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ORIGINAL PAPER

The survival of plant growth promoting microorganisms in peat inoculant as measured by selective plate counting and enzyme-linked immunoassay

Michael T. Rose · Rosalind Deaker · Sabrina Potard · Cuc Kim Thi Tran · Nga Thuy Vu · Ivan R. Kennedy

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Abstract Achieving specific counting of plant growth promoting (PGP) microorganisms maintained at high numbers in inert carriers such as peat is an important objective for the inoculation of field crops such as cereals. In this paper, methods based on selective media together with strain-specific counting using enzyme-linked immunoassay (ELISA) were examined. Selective plate counting was developed by screening four commercial PGP biofertiliser strains for resistance to antibiotics. ELISAs for each strain were developed and calibrated by purifying polyclonal antibodies, testing sample pre-treatment strategies, and investigating the effect of culture age on standard curves. Selective plate counting proved to be more accurate than the ELISA methodology, confirming that all microbial strains survived for at least 1 month in sterile peat without loss in viable numbers, and further demonstrated growth inhibition of the strain *Candida tropicalis* HY when co-inoculated with the other strains *Pseudomonas fluorescens* 1 N, *Bacillus amyloliquefaciens* E19 and *Bacillus subtilis* B9. This is the first known study to have investigated the dynamics of PGP microorganisms in multi-strain inoculants and demonstrates the utility and hitherto unmentioned drawbacks of two different low-cost counting methods for biofertiliser quality control. Such information is vital for the adoption and success of non-rhizobial PGP biofertilisers for sustainable agriculture.

Keywords Biofertiliser · Rice · Quality control · Polyclonal antibody · Antibiotics · Pseudomonas · Sustainable agriculture

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M. T. Rose · R. Deaker · S. Potard · I. R. Kennedy
SUNFix Centre, Faculty of Agriculture, Food and Natural Resources, The University of Sydney, Sydney, NSW, Australia

S. Potard
ESMISAB, University of Brest, Brest, France

C. K. T. Tran
Institute of Agricultural Sciences for Southern Vietnam, Ho Chi Minh City, Vietnam

N. T. Vu
Institute for Agricultural Environment, Tu Liem, Hanoi, Vietnam

Present Address:
M. T. Rose (✉)
Japanese International Research Centre for Agricultural Sciences, Tsukuba, Ibaraki, Japan
e-mail: mrose@affrc.go.jp; micktrose@gmail.com

Introduction

There is strong evidence that plant growth promoting (PGP) microorganisms can increase plant access to nutrients, suppress pathogens and directly stimulate plant growth; increasing crop yields and reducing requirements for chemical fertilizers and pesticides (see reviews by Vessey 2003; Bhattacharjee et al. 2008; Lugtenberg and Kamilova 2009). Successful inoculation of PGP microorganisms can result in higher economic returns to farmers and reduced losses of chemical fertilizers and pesticides to the wider environment. Nevertheless, despite tangible market interest in these organisms (Thakore 2006), the adoption of inoculant PGP technology by farmers, agronomists and government agencies has in general been

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limited (Lucy et al. 2004). There are a number of possible explanations for this, but one reason is the sheer diversity of PGP inoculant strains available combined with a lack of quality control and regulation guidelines (Kennedy et al. 2004; Berg 2009). This, along with an inherent variability in environmental response, means that PGP by microbial inoculants is currently less predictable and lacks the assured returns on investment when compared with chemical fertilizers and pesticides (Lucy et al. 2004).

The enumeration of specific PGP organisms is crucial for the evaluation of inoculant product effectiveness, with a minimum number of organisms required for effective inoculation (Deaker et al. 2004; Bullard et al. 2005). Only one literature reference could be found for the quality of a commercial, non-rhizobial inoculant product, wherein the bacteria claimed to be present could not be cultured and the product had little effect on three ornamental plant species (Elliott and Broschat 2002). Recently a number of studies have reported greater and more stable PGP when using biofertilisers containing a mixture of beneficial microorganisms rather than single strains (Nguyen et al. 2003; Domenech et al. 2006; Phan et al. 2009). Multi-strain biofertilisers can target numerous PGP pathways and are proposed to be more robust under different environmental conditions (Domenech et al. 2006). Of particular importance is the interaction and survival of strain mixtures over time in the product to determine compatibility and shelf life, and the enumeration of PGP cell numbers after application to determine their persistence in the field.

A number of methods are available for the routine identification and quantification of microorganisms. The standard method in most applied microbiology laboratories combines plate counting with selective or differential media. This requires prior knowledge of particular biochemical requirements and morphological characteristics of the organism/s of interest, in order for correct identification and quantification amidst potential background microbial populations; often antibiotics are incorporated as one of the primary selective pressures (Gomez et al. 1995). The popularity of plate counting as a standard method results from its reliability, low capital cost, low ongoing cost and ease of modification and optimization for diverse strains. A particular strength of this method is the fact that it only measures viable cells (see, for example, Pujol et al. 2006; Reichert-Schwillinsky et al. 2009). As inoculants are necessarily produced using culturable organisms, plate counting is a valid approach; however, it is labour intensive, not all viable cells are culturable and depending on the time required for growth and colony formation of specific microbial strains, assays are generally no shorter than 24 h.

