation and plating of PGPR from soil

The frozen root with bulk soil was thawed and the roots were cut using sterile scissors. The roots were teased apart and shaken to discard any excessive soil from the roots. Two grams of the root with a thin layer of soil (rhizosphere) was transferred into a sterile 50 mL Falcon tube and supplemented with sterile phosphate buffer saline (PBS, pH 7.4) to attain a volume of 20 mL. Ten sterile glass beads were added and homogenized by vortex for 1 min at the maximum speed. The tube was left standing for 5 min and the soil suspension was serially diluted (up to 10^{-5}) before plating on the MN agar plates. Diluted soil suspensions (100 μL) were spread on agar medium and incubated at 28 °C for 48 h. The PGPR in the soil suspension were detected and counted using CIB with IgG–HRP conjugate as mentioned above.

Experimental design and statistical analysis

A completely randomized design (CRD) was used for the economical membrane screening and recovery of bacteria from spiked soil samples and a randomized complete block design (RCBD) was applied for the arrangement of experimental plots. The statistical analysis employed in this work involved modelling of binomial proportions for colony recovery rate and ordinal regression analysis for both colony imprint quality and background stains scores. Recovery rate from soil spiked with inoculant PGP strains was analyzed by Student's *t* test, whereas ANOVA was carried out for enumerating inoculant PGP strains from field-grown rice.

Fig. 1. *Azospirillum brasilense* Sp 245 colony recovery rate of eight economical membranes compared with nylon membrane. FP1, FP115, FP5, and FP541, Whatman filter papers Nos. 1, 115, 5, and 541, respectively; NM, nylon membrane (Roche); GP, glossy photo paper (GBC brand); T, transparency without modification; TUS, transparency painted with white colour and blotted on unpainted side; TPS, transparency painted with white colour and blotted on painted side (all the transparencies used were Highland brand).

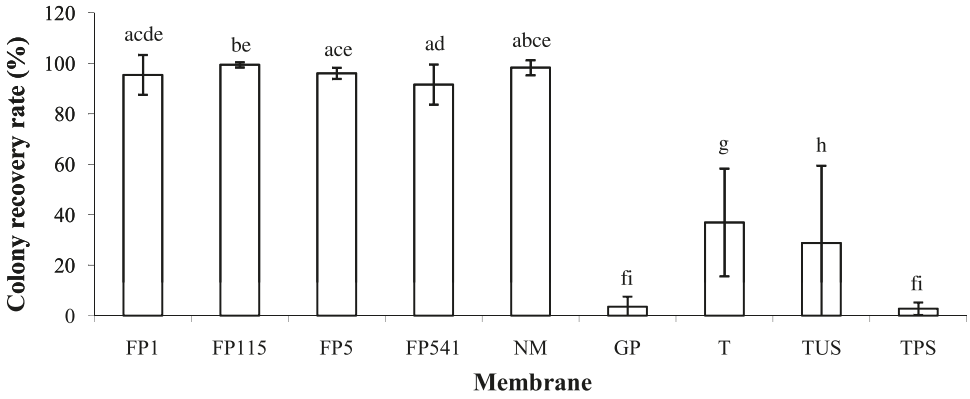
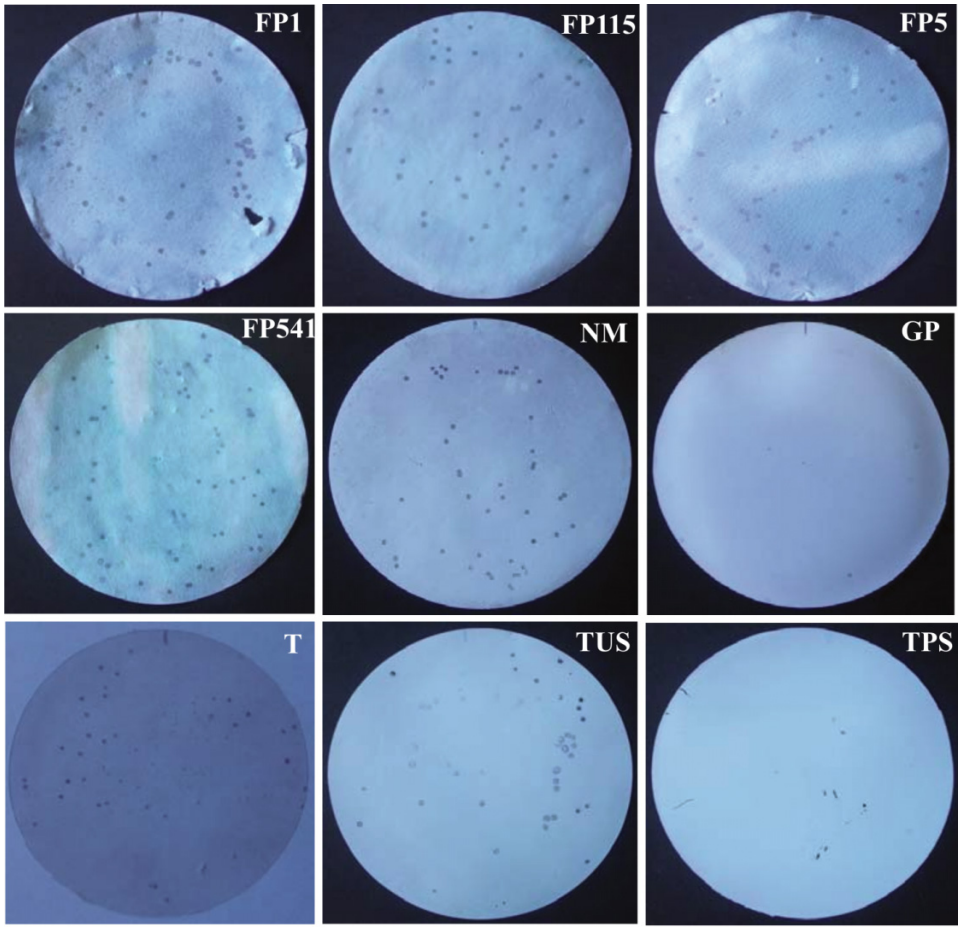


