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Role of Plant Growth-promoting Rhizobacteria in Influencing the Early Growth of Pueraria phaseoloides *and* Calopogonium caeruleum – *Inoculum Dosage and Survival in Peat-based Cultures*

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The amount of inoculum needed for a successful plant growth-response to plant growth-promoting rhizobacteria (PGPR) inoculation was determined by inoculating Pueraria phaseoloides *with five dosages of strains 7NSK2, TL3, BK1 and CR30, and* Calopogonium caeruleum *with 7NSK2 in two soils. Responses varied with crop, PGPR strain and soil factors. Under glasshouse conditions, strain TL3 caused statistically-significant yield increases of* P. phaseoloides *in Segamat series soil, at a minimum dosage level of 3.8 × 10^6 cells/g soil, whilst 7NSK2, BK1 and CR30 appeared ineffective at all dosage levels. Strain 7NSK2 caused statistically-significant yield responses from* C. caeruleum *at an inoculum dose of 5–6 × 10^8 cells/g soil in both Segamat and Beserah series soil. Sterilised, milled peat when incorporated with single strains of TL3, 7NSK2 and 34–13 proved a suitable carrier material for long-term survival of PGPR strains at high populations. The development of procedures for preserving PGPR inoculum in peat is described.*

There have been many reports in recent years on the role of plant growth-promoting rhizobacteria (PGPR) increasing growth of some agricultural crops *via* suppression of a specific deleterious rhizobacteria (DRB) in the rhizosphere or to control plant diseases caused by soil-borne pathogens[1,2,3,4]. In earlier studies, the effectiveness of PGPR strain 7NSK2 in promoting growth of *Pueraria phaseoloides* in several soils have been explored[5,6] but whether this is directly linked to an ability to colonise roots is not presently known. Inconsistent growth responses and yield increases have often been attributed to unsuccessful PGPR colonisation of the rhizosphere[1,7]. It is also well-known that different PGPR strains have different abilities to colonise a particular root niche[8]. This may be an important factor in the success of an inoculum, and may need to be resolved before PGPR strains can be used on a practical scale.

A review of published literature reveals little work with quantitative information on PGPR for rhizosphere colonisation and plant growth response. Both relative numbers and differences in colonising ability are important parameters in rhizosphere studies and increasing the inoculum load on seed or initial rhizosphere population densities means a greater probability of survival and growth as well as to colonise new sites with the growing root tip. Early bacterization trials indicated that the minimum number of viable cells needed

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for uniform colonisation lie in the region of 10^5 colony-forming units (CFU)/seed or 10^7 CFU/g dry inoculum[9,10]. Iswandi *et al.*[11], for example, found that at least 10^5-10^6 viable cells of 7NSK2 had to be applied to maize and barley grown on a sandy loam under glasshouse conditions to obtain a beneficial effect on plant growth. In another study, Ordentlich *et al.*[12] showed that the biocontrol agent *Serratia marcescens* at populations of 10^5–10^6 CFU/g soil were effective in suppressing *Sclerotium rolfsii* (up to 75% disease reduction) and damping-off caused by *Rhizoctonia solani* (by 50%) in beans.

The production and formulation of microbial inoculants is another aspect of work on biocontrol agents that has seen little research[13]. Currently, strains of *Rhizobium/ Bradyrhizobium* produced by submerged culture dominates the inoculant market, although other bacterial inoculants *(Pseudomonas fluorescens, Bacillus subtilis* and *Azospirillum* sp.) are becoming important. Formulations are usually of neutral pH and made of readily-available inexpensive local materials (peat or substitutes of organic origin). Despite the wide range of carrier materials available for production of legume inoculants, peat remained unchallenged as a carrier, with desirable attributes of high moisture-holding capacity and are commonly used without additives except $CaCO_3$[14,15]. The intent of this section is to determine the amount of inoculum for a successful plant growth-response to PGPR inoculation, and to test peat for its suitability as a carrier material for PGPR strains.

