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, sonication, and rinsing 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 3 cm of taproot and the lowermost 3 cm). Treatment with the Stomacher yielded significantly higher (P = 0.05) mean populations of GB03 compared with all other methods except significantly higher mean population of PF-5 compared with agitation with glass beads. From the lowermost 2.0 cm of taproot, populations of PF-5 recovered by the Stomacher treatment were significantly higher than all other methods. The inclusion of glass beads for agitation treatments resulted in higher absolute numbers of recovered bacteria but 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 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.


Diverses méthodes ont été utilisées pour recoller des bactéries introduites dans des racines de plantes. La présente étude a été visée à comparer systématiquement cinq méthodes : l’agitation dans un tampon, l’agitation dans un tampon avec billes de verre, l’emploi d’un mélangeur Stomacher de laboratoire, le 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 recollement de chaque méthode a été déterminée après 3 semaines, en comparant les moyennes des populations bacilles de souches bacilennes de systèmes racinaires complets, de segments individuels de racine de 2.0 cm, et de deux régions racinaires : les 5 cm les plus distaux de l’apex et les 5 cm les plus proéminents de l’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 recollées par le traitement au malaxeur des 5 cm proéminents de l’apex racinaire ont été significativement plus élevées que les autres méthodes. L’inclusion des billes de verre dans les traitements d’agitation n’a pas davantage conduit à des nombres absolu comparables plus élevés de bactéries que de réductions dans l’incertitude. 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 recollement, 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 recollement des plus grands nombres de rhizobactéries ; toutefois, ce traitement a fourni des erreurs standard moyennes plus élevées. Les recherches sur la colonisation des racines par des rhizobactéries introduites devraient inclure plusieurs méthodes de recollement pour optimiser les nombres recollements ou pour diminuer la variabilité, selon les objectifs expérimentaux.

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

[Intitulé par la réduction]

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 rhizosphere, and respond to seed exudates rich in carbohydrates and amino acids (Kloeper 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 (Kloeper 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 (Buhme et al. 1988; Kloeper et al. 1980; Polonenko et al. 1987; Anderson and Guerra 1985; Howie and Echandi 1983).

Investigations of 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

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 rhizobacteria 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 (Kloepfer et al. 1991).

Materials and methods

Bacterial cultures

Pseudomonas fluorescens strain P5-F, obtained from Charles Howell, USDA, College Station, TX, was previously reported as a biological control agent of Phytophthora ultimum (Howell and Stepanovic 1980) and Rhizoctonia solani (Howell and Stepanovic 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 (Bendet 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 P5-F (P5-F rif), using procedures described previously (Kloepfer 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, P5-F rif was removed from -80°C storage and streaked for purity onto Pseudomonas agar F (Difco) amended with 100 mg rifampicin per liter (rif-PAF).

Seed inoculation and plant growth

P5-F rif was grown in TSB amended with 100 mg rifampicin per liter for 24 h with agitation at 150 rpm at 38°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 - deinked 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 population of log 6 cfu seed⁻¹. Residual H₂SO₄ from the deinking 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 Quantum, 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 9 cfu seed⁻¹, as determined by dilution plating onto salt V-8 agar (Turner and Backman 1991) after autoclaving with cycloheximide and polymixin-B sulfate at 200 mg L⁻¹ and 3000 units L⁻¹, respectively 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) 3 cm with attached 1-cm sections of removed lateral roots and the lowermost (tip) 3 cm. Eight replicates were used for whole root systems and 2-cm segment samples, while six replicates 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 described previously, root colonization by P5-F and GB03; agitation with a wrist-action shaker (15”) for 30 min in 10 mL PB, agitation in 10 mL PB containing 0.5 mg mL⁻¹ autoclaved glass beads (140-270 mesh), sonication for 1 min in 10 mL PB using a “Microson” ultrasonic cell disruptor (Heat Systems Ultronics, Farmingdale, NY) at a 10% power setting, mixing for 1 min in 10 mL PB in a “Stomacher” blender (Fisher Scientific Products, Atlanta, GA), and trituration with a mortar and pestle. 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® 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 P5-F and GB03, respectively. The inoculation for 24 h at 28°C (P5-F) or 48 h at 30°C (GB03) bacteria were counted with a laser colony counter (Spiral Systems) for strain P5-F 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/g 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 P5-F and from log 3.5 to log 4.9 cfu g⁻¹ for strain GB03 (Table 1). Treatment with Stomacher® blender yielded the highest mean populations of both strains compared with all other methods of recovery, and this difference was significant at P = 0.03 for strain GB03 and at
Table 1. Comparison of methods for recovery of rhizobacteria from whole roots of cotton

