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ASSESSING THE POTENTIAL OF CREATING BIASED RHIZOSPHERES BASED ON INOSITOL RHIZOPINES

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ABSTRACT

ASSESSING THE POTENTIAL OF CREATING BIASED RHIZOSPHERES BASED ON INOSITOL RHIZOPINES

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Particular microbial populations may be selectively enhanced by the introduction of specific organic nutrients into the rhizosphere environment. The initial objective of this thesis was to evaluate this "biased rhizosphere" hypothesis in systems where inositol rhizopines were to be provided as the selective organic nutrient. When it became apparent that a substitute for rhizopines was required to continue this assessment, *myo*-inositol was selected as a surrogate compound, and the impact of exogenous applications of this organic nutrient on rhizosphere bacterial populations was investigated. Thus, several lines of investigation were pursued to assess the potential of creating biased rhizospheres systems and evaluating their usefulness to specifically stimulate target populations of rhizosphere bacteria.

The rhizopine synthesis (*mos*) genes from *Sinorhizobium meliloti* L5-30 were used in efforts to generate rhizopine producing transgenic plants. Rhizopines were not observed to accumulate in transformed plant tissues, and molecular analyses indicated that the cloned *mos* genes were not all properly expressed. An analysis of the cloned *mos* genes indicated that they were not sufficient for rhizopine accumulation in nodules. More information regarding the nature of rhizopine synthesis is required before a biased rhizosphere system based on rhizopine-synthesizing plants can be created.

The abundance and source of rhizopine catabolic activity in the soil and rhizosphere environment was investigated. The number of culturable bacteria capable of utilizing these

compounds for growth was one to ten percent of the number growing on complex media. A diverse set of novel rhizopine-catabolizing bacteria were isolated and characterized. Different bacteria capable of catabolizing the proposed nutritional mediators are present in the environment and may compete for these organic nutrients *in situ*.

The impact of *myo*-inositol amendment on soil and rhizosphere bacterial populations was examined in growth chamber and field experiments. Application of the nutrient resulted in increases in the numbers of culturable bacteria. ARDRA and FT-ARDRA analyses indicated that the abundance and composition of the bacterial communities changed in response to the amendment. Increases in the abundance and activity of *S. meliloti* were observed. These results indicate that nutrient amendments can be used to promote the growth and activities of targeted bacterial populations in the rhizosphere.

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"The rain drops are still falling, large, heavy, and few," said Doctor Manette. "It comes slowly."

"It comes surely," said Carton.

They spoke low, as people watching and waiting mostly do; as people in a dark room, watching and waiting for lightning, always do.

- Charles Dickens

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Chapter 1

General Introduction and Project Summary

The rhizosphere environment

The rhizosphere has been simply defined as the space in and around plant roots (40). Yet this simple definition belies the complex and dynamic nature of this environment (See Figure 1.1). In soil, roots constantly interact with a variety of abiotic and biotic factors which affect growth and activities. The structure and activities of plant roots vary according to the prevailing environmental conditions of climate and soil type, as well as to the plant's genetic characteristics (62). Ecological interactions of all types also play a major role in determining the rhizosphere environmental conditions (19, 28, 36, 42). While the disparity in scale between the macroscopic observer and the microscopic heterogeneity of relevant below ground structures and activities challenges our abilities of synthesis, we have learned much and our understanding of the rhizosphere environment is constantly being refined (9, 15, 39, 41, 43, 62, 66, 75).

Because my thesis work focused on the potential management of microbial populations in the rhizosphere, I will restrict this general introduction to the rhizosphere environment of crop plants grown in agricultural settings. Additionally, I will make some generalizations regarding the structural components of the rhizosphere and their activities, so as to underscore the basis of my research.

The environment into which a root emerges is already managed, in part, by agronomic practice (62). Generally, the soil matrix has been restructured by tillage, fertilization, and planting. The soil environment may be further modified by the application of irrigation, fertilizers, pesticides, and, on occasion, industrial wastes. In some instances, biological amendments (*e.g.* rhizobial inoculants) are also introduced for the purpose of improving the harvestable yield. In total, these agronomic practices are aimed at providing a suitable environment for plant growth and development; however, each practice will substantially impact the nature of the emerging rhizosphere somewhat differently.

The plant itself plays a central role in defining the physical, chemical, and biological characteristics of the below ground environment. Plant growth stimulates and directs the bulk flow of water and dissolved nutrients into plant tissues, primarily by evapotranspiration (5, 17). Roots

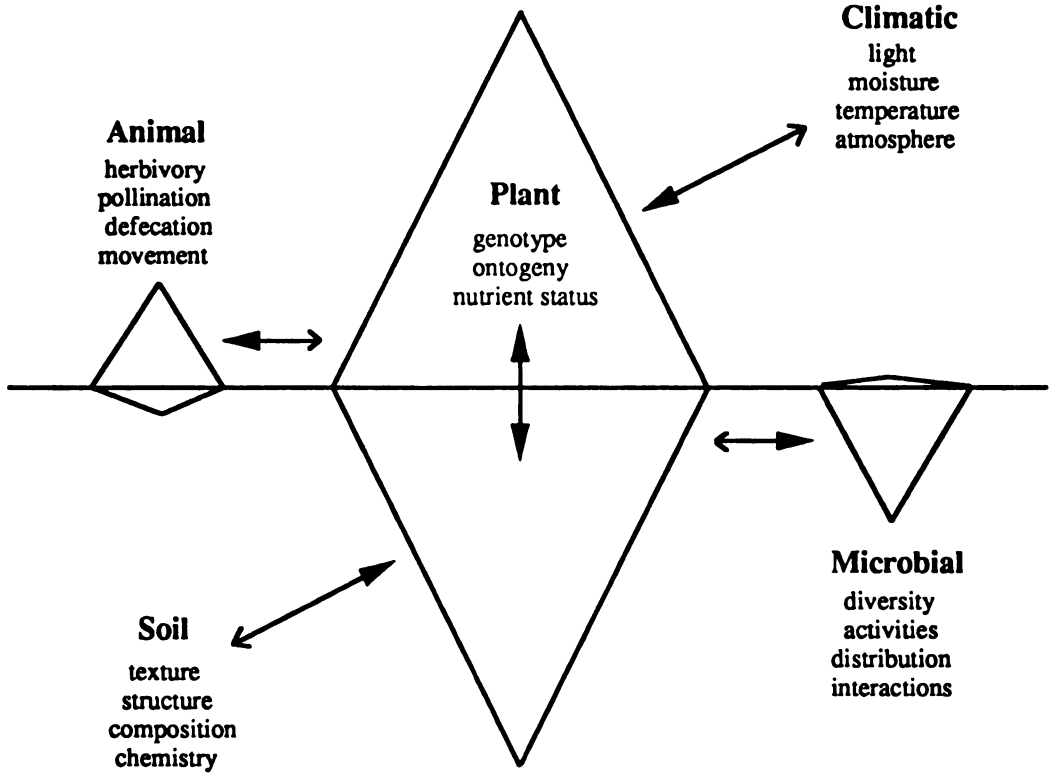

Figure 1.1: Factors determining the nature of the rhizosphere environment. Arrows represent dynamic interactions between different factors and plant roots.

consequently actively alter the soil environment through which they grow and subsequently senesce (27). Below ground, plants provide the greatest proportion of fixed carbon and nitrogen that sustains a diversity of rhizosphere organisms (40, 77). During growth the soil matrix is altered by the exudation and secretion of various molecules by the plant, particularly around active root tips (15, 28). Mature root tissues also undergo ontogenic and stochastic changes which can alter the composition of the associated microbial communities (15). During senescence, the root biomass that is not reallocated to other portions of the plant becomes available for consumption by saprophytes and detritovores. Taken together, the structures and activities of plant roots can be described as the foundation upon which rhizosphere microbial communities establish themselves.

In terms of total energy and biomass, rhizosphere microbes primarily rely on plants for fixed carbon. The occurrence, composition, and fate of plant-derived carbon compounds in the soil and rhizosphere have been well studied (15, 62, 77). In living roots, an abundance of complex polysaccharides are secreted from actively growing root tips and associated root border cells (28, 61). The components of this mucigel are synthesized intracellularly in the Golgi and secreted via membrane bound vesicles. The presumed function of this mucigel is to protect the meristematic loci from injury (*e.g.* physical abrasion, pathogen attack). A number of different organic compounds, including sugars, carboxylates, amino acids, nucleotides, vitamins, and flavonoids, have been found in root washes and leachates of various plants (see Table 1.1)(15). Environmental factors and plant genotype both affect the composition of these exudates; however, the extent to which these compounds are actively or passively exuded from the root remains unclear (15). It is estimated that, on average, about five to thirty percent of the carbon translocated to the roots is released from the roots (75). The presence of these organic compounds in the rhizosphere stimulates the growth and activity of a variety of microbes (18).

The microbial communities that inhabit the rhizosphere are diverse (15). Some of the bacteria that have been cultured from root samples include members of the genera *Agrobacterium, Achromobacter, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Enterobacter, Flavobacterium, Pseudomonas, Rhizobium*, and *Streptomyces*. Of the fungi, members of the genera *Fusarium,*

Table 1.1: Substances detected in plant root exudates (after ref. 15).

class of compound	exudate components	plants studied
sugars	glucose, fructose, sucrose, maltose, ribose, galactose, xylose, arabinose, rhamnose, raffinose, oligosaccharides	*T. aestivum, H. vulgare, P. vulgaris, Pinus* spp.
amino compounds	asparagine, alanine, glutamine, aspartate, glutamate, leucine, isoleucine, serine, aminobutyrate, glycine, cysteine, methionine, proline, phenylalanine, tyrosine, threonine, tryptophan, arginine, homoserine, cystathionine, glycine betaines	*T. aestivum, Z. mays, P. sativum, A. sativa, Trifolium* spp., *M. sativa, O. sativa, L. esculentum, Pinus* spp.
organic acids	tartarate, oxalate, citrate, malate, propionate, butyrate, succinate, fumarate, malonate, glycolate, valerate	*T. aestivum, Z. mays, P. vulgaris, L. esculentum, Pinus* spp.
fatty acids and sterols	palmitic, stearic, oleic, linoleic, linolenic, campesterol, sitosterol, campesterol	*P. vulgaris, A. hypogaea*
nulceotides and macromolecules	adenine, guanine, thiamine, uridine, cytidine, DNA, RNA, protease, phosphatase, polygalacturonase, amylase,	*T. aestivum, P. sativum, Trifolium spp., Z. mays*
secondary metabolites	flavones, flavonoids, glycoloids, hydrocyanic acid, saponins, organic phosphates, auxins, biotin, inositol, thiamine, niacin, pantothenate, choline, *p*-amino benzoic acid, nicotinic acid, bioactive compounds	*Medicago* spp., *P. sativum, Trifolium* spp., *L. esculentum, Z. mays*

Gigaspora, Glomus, Penicillium, Phytophthora, Pythium, and *Trichoderma* are well known to inhabit the rhizosphere. Not surprisingly, the pathogens and mutualists have been characterized in the most detail. Microscopic examinations of root surfaces extracted from soil have indicated that microbial populations

occur in patches, presumably in regions of high nutrient availability (20). Attempts to comprehensively catalog the diversity of microbial populations are probably incomplete due to practical and technical limitations (12, 34). Only a small percent of the total microbial population is culturable on any given medium (12) and recent studies using molecular methods based on 16S rRNA genes have resulted in the identification of phylogenetically novel rhizosphere inhabitants (6, 35). It is not clear what proportion of rhizosphere-inhabiting microorganisms significantly impacts plant growth and development. However, it is known that the abundance and/or activity of these

microbes varies spatially and temporally according to a number of factors, including plant genotype and ontogeny, soil type, soil water potential, climate, and agricultural management (15, 62).

Organic nutrients and niche expansion in the rhizosphere

Away from plant roots, soil microorganisms are thought to be carbon limited (77). The application of organic amendments to soil generally results in measurable increases in microbial respiration and biomass (23). The presence of fixed carbon in root tissues and exudates stimulates microbial activity and colonization of the rhizosphere (7). The rhizosphere effect is largely nonspecific because many different organisms are known to utilize the organic substrates released by the plant into the rhizosphere (8). In a several instances, though, connections between specific plant-derived compounds and particular microorganisms have been made. Flavonoids are known to stimulate rhizobial activity, resulting in increased growth rate, chemotactic migration to the root, production of Nod factors, and subsequent infection (74). The synthesis and catabolism of rhizopines by some rhizobial strains has been associated with nodule competitiveness (26). Opines synthesized in plant galls and hairy roots as a result of infection by strains of *Agrobacterium* can be utilized as growth substrates and can promote conjugal transfer of large plasmids carrying pathogenesis-related genes (16). It has also been shown that organic pesticides and industrial pollutants can enrich for organisms capable of utilizing them as carbon and/or energy sources (3). Additionally, the presence of auxotrophic bacteria in the rhizosphere indicates that numerous microbes may rely on specific components in root exudates for active growth (15). These observations indicate that organic nutrients may be useful to selectively manage the microbial populations in the soil and rhizosphere environments.