Fig. 2. Immunoblotting performance of eight economical membranes compared with nylon membrane. FP1, FP115, FP5, and FP541, Whatman filter papers Nos. 1, 115, 5, and 541, respectively; NM, nylon membrane; GP, glossy photo paper; T, transparency without modification; TUS, transparency painted with white colour and blotted on unpainted side; TPS, transparency painted with white colour and blotted on painted side.



Results and discussion

CIB performance of alternative consumables compared with nylon membrane

The colony recovery rate, imprint quality, and membrane background stains were evaluated on eight types of alternative consumables derived from three materials, filter papers, transparencies, and glossy photo paper in comparison with NM, as the standard commonly used for CIB.

The colony recovery rate was considered to be the most important criterion for selection of efficient economical membrane as a replacement for nylon membrane. Generally, all

Fig. 3. Scoring of *Azospirillum brasilense* Sp 245 colony imprint of eight economical membranes compared with nylon membrane. FP1, FP115, FP5, and FP541, Whatman filter papers Nos. 1, 115, 5, and 541, respectively; NM, nylon membrane; GP, glossy photo paper; T, transparency without modification; TUS, transparency painted with white colour and blotted on unpainted side; TPS, transparency painted with white colour and blotted on painted side. The colony imprints score from worst (1) to best (4). Bars represent mean ± SD of three replicates and bars with the same letter on top are not significantly different at $p = 0.05$.