Immunological techniques, such as ELISA, can be faster and less laborious than plate counting for selective enumeration (Schloter et al. 1995). Antisera raised against antigens present in microbial cells can be used directly or purified and used in qualitative or quantitative assays. ELISA-based methods are particularly popular for routine monitoring purposes in medical and food microbiology applications, because of their relatively low cost (e.g. Alinovi et al. 2009), simplicity of use and rapid turnaround (e.g. Goodridge et al. 2003). Although immunological methods do not generally distinguish between viable and non-viable cells, attempts have been made to incorporate additional steps such as vital staining (Olsen and Rice 1996) or fermentation (Mijajlovic et al. unpublished) to determine the extent of cell viability. Immunological techniques have been used to evaluate the quality of rhizobial inoculants (Nambiar and Anjaiah 1985; Lochner et al. 1988) and localize and quantify PGPR in soil–plant systems (Levanony et al. 1987; Silva-Froufe et al. 2009), but references to their use for evaluating the quality of non-rhizobial inoculants are limited (Bashan and Gonzalez 1999). Given their greater diversity, associative (rather than symbiotic) nature, and poorer nutritional and serological characterization as inoculants to date, we believe a focus on non-rhizobial compared to rhizobial inoculants currently deserves attention.

PGP microbial inoculants are often produced through the process of solid state fermentation in sterile or non-sterile peat (Deaker et al. 2004) or clay soil with a high organic content (Nguyen et al. 2003). It is essential to be able to specifically quantify the number of viable cells of each microorganism per unit weight of inoculant to determine inoculum potential at different application rates so that field results may be adequately interpreted. The aim of this experiment was to measure the survival of four commercial PGP microorganisms in peat culture, either alone or together as a mixed inoculant. A further aim was to optimize the enumeration of each strain with an ELISA-based assay, and assess the suitability of this method against a selective plate count.

Materials and methods

Bacterial strains and culture conditions

Microorganisms from the biofertiliser BioGro were obtained from Nguyen Thanh Hien, Biofertilizer Action Research Center (BARC), Hanoi, Vietnam. These were identified (Kecskés et al. 2008) as *Pseudomonas fluorescens* (1 N), *Bacillus subtilis* (B9), *Bacillus amyloliquefaciens* (E19) and *Candida tropicalis* (HY). All strains were maintained on nutrient agar or grown in nutrient broth when required for 48 h.

Antibiotic resistance of BioGro strains and development of selective media

Antibiotic resistance of BioGro strains was measured using antibiotic medium no. 3 (Oxoid, Adelaide) supplemented, after autoclaving, with filter sterilised antibiotics to achieve different final concentrations (Table 1). A bacterial suspension of each strain was prepared by adding several bacterial colonies to 1 ml of sterile deionised water and vortexing for 1 min. Agar plates were divided into sections and several strains were applied to each plate using an inoculating loop. Growth was assessed after 24 and 48 h and scored according to growth intensity.

Antisera production and IgG purification

Polyclonal antibodies against all strains were raised in New Zealand White rabbits at the Postharvest Technology Institute, HCMC, Vietnam by Nguyen Thi Trang. Broth cultures of each strain were grown for 48 h, centrifuged and the cell pellet washed 3 times in 0.85% sterile saline. The final resuspension was diluted to an optical density equivalent to 10^8 cells ml^{-1} and incubated for 1 h at 95°C. Each heat-killed suspension was subsequently emulsified in Freund's complete for the first immunisation or stored frozen in incomplete adjuvant until required for subsequent immunisations. After an initial injection, a booster injection was given monthly for 3 months. At this time, 20 ml of blood from each rabbit was collected by bleeding from the ear, centrifuged to remove cellular bodies, and the serum used to check for specific antibody production by agglutination testing against the specific whole-cell antigen. Immunoglobulins (IgG) were purified from antiserum using Protein A-Sepharose affinity chromatography provided by Sigma (Sydney, Australia).

Table 1 Bacterial growth on antibiotic-supplemented media[a]

Antibiotic	Concentration ($mg\ l^{-1}$)	HY		1 N		B9		E19	
Incubation time (h)		24	48	24	48	24	48	24	48
Ampicillin	20	3	3	3	3	0	0	0	0
	50	3	3	3	3	0	0	0	0
	100	3	3	3	3	0	0	0	0
Chloramphenicol	5	3	3	2	2	0	0	0	0
	10	3	3	1	1	0	0	0	0
	20	3	3	0	<1	0	0	0	0
Gentamicin	5	3	3	0	1	0	0	0	0
	10	3	3	0	0	0	0	0	0
Kanamycin	10	3	3	0	0	0	0	0	0
	20	3	3	0	0	0	0	0	0
	40	3	3	0	0	0	0	0	0
Spectinomycin	10	3	3	3	3	3	3	3	3
	20	3	3	2	2	2	2	2	2
	40	3	3	1	2	1	2	1	2
Streptomycin	10	3	3	3	3	1	3	0	<1
	20	3	3	2	3	1	1	0	0
	40	3	3	2	3	0	0	0	0
Tetracycline	10	3	3	0	0	0	0	0	0
	20	3	3	0	0	0	0	0	0
	40	3	3	0	0	0	0	0	0
Vancomycin	10	3	3	3	3	0	0	0	0
	20	3	3	3	3	0	0	0	0
	40	3	3	3	3	0	0	0	0
Cycloheximide	100	0	0	3	3	3	3	3	3
	200	0	0	3	3	3	3	3	3
	500	0	0	3	3	3	3	3	3

[a] 0 = no growth. Increase in numbers (from 1 to 3) indicates increase in growth intensity