MATERIALS AND METHODS

Inoculum Dosages for Growth-promotion Responses

The first experiment examined the effect of five inoculum dosages on four PGPR strains for growth-promotion responses by *P. phaseoloides* growing in two soils. The design was a randomised block of five treatment levels with four replications. In a second experiment on a different occasion, only one strain was tested on another component of the plantation cover mixture, *Calopogonium caeruleum,* in six replications.

The infertile soils used, collected from sites under natural vegetation, were an Oxisol (Segamat series) and an Entisol (Rasau series) in *Experiment 1,* and Segamat series and an Ultisol (Beserah series) in *Experiment 2 (Table 1).* The soils are generally acidic, depleted in bases and low in Bray-II P. The sieved soils (<4mm) were potted into 7.6 cm diameter plastic pots (8.5 cm diameter in *Experiment* 2), and the top half of pots stirred with Christmas Island rock phosphate (CIRP; 17.64% P) at rates equivalent to 720 kg/ha[16].

The PGPR strains used, 7NSK2 *(Pseudomonas aeruginosa),* TL3 *(Pseudomonas fluorescens),* BK1 *(Pseudomonas putida)* and CR30 (unidentified) were grown on King's Medium B (KB)[17] agar for 48h in flat medicinal bottles at 28°C, and inocula prepared by scraping colonies with sterile glass rods into 0.1*M* $MgSO_4.7H_2O$ buffer solution. Total counts were made on the resulting bacterial suspensions using a Helber counting chamber slide (1/400 mm^2, 0.02 mm depth) and appropriate dilutions made from this primary suspension to provide four inoculum dosages. Viable counts were then made on the inocula just before inoculation to provide actual counts for the following treatment levels (cells/ml): T1 (0), T2 (2.5×10^4), T3 (2.5×10^6), T4 (2.5×10^8), T5 (2.5×10^{10}) in *Experiment 1,* and T1 (0), T2 (4.4×10^4), T3 (4.3×10^6), T4 (8.1×10^8) and T5 (3.8×10^{10}) in *Experiment 2.*

Legume seeds that were surface-sterilised (conc. H_2SO_4) and pregerminated (3–4 days)

TABLE 1. PROPERTIES OF SOILS USED IN THE EXPERIMENT*

Property	Soil series		
	Rasau	Segamat	Beserah
Soil taxonomy	Typic Quartzipsamment	Tropeptic Haplorthox	Tropeptic Haplorthox
Coarse sand (%)	49.9	5.5	46.3
Fine sand (%)	36.4	4.3	10.8
Silt (%)	11.9	13.5	9.4
Clay (%)	1.3	72.6	32.6
Org. C (%)	1.51	2.60	2.60
Total N (%)	0.15	0.22	0.21
pH	5.2	4.6	5.2
Total P (p.p.m.)	101	455	141
Avail. P (p.p.m.)	7	9	7
Exch. K (m.e. %)	0.07	0.18	0.21
Exch. Ca (m.e. %)	0.26	2.84	1.87
Exch. Mg (m.e. %)	0.12	1.03	0.89
Exch. Al (p.p.m.)	83	44	33
Total Mn (μg/g)	19	147	136
Fe (%)	0.22	10.36	1.53

*from 0–15 cm depth

were planted at a depth of 1.0 cm (2 plants/pot). Nitrogen (N), at 10 μg/g as KNO_3 was applied once to all pots. The pots were watered to 70% field capacity daily by weighing. Minus-N and -P nutrient solution[18] was supplied weekly. Inoculation was by pipetting 5.0 ml of the treatment suspensions on to the base of seedlings. After one week, all plants received 1.0 ml of the appropriate bradyrhizobia to ensure nodulation: *Bradyrhizobium* sp. strain RRIM 968 *(Centrosema)* for *P. phaseoloides,* and *Bradyrhizobium* sp. strain RRIM 77 *(Calopogonium)* for *C. caeruleum,* at a population level of *ca.* 1×10^9 cells/ml. Plants were grown in the glasshouse with a temperature maximum of 39°C (day) and a minimum of 25°C (night) and harvested five weeks after sowing (seven weeks in *Experiment* 2). Shoots were cut at soil level, oven-dried (80°C, 48 h) and weighed.