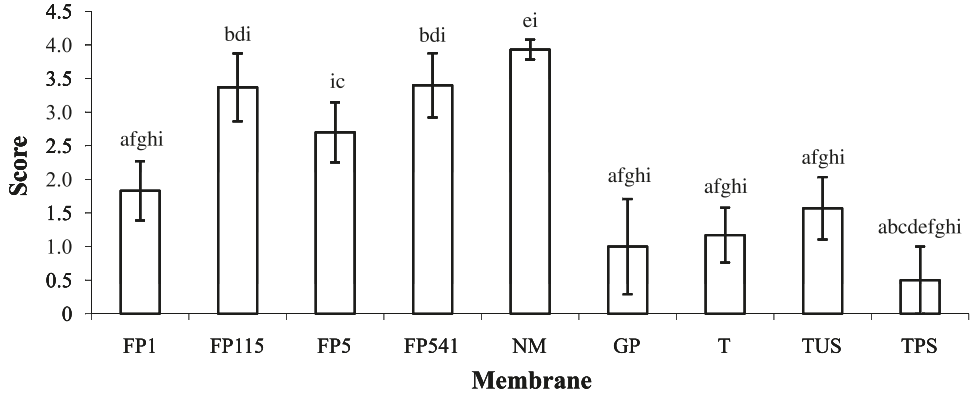


Fig. 4. Scoring of background stains of eight economical membranes compared with nylon membrane. FP1, FP115, FP5, and FP541, Whatman filter papers Nos. 1, 115, 5, and 541, respectively; GP, glossy photo paper; T, transparency without modification; TUS, transparency painted with white colour and blotted on unpainted side, TPS, transparency painted with white colour and blotted on painted side; and NM, nylon membrane. Background stain scores are as follows: 4, no stains; 3, very little stains; 2, medium level stains; and 1, intense background stains. Bars represent mean ± SD of three replicates and bars with the same letter on top are not significantly different at $p = 0.05$.

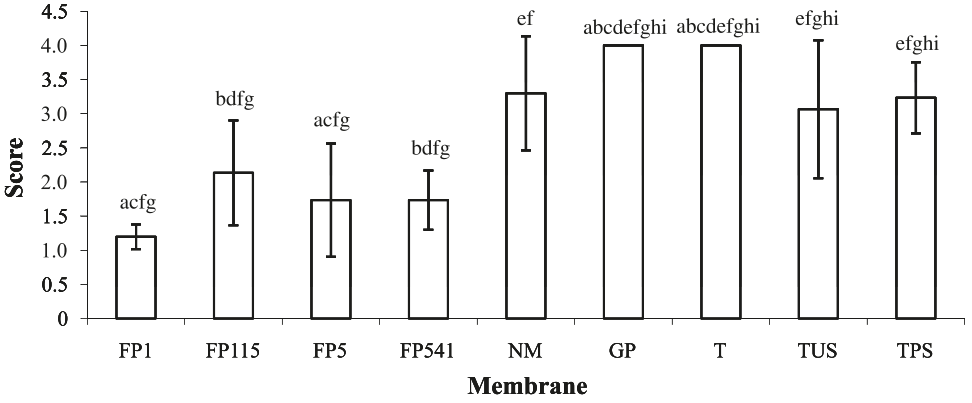
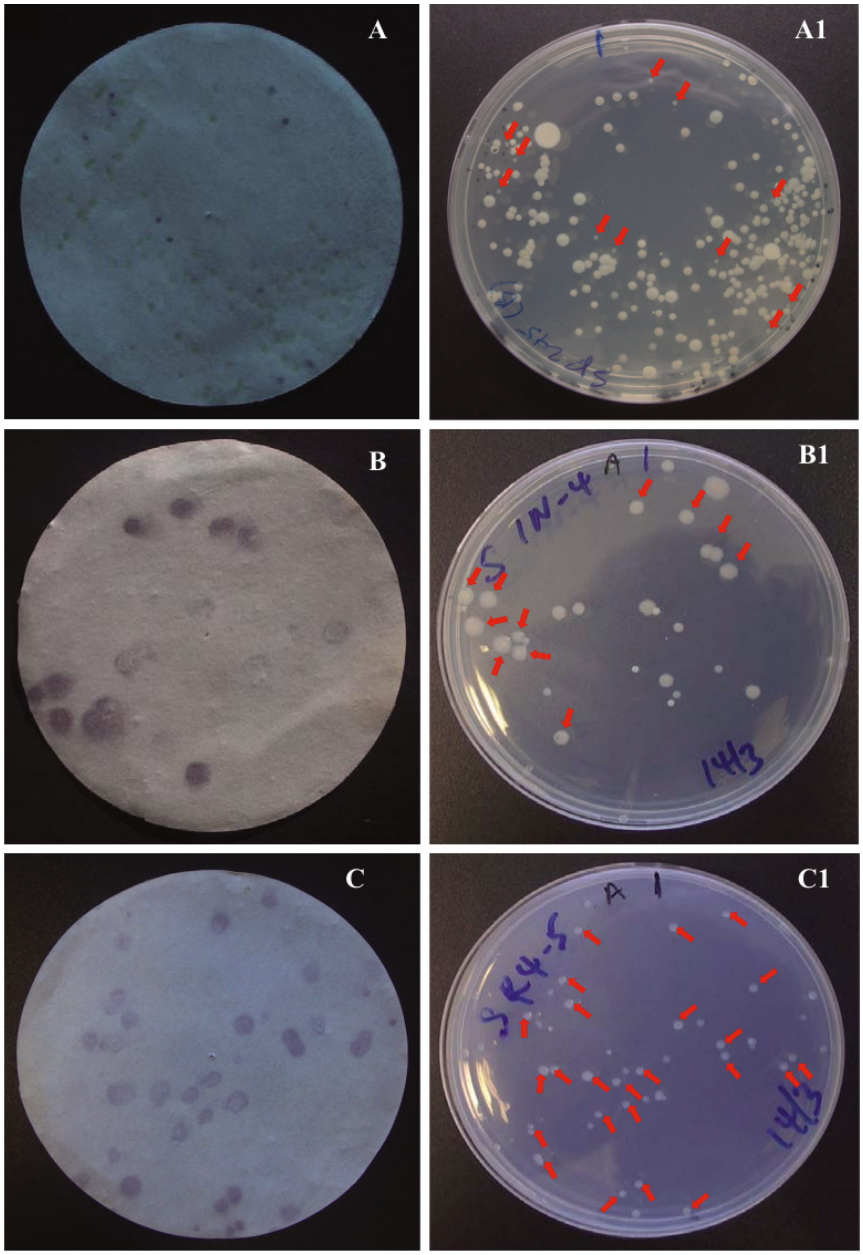