Several authors have suggested that beneficial microorganisms may be selectively managed in agricultural settings by the introduction of novel organic nutrients (7, 13, 14, 50, 60, 65). From an ecological standpoint, the presence or absence of such an organic nutrient can be used to define the niche boundaries between organisms that can or cannot utilize that nutrient. If the nutrient is novel with respect to the environment into which it is introduced and if some organisms present in that

environment can utilize it as a resource, then one can say that the niche space of those organisms has been expanded or, alternatively, that new niche space has been created. The direct result of this niche space creation is predicted to be stimulation of the metabolic activities and abundance of organisms capable of catabolizing the introduced nutrient. Indirect effects may include a variety of shifts in the population structures and activities of the communities into which the nutrient is introduced, depending on the amount of the nutrient, the relative abundance of catabolizers and noncatabolizers, and the ecological relationships between them.

To establish a practical system of nutritional mediation (*i.e.* nutrient-based niche space creation) and to validate it as an operational mechanism, several criteria must be met. First, the organic nutrient must be available to and utilized by the target microorganisms *in situ*. Second, the stimulation of the targeted microbial populations must result in increases in the desired activities (*e.g.* increased nodulation or biological control of a plant pathogen). Concomitantly, the introduced nutrient should be unavailable to organisms that would inhibit the growth and activity of the beneficial target microorganisms or that would otherwise compromise agronomic production. Correlations should be observed between the size of the created niche space (*i.e.* the amount of nutrient), the relative abundance of target microorganisms, and the amount of beneficial activity. In this work, I will describe several experiments that establish that nutrient amendment-mediated niche expansion can be used to promote the growth and activities of beneficial bacteria in the rhizosphere (see Chapter 6).

The biased rhizosphere hypothesis

Rhizosphere microbial populations vary from plant to plant (15). And it has been suggested that plants may be bred with respect to the colonizing microflora to improve crop yield (7, 50). Indeed, selection for plant resistance to microbial pathogens is a common element of plant breeding programs (1). Additionally, improvements have been made in the mutualistic plant-microbe relationship between legumes and rhizobia (10, 31, 49, 54). Such breeding programs rely on rapid assays that are dependable predictors of agronomic performance (1, 29, 63). The lack of such assays for beneficial plant-microbe interactions has limited progress towards more comprehensively

managing rhizosphere microbial populations. Our understanding of the abundance, distribution, physiology, and ecology of the majority of rhizosphere-colonizing microorganisms in different soils and their responses to agricultural practice has yet to be synthesized into a genuinely useful paradigm. In the absence of such a synthesis, efforts have focused on the responses of particular microbial populations (*e.g.* the rhizobia, mycorrhizae) to particular agronomic practices, and correlations are being established that are paving the way for a more mechanistic understanding of plant-microbe interactions.

One of these correlations involves the relationship between specific plant-derived organic nutrients and microorganisms capable of catabolizing them. In *Agrobacterium*, such a correlation exists between opines and virulent strains, and it is believed that these novel amino-acid derivatives promote the selective propagation of the inducing pathogen (16). This phenomenon is referred to as the "opine concept" (16). An analogous "rhizopine concept" is believed to describe the impact of novel nitrogen-containing inositol derivatives (*i.e.* rhizopines) on catabolizing *Rhizobium* (48, 74). A variety of other novel plant-derived secondary compounds (*e.g.* calystegin, mimosine, stachydrine, trigonelline) may also be used *in situ* as organic nutrients by rhizosphere bacteria (24, 70). The genes encoding for the catabolism of these compounds have been reported to occur on symbiotic plasmids in *Rhizobium* (25, 45, 76). Furthermore, the catabolism of rhizopines and betaines have been correlated with improved competitiveness for nodule occupancy when compared to near-isogenic transposon mutants unable to catabolize these compounds (25, 26). Thus, it appears that the ability to catabolize certain organic nutrients may play a significant role in the symbiotic success of rhizosphere bacteria.

Recently it has been shown that the presence of certain organic compounds can increase the number of rhizosphere bacteria capable of catabolizing them. Salicylate provided in irrigation water has been shown to enrich for a salicylate-utilizing inoculant bacteria in growth chamber and field experiments (13, 14). Increased numbers of culturable opine-catabolizing bacteria have been observed in the rhizosphere of plants genetically engineered to produce mannityl-opines (51, 52). Furthermore, the use of nutrient amended granules has been shown to increase the numbers of

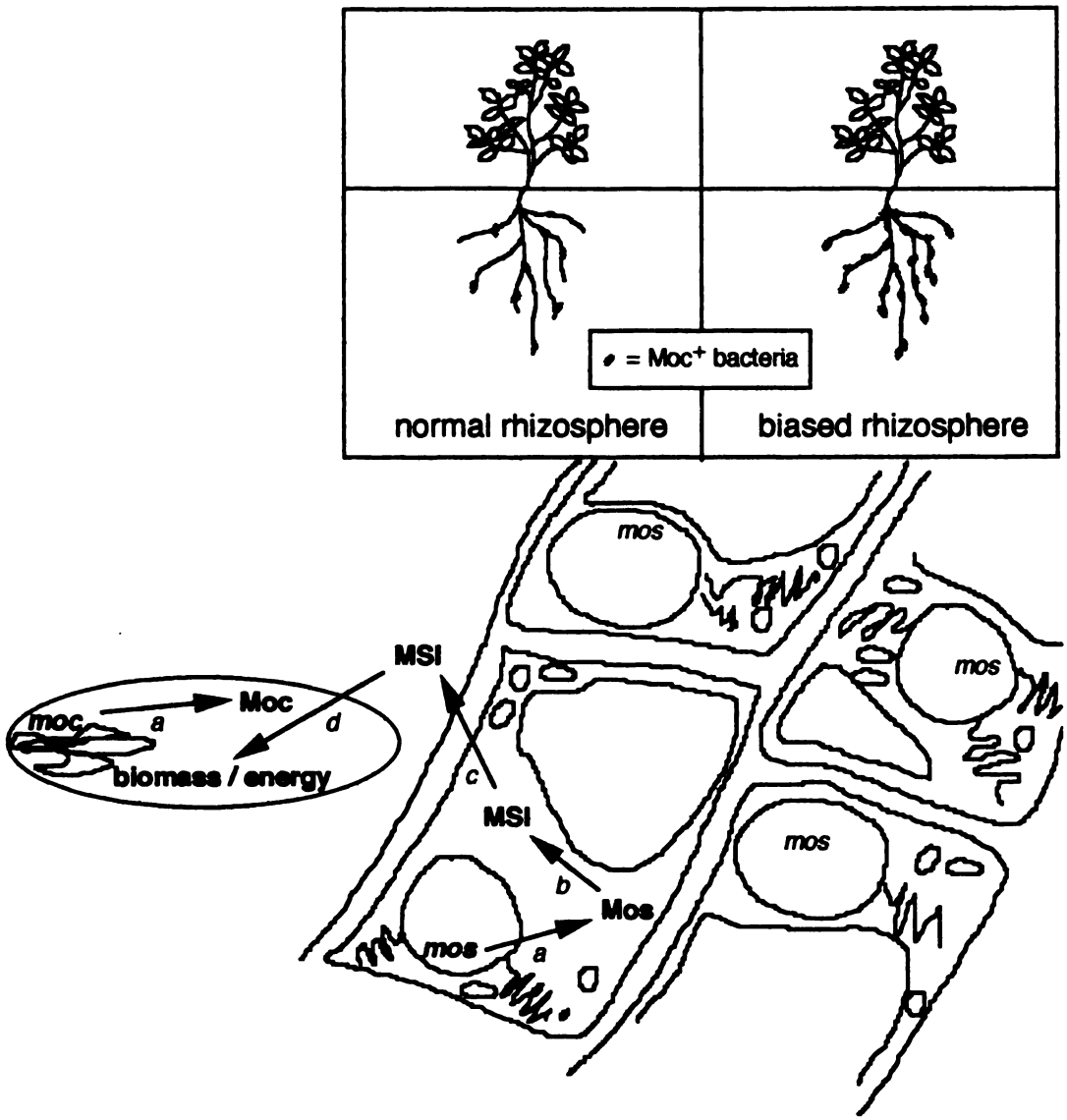

Figure 1.2: A model for a biased rhizosphere system based on 3-O-methyl-*scyllo*-inosamine (MSI) as a nutritional mediator (after ref. 50). The rhizopine is synthesized *in planta* as a result of *mos* gene expression. The compound becomes available to rhizosphere bacteria in root exudates, providing a novel source of fixed carbon and nitrogen for bacterial growth. Only bacteria harboring the *moc* genes are able to utilize the novel organic nutrient. Populations of rhizopine catabolizing bacteria increase in response to the presence of the rhizopine. Arrows indicate: gene expression (*a*), synthesis (*b*), release into the rhizosphere (*c*), uptake and catabolism (*d*).

culturable rhizosphere-colonizing *Bradyrhizobium* present in the inoculant formulation (22). These studies indicate that despite the relative abundance of organic nutrients in the rhizosphere, subpopulations of rhizosphere bacteria capable of utilizing introduced nutrients can be stimulated. This correlation provides the basis of the "biased rhizosphere" hypothesis.

This hypothesis states that increasing the concentration of a specific organic nutrient in the rhizosphere will result in an increase in the abundance and activities of microorganisms capable of utilizing it (50, 60) (See Figure 1.2). Several predictions can be made from this hypothesis. First, a monotonic correlation should exist between the amount of the organic nutrient added to the rhizosphere and the population size and activities of organisms that can catabolize it. Second, the short-term impact of adding an organic nutrient to a system will be most pronounced for organisms that utilize it as a carbon and energy source. And, third, the presence of nontarget catabolizing organisms will reduce the effectiveness with which a nutritional mediator will stimulate targeted microorganisms.

Rhizopines and inositols

My thesis research focused on assessing the potential for creating biased rhizospheres based using rhizopines and *myo*-inositol as nutritional mediators. In this section, several classes of inositol-containing compounds will be discussed with regards to the potential use of rhizopines and *myo*-inositol as nutritional mediators in the rhizosphere environment. Representative structures of these different types of compounds are presented in Figure 1.3.

