REVIEW / SYNTHÈSE

A review of issues related to measuring colonization of plant roots by bacteria

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Introduction 1220
Concepts and definitions 1220
Selection of a model test system 1221
Recovery methods 1221
Marking systems 1222
Statistical approaches 1226
Conclusion: future directions in measuring root colonization 1228

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Root colonization by introduced bacteria is an important step in the interaction of beneficial bacteria with the host plant. Investigators attempting to measure root colonization by bacteria must face several issues. A clear concept or definition of root colonization should be stated in each research summary, as several different definitions have been proposed. We consider true root colonists to be those bacteria that colonize roots in competitive conditions, i.e., natural field soils. Different methods of processing root samples are required if one is measuring external root colonization alone, internal colonization alone, or both. Given that most beneficial bacterial strains currently under investigation as root colonists are members of taxa naturally found in soils, a marking system is required to differentiate the introduced strain from members of the indigenous rhizosphere community. Spontaneous antibiotic resistance, immunological approaches, and foreign DNA sequences are among the marking systems that have been used and each has some possible advantages and disadvantages. More research is needed in the development and comparison of marking systems. The design of experiments to measure root colonization should take into account several statistical issues. One must decide what constitutes the sample unit for each replication of a given treatment, e.g., whole root systems or root segments. Consideration should also be given to how best to express the estimated population of root colonists (e.g., cfu/g fresh or dry weight root, cfu/cm root, or cfu/surface area root). Statistical analysis by standard analysis of variance tests should be used whenever possible to separate treatment means of colonization levels; however, one must determine that the underlying assumptions of these tests are correct for each experiment. Finally, in quantification of populations on roots, one will almost certainly encounter replications with no bacteria, i.e., zeros. There are several options for how to calculate treatment means when one or more replications is a zero, and the implications of these options are discussed.

Key words: biolumineseence, genetic markers, plant growth-promoting rhizobacteria, rhizosphere bacteria, root, colonization.

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La colonisation des racines suite à l'inoculation de la semence par des bactéries est une étape importante dans les interactions entre bactéries bénéfiques et la plante hôte. Les investigateurs qui désirent mesurer la colonisation des racines par les bactéries doivent envisager plusieurs avenues. Un concept clair, ou définition de la colonisation des racines, doit être énoncé dans chaque résumé, vu que différentes définitions ont été avancées. Nous considérons que les bactéries qui sont en conditions de compétition, c.-à-d. dans les sols naturels, sont les véritables colonisateurs des racines. Différentes méthodes de traitements des échantillons de racines sont possibles, selon qu'il s'agisse de ne mesurer que la colonisation externe, la colonisation interne, ou les deux. Compte tenu que la majorité des souches bactériennes bénéfiques couramment étudiées comme colonisatrices des racines sont des membres de taxons se trouvant naturellement dans les sols, un système de marquage devient nécessaire pour distinguer les souches introduites des membres des communautés indigènes de la rhizosphère. La résistance spontanée aux antibiotiques, les approches immunologiques et les séquences d'ADN étranger se trouvent parmi les systèmes de marquage qui ont été utilisés et chacun d'eux présente certains avantages ou désavantages. Il faudrait davantage de recherche sur le développement et la comparaison des systèmes de marquage. Pour mesurer la colonisation des racines, les plans expérimentaux devraient envisager plusieurs approches statistiques. Il importe de définir ce qui constitue l'unité d'échantillonnage pour chacun des traitements utilisés, par ex. : des systèmes racinaires entiers ou des segments de racines. Certaine considération devrait aussi être apportée à la meilleure façon d'exprimer les populations estimées de colonisateurs de racines (ufc/g de poids frais ou de poids sec de racine, ufc/cm de racine ou ufc/étendue de surface de racines). Les analyses de variance devraient être utilisées si possible pour séparer les moyennes des niveaux de colonisation par traitement; toutefois, il importe de déterminer si les postulats sous-jacents à ces tests sont valides pour chaque expérience. Finalement, lors de la quantification des populations sur les racines, il est presque certain que dans certaines répétitions il n'y aura aucune bactérie

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(zéro). Il existe plusieurs options pour le calcul des moyennes de traitements comportant une ou plusieurs répétitions avant la valeur zéro; les implications de ces options sont discutées.

Mots clés : bioluminescence, marqueurs génétiques, rhizobactéries favorisant la croissance des plantes, bactéries de la rhizosphère, raeines, colonisation racinaires.

[Traduit par la rédaction]

Introduction

Soil and rhizosphere bacteria have been examined for over one century as crop inoculants to improve agricultural production (Burris 1988). The first bacterial inoculants to be used in agriculture were the nitrogen-fixing rhizobia. Work initiated in Russia in the early 1900s (Mishustin 1963; Mishustin and Naumova 1962) and continued by Brown *et al.* (1964) and Rovira (1963) in the 1960s established that pseudomonads and other rhizosphere bacteria may also increase plant growth. In the past two decades, various groups of bacteria have been demonstrated as beneficial to plants, including biological control agents (Weller 1988), plant growth-promoting rhizobacteria (PGPR) (Kloepper 1991, 1992; Kloepper *et al.* 1991*a*), yield-increasing bacteria (YIB) (Mei *et al.* 1990), and *Azospirillum* spp. (Bashan and Levanony 1990). Among the groups of beneficial eubacteria, only the rhizobia form a highly specialized symbiotic host relationship that leads to morphologically distinct structures (nodules). All other groups have a loose association with the plant and are termed "associative" bacteria. To influence the plant, associative bacteria must grow in or on roots. Consequently, colonization of plant roots is of primary importance for beneficial associative bacteria, and even with rhizobia, the establishment of symbiosis is preceded by a phase of bacterial growth in the rhizosphere and attachment to roots. Hence, bacterial root colonization remains an important parameter to measure in diverse bacterial-plant systems.

How then, does one measure root colonization by introduced bacteria? While the basic answer is straightforward (perform isolations from inoculated roots and quantify the bacteria recovered) in the process, one must face several important issues, including selection of a model test system, recovery methods, marking systems, and statistical approaches. This review is designed as a "hands-on" guide for root colonization work for students and researchers who are contemplating root colonization studies as well as for those researchers already engaged in measuring colonization. The current understanding and unresolved questions related to each of the previously stated issues are summarized. Other recent reviews have focused on phenotypic (Bakker *et al.* 1991*a*; Glandorf *et al.* 1991; Parke 1991) and genotypic (Lam *et al.* 1991) bacterial traits related to root colonization, and therefore, these subjects are not discussed here.