Immunoassay reagents

Concentrated antibody stock (as prepared above) was stored in sealed plastic vials at −20°C. Working antibody solutions were prepared at concentrations as needed by diluting concentrated stock with phosphate buffer saline (PBS). PBS contained 8.7 mM Na_2HPO_4, 1.7 mM NaH_2PO_4 and 0.15 M NaCl, adjusted to pH 7.4. Carbonate buffer was prepared by dissolving 5.8 g $NaHCO_3$ and 3.2 g $NaCO_3$ in 2 l of ultrapure water. Washing solution contained 2 $g\ l^{-1}$ Tween 20 in PBS. Blocking solution contained 50 $g\ l^{-1}$ skim milk powder in washing solution. Horseradish peroxidase-conjugated swine anti-rabbit antibodies (HRP-conjugate) were purchased from DAKO, stored at 4°C and diluted to specified volumes with ultrapure water for working stock as necessary. HRP-substrate contained 2.5 $g\ l^{-1}$ β-cyclodextrin and 50 $\mu l\ l^{-1}$ 30% H_2O_2 in 0.1 M sodium acetate, adjusted to pH 5 with acetic acid. Chromogen contained 10 $mg\ ml^{-1}$ TMB (3,3′,5,5′-tetramethylbenzidine) in dimethyl sulfoxide (DMSO), and stored sealed in an amber glass vial at room temperature.

Indirect ELISA procedure

All ELISAs were conducted with Nunc Maxisorb 96-well plates. The general methodology for each ELISA involved coating the wells with 100 µl of antigen solution (standards and samples, in duplicate) and incubating for 2 h at 32°C. Subsequently, plates were washed 3 times with washing solution followed by incubation for 1 h at 32°C with 200 µl of blocking solution per well. After washing again 3 times with washing solution, 100 µl of antibody solution, diluted to an appropriate titre in blocking solution, was incubated in each well at 32°C for 1.5 h. Plates were again washed 3 times and 100 µl of swine anti-rabbit horse

radish peroxidase conjugate added to each well as a 1/1000 dilution in blocking solution from the original stock. After incubation for 1.5 h at 32°C, plates were washed 3 times with washing solution and 100 µl of chromogen:substrate solution added to generate colour. Stop solution (50 µl) was added after 15 min of incubation and the final absorbance read at 450 nm on a plate reader. A number of experiments were conducted to optimize the ELISA.

Effect of live versus heat-killed antigen on ELISA sensitivity

In preliminary experiments (not shown), live *C. tropicalis* cells demonstrated a capacity to catalyze the oxidation of TMB substrate. Because of a concern for false positive reactions, ELISAs were performed against heat-killed cells of each strain. Standard curves for live cells were constructed as above, but a replicate set was also produced and incubated at 95°C for 1 h before being used to coat wells. Mock peat samples were also treated in the same way to determine any potential matrix effects resulting from heat treatment.

Specificity and sensitivity of antiserum versus purified IgG

The specificity and sensitivity of each antiserum and IgG was initially tested using the procedure above. In this case, the antigen consisted of live 48 h broth cultures that were washed twice and resuspended in carbonate buffer. The standard curve included the initial resuspension and 1/10 serial dilutions in carbonate buffer, to 10^{-6}. Plate counts were conducted on each dilution for calibration. Both antiserum and purified IgG were diluted to 1/1000 before use in the ELISA.

Survival of biofertiliser strains in sterile peat

Commercial inoculants were prepared in a set of 15 gamma sterilized peat bags. Each bag was weighed to ensure uniformity of weight, the average being 153.36 g (±1.79). One day before inoculation, 20 ml of sterile water was injected into each bag to increase the moisture content to 20% (w/w). Broth cultures of each strain were grown with shaking overnight at 30°C and subsequently checked for purity by Gram staining. After purity was established, bags were inoculated with strains: three bags for each pure strain and three bags with a mix of the four strains. The pure strain bags were injected with 25 ml of nutrient broth culture and 75 ml of sterile water. For mixed bags, 25 ml of each broth culture was injected. After injection each bag contained 50% (w/w) moisture content. All bags were incubated at 30°C for 2 weeks after which they were stored at 4°C. The initial number of viable cells was calculated from dilution spread plates of each pure broth culture.

The survival of the microorganisms was monitored weekly for 1 month after inoculation. To determine the number of viable cells in the peat bags at each sampling time, 10 g of peat was aseptically transferred into 100 ml of sterile water. The samples were shaken for 15 min on a wrist-action shaker and serially diluted in sterile water for plate counts on antibiotic-modified agar or serially diluted in sterile carbonate buffer for ELISA. The ELISA was performed on heat-killed standards and samples; standards were derived from 48 h cultures; purified IgG was used for 1 N, E19 and B9 ELISAs; and whole antiserum was used for HY ELISAs.

Effect of standard culture age on ELISA sensitivity

Broth cultures were grown and harvested at 24, 48 and 72 h, followed by the usual washing and resuspension in carbonate buffer. All cultures were then heat-killed as above and compared for differences in sensitivity in indirect ELISA.

Statistical analyses

Standard curves for immunoassays were fit by maximum likelihood non-linear regression, using the sigmoidal function in SigmaPlot 9.0 (Systat Software). The limit of detection of microbial cells was calculated from these regressions and the absorbance corresponding to the upper value of the 95% confidence level of 8 blank readings. The significance of any main effects of enumeration method (ELISA vs. plate count) and culture conditions (pure vs. mixed), along with potential interaction, was assessed by a two factor ANOVA with the date of sampling incorporated as a random-effect (block) variable (R, 2.8.1). Subsequently, the data provided by plate counting was re-analysed by repeated measures ANOVA to determine the survival of each strain over time in the peat cultures (R, 2.8.1).

