Comparative analysis of five methods for recovering rhizobacteria from cotton roots

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A variety of methods have been used for recovering introduced bacteria from plant roots. The objective of this study was to compare systematically five methods: agitation in buffer, agitation with glass beads in buffer, mixing in a Stomacher[R] lab-blender, sonication, and trituration with mortar and pestle. Cotton seeds were treated with two previously reported rhizobacterial strains, *Pseudomonas fluorescens* strain Pf-5 and *Bacillus subtilis* strain GB03. The efficiency of recovery by each method was determined 3 weeks later by comparing average bacterial populations from whole root systems, single 2.0-cm root segments, and two root regions (the uppermost 5 cm of taproot and the lowermost 5 cm). Treatment with the Stomacher[R] blender yielded significantly higher ($P = 0.05$) mean populations of GB03 compared with all other methods and significantly higher mean populations of Pf-5 compared with agitation with glass beads. From the lowermost 5 cm of taproot, populations of Pf-5 recovered by the Stomacher[R] treatment were significantly higher than all other methods. The inclusion of glass beads for agitation treatments resulted in neither consistently higher absolute numbers of recovered bacteria nor reductions in variability. The mean standard error of each recovery method varied among root sources, and no single method consistently had the highest or lowest mean standard error. Mean standard errors for strain GB03 were generally lower than those for Pf-5 with each root source and each method of recovery. When viewed in composite, the data suggest that the Stomacher[R] treatment was the best for recovering the greatest absolute numbers of rhizobacteria; however, this treatment had high mean standard errors. Investigations of root colonization by introduced rhizobacteria should include several recovery methods to optimize recovered numbers or to decrease variability, depending on the experimental objectives.

Key words: root colonization, rhizobacteria, *Pseudomonas fluorescens*, *Bacillus subtilis*, cotton.

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Diverses méthodes ont été utilisées pour recouver des bactéries introduites dans des racines de plantes. La présente étude a visé à comparer systématiquement cinq méthodes : l'agitation dans un tampon, l'agitation dans un tampon avec billes de verre, l'emploi d'un malaxeur Stomacher[R] de laboratoire, la sonication et, finalement, le broyage par pilon dans un mortier. Des graines de coton ont été traitées avec deux souches de rhizobactéries ayant fait l'objet de publications, soit le *Pseudomonas fluorescens*, souche Pf-5, et le *Bacillus subtilis*, souche GB03. L'efficacité de recouvrement de chaque méthode a été déterminée après 3 semaines, en comparant les moyennes des populations bactériennes de systèmes racinaires complets, de segments individuels de racines de 2,0 cm, et de deux régions racinaires : les 5 cm les plus distaux des apex et les 5 cm les plus proximaux des apex. Le traitement au malaxeur a donné des moyennes de populations de GB03 significativement plus élevées ($P = 0,05$) par comparaison aux autres méthodes et des moyennes de populations de Pf-5 significativement plus élevées par comparaison à l'agitation avec billes de verre. Les populations de Pf-5 recouvrées par le traitement au malaxeur des 5 cm proximaux des apex racinaires ont été significativement plus élevées que par les autres méthodes. L'inclusion des billes de verre dans les traitements d'agitation n'a pas davantage poduit de nombres absolus concordants plus élevés de bactéries que de réductions dans la variabilité. L'erreur standard moyenne de chaque méthode a varié avec les sources de racines; aucune des méthodes n'a fourni d'erreurs standard moyennes plus élevées ou plus faibles de façon régulière. Pour chaque source de racine et chaque méthode de recouvrement, les erreurs standard moyennes pour la souche GB03 ont été généralement plus faibles que celles de la souche Pf-5. Comparées entre elles, les données suggèrent que le traitement au malaxeur a été le meilleur pour le recouvrement des plus grands nombres de rhizobactéries; toutefois, ce traitement a fourni des erreurs standard moyennes élevées. Les recherches sur la colonisation des racines par des rhizobactéries introduites devraient inclure plusieurs méthodes de recouvrement pour optimiser les nombres recouvrés ou pour diminuer la variabilité, selon les objectifs expérimentaux.

Mots clés : colonisation des racines, rhizobactéries, *Pseudomonas fluorescens*, *Bacillus subtilis*, coton.