Concepts and definitions

Before designing experiments to measure root colonization, it is desirable to decide clearly what is meant by "root colonization," as the chosen concept will influence the choice of methods. Since root colonization was first used to describe recovery of antibiotic-resistant PGPR strains from external surfaces of potato roots following application to seed pieces (Kloepper *et al.* 1980), several definitions of root colonization have been proposed (Howie *et al.* 1987; Parke 1991; Kloepper *et al.* 1991*a*; Thomashow and Weller 1990; Weller 1988). While each definition differs from the others, there is general agreement that root colonization is an active process involving growth of the introduced bacteria on or around roots and is not simply a passive chance encounter of a soil bacterium with a passing root.

Most previously published research accounts of root colonization by rhizobacteria measure external root colonization, although van Peer and Schippers (1988) reported that one pseudomonad PGPR strain colonized tomato roots internally. *Azospirillum* colonizes grass roots externally and internally (Baldani *et al.* 1986; Okon 1985; Patriquin *et al.* 1983), and therefore measurements of its root colonization capacity routinely include determinations of internal populations (Bashan and Levanony 1990; Michiels *et al.* 1989). It is important in selecting recovery methods that one decide if the goal is to measure root colonization externally, internally, or both, as each will require different recovery techniques.

The key to the concept of root colonization is that root-colonizing bacteria grow on roots in the presence of the indigenous microflora (Schroth and Hancock 1982) and thus root colonists are competitive with soil bacteria and fungi. The implication of this for measuring root colonization is that some method must be used for differentiating the introduced bacterium from indigenous bacteria of the same taxon. Marking systems are used for this purpose.

While we prefer the term root colonization with the use of "external," "internal," or "total" as descriptive qualifiers, other terms have been proposed. "Rhizosphere competence" was used by Schmidt (1979) in relation to rhizobia to describe soil microorganisms that show enhanced growth in response to developing plant roots. In this context, "rhizosphere competent" microorganisms are those that show the classical rhizosphere effect. Recently, the term rhizosphere competence has been used in relation to biological control agents, and Baker (1991) redefined it as, "the ability of a microorganism, applied by seed treatment, to colonize the rhizosphere of developing roots," a definition that does not differ substantially from that proposed by Schmidt (1979). Several reports of root colonization have used rhizosphere competence and root colonization interchangeably as synonyms (Hozore and Alexander 1991; Suslow 1982). Lynch (1990) used rhizosphere colonization as a synonym for root colonization, although no definition was proposed. Hence, several terms have been proposed as synonyms or as differential terms for root colonization, and it is important that each report of root colonization indicates which definition or concept is used. The selected definition will affect the goal of the experiment, and therefore, the choice of methods for measuring colonization. For example, if one chooses Schmidt's concept of rhizosphere competence (Schmidt 1979), then a comparison of populations in rhizospheres and root-free soil would be required to demonstrate rhizosphere competence. Alternatively, if one chooses the concept of rhizosphere competence as a synonym for root colonization, then populations of the introduced bacterium on developing roots would be required.

Selection of a model test system

Before initiating investigations of root colonization, one must select a model test system for the assays. Choices must be made regarding soil characteristics, i.e., the use of natural versus autoclaved soil, greenhouse versus field tests, soil moisture, and soil texture. As discussed above, the concept of root colonization has generally evolved to include competition of the introduced strain with indigenous soil microflora. Therefore, determinations of root colonization should be done in nonsterile field soils without autoclaving or pasteurization. Exceptions are warranted, for example, in the case where colonization in a biological control assay needs to be determined and the assay involves addition of a pathogen to pasteurized soil. However, one must not extrapolate results from pasteurized soils to conclude that similar colonization will occur in field soils. Scher *et al.* (1984) reported that two fluorescent pseudomonad strains that failed to colonize maize roots in nonsterile field soil were recovered at log 7 cfu/g root in autoclaved soil; hence, elimination of indigenous microflora from the test system could result in an artificially high assessment of root colonization capacity. Direct comparisons of root colonization capacities of bacteria in sterile and nonsterile soils will help clarify the contribution of microbial competition to colonization.

While routine testing and ranking of bacterial strains for root colonization is often done in field soils under greenhouse conditions, the results may not be consistent with those from field tests, even when the same soil is used. We are aware of no studies designed to thoroughly investigate the differences in root colonization patterns under greenhouse and field conditions using the same soil, and work is needed on this issue. However, in a study with multiple strains of *Pseudomonas putida*, *P. fluorescens*, and *Serratia* spp., Scher *et al.* (1988) reported that mean root colonization for each group was 1.0–1.5 log units lower per gram root in field trials compared with greenhouse trials. In a comparison of microcosms and field trials, *LacZY*-marked *P. fluorescens* had similar rhizosphere populations on wheat grown in microcosms and field soils (Drahos *et al.* 1988). However, van Elsas *et al.* (1990) reported that genetically marked bacteria may be recovered at higher, lower, or similar populations in microcosms and field trials. Therefore, it is not possible to make general predictions about relative colonization differences between greenhouse or microcosm and field studies.

Soil moisture and watering regimes may affect the distribution and abundance of root-colonizing bacteria. Soil moisture affects mainly bacterial population levels, while watering regimes and soil moisture, to a lesser extent, affect the distribution of bacteria along roots. Rhizobacteria can colonize roots under a range of soil moistures (Howie *et al.* 1987; Liddell and Parke 1989; Worrall and Roughley 1991), with greater colonization occurring at soil moisture levels near field capacity than at drier levels (Howie *et al.* 1987). Percolating water can aid in the distribution of bacteria along roots (Bahme and Schroth 1987; Chao *et al.* 1986; Howie *et al.* 1987; Madsen and Alexander 1982; Parke *et al.* 1986), although bacterial dispersal may occur along roots in the absence of downward-moving water (Chao *et al.* 1986; Howie *et al.* 1987; Liddell and Parke 1989). Steady-state soil moisture has been used to control variability among experiments (Liddell and Parke 1989) by removing the variable of bacterial dispersal by percolating water. However, in any agricultural system, irrigation water or rain is available, and therefore, results from steady-state soil-moisture systems may not agree with results in field trials.

The precise contribution of soil texture, which is determined by the percentages of clay, silt, and sand in a soil (Alexander 1977), to root colonization by introduced bacteria remains to be elucidated. Mahaffee (1991) compared root colonization of cotton by a *Bacillus subtilis* strain in 10 field soils and then used principal factor analysis, correlation, and multiple regression to determine which components accounted for the largest percentage of differences in root colonization among the soils. The clay content of soils appeared to be the dominant factor affecting root colonization, while sand content was negatively correlated with colonization levels. Clays interact directly and indirectly with microbial events such as growth, metabolism, nutrition, competition, predation, parasitism, transfer of genetic information, and toxicity (Stotzky 1986). The effect of clay and sand content on root colonization should also be examined more directly by amending model soils with various concentrations of clay or sand, thereby minimizing possible confounding effects of other soil factors. Until such data are available, it is premature to conclude that one soil texture is preferable to another for basic root colonization studies.