Rhizopines are inositol derivatives, the structures of which have been reported to be 3-O-methyl-*scyllo*-inosamine (MSI) and *scyllo*-inosamine (SI) (48). This class of compounds was first identified in the nodules of *Medicago sativa* infected with *Sinorhizobium meliloti* L5-30 (73) and has since been observed in the nodules of several other legumes infected with particular strains of *S. meliloti* and *Rhizobium leguminosarum* (76). Little is known about the biochemistry of rhizopines, although genes required for their biosynthesis and catabolism have been identified and characterized (45, 46, 47, 58, 59, 64). It has been proposed that, like opines (16), rhizopines may act as

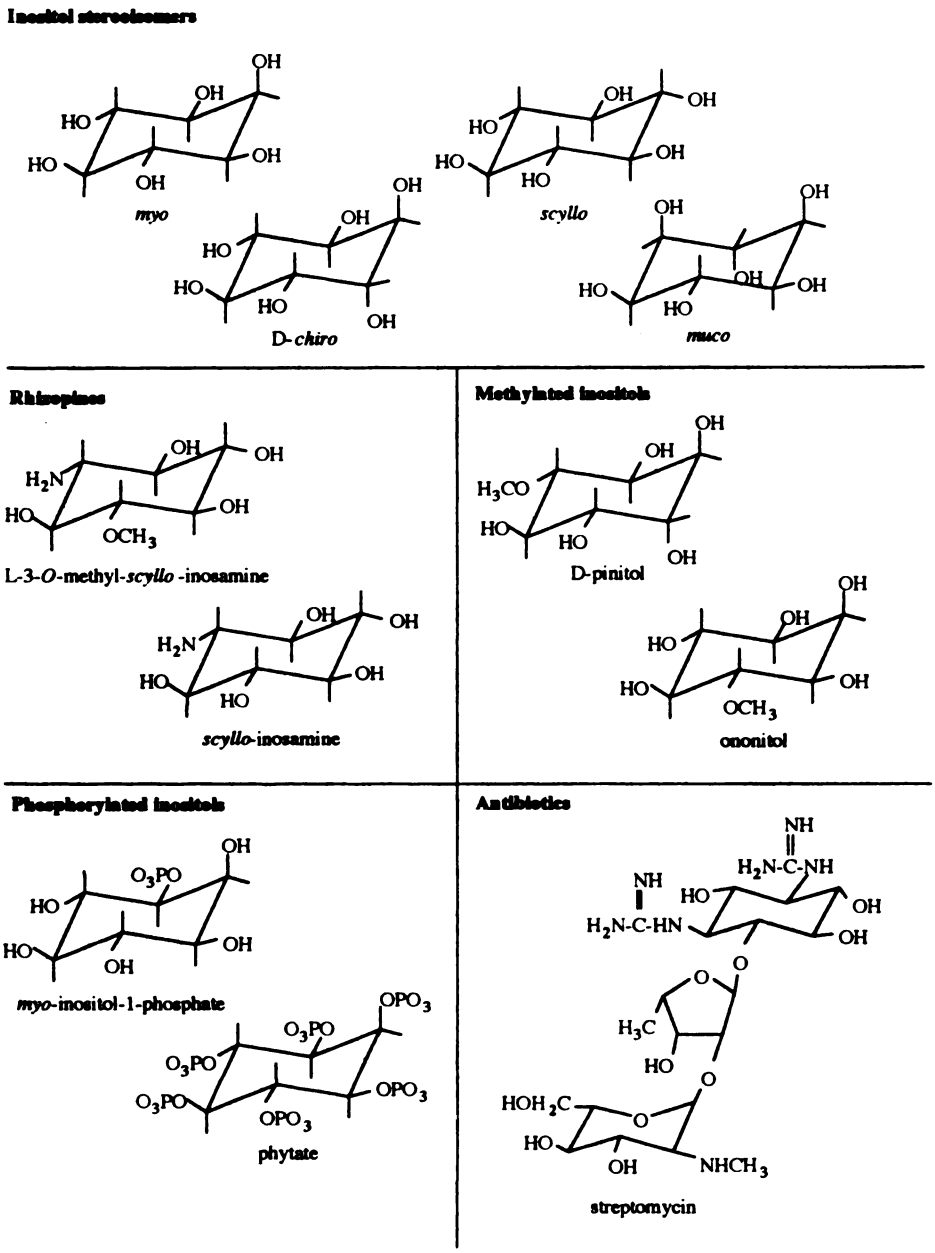

Figure 1.3: Common stereoisomers of inositol and the several classes of inositol-containing compounds discussed in the text.

"proprietary growth substrates" which can be utilized only by the bacterial strains that synthesize them *in planta* (48). Two lines of evidence support this hypothesis. First, genes for synthesis and catabolism are closely linked in the genome of rhizobia (45) and the large majority of strains that have been reported to catabolize rhizopines also synthesize them in the nodule (76). Additionally, wild-type strains capable of utilizing rhizopines out-compete near-isogenic mutants for nodule occupancy (26). However, Gordon *et. al.* have reported that rhizopine catabolism alone may not completely explain such increases in nodulation (26). They reported that the abundance of the wild-type inoculum did not increase over time relative to a mutant incapable of utilizing rhizopine despite the fact that rhizopine was being produced in the rhizosphere (*i.e.* in infected nodules). However, one could argue that such a trend does appear in their published data over the course of a single growing season. So, while rhizopines may act to some degree as nutritional mediators in nature, their role in the rhizobia-legume symbiosis is not completely understood.

Closely related to rhizopines in structure are methylated inositols. These compounds occur widely in plants, particularly in the *Leguminosae* (37). Pinitol (3-O-methyl-*chiro*-inositol) is found throughout the plant (69), and it has been used as a quantitative marker for legume abundance in mixed forage systems (68). Different methylated inositols, including pinitol, ononitol (4-O-methyl-*myo*-inositol) and O-methyl-*scyllo*-inositol, have also been identified in nodules of various legume plants (33, 67, 71, 72). In pea, the concentration of these compounds in roots and nodules has been shown to vary depending on the identity of the infecting strain of *Rhizobium* (33). Methylated-inositols are also believed to play a role in osmoregulation in plants because they increase in concentration following osmotic stress (21). By analogy, rhizopines may play some role in osmotolerance of rhizobial strains that synthesize them in the nodule environment.

Phosphate-containing derivatives of inositols occur in several forms in plants, microorganisms, and soil. Phosphotidyl inositol-containing lipids are ubiquitous in cell membranes (38). In plants, mono-, di-, and tri-phosphate derivatives also occur free in the cytosol and play a major role in calcium regulation (57). Phytate (inositol-hexaphosphate) is a major storage form of phosphorus in plants, representing 50 to 90 percent of the total phosphate in seeds and 10 to 15

percent of the phosphate in root and shoot tissues (11, 30, 55). Phytate and its partially dephosphorylated breakdown products also represent a significant proportion of soil phosphorous (4). Mycorrhizae have been reported to preferentially utilize this pool of organic phosphorous when associated with their plant symbionts (2). The introduction of additional inositol-containing compounds into the rhizosphere, then, has the potential to impact this mutualistic plant-microbe interaction.

Potentially toxic inositol-containing compounds are also likely to be present in the soil and rhizosphere environment. Several naturally occurring microbial antibiotics (*e.g.* streptomycin, gentamicin, kanamycin, and spectinomycin) contain inositol moieties (22b). While resistance to these antibiotics often involves catabolism of these molecules, the enzymatic attack of the inositol moieties are seldom implicated in such mechanisms (22b). The degree to which these compounds might act as nutrient sources for naturally resistant or insensitive bacteria is unknown.

Inositols represent a significant fraction of the total soluble carbohydrates in legume nodules and may play a role in the ecological success of infecting strains. There are approximately 2 mg of uncharged inositols per g fresh weight of nodule tissue, which represents 30 to 60 percent of the soluble carbohydrates (21, 67, 71, 72). For comparison, estimates of rhizopine concentration in alfalfa nodules infected with *Sinorhizobium meliloti* L5-30 are on the order of 0.5 mg per g (see Chapter 4). In soybeans, *myo*-inositol is the most abundant cyclitol, accounting for over 25 percent of the total extractable carbohydrate (71). In alfalfa, pinitol has been reported to be the most abundant form (21). In peas, ononitol and O-methyl-*scyllo*-inositol were reported to be the most abundant carbohydrates (67). The largest fraction of these compounds can be found in the cytosolic fraction of the plant cells as opposed to the bacteroids; however, the relative abundance does not seem to differ between the two compartments (72). Additionally, the flux of inositols between these two compartments may not be substantial (56). Nonetheless, this large pool of inositol may be available to rhizobia for utilization upon nodule senescence. In *Rhizobium*, cellulase activity is induced by *myo*-inositol as well as by a variety of polysaccharides (44), and most strains are capable of accumulating substantial intracellular carbon reserves (32, 53). Thus, it is quite possible that

infecting rhizobia utilize the large pool of inositol present in the nodule both as a signal and as a nutrient reserve to successfully migrate out of a senescing nodule to reinfect a new and conducive root segment.

Project summary

In this thesis I will assess the potential of creating biased rhizospheres in several ways. First, efforts were made to generate rhizopine-producing transgenic plants so as to directly test the biased rhizosphere hypothesis using rhizopines as the nutritional mediator. Second, the source and abundance of rhizopine catabolizing activity in the soil and rhizosphere was investigated to evaluate the potential effectiveness of rhizopines as selective agents. Lastly, the biased rhizosphere hypothesis was evaluated in growth chamber and field experiments using *myo*-inositol as the nutritional mediator. The impact of this nutrient amendment on different rhizosphere microbial populations was assessed with particular emphasis on the symbiosis between rhizobia and legumes.

In the course of conducting my research it became apparent that an alternative to rhizopine was required to continue the assessments of the biased rhizosphere hypothesis because neither rhizopine producing plants nor an adequate supply of pure rhizopine would be available. The work proceeded after finding an alternative compound that might adequately model a rhizopine-based biased rhizosphere. The criteria used for selecting *myo*-inositol as an analog for rhizopines were: (a) its structural relatedness, (b) its comparable specificity as a utilizable substrate, and (c) apparent functional relationships in catabolism of both compounds in the *Rhizobium*-legume symbiosis.

Chapter 2 describes efforts to construct rhizopine-producing plants. It was determined that rhizopine synthesis might be possible in plants because cosmids containing the rhizopine synthesis (*mos*) locus from *Sinorhizobium meliloti* L5-30 were functional in the bacteroids of a heterologous system composed of *Rhizobium loti* and *Lotus corniculatus*. Additionally, rhizopine catabolic activity was not detected in root and leaf extracts of several different plant species, indicating that the accumulation of rhizopines was not precluded in other plant tissues. A variety of plasmids containing the *mos* genes were constructed in an effort to generate rhizopine-producing transgenic plants.

Rhizopine was not observed to accumulate in any of the transformed plant lines. Transgenic *Arabidopsis* plants were obtained that accumulated transcripts corresponding to all three genes; however, the *mosA* transcripts were significantly shorter than expected. In these transgenic plants, proteins of the expected molecular weight were detected using anti-MosB antibodies, but a full-sized MosA protein was not detected. None of the transgenic lines were observed to accumulate rhizopines; however, the introduction of these constructs resulted in a distinct mutant phenotype. The occurrence of this phenotype may reflect the disruption of inositol metabolism *in planta* by the introduced *mos* genes. The functionality of the cloned *mos* ORFs was tested by reconstructing the *mos* operon on a broad-host range bacterial expression vector called pMOS27. *Medicago sativa* nodules induced by *S. meliloti* 1021 harboring this construct were not observed to accumulate rhizopines. From these experiments it was concluded that more work needs to be carried out to understand the nature of rhizopine synthesis in *S. meliloti* before rhizopine synthesizing plants can be created.

Chapter 3 briefly describes two sets of experiments that were instrumental in redirecting my research program. The first was an assessment of the abundance of culturable bacteria capable of utilizing different organic nutrients as sole carbon and/or nitrogen sources. This work indicated that the numbers of bacteria capable of catabolizing *myo*-inositol and rhizopine were roughly equivalent and represented a small fraction of the total number of culturable bacteria. The second experiment examined the impact of glucosamine amendment on inoculant and indigenous soil bacteria. It was obseved that this nutrient amendment preferentially increased the abundance of bacteria capable of growing on media containing glucosamine as sole carbon and nitrogen source. The fraction of glucosamine-utilizing bacteria represented by a single morphotype of indigenous soil bacteria increased significantly over time. These observations were consistent with the predictions of the biased rhizosphere hypothesis. Interestingly, the impact of the glucosamine amendment was greatest on the indigenous bacteria. The measured increases in the number of inoculant bacteria capable of catabolizing the compound were smaller and more transitory. This observation indicated that nutrient

amendment might have a relatively plastic effect on the microbial populations in the soil and rhizosphere environments.

In Chapter 4, the detection and isolation of novel rhizopine-catabolizing bacteria are described. This represents the first direct evaluation of the potential specificity of rhizopines as nutritional mediators. Rhizopine catabolism was detected and enumerated in soil and rhizosphere washes. A diverse set of bacteria were isolated using *scyllo*-inosamine (SI) containing media. None of the isolates contained sequences similar to the known *moc* genes as assayed by colony hybridization, indicating that they contain novel rhizopine catabolism genes. Twenty-one different strains were observed to grow on SI as sole carbon and nitrogen source and were further characterized. Six of these were found to be capable of catabolizing 3-O-methyl-*scyllo*-inosamine (MSI). Two of these were identified as *S. meliloti* strains based on their 16S rDNA sequences; however, they did not contain the highly conserved *nodBC* gene sequences and were incapable of nodulating alfalfa. It was concluded that diverse indigenous rhizopine-catabolizing microorganisms were present in the experimental soil and could compete for the nutritional mediator if introduced.

Chapter 5 describes a molecular technique called fluorescent-tag amplified rDNA restriction analysis (FT-ARDRA) that was developed for the examination of bacterial communities in soil and rhizosphere environments. The method is a modified T-RFLP analysis of amplified 16S rDNA sequences using serial dilutions of soil and rhizosphere washes. The use of different restriction enzymes results in the generation of unique community profiles that can be used to monitor changes in the dominant bacterial members. The technique was evaluated using defined mixtures of diverse isolates obtained from soil and rhizosphere samples. Examination of natural communities using this method revealed distinct differences in soil and rhizosphere samples and their response to *myo*-inositol amendment. Comparison of the FT-ARDRA profiles also indicated that some organisms respond differently to the organic nutrient amendment depending on their localization in the soil or rhizosphere. Thus, it was shown that this method could be usefully applied to the evaluation of the impact of nutritional amendments in the rhizosphere environment.