[Traduit par la rédaction]

Introduction

The term "rhizobacteria" describes rhizosphere bacteria that colonize roots (Schroth and Hancock 1982) or proliferate in soil under the influence of roots. Root colonization of introduced rhizobacteria is a process whereby the bacteria survive inoculation onto seeds or into soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids (Kloepper et al. 1985), attach to the root surface, and colonize the developing root system in the presence of native soil microflora (Schroth and Hancock 1982). Hence, rhizobacteria are efficient microbial competitors, and some specific strains may displace native rhizoplane microorganisms (Kloepper and Schroth 1981) in the root zone, often persisting through the midstages of host-plant ontogeny at population densities of log 3 to log 6 cfu · g root fresh weight^{-1} (Bahme et al. 1988; Kloepper et al. 1980; Polonenko et al. 1987; Anderson and Guerra 1985; Howie and Echandi 1983).

Investigations on the ecology of plant-root colonization by rhizobacteria are predicted on the ability to recover bacteria from roots of inoculated plants. Various methods have been used to

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remove bacteria from roots prior to plating on selective media. Most commonly, roots are shaken for a given time in tubes or flasks containing sterile water or buffer (Bahme et al. 1988; Bahme and Schroth 1987; James et al. 1985; Howie and Echandi 1983; Kloepper et al. 1980; Loper et al. 1984, 1985). Alternatively, roots can be sonicated in buffer (Parke et al. 1990), shaken in buffer with glass beads (de Weger et al. 1987; Howie et al. 1987; Weller et al. 1988; van Peer and Schippers 1988), or triturated with a mortar and pestle (Weller and Cook 1983). Recent work on the ecology of rhizobacteria has included assessments of the internal root populations (van Peer et al. 1990) using root trituration with mortar and pestle (van Peer and Schippers 1988; van Peer et al. 1990).

The method used to remove bacteria from roots may affect the estimates of population densities on or in roots. We are aware of no published study that systematically compared the calculated population densities of rhizobacteria in response to different methods of recovery from roots. The main objective of this research was to compare various methods of recovering rhizobacteria from cotton roots by determining if the calculated mean log colony-forming units per gram root or the variability within treatment methods changed with different methods. A second objective was to determine whether results varied between model *Pseudomonas* and *Bacillus* rhizobacterial strains, as these have distinct differences in tolerance to temperatures over 30°C and desiccation conditions. An abstract on part of this work has been presented (Kloepper et al. 1991).

Materials and methods

Bacterial cultures

Pseudomonas fluorescens strain Pf-5, obtained from Charles Howell, USDA, College Station, TX, was previously reported as a biological control agent of *Pythium ultimum* (Howell and Stipanovic 1980) and *Rhizoctonia solani* (Howell and Stipanovic 1979) on cotton. *Bacillus subtilis* strain GB03 was obtained from Gustafson, Inc., Dallas, TX. GB03 originated as a selection of *B. subtilis* strain A-13 (Broadbent et al. 1977) and induced plant growth promotion and biological control of *R. solani* on peanut (*Arachis hypogaea* L.) (Turner and Backman 1991) and cotton (P. A. Backman, unpublished). Bacteria were stored at −80°C in tryptic soy broth (TSB) (Difco, Detroit, MI) containing 20% glycerol between experiments.

For use in experiments, spontaneous rifampicin-resistant mutants were selected for strain Pf-5 (Pf-5 rif), using procedures described previously (Kloepper et al. 1980). A mutant strain with a similar growth rate to the wild-type strain was selected for further investigations. Prior to use in experiments, Pf-5 rif was removed from −80°C storage and streaked for purity onto *Pseudomonas* agar F (Difco) amended with 100 mg rifampicin per litre (rif-PAF).

Seed inoculation and plant growth

Pf-5 rif was grown in TSB amended with 100 mg rifampicin per litre for 24 h with agitation at 150 rpm at 28°C. The suspension was centrifuged 5 min at 10 000 × *g*, and the pellet was washed twice in 0.1 M phosphate buffer, pH 7.0 (PB) before resuspending in 20 mL sterile 1% sodium alginate. The alginate suspension was mixed with neutralized acid – delinted cotton seed (cv. DES 119) at a rate of 1.2 mL alginate suspension/100 g seed, which was determined by dilution plating on rif-PAF to result in average seed populations of log 6 cfu seed^{-1}. Residual H_2SO_4 from the delinting process on cotton seed was neutralized by shaking 300 g seed with 350 mL water and 150 g sodium bicarbonate (baking soda) for 15 min, rinsing with tap water, and drying seeds overnight at room temperature. This procedure increased seed-surface pH on average from 1.8–2.0 before treatment to 6.8 after treatment.