Recovery methods

Recovery methods for quantifying root colonization by introduced bacteria generally rely on plating techniques. Prior to plating a sample, one must first decide how the samples will be processed. One of the most frequently occurring methodological phrases in root colonization literature is the statement that, "roots were shaken (or washed) to remove loosely attached soil particles." Although this may sound like a nonprecise method that could add substantial variability into quantification of population densities, it is based on our understanding of root colonization. Conceptually, an introduced root colonist may not be restricted to the rhizoplane; however, population densities would drop sharply with distance from the rhizoplane. Loose shaking of roots retains soil particles that are embedded in the root mucigel and that, presumably, contain populations of the introduced colonist several log units greater than rhizosphere soil, which does not remain attached to roots. Parke *et al.* (1990) tested this concept by determining the relationship of soil mass adhering to roots and quantification of root colonization levels with a *P. fluorescens* strain and concluded that increasing the mass of adhering soil did not significantly affect estimation of population densities. It should be noted that this study quantified populations per centimetre root, and that soil mass may affect results if populations are expressed per gram soil (see Statistical approaches section).

One must next decide if the experimental goal is to measure only external root colonization, only internal colonization, or both, as different protocols are required for each purpose. If measurement of only external root colonization is desired, then root samples must be processed for removal of rhizoplane bacteria without tearing or cutting of tissues which would release internal colonists. Agitation of roots in sterile water or buffer with or without glass beads and sonication is frequently used for this purpose. The choice

of processing method may depend on the bacterial strain used and may affect experimental variability and the maximum recovered populations (Kloepper *et al.* 1991*b*).

For measuring only internal root colonization, it is necessary to surface-disinfest roots prior to grinding, chopping, or blending (Okon *et al.* 1977; McInroy and Kloepper 1991). Surface-disinfectants most commonly used include diluted household bleach, ethanol, and H_2O_2. Patriquin and Döbereiner (1978) treated roots with 1% chloramine for 6 h to sterilize the epidermis and outer cortex prior to isolating *Azospirillum* from the inner cortex and stele. An important control in all studies of internal colonization, which is usually not reported, is the inclusion of sterility controls. Based on work with endophytes in Kloepper's laboratory (J.A. McInroy, personal communication), no surface-disinfestation protocol results in complete killing of surface bacteria on 100% of samples without penetrating interior tissues and, thereby, killing internal colonists. Experiments should be conducted to allow checking of every sample unit (see Statistical approaches section) for sterility prior to grinding for isolation of internal colonists as was reported by Lalande *et al.* (1989).

One may also choose to measure both external and internal root colonization by some bacteria, such as *Azospirillum*, which may colonize roots externally and internally (Bashan and Levanony 1990; Michiels *et al.* 1989; Patriquin *et al.* 1983; Umali-Garcia *et al.* 1980) and may move into the stem (Rennie *et al.* 1982). For this purpose, a combination of the techniques described above will allow quantification of both kinds of colonization (Okon *et al.* 1977).

Marking systems

A major issue one faces with measuring root colonization of introduced bacteria is how to differentiate the inoculated strain from members of the indigenous rhizosphere bacterial community. Standard microscopic evaluation (Schmidt and Paul 1982) is of limited value, since most bacteria possess no distinct morphological characteristics and hence cannot be distinguished from indigenous bacteria (Gray and Williams 1971). For specific bacteria with resistance to inhibitors, quantification of root colonization can be determined by inoculating samples on selective media containing the appropriate toxic substances (Wollum 1982). Most probable number (MPN) techniques are useful for quantifying root colonization (Alexander 1982) when a characteristic and recognizable bacterial phenotype or transformation of a substrate can be observed. Quantification can also be performed using plate counts with a level of detection between 1–100 colony forming units (cfu)/mL sample (Stewart-Hull 1988) and 10–100 cfu/g soil (Fredrickson *et al.* 1988). With both MPN and plate counts, the selectivity from indigenous bacteria may be based on a combination of nutritional specificity (Saint-Pierre and Dion 1988; Höfte *et al.* 1990), characteristic phenotypes (Bashan *et al.* 1991*b*; Schaad 1988), biochemical alteration of a substrate (Schaad 1988; Wollum 1982) or dye (Drahos *et al.* 1986; Wollum 1982), antibiotic resistance (Stewart-Hull 1988; Wollum 1982), heavy metal resistance (King *et al.* 1991; Stewart-Hull 1988), and siderophore utilization (Raaijmakers *et al.* 1991). Among these approaches, antibiotic-resistance is the most frequently used method for obtaining selectivity.

As the preceding discussion indicates, most techniques for quantifying root colonization require bacterial growth for enumeration and differentiation from indigenous microorganisms. However, it is well established that plate counts underestimate the actual number of bacteria present in soil ecosystems (Gray and Williams 1971) for several reasons. Physical attachment of bacteria to soil particles and aggregation of soil particles will lead to underestimation. In addition, some bacteria may be killed in the dilution medium used and others may fail to grow on the selective medium used for enumeration owing to physiological modifications that occurred in soil. After a period of starvation, some bacteria do not grow on nutritionally rich media. Colwell *et al.* (1985) demonstrated that nonculturable *Vibrio cholerae* were still viable based on enrichment through a host prior to isolation. The general occurrence of nonculturable bacteria has been demonstrated by direct microscopy (Gray and Williams 1971), immunofluorescence (Postma *et al.* 1988), and DNA technologies (Pillai and Pepper 1991). Even though it is often difficult to distinguish between nonculturable and dead (nonviable) bacteria, the latter are considered to disintegrate within 2 weeks in soil and, therefore, could not account for major enumeration differences between immunofluorescence and plate counts (Postma *et al.* 1988). Most immunofluorescence microscopy and nucleic acid hybridization techniques estimate higher population densities of bacteria in soil ecosystems than techniques that require bacterial growth. These differences are partly accounted for by nonculturable starved bacteria that do not grow on media but which react with probes, and partly, by DNA released from lysed bacteria (Pillai *et al.* 1991).