In Chapter 6, the biased rhizosphere hypothesis is evaluated with respect to the impact of *myo*-inositol amendment on bacterial populations in the rhizosphere. The relative ability of two *S. meliloti* strains to colonize the roots of *Medicago sativa* was compared under gnotobiotic conditions. In these experiments, a wild-type strain outcompeted a near-isogenic mutant unable to utilize *myo*-inositol for rhizosphere colonization only when the compound was added to the growth medium. From this it was concluded that *myo*-inositol can be used to expand the niche space of catabolizing bacteria in the rhizosphere. In experiments using non-sterile agricultural soil, it was observed that the nutrient amendment increased the number of culturable microorganisms present in the rhizosphere of alfalfa. ARDRA and FT-ARDRA of the most abundant culturable microorganisms indicated quantitative shifts in the composition of these fast-growing bacterial populations. The observed increases were greatest for organisms that could utilize *myo*-inositol as sole carbon source to support growth. Because *Sinorhizobium meliloti* strains are known to be generally capable of utilizing *myo*-inositol for growth (32, 51), it was hypothesized that this compound may be used as a nutritional mediator to stimulate populations of this beneficial soil bacteria in an agricultural setting. FT-ARDRA profiles of rhizosphere samples indicated that there was a strong correlation between the amount of *myo*-inositol amendment and fluorescent signals corresponding to those predicted for *S. meliloti* one week after germination of the planted *M. sativa*. The amount of applied organic nutrient was likewise strongly correlated with nodulation of *M. sativa* in both growth chamber and field experiments. Therefore, *myo*-inositol amendment was able to stimulate the abundance and activities of a targeted microbial population. Additional changes in the FT-ARDRA profiles indicated that the structure of the rhizosphere microbial communities was altered by the nutrient amendment. Given the complex nature of soil and rhizosphere microbial communities, it is not possible to conclusively demonstrate the mechanism of nutritional mediation for any particular organism in natural settings, however, the results of these experiments are largely consistent with it in the case of *myo*-inositol-mediated increases in *S. meliloti* abundance and nodulation activity.

In conclusion, evidence supporting the biased rhizosphere hypothesis is presented, and the potential for creating practical systems based on nutritional mediation is indicated. Although it is

uncertain if systems based on rhizopines as the nutritional mediator can be successfully engineered, it should be possible to find alternative compounds (*e.g. myo*-inositol) with equivalent specificity. It might be most desirable to alter the composition of plant root exudates to deliver the organic nutrient into the rhizosphere, both in terms of the amount that may be supplied and the efficiency with which it is delivered. However, it seems that exogenous amendments can be used to effectively alter rhizosphere microbial communities. The prediction that a nutritional mediator can specifically increase the abundance and activities of microorganisms capable of utilizing it in the rhizosphere is supported by both the glucosamine and inositol amendment studies. Indeed, the results of the field tests indicate that practical applications of biased rhizosphere systems may be found because targeted populations of beneficial microorganisms can be stimulated. Alteration of the nutrient balance will undoubtedly impact the microbial communities which inhabit the rhizosphere, but the extent to which such alterations impact plant growth and yield potential still needs to be investigated. The development of new experimental tools, such as FT-ARDRA, should facilitate the extensive analyses that need to be done to evaluate the dynamic nature of rhizosphere microbial communities and their responses to various agricultural practices. With the information derived from such investigations, the management of rhizosphere microbial communities may play an even greater role in the development of more productive and sustainable agricultural systems.

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Chapter 2

Efforts to Construct Transgenic Plants That Produce Rhizopine

Abstract

Rhizopine synthesis (*mos*) genes from *Sinorhizobium meliloti* L5-30 were used in attempts to produce genetically-engineered plants that accumulate rhizopine. It was hypothesized that this approach might be feasible beacause the *mos* genes functioned in a heterologous system, and plant tissues were not observed to contain rhizopine-catabolizing activity. The initial efforts to transform plants with the *mos* genes indicated that hygromycin-based selection, DNA-transfer of the pPCV91-derived vectors into the plant genome, and/or the structure of the constructs were inadequate to generate rhizopine producing plants. An alternative cloning strategy dependent on PCR amplification of the three predicted *mos* open reading frames, *mosA*, *mosB*, and *mosC*, was subsequently carried out. Amplified DNA corresponding to the *mos* genes were introduced into bacterial over-expression vectors, and proteins corresponding to MosA and MosB, but not MosC, were isolated. MosA and MosB specific antisera were generated and used to examine the expression of these genes in transformed plants. The three amplified *mos* open reading frames were inserted into a plant expression vector called pMOS7B1. Plants transformed with pMOS7B1 displayed a visibly altered growth phenotype characterized by enlarged leaves and suppression of flowering. Northern blot analyses indicated that all three *mos* open reading frames were transcribed in several transformed lines, but the *mosA* transcripts were smaller than expected. Western blot analysis indicated that MosB but not MosA proteins accumulated in these transformed lines. High-voltage paper electrophoresis analyses of extracts of transgenic tissues failed to detect any accumulation of rhizopines. The functionality of the cloned *mos* ORFs was tested by reconstructing the *mos* operon on a pTE3-derived vector under the control of the *trp* promoter. *Medicago sativa* nodules induced by *S. meliloti* 1021 harboring this reconstructed operon were not observed to accumulate rhizopines.

Introduction

The biased rhizosphere hypothesis predicts that the introduction of a nutritional mediator into the rhizosphere will result in an increase in microbial populations capable of catabolizing it (26). A spatial and temporal association between the compound and the organism which can utilize it are

required for the phenomenon of a biased rhizosphere to occur. One means of creating such an association is to genetically engineer the plant to produce the nutritional mediator.

Recently, several reports have substantiated the biased rhizosphere hypothesis in systems using transgenic plants engineered to produce opines. It has been reported that such transgenic plants can synthesize significant quantities of opines, up to 150 mg per g dry weight of tissue (29). These plants can also support a greater fraction of genetically engineered opine-catabolizing bacteria under gnotobiotic conditions when co-inoculated with near-isogenic non-catabolizing strains (8, 30). Perhaps most significantly, it has been shown that opine-producing plants can specifically increase the populations of culturable opine-utilizing bacteria in non-sterile soil (20, 21). These results suggest that plant-produced nutritional mediators can be used to effectively bias rhizosphere microbial populations.

Interest in rhizopine as a nutritional mediator (16, 26) prompted me to try to genetically-engineer plants to produce and accumulate rhizopines using the *mos* genes from *Sinorhizobium meliloti* L5-30. These genes are required for the synthesis of 3-O-methyl-*scyllo*-inosamine (MSI) in nodules infected with *S. meliloti* (14, 17). An analysis of the DNA sequence of this region identified four open reading frames (ORFs), three of which are thought to be indispensable for rhizopine synthesis (17). No studies have been done on the biochemistry of these proteins, and the only available information is based on DNA sequence analysis. It has been suggested that the *mosA* product is involved in the methylation of *scyllo*-inosamine (SI), because SI accumulates in nodules infected by a strain of *S. meliloti* lacking *mosA* sequence in its otherwise homologous *mos* locus (27). The deduced *mosB* product contains a putative DNA-binding motif, which may indicate some regulatory function. The deduced *mosC* product has been predicted to be a membrane spanning protein, possibly involved in either importing a precursor or exporting rhizopine. The *mosA, B,* and *C* genes are likely expressed from a common *nifA*-dependent promoter (15, 17). It was postulated that the introduction of these genes into the plant genome would result in rhizopine production in plant tissues and such rhizopine-producing plants could be used to test the predictions of the biased rhizosphere hypothesis (26).

Materials and Methods

Bacterial strains and culture media

The strains used in this work are listed in Table 2.1. *Escherichia coli* were cultured on LB media supplemented with either ampicillin (100 ug/ml), carbenicillin (100 ug/ml), kanamycin (50 ug/ml) or tetracycline (10 ug/ml) as needed to maintain introduced plasmids. *Agrobacterium* strains were maintained on either YEB or LB media supplemented with rifampicin (100 ug/ml) and either carbenicillin (100 ug/ml), gentamycin (25 ug/ml), and/or kanamycin (50 ug/ml) to maintain introduced plasmids. *Sinorhizobium meliloti* and *Mesorhizobium loti* strains were maintained on TY media supplemented with streptomycin (100 ug/ml) and/or tetracycline (10 ug/ml).

Molecular methods

Molecular clonings were carried out according to standard methods (28). Plasmids were isolated using alkaline-lysis based mini-preps and maxi-preps of DNA. $CaCl_2$-mediated transformation or electroporation was used to introduce the DNA constructs into *E. coli*. Plasmids were introduced into *Agrobacterium* strains by electroporation or tri-parental mating using pRK2013 as the helper plasmid. Restriction mapping was used to verify the structure of recombinant plasmids.

The polymerase chain reaction (PCR) was also used to clone the *mos* open-reading frames (ORFs). Primers were designed to contain specific restriction sites so that the PCR products could be conveniently cloned into three different vectors. The primers used and their DNA sequences are listed in Table 2.2. Amplification conditions were as follows. Each 50 ul reaction mix included 1x Gitchier buffer (24), 160 ug/ml BSA, 200 uM dNTPs, 5% DMSO, 50 pmoles of each primer, and 100 ng of linearized template plasmid (pAG8523, pAG8525, or pAG8527). Two units of Ampli-Taq Polymerase were added after pre-heating sample mixtures to 95°C. Reactions were cycled 15 times; 94°C for 1 minute, 50°C for 1 minute, and 68°C for 2 minutes. After the fifteenth cycle, a 5 minute extension cycle at 72°C was included, after which samples were cooled and stored at 4°C. The amplified products were digested and gel-purified prior to cloning.

Table 2.1: Strains and plasmids used in this work.

strain #	species	plasmids	*mos genes*
Testing of mos genes in Rhizobium loti			
8544	E. coli / HB101	pPM1071	ABC
8545	E. coli / HB101	pPM1062	ABC
8547	E. coli / HB101	pPM1031	
7011	E. coli / HB101	pRK2013	
6023	**Rhizobium loti / PN184**	-	
6078	**Rhizobium loti / PN184**	**pPM1031**	
6079	**Rhizobium loti / PN184**	**pPM1062**	**ABC**
6080	**Rhizobium loti / PN184**	**pPM1071**	**ABC**
Initial constructs used for plant transformation			
7011	E. coli / HB101	pRK2013	
7137	E. coli / HB101	pBS	
7139	E. coli / HB101	pPCV91	
8523	E. coli / HB101	pAG8523	A
8525	E. coli / HB101	pAG8525	B
8527	E. coli / HB101	pAG8527	C
8558	E. coli / DH5alpha	pBS-mosA(s)	A
8559	E. coli / DH5alpha	pBS-mosB(n)	B
8561	E. coli / DH5alpha	pPCV91-C / pMOS3	C
8562	E. coli / DH5alpha	pPCV91-AC / pMOS5	A, C
8563	E. coli / DH5alpha	pPCV91-BC / pMOS6	B, C
8564	E. coli / DH5alpha	pPCV91-ABC / pMOS7	A, B, C
4062	A. rhizogenes / GV3101CT	pRi15834	
4063	A. tumefaciens / GV3101CT	pTiBo542	
4600	**A. rhizogenes / GV3101CT**	**pRi15834, pPCV91**	
4601	**A. rhizogenes / GV3101CT**	**pRi15834, pMOS7**	**A, B, C**
4602	**A. rhizogenes / GV3101CT**	**pRi15834, pMOS6**	**B, C**
4604	**A. tumefaciens / GV3101CT**	**pTiBo542, pPCV91**	
4605	**A. tumefaciens / GV3101CT**	**pTiBo542, pMOS7**	**A, B, C**
PCR-based cloning and plant transformation			
7011	E. coli / HB101	pRK2013	
7137	E. coli / HB101	pBS	
7139	E. coli / HB101	pPCV91	
8624	E. coli / HB101	pBS-A*	A*
8625	E. coli / HB101	pBS-B*bl	B*
8627	E. coli / HB101	pBS-C*	C*
8628	E. coli / HB101	pBS-C*249	C*
8630	E. coli / HB101	pBS-A*244	A*
8633	E. coli / HB101	pBS-deltaBN	
8634	E. coli / HB101	pBS-deltaBN258	
8637	E. coli / HB101	pBS-A*C*244	A*, C*
8640	E. coli / HB101	pPP	