Results

Antibiotic testing for selective media

Bacillus species B9 and E19 were sensitive to all tested antibiotics except for spectinomycin and streptomycin (B9 only) at low concentrations (Table 1). As expected, all bacterial strains were resistant to cycloheximide and the yeast HY was sensitive. HY was resistant to all other antibiotics at all concentrations and could be differentiated from bacterial species through resistance tetracycline and

kanamycin whereas the bacteria were sensitive to these antibiotics. *Pseudomonas* 1 N could be clearly differentiated from B9 and E19 by resistance to vancomycin and ampicillin at all concentrations and exhibited more resistance to streptomycin at low concentrations. Growth of 1 N on chloramphenicol was reduced compared to the control without antibiotics but some growth occurred at low concentrations in contrast to complete inhibition of B9 and E19. 1 N, B9 and E19 had similar growth responses to spectinomycin.

As a result selective media for 1 N and HY could be designed based on differential patterns of antibiotic resistance. Media for 1 N was designed to contain vancomycin (10 mg/l) and cycloheximide (100 mg/l) and media for HY could contain tetracycline (10 mg/l) or kanamycin (10 mg/l). Selection for growth of B9 and E19 using antibiotics was not possible because of poor resistance by these species. These species could, however, be identified by their distinctive colony morphology.

Effect of live versus heat-killed cells on ELISA sensitivity

Although heat-treated supernatant of liquid culture of HY had higher mean absorbance values at high cell densities, there were no significant effects observed on ELISA sensitivity after heating of either standard liquid cultures (Fig. 1) and peat cultures (Table 2) of 1 N and HY. As such, it was decided that samples undergoing routine testing should be boiled to reduce the likelihood of false positives caused by potential catalase-positive contaminants, and to allow standards to be kept frozen as a homogeneous matrix. All samples in subsequent experiments were treated in this manner.

Specificity and sensitivity of antiserum versus purified IgG

The sensitivity and specificity of each antiserum was variable for each strain (Fig. 2). Each of the antisera raised against the three bacteria showed some activity against HY, despite the rabbits being injected with heat-killed cells derived from pure bacterial cultures. Nevertheless, the antisera raised against 1 N and E19 were more sensitive to their respective bacterial antigens than the HY antigens. The antisera raised against B9, however, was not very sensitive and cross-reacted with both HY and E19 to a similar extent as its reactivity against B9.

After IgG purification and use of these fractions in the ELISA, the cross-reactivity against HY previously observed for all antisera was removed (Fig. 3). The purification step also rendered the antisera raised against HY unreactive against heat-killed HY cells. The sensitivity of the assays against 1 N and E19 using their respective IgG fractions, despite being reduced, were still sufficient at a 1/1000 dilution. This was not the case for the IgG raised against B9, which decreased significantly in sensitivity at a 1/1000 dilution. All subsequent assays were thus performed using a 1/100 dilution of the purified IgG against B9. The limits of detection for each of these strains in pure liquid culture were calculated as 4.3 (1 N), 2.7 (HY), 2.8 (B9) and 4.0 (E19) $\log_{10}$(CFU) ml^{-1}, corresponding to a limit of detection in pure peat culture as 5.3 (1 N), 3.7 (HY), 3.8 (B9) and 5.0 (E19) $\log_{10}$(CFU) g^{-1}. In mixed peat culture, the LODs were the same for 1 N and HY, as no cross-reaction was observed with the other strains; however, because of the cross-reaction between antisera raised against the two bacilli, B9 and E19, the LODs could not be calculated.

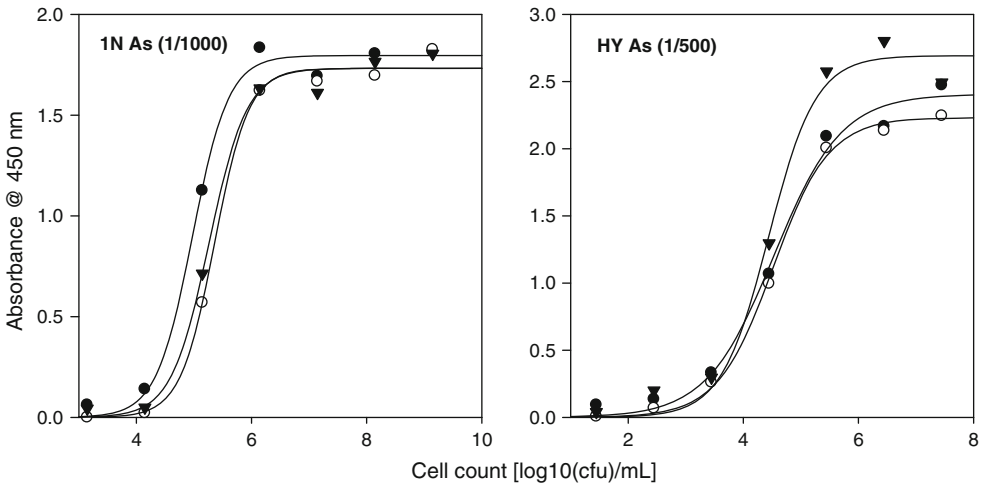

Fig. 1 Sensitivity of indirect ELISAs against bacterial antigens after different treatments: (*filled circle*) live, (*open circle*) heat-killed, (*filled down triangle*) heat-killed supernatant

Table 2 Effect of different treatments on peat culture ELISA counts

	HY			1 N		
	Live	Boiled	Boiled supernatant	Live	Boiled	Boiled supernatant
Count (CFU g^{-1})	6.04	5.97	5.86	7.04	7.32	7.18
95% confidence level ($n = 6$)	0.25	0.03	0.07	0.05	0.25	0.15