In contrast with direct observation and isolation techniques, immunological (Schmidt and Paul 1982), nucleic acid restriction profiles (Brown *et al.* 1990; Gibson *et al.* 1991; Stewart-Hull 1988), and nucleic acid hybridization (Trevors and van Elsas 1989; van Elsas and Waalwijk 1991) techniques can be used without requiring bacterial growth. With these procedures, antibodies and specific nucleic acid sequences are used as probes, allowing specific detection of the inoculated bacteria. While microscopic enumeration of target bacteria by immunofluorescence is time consuming (van Vuurde 1991), it can be used to confirm *in situ* colonization. Underberg and van Vuurde (1990) used immunofluorescence and immunogold staining to visualize *in situ* colonization of potato roots by *Erwinia chrysanthemi*. The precision of immunofluorescence techniques can be improved by increasing the bacterial concentration in a sample or amplifying the antibody reaction (van Vuurde 1991). Under conditions of low numbers of *Azospirillum* in samples, Bashan *et al.* (1991*b*) combined a selective enrichment step with the ELISA procedure to increase the accuracy of *Azospirillum* quantification.

The utilization of DNA technologies in the rhizosphere is currently under development and will likely be useful for measuring root colonization in the future. Radioactive or nonradioactive labelled nucleic acid sequences can be used for DNA hybridization by assaying samples with dot, colony, or Southern blots (Audy *et al.* 1991; Lewin 1990; Trevors and van Elsas 1989). Restriction fragment length polymorphisms (Brown *et al.* 1990), cloned sequences (Steffan and Atlas 1988), and gene fragments (Tsai and Olson 1992) can be used for strain identification. Using DNA probes, detection of approximately 10^4 bacteria/g soil can be obtained within 24 h (Holben *et al.* 1988). This lack of sensitivity may be explained by the presence of soil

TABLE 1. Genetic markers of potential use for root colonization studies

Vector	Marker genes[a], proteins	New phenotypes	Reference(s)
Bacteriophage			
Mu d	*lacZ*, Km^r, β-galactosidase	Cleave X-Gal to yield blue colonies	Höfte *et al.* 1990
lambda:pHC79-cosmid vector of 25 md fragments of DNA	Not available, Ap^r, prodigiosin biosynthesis pathway	Red colonies	Dauenhauer *et al.* 1984
Transposon			
Tn*5*	Km^r, Nm^r	Antibiotic resistances	Bakker *et al.* 1991*a*
Tn*7*	*lacYZ*, lactose permease and β-galactosidase	Catabolise lactose and cleave X-Gal to yield blue colonies	Drahose *et al.* 1986 Kluepfel *et al.* 1991
Tn*5*	*lac*, Km^r, β-galactosidase	Cleave X-Gal to yield blue colonies	O'Sullivan and O'Gara 1988
Not available	*GusRAB*, β-glucuronidase	Cleave X-Glue to yield indigo colonies	Wilson and Jefferson 1990
Tn*5*	*GusA*, β-glueuronidase		Hardarson *et al.* 1991
Tn*5*	Yio^r, Km^r, violacein biosynthesis pathway	Purple colonies	Pemberton *et al.* 1991
Tn*4431*	*luxABCDE*, Ap^r, Cm^r, Km^r, Te^r, luciferase, aldehyde biosynthesis	Bioluminescent colonies	Shaw *et al.* 1987
Tn*5*	*luxAB*, Km^r, luciferase	Bioluminescent colonies	Boivin *et al.* 1987
Plasmid			
Tol plasmid pWWO-EB62	*xylAEMRS*, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase recognizing *m*- and *p*-nitro-substituted compounds	Catabolize *m*-xylene, *m*-toluate or *p*-ethylbenzoate as earbon source	Ramos *et al.* 1991
pLV1016 and PLV1017	*xylE*, Ap^r, Km^r, Tc^r, catechol-2,3-dioxygenase	Produce hydroxymueonic acid, which reacts with catechol to yield yellow colonies	Winstanley *et al.* 1991
pR0103	*tfd*, Tc^r, deregulated 2,4-dichlorophenoxyacetate (TFD) monooxygenase	Convert phenoxyacetate to phenol, which reacts, with 4-aminoantipyrine dye to yield red colonies	King *et al.* 1991

[a]Ap, ampicilin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Tc, tetracycline.

that adsorbs DNA (Tsai and Olson 1991), impurities that affect restriction endonucleases (van Elsas and Waalwijk 1991) and amplifying polymerase (Tsai and Olson 1992), and DNA hybridization efficiency (Tsai and Olson 1991). In addition, sand may shear DNA during the extraction process (Tsai and Olson 1991). Despite these problems, DNA extraction procedures are improving, and 1-g samples can now be used to recover 12 μg DNA/g soil within 7 h (Tsai and Olson 1991). To increase the sensitivity of detection based on hybridization of extracted DNA with probes, the specific nucleic acid sequence of interest may be amplified exponentially using the polymerase chain reaction (PCR) (van Elsas and Waalwijk 1991). Using PCR, Steffan and Atlas (1988) reported a detection limit of *P. putida* of 100 bacteria/100 g sediment (i.e., 1 bacterial cell/g). Improvements in DNA extraction methods to decrease soil impurities, coupled with amplified specific nucleic acid using PCR, will likely lead to better detection of a particular strain with a limited amount of sample; however, it is unlikely that these procedures will allow routine quantification of bacteria on roots from large numbers of samples in the near future.

Immunofluorescent colony staining (IFCS) and colony immunoblot assays represent intermediate approaches to quantification that apply selectivity of immunological and hybridization techniques with the advantages of colony isolation. IFCS was used by van Vuurde (1991) to detect *E. chrysanthemi*, at a detection limit of 1–5 cfu/g, in cattle manure slurry and potato tuber extracts (van Vuurde and Roozen 1990) in the presence of large populations of saprophytic bacteria. With this approach, semiselective pour plating and root embedding were used to allow confined growth of bacteria, and subsequent staining with fluorescent antibodies allowed the detection of fluorescent *E. chrysanthemi* colonies against nonfluorescent colonies of saprophytes. Leeman *et al.* (1991) also used IFCS and reported a detection limit of 20 cfu/g soil for *P. fluorescens* strain WCS374. Colony immunoblot assay with amplified antibody reaction gave comparable estimates of *Rhizobium* populations on seeds to plate counts or MPN (Olsen and Rice 1991).

Despite the relatively long list of theoretical approaches to obtaining specificity, these current techniques are still limiting our ability to quantify efficiently root colonization. With the exception of plate isolation procedures, these methods are relatively laborious, which reduces the number of samples one can test. This may account for the predominant use of spontaneous antibiotic-resistance as a marker for measuring root colonization. Efforts to measure root colonization of individual components in a mixed inoculant would require different specific markers for each strain. Genetic recombination to introduce foreign genes ("marker genes") to a root-colonizing bacterium, thereby allowing its differentiation from indigenous bacteria, has

been suggested as an alternative to spontaneous antibiotic resistance (Hemming and Drahos 1984; Drahos *et al.* 1986; Kluepfel *et al.* 1991). Evaluation of marker genes is a rapidly growing research area, and those marker genes available to date are summarized in Table 1.