Table 2.1:

strain #	species	plasmids	*mos genes*
8646	E. coli / HB101	pPP-C*	C*
8647	E. coli / HB101	pPP-C*A*	A*, C*
8648	E. coli / HB101	pPP-C*A*B*	A*, B*, C*
8649	E. coli / DH5alpha	pBIN19-PPC*A*B*/pMOS7B1	A*, B*, C*
8650	E. coli / HB101	pPCV91-C*	C*
8651	E. coli / HB101	pPCV91-C*A*	A*, C*
8652	E. coli / DH5alpha	pPCV91-C*A*B*/pMOS7A	
4542	A. tumefaciens GV3101	pMP90RK	
4070	A. tumefaciens GV3101	pMP90RK, pPCV91	
4071	A. tumefaciens GV3101	pMP90RK, pMOS7A	A*, B*, C*
4072	A. tumefaciens / C58C1	pMP90	
4073	**A. tumefaciens / C58C1**	**pMP90, pBIN19**	
4074	**A. tumefaciens / C58C1**	**pMP90, pMOS7B1**	**A*, B*, C***
Overexpression of Mos proteins			
7148	E. coli / DH5alpha	pET15b	
8653	E. coli / HB101	pET15b-A*	A*
8654	E. coli / HB101	pET15b-B*	B*
8636	E. coli / HB101	pET15b-C*	
8655	**E. coli / BL21(lDE3)pLYS S**	**pET15b**	
8656	**E. coli / BL21(lDE3)pLYS S**	**pET15b-A***	**A***
8657	**E. coli / BL21(lDE3)pLYS S**	**pET15b-B***	**B***
8658	**E. coli / BL21(lDE3)pLYS S**	**pET15b-C***	**C***
Test of reconstructed operon			
8642	E. coli / HB101	pTE3-245	
8643	E. coli / HB101	pBS-A*B*C*244 / pMOS17	
8644	E. coli / HB101	pMOS27	
8645	E. coli / HB101	pMOS72	
3169	**S. meliloti 1021**	**pPM1093**	**ABC**
3247	**S. meliloti 1021**	**pTE3-245**	
3249	**S. meliloti 1021**	**pMOS72**	**C*B*A*(-)**
3250	**S. meliloti 1021**	**pMOS27**	**A*B*C***

Table 2.2: Oligonucleotide linkers and PCR primers used in this work.

oligo #	description	sequence
Linkers		
DB130	ss adaptor PstI->NotI	CAG CGG CCG CTG TGC A
NEB1071	ds BamHI	CGG GAT CCC G
NEB1148	ds SalI	CGG TCG ACC G
DB244	ss adaptor SpeI->SacI	CTA GCG AGC TCG
DB245	ss adaptor PstI->SacI	GGA GCT CCT GCA
DB249	ss adaptor BamHIx->SacII	GAT CAC CGC GGT
PCR primers		
DB238	mosA*forward	CAG GCC TCT AGA TCT CCA TCC ACA ATT C
DB239	mosA*reverse	GCG TGT CTG AGA TCT AGA CGG CAC CTG
DB240	mosB*forward	CGG GAT GCA TAT GCG GCC GCT GGT CTT ACA G
DB241	mosB*reverse	GGA GCA CAT ATG CGG CCG CTT GTC CTG GTC
DB242	mosC*forward	CGT CAT CCC GCG GTC GAC GAA ACC AAG CGG AG
DB203	mosC*reverse	CGC CAA GGA TCC GCG CAT GGG ATG

The preparation of antisera followed standard protocols (28). The induction and isolation of the over-expressed Mos proteins from *E. coli* were performed according to the protocols provided by Novagen (Madison, WI). The expressed His-tagged proteins were isolated from *E. coli* using a Ni-binding column. Protein purification was monitored by SDS-PAGE and Coomassie staining of various fractions. Rabbits were maintained, immunized, and bled by employees of the Michigan State University Animal Care Facilities.

The molecular analyses of plant tissues were carried out using standard protocols (28). For Northern blot analyses, total RNA was isolated using a hot-phenol-based method (33), except that the buffer of Hall *et al.* was used (9). RNA was separated on formaldehyde-containing gels and transferred onto MagnaGraph nylon membranes (MSI Scientific, Westborough, MA). Random-primed DIG-labeled probes were prepared with the Genius I Kit (Boehringer Mannheim Biotechnologies, Indianapolis, IN) according to the manufacturers instructions. The hybridization solution contained 45% deionized formamide, 4.5x SSC, 45 mM $NaPO_4$, 1.8% blocking reagent (BMB, Indianapolis, IN), and 1.8% SDS, pH 7.0. Hybridizations were performed overnight at 68°C and blots were subjected to two high stringency washes (0.1x SSC, 0.1% SDS, $\geq$ 37°C). Detection of bound hybrids involved alkaline-phosphatase mediated staining following the manufacturer's instructions. For Western blot analyses, proteins were isolated by precipitation with 70% acetone. The protein concentration was estimated using the Bio-Rad assay (Hercules, CA). Proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were treated with Blotto to prevent non-specific binding, and incubated with 1:500 dilutions of crude anti-sera. Detection of bound anti-Mos anti-bodies relied on alkaline-phosphatase-conjugated secondary antibodies and colorimetric detection using NBT and BCIP.

Plant transformation

The vectors used for plant transformation were based on pPCV91 or pBIN19. The vector pPCV91 was obtained from Drs. C. Koncz and N. Strizhov (Max-Planck-Institute, Koln, Germany). This plasmid contains three different promoters followed by unique restriction sites and thus allows

for the concurrent expression of *mosA*, *mosB*, and *mosC*. The promoters on pPCV91 are an enhanced 35S promoter of the cauliflower mosaic virus and the 1' and 2' promoters from the mannopine synthase (*mas*) genes (34). The *mas* 1' and 2' promoters have been shown to direct expression of genes predominantly in the basal parts of plants (13, 31). In addition, pPCV91 contains the *hpt* gene (7), encoding hygromycin phosphotransferase, as a plant selectable marker and the β-lactamase gene as a selectable marker in bacteria (28). It also contains the left and right border of the T-DNA of *A. tumefaciens*, thus enabling the transfer of DNA between the two borders into the plant genome, when the virulence functions are provided in *trans*. The plasmids pBIN19 and its derivative pBIB have been described elsewhere (2, 3).

Plant transformations were mediated by *Agrobacterium* using standard protocols. Transformation of *Lotus corniculatus* was accomplished by *Agrobacterium*-mediated hairy root induction of infected hypocotyls (23). For *Nicotiana tabacum* co-cultivation of *Agrobacterium tumefaciens* with leaf explants was used to generate transformed callus from which plants were regenerated (19). *Arabidopsis thaliana* var. Columbia was transformed via vacuum infiltration of *Agrobacterium tumefaciens* (1, 11). Selection of transformants was based on resistance to the antibiotics hygromycin B or kanamycin, depending on the construct. All plants and tissues were incubated in growth chambers.

Assays for rhizopine synthesis and catabolism

Preparation and analysis of plant extracts followed a published procedure for detection of rhizopine from nodule tissues (25). Briefly, plant material was crushed in sterile distilled water, centrifuged to remove debris, and then dried in a Speed-Vac. Samples were resuspended to a final concentration of 1 mg fresh weight per ul in sterile distilled water. Samples were stored at -20°C prior to analysis. Five to twenty microliters of each sample was loaded on 3MM Whatman paper for high-voltage paper electrophoresis (HVPE). Electrophoresis was performed at 3000 V in 1.1 M acetic acid/0.7 M formic acid buffer. Visualization of α-diol-containing compounds, including MSI, resulted from staining with alkaline $AgNO_3$ following Dessaux *et. al.* (4). MSI was detected as a

dark spot running at a characteristic distance relative to orange G ($R_{orange\ G}$ = - 0.9). Extracts from nodules containing the MSI rhizopine were used as positive controls.

For the rhizopine catabolism assays, approximately 100 mg (fresh weight) of plant tissue was crushed with a plastic pestle in a tube containing 1 ml of sterile distilled water. Plant debris was removed by centrifugation. 100 ul of supernatant was incubated with 900 ul of nodule extract containing MSI rhizopine for 3 days at 28°C. The assay mix was then concentrated, stored, and analyzed as described above.

Results

Rhizopine synthesis and the absence of catabolism in plants

Rhizopine synthesis is known to occur only in legume nodules infected by rhizobia containing the *mos* genes (18). To determine whether rhizopine synthesis could occur in other plants, such as *Lotus corniculatus*, cosmids containing the *mos* and *moc* loci from *Sinorhizobium meliloti* L5-30 were introduced into *Mesorhizobium loti* PN184 by tri-parental mating. The presence of the constructs in the putative transconjugants was confirmed by reisolating the cosmids and subsequent restriction analysis (data not shown). All of the strains nodulated *Lotus corniculatus* plants under gnotobiotic conditions. Nodules infected by *M. loti* transconjugants harboring the *mos* locus, alone or in combination with the *moc* locus, accumulated the rhizopine, 3-O-methyl-*scyllo*-inosamine (MSI) (see Figure 2.1).

Different plant tissues were screened for activity that would degrade rhizopines. Extracts from *Arabidopsis thaliana*, *Nicotiana tabacum*, *Medicago sativa*, and *Lycopersicon esculentum* were examined using the standard assay for rhizopine catabolism. No catabolism of the the MSI rhizopine was detected in extracts from any of the plant species tested (data not shown).

Initial construction of pMOS plant expression vectors

Restriction fragments containing the *mos* genes, required for the production of 3-*O*-MSI in nodules by *S. meliloti*, were initially cloned from pPM1093 (A. Gufstafson, S. Rossbach, F.J. de

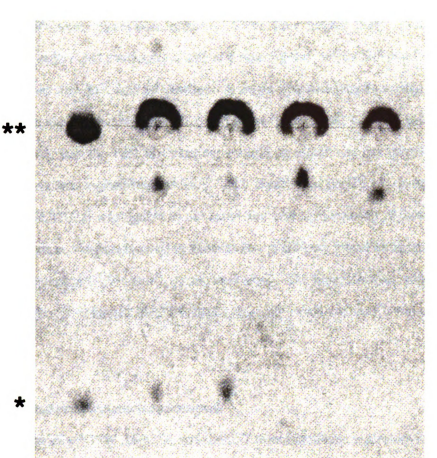

Figure 2.2: Accumulation of rhizopine in nodules of a heterologous symbiotic system. *Rhizobium loti* PN184 containing plasmids containing the *mos* and/or *moc* loci from *Sinorhizobium meliloti* L5-30 were inoculated onto *L. corniculatus* plants. Extracts from nodules induced by PN184(pPM1071) (*mos, moc*) (1), PN184(pPM1062) (*mos*) (2), or PN184(pPM1031)(*moc*) (3) were examined by HVPE analysis. Extracts from nodules induced by PN184 were used as a negative control (4). Extracts of *M. sativa* nodules induced by L5-30 are presented as a positive control (L). Uncharged a-diol-containing compounds are visible at the origin (**). The positively staining rhizopine migrates toward the negative electrode (*).

Bruijn, unpublished data). The *mos*-containing inserts of pAG8523, pAG8525, and pAG8527 were modified by linker ligations so that the fragments containing the three open-reading frames could be inserted into the plant expression vector pPCV91 (see Figure 2.2). A double-stranded oligonucleotide (NEB1148) containing a *Sal*I site was ligated to the *Sal*I-*Xba*I fragment from pAG8523, digested with *Sal*I, and gel purified. A single-stranded oligonucleotide linker, DB130 (*Pst*I->*Not*I), was used to modify pAG8525. The newly generated plasmid, pAG8525(n), was digested with *Not*I, and the 1.84 kb fragment containing *mosB* was gel purified. The *mosC* containing fragment was excised from pAG8527 with *Bam*HI and *Eco*RV. A double-stranded (ds) *Bam*HI linker (NEB1071) was ligated to the insert and was subsequently digested with *Bam*HI before gel purification. Fragments carrying the three *mos* genes were introduced sequentially into the plant expression vector pPCV91: *mos*C, *mos*A, then *mos*B. The final construct, pMOS7, contained all three *mos* genes. The plasmid pMOS 6, containing only *mos*B and *mos*C, was constructed in a similar fashion.