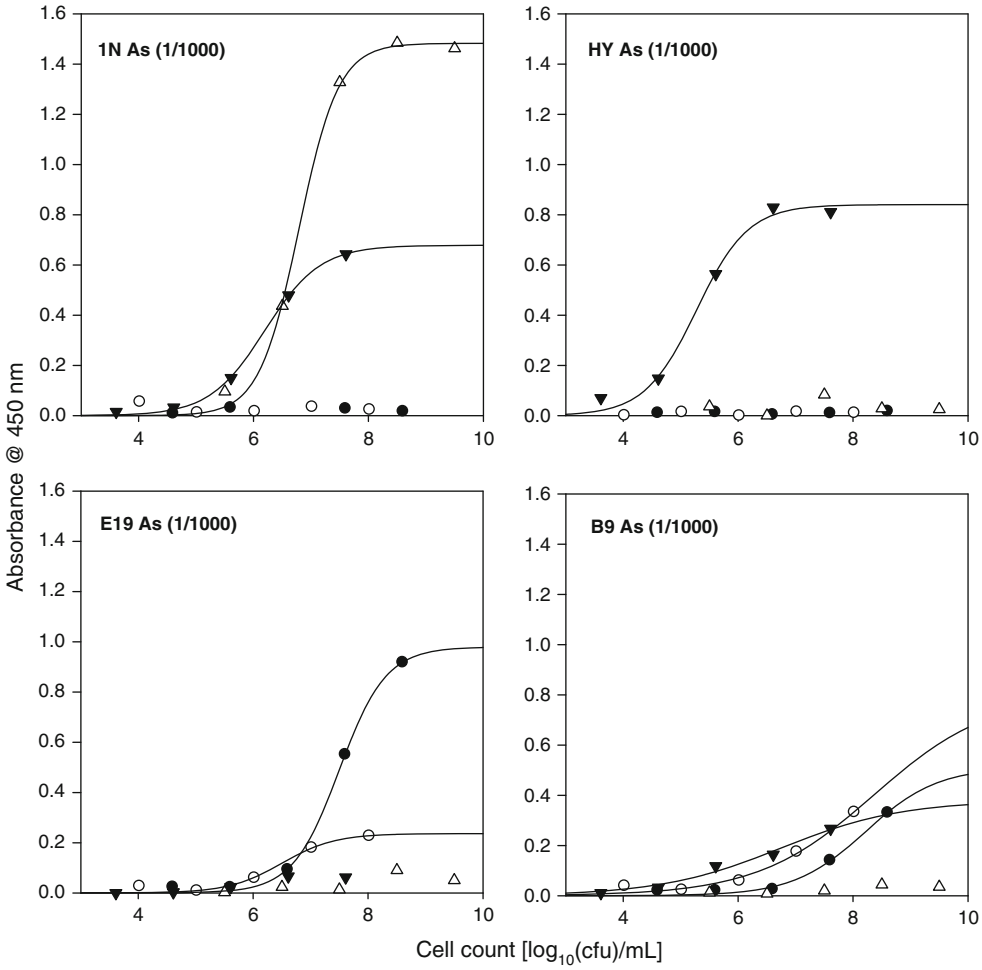

Fig. 2 Specificity and sensitivity of 1/1000 dilutions of unpurified antisera against heat killed 48 h pure culture antigen dilutions. Strains: (*filled circle*) E19, (*open circle*) B9, (*filled down triangle*) HY, (*open triangle*) 1 N

Comparison of ELISA and plate counting methods

The ELISA counting method was tested against the antibiotic plate counting method using samples taken weekly from the peat cultures. Strains E19 and B9 could not be individually quantified by ELISA because of the cross-reactivities of their corresponding IgG detection antibodies. Furthermore, the absorbance values gained when using the ELISA to determine their combined number were too high for the standard curves. This anomaly was addressed in a subsequent experiment (see below).

For both 1 N and HY, no significant interaction was observed between the method of enumeration and the type of peat culture, indicating that each method performed consistently for counting a particular strain within a mixed culture (Table 3). However, both immunoassays underestimated the actual number of cells of each target strain when compared with the plate counts. For 1 N, there was no significant difference in the grouped cell numbers when grown as a pure or mixed peat culture. In contrast, lower numbers of strain HY were recorded when it was grown in combination with the other three strains, compared to when it was grown in peat alone.

Effect of standard culture age on ELISA sensitivity

As a consequence of the excessively high numbers of E19 and B9 estimated by the ELISAs conducted on peat