Marker genes may be introduced into recipient root-colonists by bacteriophages, transposons, and plasmids. DNA can be exchanged by transformation, transduction, and conjugation. Transformation can be achieved by chemically altering bacterial membranes (Maniatis *et al.* 1982; Chang and Cohen 1979) or by electroporation (Miller *et al.* 1988; Wirth *et al.* 1989). When DNA vectors are carried by host cells, recipients must have distinguishable characteristics such as spontaneous or intrinsic antibiotic resistance, resistance to heavy metals (King *et al.* 1991; Stewart-Hull 1988) or catabolism of specific carbon sources (Boivin *et al.* 1988) to allow differentiation from donor cells. Following integration of donor DNA into the recipient, a novel DNA sequence is maintained in the recipient genome by homologous recombination (Lewin 1990) or by autonomous replication of a plasmid (van Elsas and Waalwijk 1991). The result is a transformed root colonist that now expresses foreign genes, thereby conferring the ability to differentiate the transformant from indigenous bacteria.

Bakker *et al.* (1991*b*) used kanamycin and streptomycin resistance, mediated by Tn*5*, to study root colonization of *P. fluorescens* strain WCS374. Even though the transposon was stable under field conditions, the presence of indigenous pseudomonads with resistance to the antibiotics interfered with measuring root colonization by the transformed strain. This study demonstrates that Tn*5* selectivity per se may not be specific enough for discriminating marked strains from background populations of indigenous bacteria.

One focus of marker gene research is to combine marker gene expression with nutritionally selective media to isolate transformed bacteria. Resistance to the herbicide phosphinothricin, combined with catabolism of toluene derivatives, allows specific recovery of engineered *P. putida* from environmental samples (Ramos *et al.* 1991). Catabolism of specific carbon sources, combined with cleavage of dyes, allows the visual detection of an engineered strain. The *lacZ* gene, which encodes β-galactosidase, was used to recover engineered pseudomonads from roots (Drahos *et al.* 1986; Höfte *et al.* 1990; Kluepfel *et al.* 1991). When plated onto media containing the dye 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-Gal), engineered $lacZ^+$ or $lacZY^+$ pseudomonads cleave this dye to yield a blue color. In addition, these engineered pseudomonads utilize lactose as a carbon source, a characteristic naturally absent in pseudomonads (Drahos *et al.* 1986). However, the medium used for isolating these engineered pseudomonads (M9 amended with lactose and X-Gal) allows growth of other aerobic lactose-utilizing bacteria. Therefore, quantification of marked pseudomonads in the presence of indigenous soil bacteria required a medium that contained X-Gal and allowed production of fluorescent pigments. When high populations of nonpseudomonad lactose-utilizing bacteria were encountered, the estimated populations of marked pseudomonads were reduced. The addition of the antibiotics rifampicin and nalidixic acid into the recovery medium overcame this problem (Drahos *et al.* 1986; Kluepfel *et al.* 1991).

Höfte *et al.* (1990) developed a variation of this method by engineering pseudomonad strain 7NSK2 to constitutively express β-galactosidase. This allowed growth of blue colonies of the marked strain on media without lactose but containing X-Gal. Sebatic acid was used as a selective carbon source for recovery of strain 7NSK2 on M9/sebatic acid/X-Gal/kanamycin medium from soil (Höfte *et al.* 1990) and rhizosphere (Seong *et al.* 1991) samples without growth of background bacteria. Hence, a combination of different techniques has frequently been required to fully use the *lacZ* as a marker gene for measuring root colonization. It is interesting to note that when bacterial growth is required to identify a bacterial phenotype, antibiotic resistance is still required. Thus, the same selectivity may be achieved with spontaneous antibiotic resistance alone, although pleiotropic mutations may be more common with spontaneous mutants.

The β-glucuronidase (*Gus-A*) gene is also used for visual detection of Tn*5-GusA* transformed *Rhizobium* strains in nodules or on Petri dishes, when 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) is oxidized to an indigo dye (Wilson and Jefferson 1990; Hardarson *et al.* 1991). Even though the *Gus* genes are present in some *Enterobacteria*, they may be more specific than the *lacZ* genes. β-Glucuronidase activity has not been reported in most plants (Jefferson 1987), bacteria, or fungi (Wilson and Jefferson 1990). Therefore, members of the indigenous microbial community may create less interference when counts are performed on media. With nodulated legumes, selection of Tn*5–GusA* engineered *Rhizobium* strains is performed by the host, since *Rhizobium* is host-specific.

Another approach for using marker genes is to introduce genes that encode readily distinguishable phenotypes such as production of a new colony colour for the recipient bacterial strain. The expression of prodigiosin genes in engineered *Escherichia coli* supplied with 2-methyl-3-amylpyrrole or 2,2′-bipyrrole-5-carboxyaldehyde yields red colonies (Dauenhauer *et al.* 1984). In a similar approach, genes encoding for production of the purple pigment violacein may be useful genetic markers. Pemberton *et al.* (1991) reported that following conjugation with a strain of *E. coli* carrying violacein genes, strains from six Gram-negative genera produced purple colonies. A third colorimetric marker involves the use of *XylE* genes. Colonies of *E. coli*, *Pseudomonas* spp., *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Serratia rubidaea*, and *Acinetobacter calcoaceticus* engineered with *XylE* turned yellow when sprayed with catechol (Winstanley *et al.* 1991). Finally, King *et al.* (1991) transformed *P. aeruginosa* and *P. putida* strains to constitutively express a 2,4-dichlorophenoxyacetate monooxygenase gene from *Alcaligenes eutrophus* (Don *et al.* 1985). When grown on pheoxyacetate-containing media and sprayed with the dye 4-aminoantipyrine, the recombinant strains turned red. The utility of these chromogenic marker genes must still be proven for root colonization studies. The presence of red and purple indigenous rhizosphere bacteria may interfere with recovery of transformed colonists in some soils. Under these conditions, combining the use of colored phenotypes with selective media or distinctive colony morphology may overcome interference from indigenous bacteria.