Table 1. The recovery rate of added plant-growth-promoting rhizobacteria (PGPR) inoculants on rice field soil using total plate count and colony immunoblotting (CIB).

Method	Treatment	*Azospirillum brasilense* Sp 245	*Pseudomonas fluorescens* 1N	*Rhizobium leguminosarum* bv. *trifolii* R4
	PGPR added (CFU·3 mL^{-1})	4.71×10^8	6.0×10^8	8.91×10^9
Plate count	Inoculated (T_{pt})	4.45×10^8	4.5×10^7	5.65×10^9
	Uninoculated (T_{pc})	3.0×10^7	2.7×10^7	3.6×10^8
	Recovery (%)[a]	88.0	3.0	59.4
CIB	Inoculated (T_{ct})	2.44×10^8	1.71×10^7	2.82×10^9
	Uninoculated (T_{cc})	1.2×10^7	0	0
	Recovery (%)[b]	49.3	2.9	31.6
Percentage of total plate count as a positive CIB detection (%)[c]				
		60	95	53

[a]Calculated as $[(T_{pt}–T_{pc})/\text{added PGPR}] \times 100\%$.
[b]Calculated as $[(T_{ct}–T_{cc})/\text{added PGPR}] \times 100\%$.
[c]Calculated as $[(T_{ct}–T_{cc})/[(T_{pt}–T_{pc})] \times 100\%$.

Fig. 5. Recovery of biofertilizer strains from soil spiked with biofertilizer inoculant. Immunoblot of (A) *Azospirillum brasilense* Sp 245, (B) *Pseudomonas fluorescens* 1N, and (C) *Rhizobium leguminosarum* bv. *trifolii* R4 using FP541. The corresponding colonies on the plate media for the immunoblot were given at A1, B1, and C1, respectively, for strains *A. brasilense* Sp 245, *P. fluorescens* 1N, and *R. leguminosarum* bv. *trifolii* R4. The respective colonies on plate media that produced a purple imprint (positive test) were marked with arrows.



the filter papers tested, FP1, FP115, FP5, and FP541, gave good colony recovery rates comparable with the standard NM (Figs. 1 and 2). The colony recovery rate of filter papers ranged between 91% and 99.9% with FP115 demonstrating the best performance. Differences in colony recovery rates between filter papers and control NM were as low as 3.6%.