GB03 inoculum consisted of a commercial spore preparation (Quantum 4000 HB), which was obtained from Gustafson, Inc. Quantum 4000 was applied as a dust at a rate of 7.5 g · kg seed^{-1} by shaking neutralized seeds with Quantum 4000 in a plastic bag. This resulted in an average seed population of log 6 cfu seed^{-1}, as determined by dilution plating onto salt V-8 agar (Turner and Backman 1991) amended after autoclaving with cycloheximide and polymixin-B sulfate at 200 mg L^{-1} and 3000 units L^{-1}, respectively (modified salt V-8 agar). Modified salt V-8 agar was selected because Turner and Backman (1991) discovered that this medium is semiselective and differential for strain GB03.

Five inoculated cotton seeds were planted per 10-cm square plastic pot containing field soil from Headland, AL, which was a typical coastal plains sand (87% sand, 8% silt, and 5% clay with 0.6% organic matter). Pots were maintained in the greenhouse at 20–30°C and were watered every 2 days. Root samples were taken for determining populations of introduced rhizobacteria 3 weeks after planting, which corresponded to the first true-leaf stage.

Comparison of methods for recovery from plants

Populations were estimated for each of three types of root samples: whole root systems, 2-cm segments, and main root sections. The 2-cm segments were taken from the taproot 5–7 cm below the soil line. Main root sections included both the uppermost (crown region) 5 cm with attached 1-cm stumps of removed lateral roots and the lowermost (tip) 5 cm. Eight replications were used for whole root systems and 2-cm segment samples, while six replications were used for main root region samples. Root fresh weights were recorded for whole root systems (mean weight, 2.1 g per sample) and main root sections (mean weight, 0.4 g per sample). All root samples were prepared by removing roots from pots and shaking off loosely attached soil without washing.

The following five recovery methods were used as treatments to assess root colonization by Pf-5 and GB03: agitation with a wrist-action shaker (15°) for 30 min in 10 mL PB, agitation in 10 mL PB containing 0.5 g mL^{-1} autoclaved glass beads (140–270 mesh), sonication for 1 min in 10 mL PB using a "Microson™ ultrasonic cell disruptor" (Heat Systems Ultrasonics, Farmingdale, NY) at a 10% power setting, mixing for 1 min in 10 mL PB in a "Stomacher^R laboratory blender" (Fisher Scientific Products, Atlanta, GA), and trituration with mortars and pestles. The sonication level (percent power) and time were the greatest values that did not cause decreases in viable bacteria in buffer suspensions, as determined in a separate study. A mixing time of 1 min with the Stomacher^R blender was found to yield the optimum recovery of bacteria from roots.

After treatment, all root samples were serially diluted and spiral plated (Spiral Systems, Inc., Bethesda, MD) onto rif-PAF agar and modified salt V-8 agar for quantification of Pf-5 and GB03, respectively. After incubation for 24 h at 28°C (Pf-5) or 48 h at 30°C (GB03), bacteria were counted with a laser colony counter (Spiral Systems) for strain Pf-5 or hand counted (GB03) based on the distinctive colony morphology described by Turner and Backman (1991), and number of colony-forming units per millilitre was determined using Bacterial Enumeration software (Spiral Systems). No "background" colonies developed on rif-PAF inoculated with suspensions of nonbacterized roots after 24 h incubation. Mean population densities (cfu · g fresh root^{-1} or cfu · cm root^{-1}) were calculated by averaging log values for each replication and were compared among recovery methods for each type of root sample using the general linear models procedure and calculating LSD values at $P = 0.05$ and $P = 0.10$ for experiments with a significant F value. All statistical analyses were conducted using SAS software (SAS Institute, Inc., Cary, North Carolina). The experiments were repeated once.

Results

Mean populations of rhizobacteria from whole root systems estimated using the five methods of recovery ranged from log 2.6 to log 3.9 cfu g^{-1} for strain Pf-5 and from log 3.5 to log 4.9 cfu g^{-1} for strain GB03 (Table 1). Treatment with the Stomacher^R blender yielded the highest mean populations of both strains compared with all other methods of recovery, and this difference was significant at $P = 0.05$ for strain GB03 and at

TABLE 1. Comparison of methods for recovery of rhizobacteria from whole roots of cotton

	Mean population recovered and variability within treatments[a]			
	Pseudomonas fluorescens strain Pf-5		*Bacillus subtilis* strain GB03	
Method	Log cfu/g	MSE	Log cfu/g	MSE
Agitation	3.1	0.21	3.7	0.11
Agitation with glass beads	2.6	0.16	4.0	0.15
Stomacher®	3.9	0.37	4.9	0.42
Sonicator	3.2	0.28	3.7	0.19
Mortar and pestle	3.3	0.17	3.5	0.07
$LSD_{0.05}$	0.7		0.6	
$LSD_{0.10}$	0.6		0.5	
CV	24.3		1.35	

[a]Means were calculated from eight replications with two plants per replication.