Similar to the use of chromogenic marker genes is transformation with *lux* genes for bioluminescence (Legocki *et al.* 1986; Shaw and Kado 1986). Bacterial bioluminescence genes have been cloned from *Vibrio fischeri* (Engebrecht *et al.* 1983), *V. harveyi* (Belas *et al.* 1982), *Photobacterium*

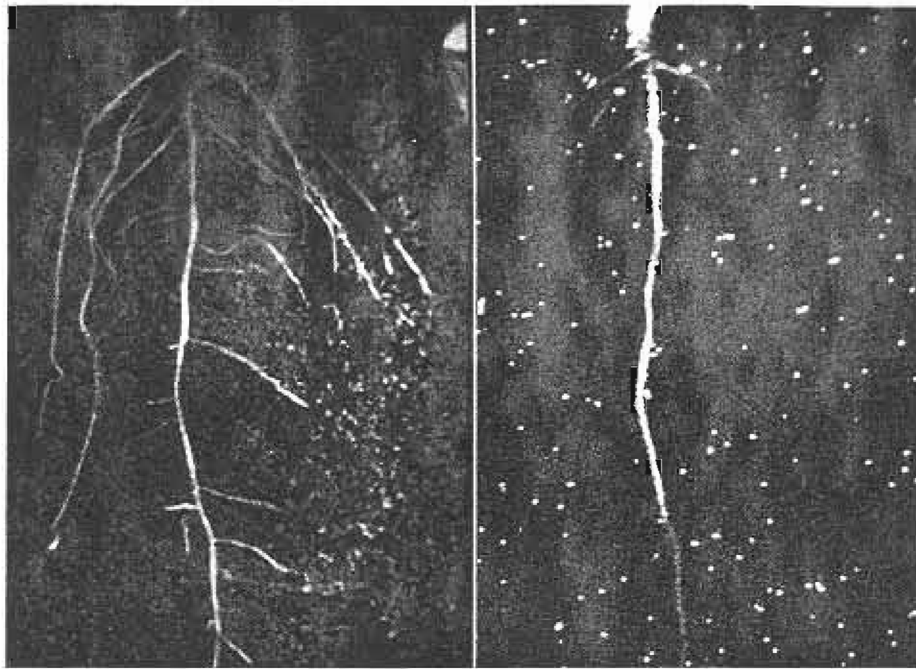

FIG. 1. Use of bioluminescence as a marker for visualizing root colonization by rhizobacteria. Both photographs are of the same 8-day-old soybean seedling that was inoculated with rhizobacterial strain GR7-4L (*LuxAB* transformant) by dipping seeds into a bacterial suspension. Seedlings were grown in nonsterile field soil in plexiglass chambers. Root colonization is evident by observing bioluminescence in a dark room (right) and comparing with root structure seen with incident light (left). The pattern of root colonization can be seen to include root branches and the root tip.

sp. (Delong *et al.* 1987), and *Xenorhabdus luminescens* (Frackman *et al.* 1990). The *lux* operon consists of five genes in the bioluminescence systems of *Vibrio* and *Xenorhabdus* (Meighen 1991). *LuxA* and *luxB* encode the subunits of the luciferase enzyme, while *luxC*, *luxD*, and *luxE* genes encode for functions for production of and aldehyde, which is the substrate that luciferase operates on (Meighen 1991).

Most bioluminescent bacteria have been isolated from marine environments, except *X. luminescens*, which has been isolated from human wounds (Colepicolo *et al.* 1989) and insect-pathogenic nematodes (Schmidt *et al.* 1989). Even though *X. luminescens* is a terrestrial bacterium, living in symbiosis with nematodes, it cannot be isolated directly from soil samples (Colepicolo *et al.* 1989). To date, no bioluminescent bacteria have been reported as natural constituents of rhizosphere bacterial communities based on spread plate or broth enrichment techniques (Beauchamp *et al.* 1991*a*; C.J. Beauchamp, unpublished), suggesting that *lux* may be a practical marker gene for measuring root colonization.

Intensity of light emitted by engineered strains depends on the expression level of the *lux* genes, the number of gene copies, and the physiological status of the recipient strain (Shaw *et al.* 1987). Transposon vector Tn*4431*, carrying the *luxCDABE* genes must be inserted behind a promoter to be expressed in recombinant strains (Shaw *et al.* 1986). In contrast, strains engineered with Tn*5*-*luxAB* emit light as soon as aldehyde is added, as the *luxCDE* genes, which encode aldehyde production, are lacking.

Bacterial bioluminescence is detected visually (Shaw *et al.* 1987; Beauchamp *et al.* 1991*a*, 1991*b*; Legoki *et al.* 1986; O'Kane *et al.* 1988), by imaging (de Weger *et al.* 1991; Fravel *et al.* 1990; Legoki *et al.* 1986; Mahaffee *et al.* 1991; O'Kane *et al.* 1988; Shaw *et al.* 1992), by luminometer counts (Shaw *et al.* 1987; Shaw *et al.* 1992), or by a scintillation counter (Boivin *et al.* 1988; Fravel *et al.* 1990; Mahaffee *et al.* 1991), which allows quantification of light intensity.

Photographs of engineered bioluminescent bacteria can be taken using regular 35-mm film (100–1600 ASA) with long exposure times, or autophotographs can be taken using X-ray film (De Weger *et al.* 1991; Shaw *et al.* 1987). The required exposure time can be reduced using a charge coupling device (CCD) camera (Fig. 1) (Shaw *et al.* 1992; Mahaffee *et al.* 1991). With a CCD camera and statistical software, Shaw *et al.* (1992) correlated light intensity units to the number of bioluminescent *Xanthomonas campestris* pv. *campestris* in cabbage leaves.

As a marking system, bioluminescence eliminates the need for plating when qualitative information is required, i.e., bacteria are present or absent on roots. In addition, the use of imaging allows visualization of *lux*-marked colonists directly on roots, which is useful for comparing gross colonization patterns among strains or among various plant hosts (Beauchamp *et al.* 1991*b*). Mahaffee *et al.* (1991) used a CCD camera to visualize *lux*-marked rhizobacteria on roots. Embedding roots into selective media increases the number of bacteria on the root surface, thereby allowing detection of bioluminescence by eye (Beauchamp *et al.* 1991*b*) or following exposure to X-ray film (Fravel *et al.* 1990). Further improvements in luminometry techniques for estimating root colonization may lead to indirect quantification of bacteria using light intensity.

Use of all the indirect marking systems described above for quantifying root colonization levels is predicated upon a linear relationship between bacterial populations in a sample and the amount of product produced by the transformed bacterial colonist. Accordingly, this relationship

should be validated with each system. King *et al.* (1991) correlated phenol concentration with bacterial populations using ultraviolet spectrophotometry, gas chromatography, and high-performance liquid chromatography techniques. With spectrophotometry of the red dye - phenol complex, the detection limit was approximately log 3 cfu/mL.