Survival in Peat Culture

The methods of incorporating PGPR into peat is based on the technology developed for *Rhizobium*[19].

Peat was taken from Klang, Selangor and dried to a moisture content of 4%. Samples were ground in a hammer mill, passed through a 75-μm mesh sieve and limed to pH 6.5 before being distributed as 50 g amounts into 250 ml conical flasks. The prepared samples were steam-sterilised (121°C, 60 min).

Cultures of strains 7NSK2, TL3 and 34–13 (*P. fluorescens,* supplied by Esso Ag. Biologicals – Esso Chemical Canada) were grown separately in KB broth to turbidity. The broth cultures were separately added to the sterilised peat to give a final moisture content

of 40%–60% along with a drop of a 0.5% surfactant, and mixed using sterile glass rods in a laminar flow cabinet. The flasks were covered with polythene film to reduce moisture loss since preliminary work revealed that drastic drops of viable populations were associated with moisture losses. The treated samples were stored at 30°C for a period of 16 (7NSK2) and 24 (TL3, 34–13) weeks.

Counts of viable PGPR were determined monthly by suspending 10 g of peat samples in 90 ml of a 0.1 *M* $MgSO_4.7H_2O$ buffer solution in 250 ml Duran bottles. The samples were processed by agitating the bottles flat on a reciprocating shaker (230 cycles/min) for 10 min. Aliquots from each of the serial 10-fold dilution were plated onto KB agar plates in duplicates and colony counts read after 3 days of incubation. The remaining peat in the flask were used to determine the moisture content. Initial counts were made 1 h after inoculation.

RESULTS

Inoculum Dosages for Growth-promotion Responses

Experiment 1. Strains 7NSK2, BK1 and CR30 were ineffective at improving shoot dry weight (DW) of *P. phaseoloides* at all dosages in both soils *(Table 2).* In Rasau series soil, BK1 at the highest dose increased shoot DW of the legume over the uninoculated controls but this was only significant at $P<0.10$. On the other hand, TL3 significantly increased shoot weights (31%–32%, $P<0.01$) at the two higher dosages (T4,T5) in Segamat series soil.

Experiment 2. The treatment effects were highly significant ($P<0.01$) for *C. caeruleum* shoot in both Segamat and Beserah series soils *(Table 3).* In Beserah series soil, plants responded to the highest inoculum dose with a 168% increase in shoot weights over the uninoculated controls. In Segamat series soil, plants responded to the same dosage with a 58% increase in shoot weight.

Survival in Peat Culture

Populations of TL3 oscillated about a mean of 4×10^{10} cells/g dry peat for 24 weeks, from 2.1×10^{10} at inoculation. Maximum growth was achieved at 12 weeks (1.5×10^{11} cells/g dry peat) but numbers declined thereafter (9.1×10^{9} cells/g dry peat) by 24 weeks *(Figure 1).* Strain 34 –13 showed a steady increase in growth (3×10^{11} cells/g dry peat at 16 weeks) from 2.5×10^{8} cells at inoculation but began to decline thereafter. Unlike TL3 and 34–13, 7NSK2 achieved maximum growth within two weeks (*ca.* 8.3×10^{10} cells/g dry peat) but declined slowly by 16 weeks (3.2×10^{10} cells/g dry peat). For all peat treatments, the mean moisture contents declined from 39% to 23% by 24 weeks.

DISCUSSION

The plant experiments indicate that there was a minimum population density on roots to cause statistically-significant growth responses from PGPR inoculation. This may vary with the crop, PGPR strain and edaphic factors. In general, it may be difficult to speculate on the minimum population thresholds on roots required for effectiveness since strains of PGPR may differ sufficiently in their ability to colonise roots[8]. Thus while strain TL3 could elicit responses from *P. phaseoloides* at the two highest dosages in Segamat series soil under glasshouse conditions, the remaining three strains could not. The introduced dosages corresponded to levels of $3.8 \times 10^{6} - 3.8 \times 10^{8}$ cells/g soil, assuming an even distribution of the population throughout soil in the pots. On the other hand, 7NSK2 caused growth responses of *C. caeruleum* in both Segamat