Lotus and Nicotiana transformations and analysis

The plasmids pPCV91, pMOS6, and pMOS7 were introduced separately into two different strains of *Agrobacterium*. These strains have isogenic chromosomal backgrounds, GV3101CT, containing helper functions necessary for the maintenance of pPCV91-derived plasmids in *Agrobacterium* (12). However, the strains differ in their plasmid content; strain 4062 contains pRi15824 (22), while strain 4063 contains pTiBo542 (10). Both strains grow well on YEB agar containing rifampicin (100 ug/ml), and are sensitive to carbenicillin ($\geq$ 75 ug/ml), but not ampicillin ($\leq$ 250 ug/ml). It was found that pPCV91 derived vectors could be adequately maintained in both strains in the presence of carbenicillin at a level of 100 ug/ml.

Transformation of *Lotus corniculatus* was performed via *Agrobacterium*-mediated hairy root induction. Hypocotyls were infected with strain 4062 harboring the three different plasmid constructs. Hairy roots were generated and removed, however shoots could not be regenerated in the presence of hygromycin, even at low concentrations (10 ug/ml). Because hygromycin selection has

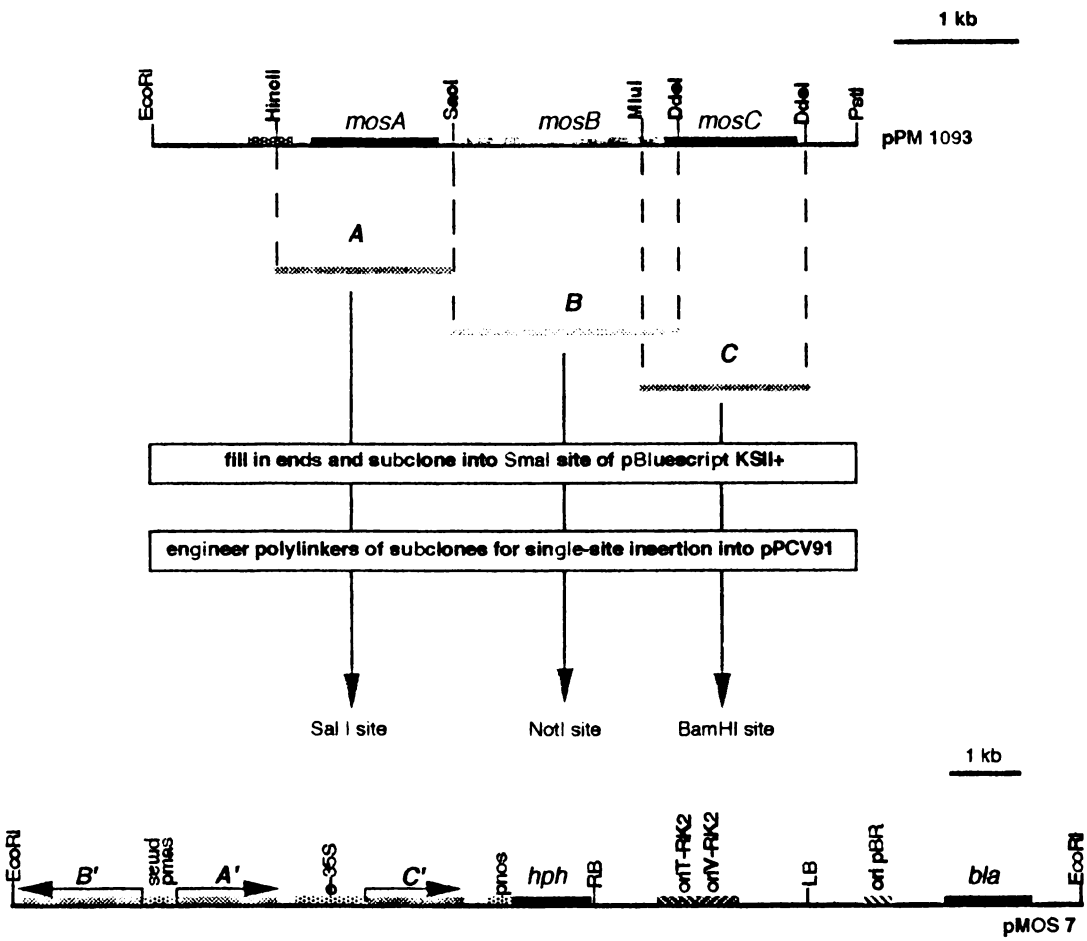

Figure 2.2: Initial strategy for inserting the *mos* ORFs into pPCV91.

not been used in any published report of *Lotus* transformation, and the fact that hairy roots are themselves transformed (32), regeneration of plants proceeded in the absence of selection.

Transformation of *Nicotiana tabacum* was mediated by *Agrobacterium*-induced callus formation. Leaves were infected with strain 4063 harboring either pPCV91 or pMOS7. Hygromycin tolerant calli were generated; however, no hygromycin resistant shoots were obtained from these calli.

During the course of the transformation procedures, samples of calli and hairy roots were taken for HVPE analysis. No difference between control and experimental tissues was observed (data not shown). Regenerated *L. corniculatus* were also screened after approximately six months of greenhouse growth. In wild-type plants, no stainable compound that co-migrated with rhizopine was observed in extracts prepared from root, leaf, or flower tissue. Similarly, no rhizopine-like signal was observed in extracts of any of the plants regenerated from hairy roots.

Evaluation of the lack of hygromycin resistant plants

Hygromycin-resistant plants were not obtained in the experiments described above. Two control experiments were carried out to evaluate the cause(s) of this unexpected result. In the first experiment, strain 4063(pPCV91) and LBA4404(pBIB) were used to transform *N. tabacum.* Hygromycin-resistant tobacco plants could only be regenerated from the latter combination. Subsequently, pPCV91 was introduced into *A. tumefaciens* GV3101- (pMP90RK). However, none of the seeds obtained from plants infiltrated with this strain germinated in the presence of hygromycin. It was concluded that the rate of effective plasmid transfer and/or the expression of the *hpt* gene *in planta* was significantly reduced in the case of the pPCV91 vector system.

Construction of new vectors using PCR-amplified mos ORFs

The strategy for cloning and expressing the *mos* genes was reevaluated after the initial screens of primary transformants were found to be negative in terms of rhizopine production. A review of the DNA sequence data of the *mos* locus revealed the presence of a number of potential

translational start sites upstream of the coding regions of all three *mos* ORFs. Several of these ATG codons are contained within the fragments used to generate pMOS7, are out of frame with the coding sequences, and may preclude the functional expression of the *mos* genes *in planta* (17). Since no convenient restriction sites exist between these out of frame ATG codons and the correct translational start, a PCR-based cloning procedure was pursued. Figure 2.3 outlines the cloning strategy used to prepare the different types of vectors described below.

Amplification of the *mos* ORFs using *mosA*-, *mosB*-, or *mosC*-specific primers produced products of the expected sizes (data not shown). These PCR products were inserted separately into pBluescript II SK (pBS) vectors (see Figure 2.4). The *mosA* PCR product was digested with *Xba*I and inserted into the polylinker of the vector in both orientations (pBM8623 and pBM8624). The *mos*B PCR product was treated with the Klenow fragment of DNA polymerase and T4 polynucleotide kinase prior to being inserted into the *Sma*I site of the polylinker (pBM8625 and pBM8626). The *mos*C PCR product was digested with *Sac*II and *Bam*HI and inserted directionally into the polylinker of pBS (pBM8627). These plasmids provided the *mos* gene fragments for all further molecular cloning.

Expression of the amplified mos genes in E. coli

The three *mos* ORFs were inserted separately into the bacterial expression vector pET15b (see Figure 2.5). The forward primers used in the PCR reactions contained restriction sites that allowed for the generation of in-frame gene-fusions between the polyhistidine encoding leader and each of the *mos* genes. The *Bgl*II-*Xba*I fragment from pBM8624 containing *mosA* was inserted into the *Bam*HI site of pET15b to yield pBM8653. Likewise, the *mosB* gene from pBM8625 was inserted into the *Nde*I site to yield pBM8654, and the *Sal*I-*Xho*I fragment from containing *mosC* from pBM8628 was inserted into the *Sal*I site to yield pBM8636. These constructs were introduced into *E. coli* BL21(λDE3)(pLysS) for induction and isolation. Induction of gene expression by the addition of IPTG resulted in the production of proteins of the expected size for two of the three genes (see Figure 2.6). Both the putative MosA and MosB proteins accumulated to a significant percentage

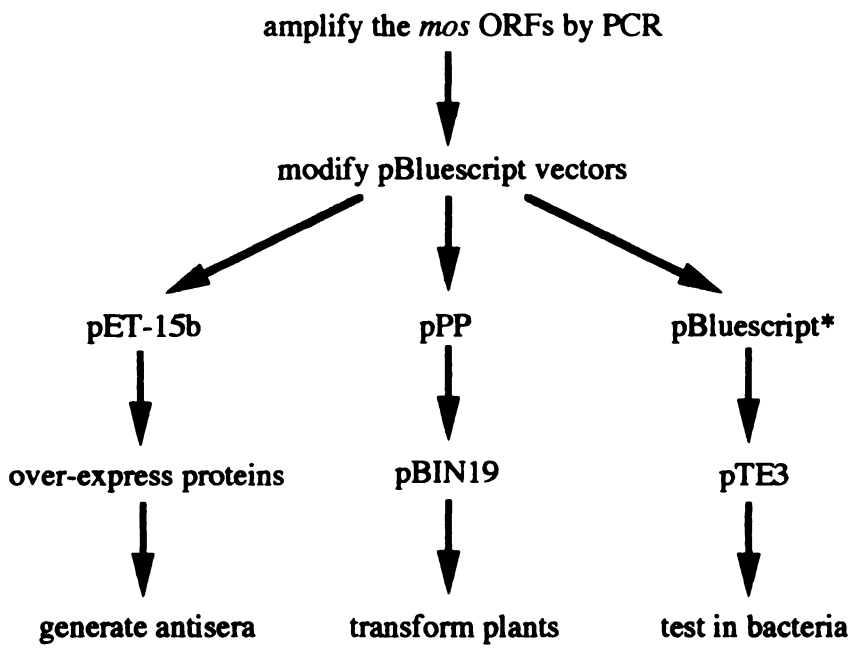

Figure 2.3: Flow chart of PCR-based *mos* gene cloning strategy and applications of various constructs.

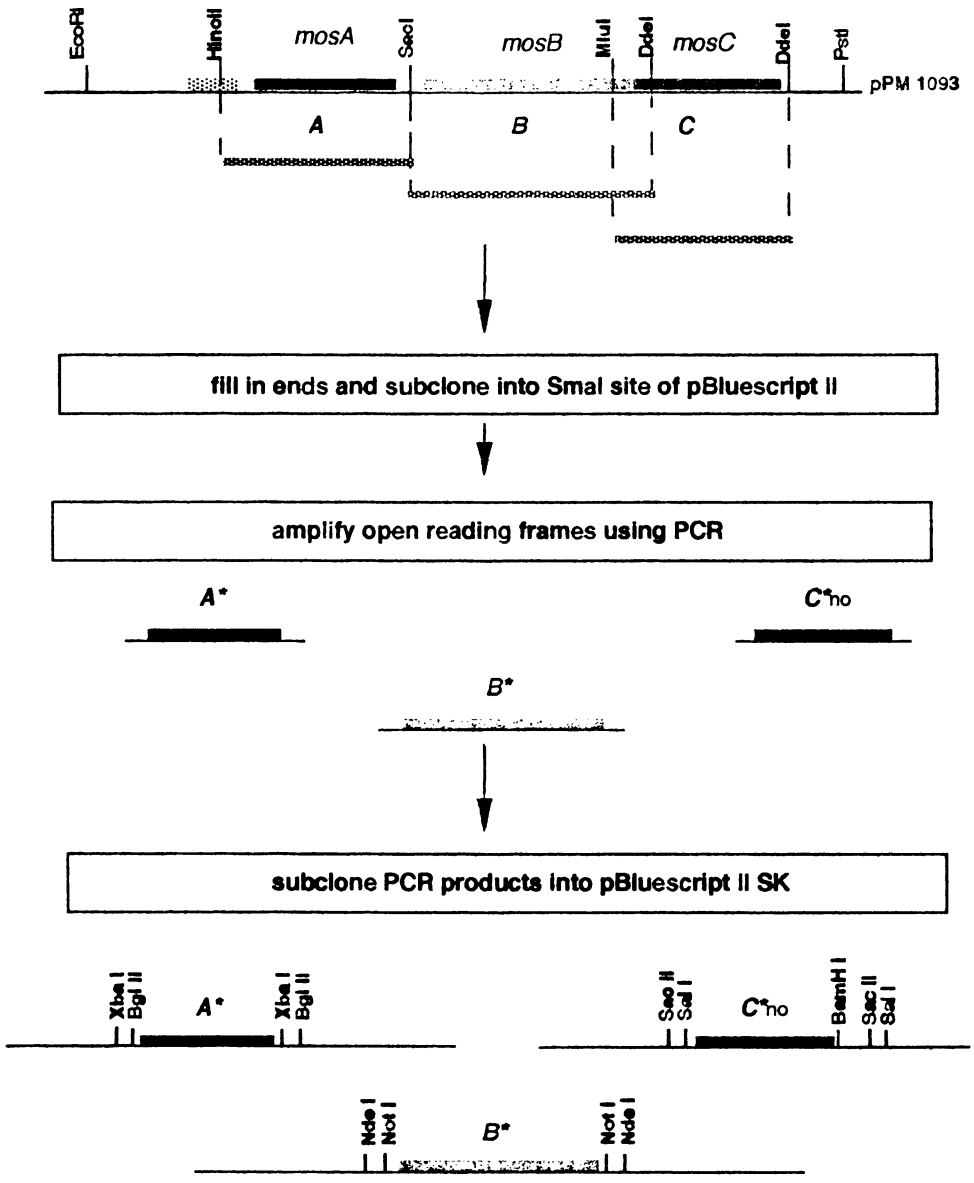

Figure 2.4: PCR-based cloning of *mos* ORFs.