Each of the marking systems discussed here has advantages and disadvantages. The choice of systems partially depends on the intended use. In general, when one is measuring colonization in soil systems, interference may result. Clay particles and humic matter may interfere with antigenic reactions and recovery of nucleic acids. With serological techniques, quantification is dependent on dispersion and flocculation methods, as well as the quality of the antigens used for antisera production (Schaad *et al.* 1990). Plant tissues and soil may interfere with the development of colorimetric reactions associated with the cleavage of a dye (e.g., *lacZY* and *GusA* genes) and the light intensity for bioluminescent microorganisms. In the selection of marking systems, one should take these limitations into consideration.

At the present time, there is no single marking system that can be considered best for multiple uses. More research is needed to develop alternative markers and to compare different marking systems for measuring root colonization. Strategically, both nonengineered marking systems and the use of marker genes have some drawbacks. Nonengineered markers must be stable over time in the rhizosphere. Colony morphology and intrinsic antibiotic resistance profiles can be modified after a bacterial strain is introduced into the rhizosphere (Brunel *et al.* 1988; Lindström *et al.* 1990). Although serological markers are generally stable, modifications can result from alterations of major lipopolysaccharides (Gibson *et al.* 1991). In contrast, DNA restriction profiles appear to be stable (Gibson *et al.* 1991), and the development of short oligonucleotide probes from intergenic regions of ribosomal genes may lead to specific strain detection (Gill *et al.* 1991). Although *lacZY* marker genes did not affect root colonization of recombinants compared with wild-type strains with spontaneous rifampicin-resistance (Cook *et al.* 1990; Kluepfel *et al.* 1990), introduction of foreign DNA sequences can reduce the competitiveness of recipient bacteria (Schroth and Becker 1990; van Elsas *et al.* 1991). Brockman *et al.* (1991) reported that 7 of 10 Tn*5* recombinants of *Rhizobium leguminosarum* exhibited altered phenotypic traits, including growth rate in culture, symbiotic effectiveness, and nodule occupancy rates. Introduced marker genes may constitute an important energetic sink at the expense of other essential metabolic pathways (McCormick 1986). In addition, the insertion of marker genes into the bacterial genome may interrupt genes required for normal metabolic pathways.

Statistical approaches

One of the first issues related to statistical approaches to measuring root colonization is considering what constitutes a sample unit. Historically, the sample unit for root colonization has consisted of whole root systems or root segments (Bahme and Schroth 1987; De Weger *et al.* 1987; Kloepper *et al.* 1991*b*; Parke *et al.* 1990). Whole root systems constitute a logical sample unit; however, while sampling whole roots is feasible for seedlings, it is impossible for mature field-grown crops. Use of root segments offers a reasonable alternative to whole roots when consistency is used in selecting the individual segments. Root segments may have the additional advantage of increasing the precision for differentiating relative colonization capacities of different bacterial strains, as shown in the following example. Several investigations have been conducted to ascertain whether or not bacterial motility aids in root colonization. Scher *et al.* (1988) used whole roots as the sample unit and found no relationship between root colonization capacity and bacterial motility. De Weger *et al.* (1987) used root segments at different distances from the inoculation point as the sample unit. For root segments within 1 cm of the inoculation point, their results agreed with Scher's; however, at 8 cm from the source, nonmotile mutants colonized roots at significantly lower populations than the motile wildtype. One disadvantage of using root segments is that variability among replications is usually higher than that for whole roots (Kloepper *et al.* 1991*b*). The maximum amount of information can be obtained from root colonization experiments by using both whole root systems and root segments whenever feasible.

Having selected a sample unit, one must then confront the issue of how to express the measured population. Classically, root colonization is expressed as colony-forming units (cfu) per gram root fresh or dry weight or as cfu per centimetre root, and arguments can be made for each of these options. Determination of dry weight provides an assessment of root biomass available for supporting bacterial growth through metabolic activity, and therefore, expressing colonization as cfu per gram root relates the population to root biomass and indirectly to root metabolism. In contrast, expression of populations as cfu per centimetre root reflects an attempt to relate populations to root-surface microbial niches, which are scattered along the root. Sampling error related to soil moisture may be greater when expressing populations as cfu per gram than cfu per centimetre, since soil adherence to root samples will be greater in moist than dry conditions (Liddell and Parke 1989). In moist soils, therefore, a smaller percentage of the sample (root + attached soil) weight consists of root, and since the greatest colonization is at the root surface (Liddell and Parke 1989), the calculated population of bacteria on roots may be underestimated one or more log units when expressed as area per gram.

Root architecture, i.e., kind of tap root, pattern of lateral branches, and size and frequency of root hairs, may relate directly to maximum populations of colonists that can be obtained. Neither the use of cfu per gram nor cfu per centimetre accounts for differences in root architecture among cultivars or crops, and the alternative expression of cfu per root surface area may be best for this application. Root surface area has been calculated by a titration method (Wilde and Voigt 1949) and a gravimetric method (Carley and Watson 1966); however, both methods are designed for larger root samples, typically whole root systems, than are generally used for measuring root colonization. Future improvements in image analysis techniques (Coles *et al.* 1991; Zoon and van Tienderen 1990) will likely be applicable for determining root surface area.

If one is specifically measuring internal root colonization, it is not particularly meaningful to express populations as cfu per centimetre root, since colonization of a three-dimensional volume is not strictly proportional to root length, although this has been used for measuring root sur-

face colonization of *Azospirillum brasilense* strain Cd (Bashan *et al.* 1991*a*). Internal root colonization by *Azospirillum* is typically expressed as cfu per gram root dry weight (Okon *et al.* 1977) or cfu per gram root fresh weight (Bashan *et al.* 1990). Expression of bacterial populations inside roots as cfu per volume root would, perhaps, most accurately relate populations to the microbial niche available for colonization. At this time, however, we are not aware of any rapid procedures for assessing root volume that would be applicable to the typical root sample size used in colonization determinations.

Statistical analysis of root colonization levels is often appropriate to test the hypothesis that a particular treatment results in statistically higher or lower colonization levels than a control or a different treatment. Separation of treatment mean colonization levels for this application is done by standard analysis of variance (ANOVA) procedures. When conducting ANOVA tests, one must remember that ANOVA is valid only when the four underlying assumptions are correct: the error terms are randomly, independently, and normally distributed; the variances of different treatments are homogeneous; variances and means of different samples are not correlated; the main effects are additive (Gomez and Gomez 1984*a*; Little and Hills 1978). The first and third assumptions are normally evaluated by logic. The assumption related to the error terms is normally fulfilled when treatments are randomized. Similarly, a rapid examination of treatment means and variances enables one to determine if they are correlated, e.g., a high mean linked with a large variance.