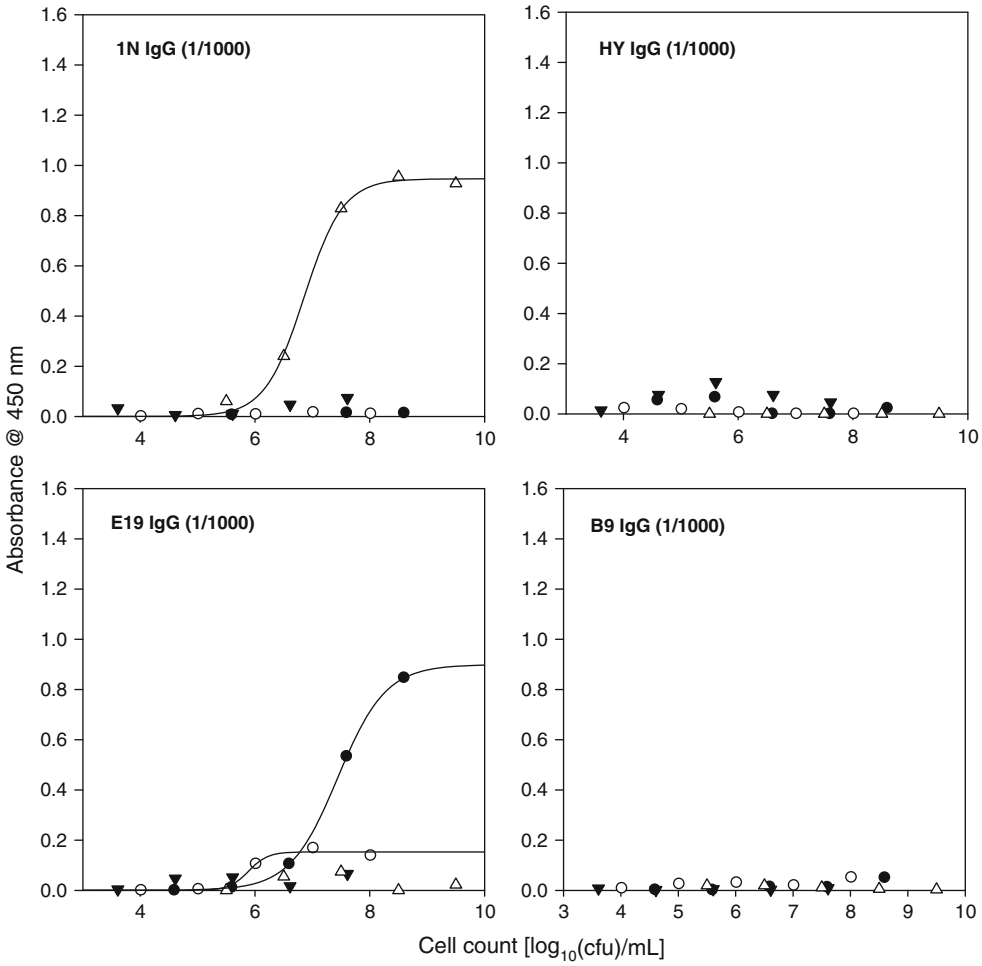

Fig. 3 Specificity and sensitivity of 1/1000 dilutions of purified IgG against heat killed 48 h pure culture antigen dilutions. Strains: (*filled circle*) E19, (*open circle*) B9, (*filled down triangle*) HY, (*open triangle*) 1 N

Table 3 Results of a two-factor ANOVA analysis comparing the enumeration of biofertiliser strains by two methods (ELISA vs. plate count) and two culture conditions (pure vs. mixed populations)

Factor	Value	1 N	HY
Method:culture	Probability of significant interaction (t < T)	0.578	0.488
Method	Probability of significant effect (t < T)	<0.001	<0.001
Plate count	Mean count (log(cfu) g^{-1}) (Std error)	8.84 (0.04)	6.8 (0.2)
ELISA	Mean count (log(cfu) g^{-1}) (Std error)	7.30 (0.06)	5.4 (0.1)
Culture	Probability of significant effect (t < T)	0.484	<0.001
Mix	Mean count (log(cfu) g^{-1}) (Std error)	8.0 (0.2)	6.6 (0.2)
Pure	Mean count (log(cfu) g^{-1}) (Std error)	8.1 (0.2)	5.7 (0.2)

cultures, the effect of standard culture age on the ELISA sensitivity was investigated for each strain. No significant difference was observed between standard curves constructed for 1 N and HY using cultures of different age (Fig. 4a, b). In contrast, the assay sensitivity for B9 and E19 increased concurrently with age of the culture used to create the standard curve (Fig. 4c, d).

Survival of biofertiliser strains in sterile peat

The survival of the strains was monitored in the pure and mixed peat cultures over 1 month and the plate count data analysed by a repeated measures ANOVA. The total counts of all bacterial strains in pure and mixed cultures significantly increased in density in within the first week, but