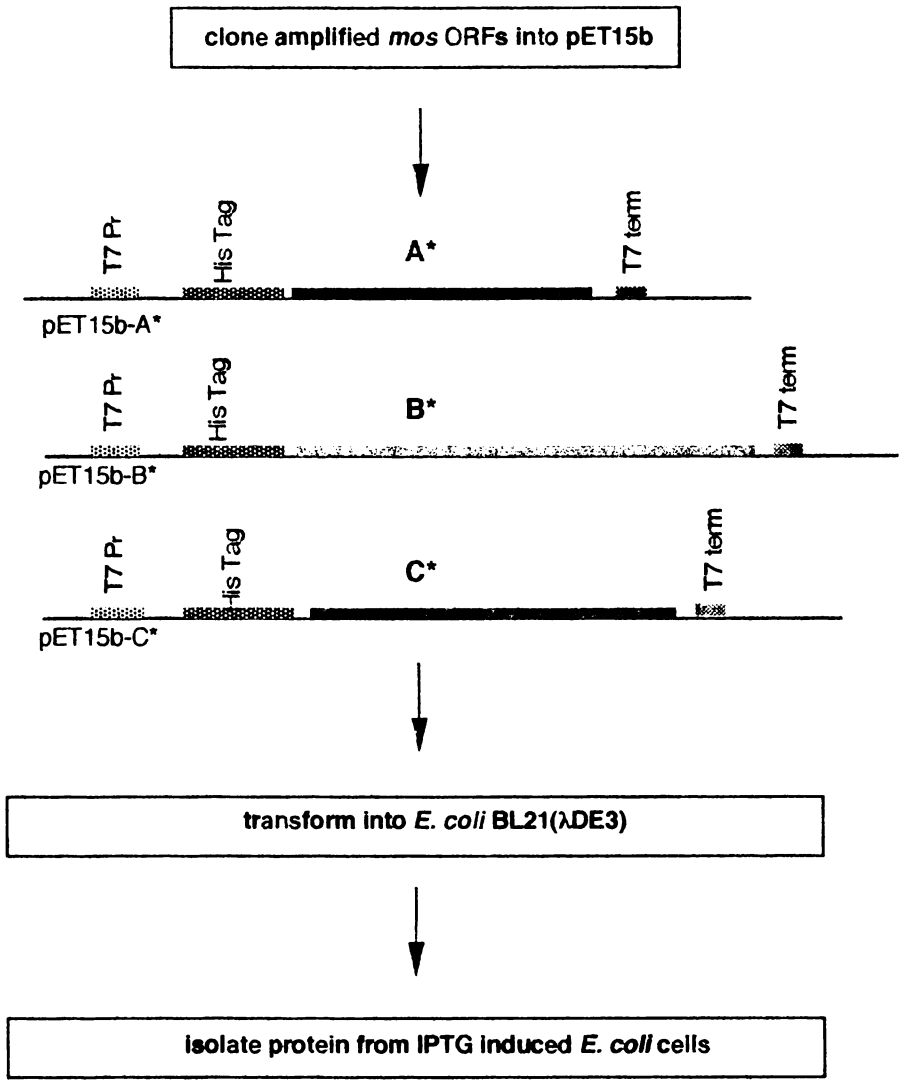

Figure 2.5: Cloning of PCR-amplified *mos* ORFs into bacterial expression vectors.

of the total cellular protein, and were present exclusively in the cell pellet fraction. The putative *mos*C protein, predicted to run at 46 kDa, was was not observed to accumulate. The purified MosA and MosB proteins were injected into rabbits and antisera were obtained. Western blot analyses of proteins extracted from nodules infected with *S. meliloti* L5-30 indicated that the antibodies detected the wild-type MosA and MosB proteins (see Figure 2.10).

Construction of pMOS7B1

Because of the problems regarding selection of hygromycin resistant plants mentioned above, I decided to create a hybrid vector containing the three promoter and terminator combinations from pPCV91 (for expression of the *mos* ORFs) and the *npt*II gene from pBIN19 (for selection of transformed plants based on resistance to kanamycin) (see Figure 2.7). First, a modified pBluescript SK vector was constructed, lacking the *Bam*HI, *Not*I, and *Sal*I sites from its polylinker. The vector was digested with *Bam*HI and *Not*I, treated with the Klenow fragment to fill in the overhanging ends, and religated to form pBM8633. The *Sal*I site of this vector was changed to a *Sac*I site by inserting the oligo linker DB258, resulting in pBM8634. Then, the 3.5 kb *Eco*RI fragment from pPCV9I, containing the three unique cloning sites with their associated promoters and terminators, was inserted into pBM8634 to generate pPlantPrep.

The three *mos* open reading frames were sequentially inserted into pPlantPrep. The 1.28 kb *mosC*-containing insert from pBM8628 was inserted in the proper orientation to generate pPPC. Then the 1.06 kb *Bgl*II-*Xba*I *mosA*-containing fragment from pBM8630 was inserted to yield pPPCA. Subsequently, the 1.60 kb *mosB* containing *Not*I fragment from pBM8626 was inserted to yield pPPCAB. Similar constructions were undertaken in parallel using pPCV91, giving rise to pMOS7A.

To complete the construction of the plant transformation vector, the 7.5 kb *Bss*HII fragment containing the three *mos* genes was rendered blunt-ended with mung bean nuclease, gel purified, and inserted into the *Sma*I site of pBIN19. This construct is referred to as pMOS7B1.

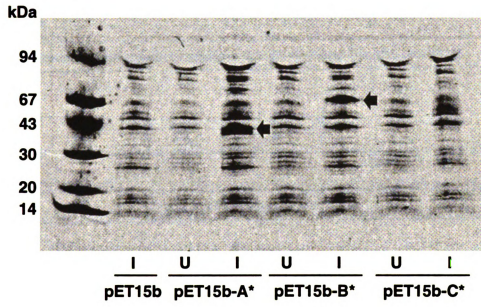

Figure 2.6: SDS-PAGE gel of protein extracts from *E. coli* containing different *mos* gene constructs. Cells were either uninduced (U) or induced (I) with 1mM IPTG to express the plasmid encoded proteins. Arrows indicate expressed MosA and MosB proteins.

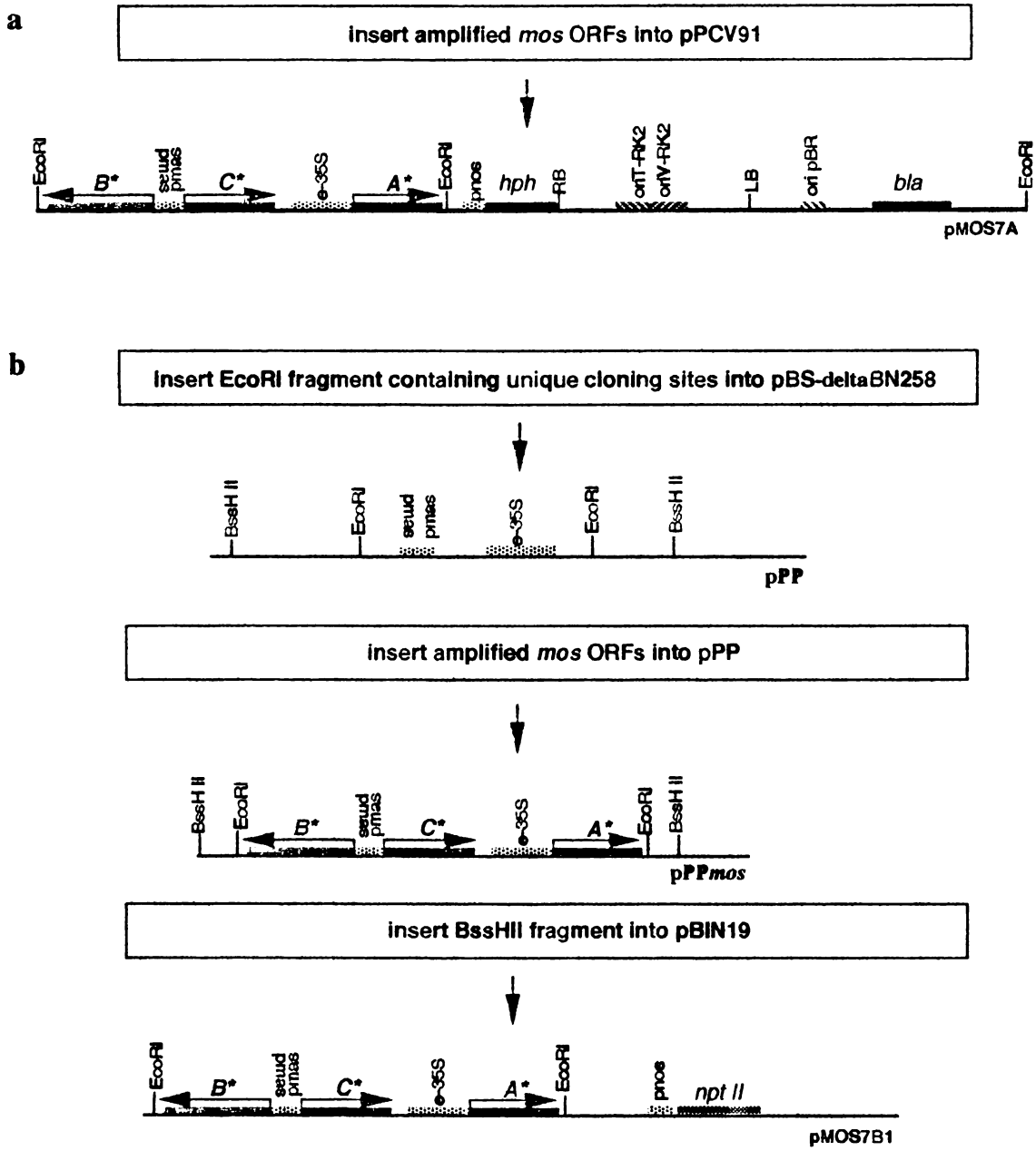

Figure 2.7: Construction of pMOS7A(a) and pMOS7B1(b).

Arabidopsis transformation and analysis of transgenic tissues

The plasmids pBIN19 and pMOS7B1 were introduced into *A. tumefaciens* C58C1. Restriction analysis of the plasmids reisolated from the transconjugants indicated that their structure was stabily maintained in these strains (data not shown). *Arabidopsis* plants were infected with these strains by vacuum infiltration, and T1 seed, representing at least twelve independent lines, was obtained from separate plants. Selection of transformed plants was made on the basis of resistance to kanamycin (100 ug/ml). Notably, plants infected with C58C1(pMOS7B1) produced much less seed than those infected with the control strain C58C1(pBIN19) (data not shown).