Because root colonization partially involves multiplicative bacterial growth, the assumption of additivity of main effects is generally invalid. Loper *et al.* (1984) suggested that the lognormal distribution most accurately explained root colonization data in the same way that phylloplane bacterial colonization is usually lognormally distributed (Hirano *et al.* 1982). There are two important implications of lognormal distribution for root colonization studies. First, all data should be logarithmically transformed before determining means (Baker 1985), as failure to do so results in overestimation of mean colonization values (Loper *et al.* 1984). Secondly, each replication should consist of a single sample from a single plant rather than "batch" samples from multiple plants. Batch sampling results in an arithmetric averaging of lognormally distributed bacterial populations, thereby overestimating the true mean population (Loper *et al.* 1984).

While bacterial colonization of plant tissues is usually explained best by the lognormal distribution, other distribution patterns may better fit some experimental conditions. Ishimaru *et al.* (1991) found that bean leaf colonization by *Xanthomonas campestris* pv. *phaseoli* was better explained by the flexible Weibull distribution than by lognormal distribution. Intuitively, one may envision experimental conditions where multiplicative bacterial growth is not occurring, and hence, the lognormal distribution would not be appropriate. Flowering or pod-fill stages of maturity are associated with lowered levels of root exudates (Curl and Truelove 1986), which could result in cessation of bacterial growth. Similarly, a mid-season drought could result in declining populations of Gram-negative bacterial colonists and in conversion of *Bacillus* sp. colonists from vegetative cells to spores, and in each case, the lognormal distribution would not best account for the changes in population dynamics. Researchers involved in measuring root colonization should watch the literature for future reports dealing with distribution analyses of root colonization.

Another of the underlying assumptions for correct use of ANOVA is that the variances of treatment means are homogeneous (Gomez and Gomez 1984*a*; Little and Hills 1978). In lay terms, this means that the variability among replications within each treatment is similar to that of other treatments. If the variances are not homogeneous, then the ANOVA should not proceed. Little and Hills (1978) present the following example of how failure to check for homogeneity of variances prior to proceeding with ANOVA can lead to false conclusions. In a four-treatment experiment with five replications per treatment, the treatments had the following values for mean/variance: 3/2.5, 6/2.5, 9/22.5, 14/22.5. When ANOVA was done without testing for homogeneity of variances, the conclusion was made that treatments 1 and 2 were not significantly different but treatments 3 and 4 were (based on calculation of a significant F value and $LSD_{0.05}$). After testing for homogeneity of variance, it was determined that the variances were heterogeneous and therefore, treatments 1 and 2 were analyzed separately from 3 and 4. The conclusion after reanalysis was the opposite, i.e., treatments 1 and 2 were significantly different but treatments 3 and 4 were not. With root population data, it is reasonable to expect frequent situations where the variances are heterogeneous, and variance may even be strain-dependent in some cases. Therefore, it is obligatory that a test for homogeneity of variances (e.g., Bartlett's test) (Gomez and Gomez 1984*a*; Little and Hills 1978) be used prior to ANOVA.

While several other statistical issues could be discussed, we will conclude with consideration of how to handle "zeros." We will use the following two examples of data from a single bacterial treatment, replicated five times, for this discussion:

> example 1 (log cfu/cm root for each replication): 1.1, 1.8, 0, 0, 1.2;
> minimum detectable population = log 1.0 cfu/cm root
>
> example 2 (log cfu/cm root for each replication): 4.3, 3.2, 2.8, 0, 2.9;
> minimum detectable population = log 2.0 cfu/cm root

Both examples are from actual experiments in our laboratory, where we were measuring colonization of root branches in example 1 and colonization of whole root systems in example 2. In these examples, a value of zero does not really indicate that there were no bacteria on the root, but rather that the population was below the minimum detectable level. An informal poll of laboratory groups engaged in root colonization research revealed that five different approaches are used for determining the mean: (*i*) replace the zeros with values calculated from a censored data program (Rouse *et al.* 1985); (*ii*) replace the zeros with values calculated by the missing data procedure; (*iii*) use the zeros in calculation of the mean; (*iv*) replace the zeros with the minimum detectable population; and (*v*) discard the zeros and determine the mean of the remaining replications. Approaches *i* and *ii* appear to have limited usefulness for root colonization analyses. Utilization of programs for censored data (Rouse *et al.* 1985) requires an estimation of variance that is nearly

impossible for root colonization, given that colonization is innately highly variable on a single root and among roots (Loper *et al.* 1984). While calculation of missing data is statistically valid, recording a zero is not really the same as completely missing data owing, for example, to loss of a sample. In addition, the missing data value will be based on the data from the remaining replications, and hence, the mean may be an overestimation of the true mean if the true colonization level for the "missing" replication was closer to zero than to the other replications. When the missing data value is used in analysis of variance, one must reduce the overall degrees of freedom by one for each "missing" data point, thereby lowering the power of the analysis to detect significant differences among treatment means. Finally, the missing data calculation can only be used for one replication per treatment (Gomez and Gomez 1984*b*), and therefore, it could not be used in example 1.

Let us now consider what happens when we use approaches *iii–v* for calculating means in examples 1 and 2. With example 1, approaches *iii*, *iv*, and *v* calculate means of 0.8, 1.22, and 1.37, respectively, while with example 2, these same approaches yield means of 2.64, 3.04, and 3.3, respectively. Thus, the calculated mean of the same treatment varies as much as 0.66 log units with these examples. Approach *v* (discarding the replications with zeros) is probably not a good method, since this will consistently result in an overestimation of the mean. Selecting between approaches *iii* and *iv* partially depends on whether one expects "true zeros" in the system. With example 1, 1.0-cm segments of root branches are the sample units, and hence, one would expect no colonization (true zeros) on some samples. However, in example 2, because whole root systems are being sampled and only one replication had no colonies, it is probably more reasonable to use the minimum detectable population instead of zero (approach *iv*) in mean calculation. Recognizing that the approach used in calculation of means may greatly affect the estimated mean, all reports of measuring root colonization should include in the Methods section details of how zeros were treated.

Conclusion: future directions in measuring root colonization

Measurement of root colonization by introduced microorganisms will continue to be an important aspect of investigations focused on improving crop productivity through growth promotion or biological disease control. To increase understanding among different laboratory groups, each report of root colonization work should clearly define or delineate the concept of root colonization used in the study. One of the next areas for advancement in our understanding of root colonization will be how populations of mixed inocula interact during colonization. Such investigations of mixed inocula are currently limited by the number of well-characterized marking systems. Significantly more research is needed to develop and compare multiple markers for efficiencies of recovery. To ensure proper interpretation of results from studies quantifying root colonization, researchers should fully describe the statistical procedures and analytical methods used. Finally, quantitative ecological studies should continue to determine if distribution patterns other than the lognormal distribution explain colonization dynamics in particular situations.

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