<table>
<thead>
<tr>
<th>Method</th>
<th>Pseudomonas fluorescens strain PF-5</th>
<th>Bacillus subtilis strain GB03</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log cfu/g</td>
<td>MSE</td>
</tr>
<tr>
<td>Agitation</td>
<td>3.1</td>
<td>0.21</td>
</tr>
<tr>
<td>Agitation with glass beads</td>
<td>2.6</td>
<td>0.16</td>
</tr>
<tr>
<td>Stomacher*</td>
<td>3.9</td>
<td>0.37</td>
</tr>
<tr>
<td>Sonicator</td>
<td>3.2</td>
<td>0.28</td>
</tr>
<tr>
<td>Mortar and pestle</td>
<td>3.3</td>
<td>0.17</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>LSD0.10</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>24.3</td>
<td></td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Method</th>
<th>Pseudomonas fluorescens strain PF-5</th>
<th>Bacillus subtilis strain GB03</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log cfu/g</td>
<td>MSE</td>
</tr>
<tr>
<td>Agitation</td>
<td>2.9</td>
<td>0.25</td>
</tr>
<tr>
<td>Agitation with glass beads</td>
<td>2.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Stomacher*</td>
<td>2.7</td>
<td>0.31</td>
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<tr>
<td>Sonicator</td>
<td>3.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Mortar and pestle</td>
<td>2.8</td>
<td>0.42</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>ns</td>
<td>0.4</td>
</tr>
<tr>
<td>LSD0.10</td>
<td>ns</td>
<td>0.3</td>
</tr>
<tr>
<td>CV</td>
<td>31.4</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*Means were calculated from six replicate plants per.

The overall populations of PF-5 were recovered from the 2 cm long segments, with mean populations ranging from \(<\log_{10} 1.0 \text{ to } \log_{10} 1.9 \text{ cfu cm}^{-1}\) (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* 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* treatment recovered a mean population of \(\log_{10} 3.4 \text{ cfu cm}^{-1}\), 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* of cotton

<table>
<thead>
<tr>
<th>Method</th>
<th>Pseudomonas fluorescens strain Pf-5</th>
<th>Bacillus subtilis strain GB03</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log cfu/g</td>
<td>MSE</td>
</tr>
<tr>
<td>Agitation</td>
<td>&lt;1.0</td>
<td>—</td>
</tr>
<tr>
<td>Agitation with glass beads</td>
<td>1.7</td>
<td>0.33</td>
</tr>
<tr>
<td>Stomacher*</td>
<td>1.1</td>
<td>0.42</td>
</tr>
<tr>
<td>Sonicator</td>
<td>1.6</td>
<td>0.75</td>
</tr>
<tr>
<td>Mortar and pestle</td>
<td>1.9</td>
<td>0.29</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>ns†</td>
<td>ns†</td>
</tr>
<tr>
<td>LSD0.10</td>
<td>0.6</td>
<td>ns†</td>
</tr>
<tr>
<td>CV</td>
<td>28.7</td>
<td>18.7</td>
</tr>
</tbody>
</table>

*The 2-cm segments were from the main root 5–7 cm below the soil line.
†Means were calculated from eight replications with two plants per replication.
*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 populations 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 rhizobacteria 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 3-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 X 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 apparent 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⁻²·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.