The other economical membranes, transparency (T), surface modified transparencies (TUS and TPS), and glossy photo paper (GP) did not perform well, exhibiting extremely low colony recovery rates between 2.8% and 36.9%. All of these were significantly lower than the recoveries observed for nylon and the filter papers.

To a certain extent, this result confirms that of Rodrigue and Lavoie (1990) who reported that filter papers Nos. 1 (FP1) and 5 (FP5) could obtain recovery rates as high as 97% and 98%. In our work, all the filter papers tested recorded a similar level of recovery; however, we obtained higher values, 98.3% (Roche) vs. 66% (Nylon 66) for the nylon membranes (Rodrigue and Lavoie 1990).

Transparencies (with and without surface modified) and glossy photo paper did not perform well. Most of the colonies on these membranes detached either during shaking in solution (blocking, washing, and primary and secondary antibodies reactions) or during final rinsing with water. We ex-

Table 2. *Pseudomonas fluorescens* 1N count in rhizospheric soil from rice plants from plots inoculated with *P. fluorescens* 1N and a control plot sampled 15, 117, and 147 DAS.

	PGPR count using CIB (log_{10} CFU·g^{-1} rhizospheric soil)		
Treatment	15 DAS	117 DAS	147 DAS
Control[a]	3.63±3.13	3.54±3.07	3.54±3.07
Treatment 1[b]	5.53±0.33	5.49±0.23	5.76±0.23
Treatment 2[c]	5.53±0.28	6.11±0.04	6.17±0.41

Note: Values represent mean ± SD of three replicates. PGPR; plant-growth-promoting rhizobacteria; CIB, colony immunoblotting; DAS, days after sowing.

[a]*Pseudomonas fluorescens* 1N from control plots (no biofertilizer application).

[b]*Pseudomonas fluorescens* 1N from treatment 1 (plot was kept wet up to 2 weeks).

[c]*Pseudomonas fluorescens* 1N from treatment 2 (plot was flooded after the seeds germinated).

pect that the smooth surface and hydrophobicity of these membranes did not provide enough adhesion to retain the colonies.

Good colony imprint colouration and contrast on a stainless background are also important qualities for accurate immunoblotting. As such, the imprint quality and the background stain intensity were scored by five respondents during the screening of alternative membranes. Results from these assessments are given (Figs. 2 and 3). The best colony imprints of *A. brasilense* Sp 245 were observed on NM, followed by FP115 and FP541, with qualitative scores above 3. In contrast to the study of Rodrigue and Lavoie (1990), FP5 did not perform well with a lower score of 2.7. All other membranes, filter papers FP1 and FP5, transparencies, and glossy photo paper recorded scores below 2.7. The poor imprint colouration of the transparencies and glossy paper resulted from substantial colony detachment, which left very little residue for substrate and chromogen reaction. Low membrane background stain intensity is important for being able to differentiate positive and negative colouration in CIB. In our previous work on soil spiked with target bacterial cells (Krishnen et al. 2007), purple imprints indicated a positive test whilst pale green or colourless imprints indicated a negative test. Stains developing on the nonimprinted background could therefore hinder positive test detection.

The membrane background stain for all tested membranes is given in Fig. 4 and the respective photos are given in Fig. 2. All the filter papers recorded scored poorly, showing medium or intense green background (scores between 1.2 and –2.1). All other materials, including the transparencies, glossy photo paper, and nylon (control) showed less background stain, with scores ranging from 3.1 to 4.0. The best membranes without any stains were transparency (T) and glossy photo paper with scores of 4.0. Rodrigue and Lavoie (1990) encountered intense bluish background stains that interfered with the detection of a positive test. Similar findings were also reported by Moutouvirin (2007) on filter paper membrane with intense green stains. Surprisingly, nylon 66 recorded the most intense background stains in Rodrigue and Lavoie (1990), which contrasts with our results that nylon (Roche) was one of the better performing membranes with very low stains. Removal of these background stains was deemed crucial for efficient positive detection.