TABLE 2. Comparison of methods for recovery of rhizobacteria from taproot sections of cotton

	Mean population recovered and variability within treatments[a]							
	Pseudomonas fluorescens strain Pf-5				*Bacillus subtilis* strain GB03			
Method	Log cfu/g	MSE	Log cfu/g	MSE	Log cfu/g	MSE	Log cfu/g	MSE
Agitation	2.9	0.25	3.0	0.21	3.9	0.14	4.2	0.12
Agitation with glass beads	2.2	0.40	2.5	0.39	4.0	0.19	4.1	0.36
Stomacher®	2.7	0.31	3.4	0.13	4.0	0.15	4.1	0.23
Sonicator	3.0	0.38	1.8	0.15	3.7	0.18	3.6	0.23
Mortar and pestle	2.8	0.42	2.8	0.96	3.7	0.25	3.8	0.22
$LSD_{0.05}$	ns[d]		0.4		ns		ns	
$LSD_{0.10}$	ns		0.3		ns		0.5	
CV	31.4		7.2		18.7		12.2	

[a]Means were calculated from six replicate plants per.
[b]The uppermost 5 cm of main root with attached 1-cm length of lateral roots.
[c]Tips were the lowermost 5 cm og main root with attached 1-cm length of lateral roots.
[d]ns, not significant.

$P = 0.10$ for strain Pf-5. With the upper 5 cm of taproot (Table 2), Stomacher^R treatment and agitation with glass beads yielded higher GB03 populations than all other treatments. Populations of Pf-5 on the lower 5 cm of taproot were significantly higher than all other recovery methods with Stomacher^R treatment, and this treatment had the lowest mean standard error (MSE) of all recovery methods. Mean populations of GB03 were higher with Stomacher^R treatment than all other recovery methods with the 2-cm segments (Table 3).

The lowest overall populations of Pf-5 were recovered from the 2 cm long segments, with mean populations ranging from <log 1.0 to log 1.9 cfu · cm root^{-1} (Table 3). However, populations of GB03 on these segments were similar to populations recovered from other root sources. While mean population differences among recovery methods were not statistically significant, the highest populations of strain Pf-5 were recovered with the mortar and pestle treatment, and Stomacher^R treatment recovered the highest populations of strain GB03.

The estimated populations of Pf-5 and GB03 from the upper 5 cm of taproot and GB03 from the lower 5 cm of taproot were not significantly different among recovery methods (Table 2). With strain Pf-5 on the lower 5 cm of taproot, Stomacher^R treatment recovered a mean population of log 3.4 cfu cm^{-1}, which was significantly higher than mean populations with all other methods of recovery.

The MSE of each recovery method varied among root sources sampled, and no single method consistently had the highest or lowest MSE (Tables 1–3). MSE values for strain GB03 were generally lower than those for Pf-5 with each root source and each method of recovery.

Discussion

The most definitive results, based on detection of statistically significant differences in mean populations among treatments, occurred with whole root systems, probably because the greater root mass resulted in lower plant to plant variation than with smaller root samples. MSE values for recovery methods of Pf-5 were lower in most cases with whole root systems (Table 1) than 5-cm taproot segments (Table 2) or 2-cm segments (Table 3). This suggests that studies with seedling root colonization by rhizobacteria may gain statistical precision by using whole root samples. Additional replications would be required to have similar precision in separation of means from those root sources with higher variability (higher MSE values). In a study by Loper

TABLE 3. Comparison of methods for recovery of rhizobacteria from 2-cm root segments[a] of cotton

Method	Mean population recovered and variability within treatments[b]			
	Pseudomonas fluorescens strain Pf-5		*Bacillus subtilis* strain GB03	
	Log cfu/g	MSE	Log cfu/g	MSE
Agitation	<1.0	—	3.7	0.18
Agitation with glass beads	1.7	0.33	3.4	0.21
Stomacher®	1.1	0.42	4.0	0.31
Sonicator	1.6	0.75	3.6	0.18
Mortar and pestle	1.9	0.29	3.3	0.20
$LSD_{0.05}$	ns[c]		ns	
$LSD_{0.10}$	0.6		ns	
CV	28.7		18.7	

[a]The 2-cm segments were from the main root 5–7 cm below the soil line
[b]Means were calculated from eight replications with two plants per replication
[c]ns, not significant.

et al. (1984) on root colonization by two fluorescent pseudomonad rhizobacterial strains, an average of 14 and as many as 36 replications were needed to detect a mean population difference of 0.5 log units at $P = 0.05$.