TABLE 2. SHOOT DRY WEIGHT OF *PUERARIA PHASEOLOIDES* DUE TO INOCULATION WITH DIFFERENT DOSAGES OF PGPR STRAINS *(EXPERIMENT 1)**

Treatment	No. cells/pot**	Shoot dry weight, g/pot							
		BK1		TL3		7NSK2		CR30	
		Segamat	Rasau	Segamat	Rasau	Segamat	Rasau	Segamat	Rasau
1	0	0.666	0.208	0.698bc	0.233	0.523	0.290	0.580	0.215
2	1.25×10^5	0.612	0.253	0.491c	0.215	0.582	0.192	0.392	0.205
3	1.25×10^7	0.453	0.183	0.816ab	0.187	0.682	0.227	0.354	0.212
4	1.25×10^9	0.640	0.225	0.917a	0.188	0.608	0.272	0.394	0.203
5	1.25×10^{11}	0.504	0.310	0.921a	0.206	0.691	0.287	0.415	0.231
S.E.		±0.067	±0.031	±0.058	±0.027	±0.055	±0.034	±0.072	±0.034
L.S.D.(P<0.05)		0.205	0.095	0.180	0.083	0.169	0.104	0.221	0.105
C.V. (%)		23	26	15	26	28	27	35	32

*Means of 4 replicate pots, 2 plants/pot, after 5 weeks growth. Means within a column not followed by common letters are different (P<0.05)

**Amount of soil in pots: Segamat, 330g; Rasau, 445g

TABLE 3. SHOOT DRY WEIGHT OF *CALOPOGONIUM CAERULEUM* DUE TO INOCULATION WITH DIFFERENT DOSAGES OF STRAIN 7NSK2 *(EXPERIMENT 2)**

Treatment	No. cell/pot**	Segamat Shoot DW g/pot	Beserah Shoot DW g/pot
1	0	0.050 (0.0212)b	0.048 (0.0146)b
2	2.2×10^5	0.039 (0.0167)b	0.066 (0.0170)b
3	2.2×10^7	0.033 (0.0139)b	0.055 (0.0166)b
4	4.1×10^9	0.039 (0.0166)b	0.056 (0.0182)b
5	1.9×10^{11}	0.080 (0.0330)a	0.129 (0.0335)a
S.E.		±(0.0038)	±(0.0023)
L.S.D.(P<0.05)		(0.0092)	(0.0056)
C.V. (%)		38	8

*Means of 6 replicate pots, 2 plants/pot; plants harvested after 7 weeks. Means within a column not followed by common letters are different (P<0.05), on a log (X+1) transformed basis (in brackets)

**Amount of soil in pots: Segamat, 317 g; Beserah, 354 g

and Beserah series soils at the highest dose, corresponding to levels of 5–6 × 10^8 cells/g soil. These figures appeared much higher than levels reported by other workers for roots of other crops, for example, Iswandi *et al.*[11] used 10^5–10^6 viable cells to maize and barley for a beneficial effect on plant growth, and Keel *et al.*[20] added *P. fluorescens* strain CHA0 suspensions at 6 × 10^6 CFU/g soil to suppress tobacco black root rot. The unpredictability of 7NSK2 effectiveness on *P. phaseoloides* in the same soils over different occasions were highlighted in a previous study[6] and the reasons were not presently clear. Unsuccessful colonisation of the rhizosphere is one possibility but this need to be checked using