This work was designed to select the most economical membrane material that could also match the criteria of a high colony recovery rate and high colony imprint resolution with low or no background stains at all. Unfortunately, none of the membranes under consideration fulfilled all three criteria. Therefore, alternative membranes were selected based primarily on the highest performance in colony recovery and imprint. Despite their intense background stains, membranes FP115 and FP541 were short listed as economical candidates because these two materials performed as well as the standard NM with respect to high colony recovery and good imprint quality. The membrane FP541 was chosen for further study to remove background stains because FP115 was no longer commercially available.

Enhanced CIB protocol for using FP541

Various changes in the immunoblotting protocol were imposed to remove the intense green residual stain from the background of FP541. Of the protocol modifications investigated, the only positive result was the elimination of the green background stains when the membrane was rinsed with dilute H_2SO_4. Rinsing the membrane with any concentration of H_2SO_4 exceeding 0.001 mol·L^{-1} eliminated the background stains whilst the colony imprints remained purple in colour. Any increase in acid concentration over 0.1 mol·L^{-1} resulted in brown stains, due to the excess residual substrate–chromogen solution on the membrane. The optimum FP541 colony immunoblot was obtained when the material was rinsed with 0.01 mol·L^{-1} acid, showing easily resolved colony imprints on a stainless background.

FP541 based CIB enumeration of spiked inoculant-PGP bacteria from soil

Based on conventional plate counting, the recovery for each strain ranged from 3% for *P. fluorescens* to 88% for *A. brasilense* Sp 245 (Table 1). The low recovery rate, particularly for *P. fluorescens* 1N, may be the result of a number of reasons. First, it was observed that during inoculation the soil absorbed the liquid inoculant quickly and became very sticky, hindering an even distribution of the biofertilizer strains in clay soil. Furthermore, it is possible that clumping of bacterial cells together, along with adherence to soil particles, may reduce the extraction efficiency and apparent recovery. For example, Ozawa and Yamaguchi (1986) observed that *Bradyrhizobium japonicum* strains have a strong tendency to bind to clay particles. Indeed, Picard et al. (1992) recorded 20%–120% bacterial recovery when PGPR were spiked on silt loam soil and counted by most probable number–PCR from the DNA extracted from soil. It is possible that better recoveries could be obtained on sandier soil types, rather than the clay that was used in this experiment. Nevertheless, future investigations could explore other options for increasing the extraction efficiency of viable cells from all soils, through modifications to the extraction buffers and (or) homogenization procedures.

Despite the poor overall recovery as indicated by the plate counts, the modified CIB protocol was able to effectively distinguish the spiked strains from the indigenous soil microbiota by the formation of purple colony imprints (Fig. 5). Interestingly, positive detections for *A. brasilense* Sp 245 were made in the nonspiked control soils. It is likely that the

Table 3. Cost of single immunoblotting assay using economical FP541 membrane compared with commercial nylon membrane using indirect and direct ELISA.

	Indirect ELISA based CIB			Direct ELISA based CIB	
		Price (cents)			Price (cents)
	Amount or volume	Nylon	FP541	Amount or volume	FP541
Membrane	1 unit	625	36	1 unit	36
Subtotal		625	36		36
Reagents and chemicals					
Blocking solution	24 mL	3.3	3.3	8 mL	1.1
Washing solution	8 mL	0.8	0.8	8 mL	0.8
PBS	4 mL	0.2	0.2	4 mL	0.2
Primary antibody (polyclonal)	1 µL	19	19	—	—
Secondary antibody (HRP)	3 µL	58.5	58.5	—	—
Antibody–HRP conjugate	—	—	—	20 µL	65
Substrate	2.85 mL	2.8	2.8	2.85 mL	2.8
Chromogen	0.15 mL	9.5	9.5	0.15 mL	9.5
Sulphuric acid (1 mol·L^{-1})	0.5 mL	0.1	0.1	0.5 mL	0.1
Sulphuric acid (0.01 mol·L^{-1})	20 mL	—	0.04	20 mL	0.04
Subtotal		94.24	94.244		79.54
Total		719.24	130.24		115.5

Note: All the dollar values are given in Australian currency. HRP, horseradish peroxidase.

antiserum used contained some cross-reactivity against other related strains and, therefore, was able to detect indigenous azospirilla existing in the rice field soil used in this experiment. A range of strains of azospirilla have previously been isolated from various locations at New South Wales, Australia (Han and New 1998), supporting this possibility.