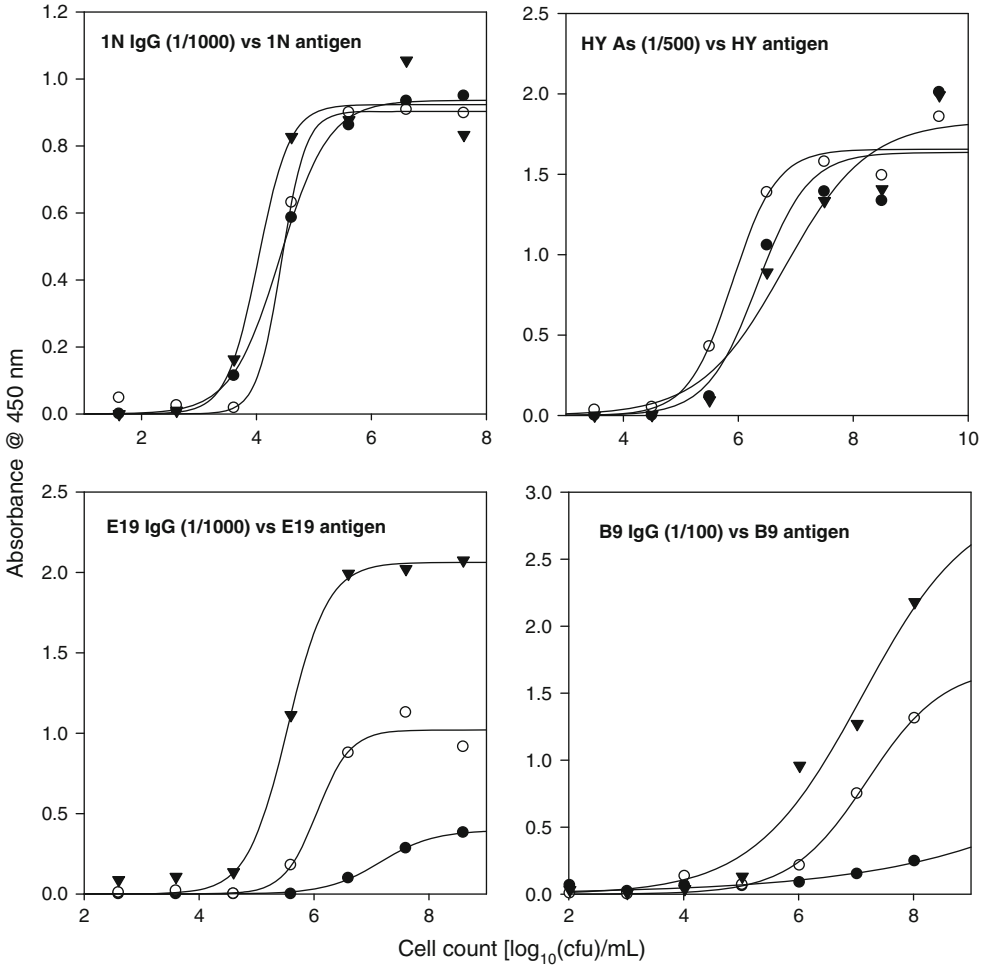

Fig. 4 Sensitivity of standard heat-killed cells as antigen after growth in broth culture for different times: (*filled circle*) 24 h, (*open circle*) 48 h, (*filled down triangle*) 72 h

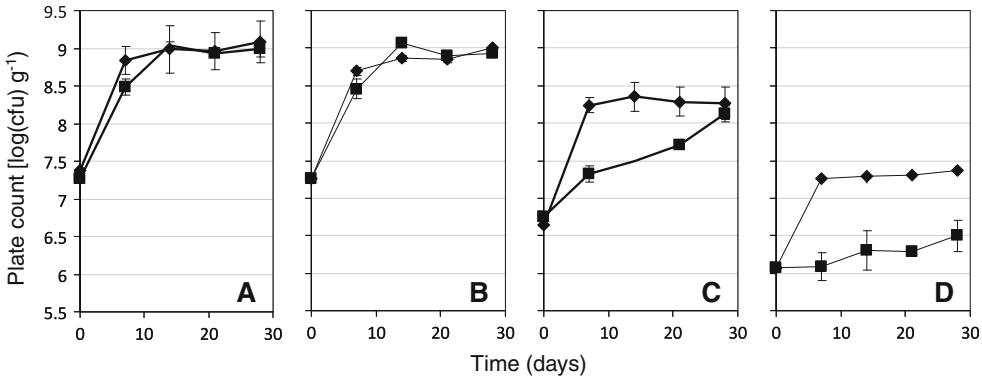

Fig. 5 The growth and survival of BioGro strains in pure or mixed peat cultures. **a** Sum of all strains, **b** strain 1 N, **c** the sum of strains B9 and E19, and **d** strain HY. *Error bars* represent 95% confidence levels. Culture: (*filled circle*) pure, (*filled square*) mixed

thereafter reached a plateau (Fig. 5a), mirroring the growth and survival of the dominant strain 1 N (5B). There were no significant differences between these growth/survival curves. The densities of strains E19, B9 and HY followed a similar trend to that of 1 N in pure peat culture, but showed significantly different growth rates in mixed peat cultures after the initial inoculation (Fig. 5c, d). The sum of strains B9 and E19 in mixed cultures eventually reached a similar number to that of pure cultures, however strain HY was clearly inhibited when grown mixed together with the other three biofertiliser strains.

Discussion

An important aspect of ELISAs for enumerating bacteria is the need for validation against direct cell counts or another standard method. For every batch of samples being tested,

at least one standard curve should also be run to account for slight differences in experimental conditions between each batch. In this study, high density bacterial cultures were grown, enumerated by plate counting, heat-killed and then kept frozen in smaller aliquots for generating a standard curve when required. From our experience these standards could be kept frozen for up to 3 months without any appreciable loss of antigenic activity. Using heat killed cells overcame the possibility of false-positives when using live cells in ELISAs; which could otherwise result from the presence of microorganisms producing extracellular or membrane bound catalases which can oxidize the TMB substrate.

ELISA characterisation of the unpurified antisera raised against the strains *P. fluorescens* 1 N and *B. subtilis* B9 showed some cross-reaction against the *C. tropicalis* HY yeast cells (Fig. 1). Although the exact antigenic residues were not determined, the cross-reactivity was removed after purification on Protein A Sepharose (Fig. 2). However, the antiserum raised against *C. tropicalis* HY also lost most of its sensitivity after purification as IgG on the Protein A Sepharose column. Previous medical studies have shown that IgE components of mammal antisera are responsible for allergenic responses to yeast infections (Doekes and van Ieperen-van Dijk 1993), and that there is a high cross-reactivity of IgE components among different yeasts (Kanbe et al. 1997; Savolainen et al. 1998). Sensitisation to common airborne allergens, such as dust mites, pollen and animal dander, can cause increased levels of total and specific IgE antibodies in animals (Doekes et al. 1996), and this could be the cause of multiple yeast cross-reactivities in the rabbit antisera produced against the BioGro strains. Because the binding affinity of IgE to protein A is much lower than IgG, purification on Sepharose A column removes over 90% of IgE components (Peng et al. 1994) would thus eliminate any IgE antibodies specific against the HY strain from purified IgG fractions. Unfortunately this hypothesis could not be further investigated in this study. Regardless, the whole antisera raised against *C. tropicalis* HY remained highly sensitive to strain HY without cross-reacting against the other biofertiliser strains, and could therefore be used for method calibration in sterile peat.