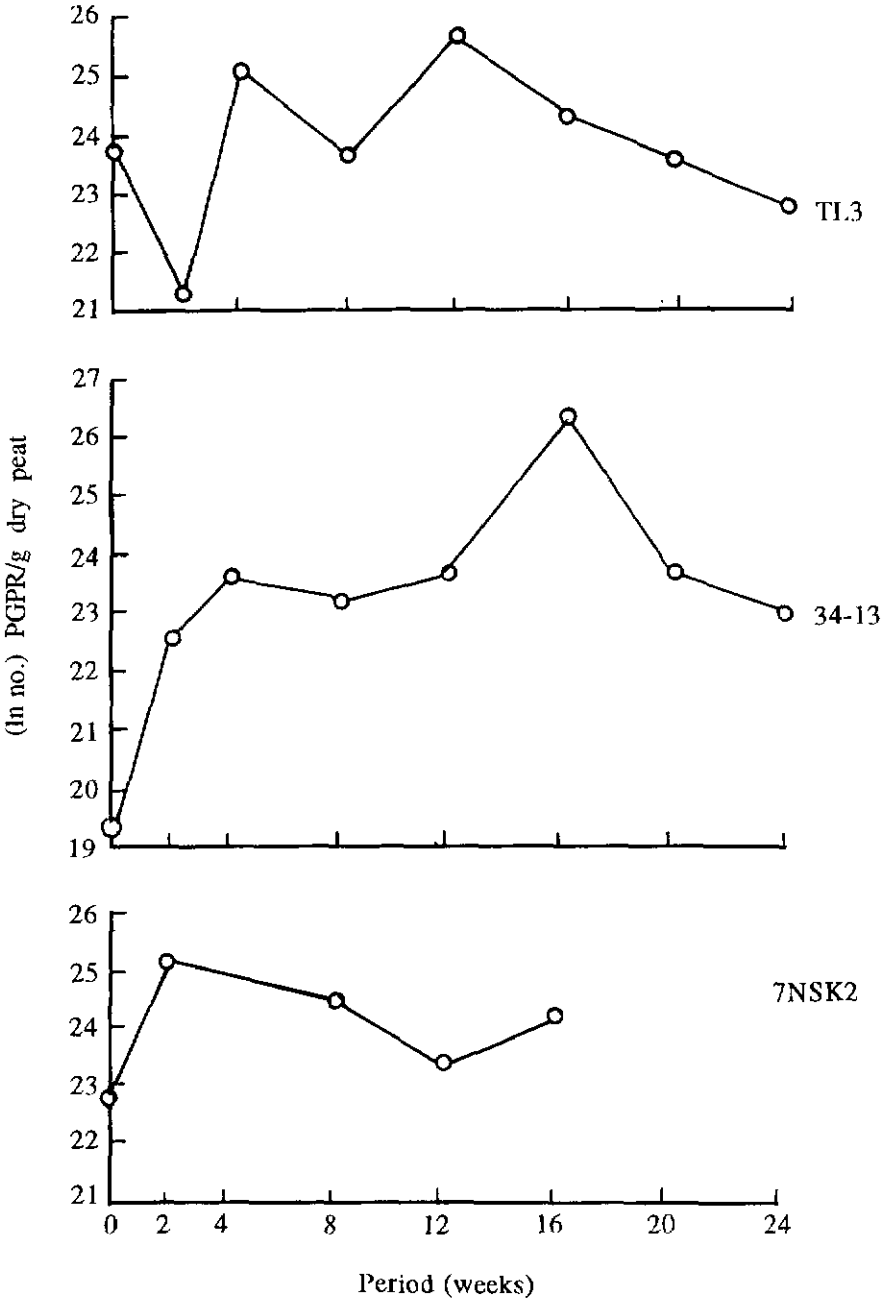

Figure 1. Effect of storage duration on survival of PGPR strains TL3, 34-13 and 7NSK2 in sterilised peat.

marked strains in further root colonisation studies. The specificity of PGPR strains for certain soils had already been shown for sugarbeet cultivation but the use of compatible mixtures of strains effective in different soil types or on a variety of crops may increase the applicability of PGPR inoculation[21].

The carrier material experiments also indicate that sterilised milled peat was a suitable material to provide long-term survival of beneficial rhizobacterial strains at high populations. Although initial rapid growth in peat varied between strains, all strains were capable of maintaining populations in excess of 10^9 cells/g peat by the end of 16 or 24 weeks. In the case of rhizobial culture, the factors affecting growth and survival in peat during storage are moisture content, gaseous exchange, incubation temperatures and storage duration[14]. The decline in numbers for cultures stored at 30°C in this study was expected since temperature effects were frequently confounded with effects of moisture loss.

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