Tracking of inoculated biofertilizer strain *P. fluorescens* 1N from field-grown rice

The fate of biofertilizer strain *P. fluorescens* 1N applied on the field-grown rice was tracked using FP541 based CIB. The *P. fluorescens* 1N population in the rhizosphere of plants from both treatment 1 and 2 were similar after 15 DAS with ~$\log_{10}$ 5.53 CFU·g^{-1} rhizospheric soil being recovered (Table 2). However, after 117 and 147 DAS, the bacterial population in the rhizosphere was slightly higher for treatment 2 compared with treatment 1. The *P. fluorescens* population in the control plot remained unchanged and was persistently low in comparison with treatments. The similar population count for 15 DAS probably relates to a similar colonization rate of the rice rhizosphere. The higher population of *P. fluorescens* 1N in treatment 2 on 117 and 147 DAS suggests that the continuously wet condition may improve bacterial survival and multiplication.

Such a result is in contrast with the continuous decrease in the *Pseudomonas fluorescens* WCS374 population from 10^8 to 10^6 cells that was observed on wheat cultivated silt loam (van Elsas et al. 1986), which was kept wet throughout the experiment. However, the persistence of the population number in treatments 1 and 2 for 147 days is a good sign that the bacteria survived in the rhizospheric soil similarly to other published works on different PGPR strains (van Elsas et al. 1986; Araujo et al. 1994; Mubassara et al. 2008; Kecskés et al. 2009).

Costing of immunoblotting

Immunoblotting is regarded as a reliable method for differential enumeration of bacterial populations in the various microniches such as animal and human faeces (Duez et al. 2000; Szakál et al. 2003; Grimm et al. 2007), food samples (Carroll et al. 2000), bio-inoculant (Olsen and Rice 1989), and soils (Kecskés et al. 2007). Nylon membranes have already shown their adequacy for this purpose, but their elevated price is a major drawback for routine application. The ultimate goal of this study was to screen economical membranes as replacements to nylon and reduce the total cost of immunoblotting. Table 3 shows the cost comparison of immunoblotting when using nylon vs. FP541 membrane, both using indirect and direct ELISA. The cost for all reagents used in both nylon and FP541 based immunoblotting totalled $0.94 per assay for indirect ELISA and $0.80 for direct ELISA. The cost of a single NM was $6.25, while the FP541 only cost $0.36. The total cost for a single immunoblot assay using nylon and FP541 membranes were $7.20 and $1.30, respectively, for indirect ELISA based CIB. The cost for direct ELISA based CIB using FP541 only cost $1.16. The new protocol developed here thus represents a cost saving of over 80% for routine detection of bacteria using immunoblotting procedures.

Conclusion

In conclusion, filter paper Whatman No. 541 proved an economical replacement for the NM, showing repeatable colony recovery and clear imprint quality. The green background stains that develop on this alternative material under current protocols, interfering with determination of a positive test, were eliminated by the addition of a 0.01 mol·L^{-1} H_2SO_4 acid rinse step. Our aim to reduce the CIB cost was achieved by replacing the use of NMs with FP541, reducing

the cost to one-fifth the original value without a loss of performance. Further, the usage of IgG–HRP conjugate, rather than a second anti-rabbit–HRP conjugate, made this modified CIB more rapid. We showed that the optimized CIB method can be used for field-level monitoring. Because the optimized CIB assay is a relatively simple and cost-effective technique, we believe it will be useful for future studies in which selected microbial strains are augmented into environmental matrices outside the laboratory.

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