The results indicate that the recovery method can have a significant effect on the estimated popuations of rhizobacteria. When viewed in composite, the data suggest that the Stomacher® treatment was the best for recovering the greatest absolute numbers of rhizobacteria; however, this treatment had high MSE values for whole root systems. In contrast, the lowest variability occurred with agitation using glass beads.

The most commonly used method for recovering rhizobacteria from roots is agitation in buffer. This method yielded the highest estimated mean populations from only one root source with strain Pf-5 and from none of the sources for strain GB03; however, MSE values with agitation were low.

There was no consistent benefit in either absolute numbers or reduction in variability to agitation with glass beads compared with agitation in buffer alone with either strain. These results contrast to the report of Geels and Schippers (1983) that recovery of fluorescent pseudomonads from potato periderm was enhanced 100% by agitation of periderm disks with glass beads as compared with agitation without beads. Geels and Schippers (1983) also reported that trituration of periderm disks with mortar and pestle did not result in recovery of more bacteria than agitation with glass beads. In our study, trituration with mortar and pestle yielded slightly higher populations of Pf-5 than agitation with glass beads for all root sources, while the GB03, populations recovered by trituration with mortar and pestle were slightly lower than after agitation with glass beads. Because these differences were generally not significant, one should conclude that in the current study there were no overall differences between agitation with glass beads and trituration with mortar and pestle. Treatment with both the Stomacher® and mortar and pestle would allow liberation of internal root populations of bacteria (van Peer et al. 1990), if the rhizobacterium used exhibits internal colonization. In this study, recovered populations were generally lower with mortar and pestle than with Stomacher® treatment. While the reasons for this are currently unknown, mortar and pestle may result in more complete trituration of plant tissues, thereby releasing plant inhibitors that could reduce bacterial viability.

Sonication of whole root systems recovered Pf-5 and GB03 at levels intermediate between agitation with glass beads and trituration with mortar and pestle. With the lowermost 5 cm of taproot (Table 2), sonication recovered a significantly lower mean population of Pf-5 compared with all other recovery methods. MSE values for sonication were generally intermediate among MSE values of all five recovery methods. Hence, in this study, no clear advantage to sonication was noted compared with the other recovery methods.

Calculation of mean standard errors allowed a quantitative measure of variability among replications within treatments, which in the case of this study, measured variation in populations of rhizobacteria among plants. MSE values ranged up to 0.96 for Pf-5 on the lowermost 5-cm root segments (Table 2), indicating considerable plant to plant variability in colonization levels, which agrees with previous reports in other crop systems. Bahme and Schroth (1987) reported up to a 2.8 log unit difference in populations of *Pseudomonas fluorescens* strain A1-B on equivalent sections of potato root among plants. Similarly, Loper et al. (1984) detected high variability in populations of rhizobacteria on roots among sugar beet plants, with up to a 2.76 log unit difference with fluorescent *Pseudomonas* strain SH5. Such variability in populations results, in part, from the frequency with which rhizobacteria colonize individual root segments. Bahme et al. (1988) reported that detection frequencies (percentage of root segments with any recoverable rhizobacteria) decreased from 70–100% for segments 0–16 cm from the inoculated potato seed piece to 30% for segments over 24 cm from the seed piece.

MSE values differed between bacterial strains. GB03 had lower MSE values than Pf-5 for 16 of the 20 estimated populations (five treatments × four root sources). Similar strain-dependent variability in root colonization was noted by Loper et al. (1984). As indicated in the Materials and methods section, the root samples included adhering rhizosphere soil, and therefore, it is possible that some of the differences in variability relate to the mass of adhering soil. We consider that this possibility does not account for the main variability in these experiments for the following reasons. First, because the soil was predominantly

sand, most of the soil was easily shaken from the roots, and roots had a uniform-appearing amount of soil adhering before processing for population estimations. Second, with a sandy soil very similar in texture to the one used in this study, Park et al. (1990) reported that the amount of adhering soil (from 0 to 1200 mg · cm root^{-1}) on roots did not significantly affect the estimation of root populations with a *P. fluorescens* rhizobacterium.

The results reported here indicate that the methods used to recover root-colonizing bacteria affect estimates of bacterial populations on roots. Therefore, studies of root colonization by introduced bacteria should examine several different recovery methods to maximize the estimated bacterial populations or to minimize variability within treatments, depending on the objectives of the particular investigation.

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