Nevertheless, under the conditions described in this paper, reliable quantification of cell numbers by ELISA was not achieved because of matrix effects. The ELISA methodology underestimated the cell densities of strains HY and 1 N by approximately 1.5 log units as compared with the plate counts. The underestimation of the 1 N and HY counts may be related to the binding of antigenic components by colloidal particles in the peat, which could either inactivate the antigen or increase the likelihood of wash-off. In contrast, the ELISA method over-estimated the cell counts of E19 and B9, to the extent that the absorbance values recorded for the peat samples did not fall on the standard curve. After a subsequent experiment, it was found that the growth stage of the initial culture significantly affected the ELISA results for these two bacilli. It is presumed that as the cultures ages, either more antigenic compounds are produced extra-cellularly or persist after cell death or sporulation, resulting in a higher signal per unit colony count. A similar phenomenon was observed by Schwebach et al. (2001), who found that the surface expression of an arabinomannan antigen produced by *Mycobacterium tuberculosis* increased with culture age in vitro, thereby increasing antibody binding. Consequently, in our case, three-day old cultures were found to be more appropriate for calibrating the cell counts for B9 and E19 rather than 24 h cultures, returning a more accurate count of the aged population within peat samples.

Along with these general issues, the specific differentiation of the two closely related PGP strains *B. subtilis* B9 and *B. amyloliquefaciens* E19 also proved challenging. Although both strains had similar antibiotic resistance patterns, they could be identified in the sterile peat because of a difference in their colony morphologies. However, in other circumstances this may not be possible; in which case it will be necessary to undertake further research into phenotypic differences that will allow differentiation. Presumably, the inclusion of a strain in a mixed biofertiliser will be because of a specific functional attribute not possessed by any of the other strains, for example, nitrogen fixation, biocontrol activity, phosphorus solubilisation, auxin production and so on. In this case, it should be possible to design biochemical tests for those functions that allow differentiation on culture plates (Deaker et al. 2010). With regards to the differentiation of strains showing cross-reaction to antisera, the specific adsorption of cross-reacting antibodies onto immobilized cells of the cross-reacting strain can be used to increase ELISA sensitivity. Such an approach has been used successfully previously to differentiate closely related rhizobial strains (Olsen et al. 1983).

Despite the current issues regarding the highly accurate quantification of biofertiliser strains by direct ELISA, the general methodology still shows promise for use as a rapid, *semi-quantitative* screening test in quality control procedures. Previous research has highlighted the speed and effectiveness of some commercial kits based on immunological techniques, but the presence of false-negative and positive assay results has been acknowledged as a limitation (Lochner et al. 1988). Further research is necessary to define the culture conditions required to produce realistic cells for generating standard curves, along with validation of the method under non-sterile conditions. Until then, calibrating the ELISA response against a direct plate count from a ‘standard’ pure culture peat inoculant can be used to

quantify strains in unknown sample bags. A combination of primary labeled IgG and optimized standards should facilitate the development of 'dip-stick' technology, allowing rapid screening of commercial product.

Meanwhile, the survival of the bacterial strains comprising BioGro in sterile peat in this experiment was accurately quantified using direct plate counts. After introduction into sterile peat as single inoculants, all strains rapidly increased in number, presumably continuing to grow on broth culture nutrient sources along with extra carbon present in the peat. However, notably different growth curves were visible when all of the strains were co-inoculated into the same peat bags. Under these conditions, 1 N remained unaffected and grew at a similar rate and to a similar extent as in pure culture conditions. This suggests that strain 1 N can rapidly reproduce under nutrient rich conditions thereby out-competing slower growing bacteria for resources. In contrast, the growth of the other biofertiliser strains was clearly hindered by this competition, particularly the slow-growing yeast HY, which did not significantly increase in number over the 30 days. Interestingly, the rate of growth under competitive conditions ($1\ N > B9 = E19 > HY$) corresponds roughly with the inverse of cell size ($1\ N < B9 = E19 < HY$), suggesting an inhibition mechanism dependent on the surface area to volume ratio, such as carbohydrate/nutrient limitation. This aside, it is recommended that BioGro strains are individually cultured and stored, with mixing directly prior to field application to maximize the cell numbers of all strains being applied to the crop. More research is needed into the long term viability of these strains and growth after application to crops.

It is concluded that the current ELISA methodology still requires further development to be used as a routine, quantitative tool for the enumeration of mixed biofertiliser microorganisms. Nevertheless, progress has been made in this study by identifying reasons for the variability in ELISA performance, including the presence of non-specific cross-reactivities of antibodies raised against bacteria with yeast antigens, and an increased sensitivity of ELISAs against bacilli as a result of culture aging. These rapid ELISA tests are still a valid option for the semi-quantitative rapid screening of biofertiliser products if suitable standard curves are generated to reflect the culture conditions. Plate counts using antibiotic selective media enabled the accurate quantification of the growth and survival of mixed bacterial peat cultures, despite being slightly higher in cost (Supplementary Table S1). Competition was observed between strains; a fact which needs to be considered when producing mixed microbial inoculants for plant growth promotion. We have shown that using selective media based on differential antibiotic resistance, plate counts to determine viable numbers and ELISA for confirmation of identity may together provide a useful methodology for quality control of multi-strain PGP inoculants such as BioGro.

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