Plants transformed with the pMOS7B1 construct generally displayed an altered growth phenotype (see Figure 2.8). Under normal light conditions (16 hr light: 8 hr dark), these transgenic plants displayed enlarged and more numerous rosette leaves. Furthermore flowering was generally delayed or entirely suppressed. This contrasted with wild-type plants and those transformed with pBIN19 that initiated flowering two to three weeks after germination under the same light conditions. The aberrant phenotype was observed to be partially suppressed under 24 hour light conditions. Under such conditions, the plants transformed with pMOS7B1 flowered, but the emerging bolts were generally fewer and greatly enlarged and the plants retained the enlarged rosette leaves. The initial seed pods were greatly enlarged and contained no viable seed; however, more apical pods gave rise to viable seed.

Molecular and biochemical analyses indicated that the *mos* construct, pMOS7B1, was insufficient for the production of rhizopine *in planta*. Leaf tissues of T1 plants and whole seedlings of T2 plants were assayed for the accumulation of rhizopine using the HVPE assay, but no rhizopine (*i.e.* $\leq$ 30 ppm) could be detected in these tissue extracts (see Figure 2.9). Northern and Western blot analyses were performed on select lines displaying variations in their growth phenotype (see Figure 2.10). Transcripts corresponding to all three *mos* genes were detected in three of the four selected lines that had been transformed with pMOS7B1. Interestingly, only the lines displaying a visibly mutant phenotype were observed to contain the *mos* transcripts. The sizes of the hybridizing bands were estimated relative to the migration pattern of the 28S and 18S rRNA. The *mosB* and

Figure 2.8: Phenotypes of *Arabidopsis thaliana* plants transformed with pBIN19 (1) **or pMOS7B1** (2). Plants are shown after 3 weeks of incubation under normal light conditions (panel a) and after 6 weeks under 24 hour light conditions (panel b).

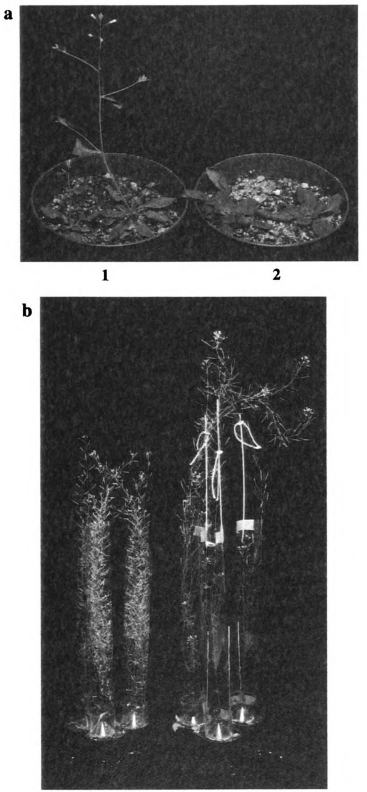

Figure 2.8

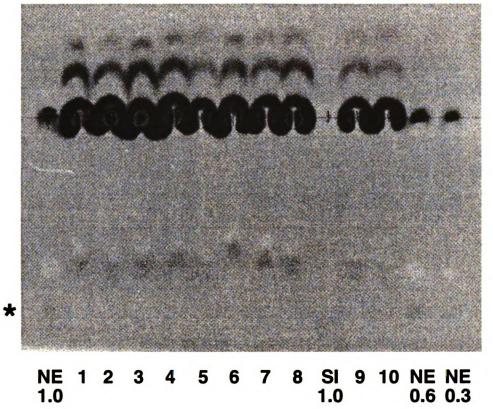

Figure 2.9: HVPE analysis of extracts of transgenic *Arabidopsis thaliana* plants. The independent lines of plants displayed were transformed with either pMOS7B (lanes 1 - 8) or pBIN19 (lanes 9 - 10). Nodule extracts containing MSI (NE) and synthesized SI (SI) were included as positive controls.

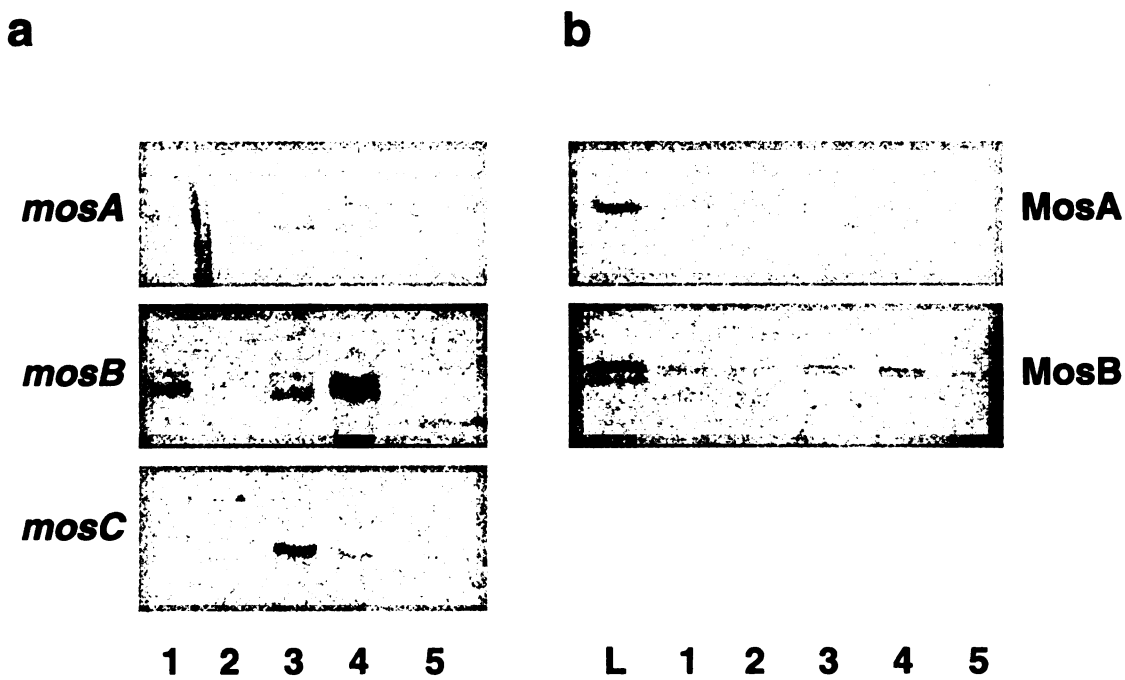

Figure 2.10: Analysis of *mos* gene expression in transgenic *Arabidopsis thaliana* plants. (a) Northern blot analysis using DIG-labeled probes containing *mosA*, *mosB*, and *mosC* DNA sequences. (b) Western blot analysis using anti-MosA and anti-MosB antisera. The independent lines of plants displayed were transformed with either pMOS7B (lanes 1 - 4) or pBIN19 (lane 5). Total protein from nodules infected with *S. meliloti* L5-30 (L) were included as a positive control.

mosC transcripts were observed to be 1.79 and 1.49 kb respectively, approximately 190 bp longer than the corresponding open reading-frames. The increased length of these mRNAs probably results from the presesence of 3' untranslated sequence in the *nos* terminator regions adjoining each insertion site in the vector. In contrast the detected *mosA* transcript was observed to be 1.03 kb in length, approximately 220 bp shorter than expected. The anti-MosA antisera could detect proteins of the size expected for MosA in nodules infected with *S. meliloti* L5-30, however, no protein corresponding to MosA was detected in extracts of the transformed plants. Two bands, approximately 60 kDa in size, similar to the size predicted for MosB, were detected both in L5-30 infected nodules and transgenic plant tissues using the antiserum specific for MosB. At least one comigrating band was observed to be faintly present in samples of control plants transformed with pBIN19. Neither of these signals was detected in replicate blots hybridized with pre-immune sera (data not shown). Line 4 which displayed the greatest amount of *mosB* transcript also contained the greatest amount of MosB protein.

Evaluation of the functionality of the amplified mos genes

To investigate whether or not the PCR-amplified *mos* genes were sufficient for rhizopine synthesis, the three ORFs were reorganized into an operon structure similar to that of the wild-type *mos* locus (see Figure 2.11). First, the plasmid pBM8627 was modified using DB249 (*Bam*HI->*Sac*II), resulting in pBM8628. In parallel, plasmid pBM8624 was modified with the linker DB244 (*Spe*I->*Sac*I), resulting in pBM8630. The *mos*C-containing *Sac*II fragment of pBM8628 was inserted into the corresponding unique site in pBM8630 to yield pBM8637. The *mos*B-containing *Not*I fragment from pBM8626 was subsequently inserted into the unique site of pBM8637 producing pMOS17. The *Sac*I insert of pMOS17, containing all three open reading frames (in the order *mosA*, *mosB*, *mosC*), was then cloned into the unique *Sac*I site of pTE3-245. The broad host-range plasmid pTE3 contains the *trp* promoter 5' to the unique cloning site, and expression from this promoter has been suggested to be constitutive in *S. meliloti* (6). The *mos* operons constructed are referred to as pMOS27 and pMOS72, with the inserts in the (+) and (-) orientations respectively.

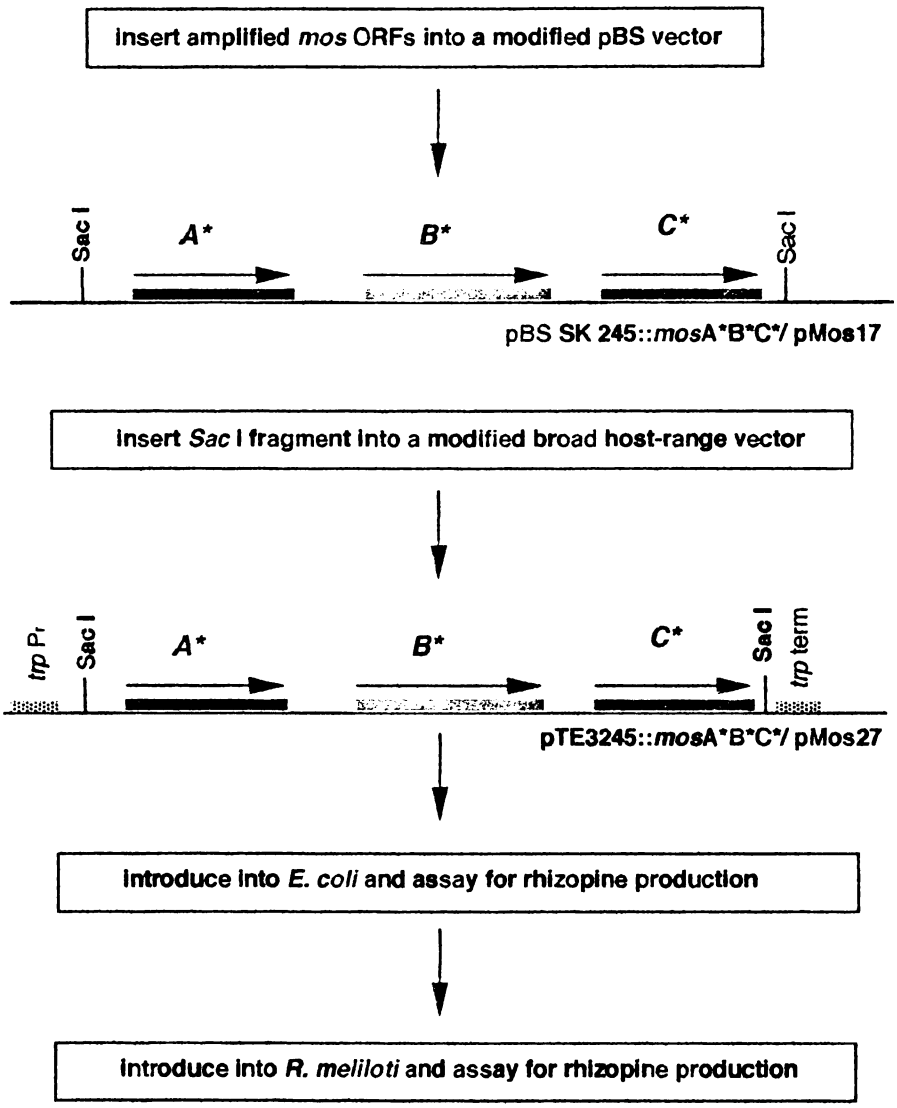

Figure 2.11: Reconstruction of a *mos* operon using PCR-amplified *mos* open-reading frames.

These reconstructed operons were used to determine the functionality of the amplified *mos* ORFs in nodules. The three pTE3-derived constructs and pPM1093 were mobilized separately into *S. meliloti* 1021 by tri-parental mating, giving rise to strains 3247, 3249, 3250, and 3169 (see Table 2.1). *M. sativa* were inoculated separately with each of these strains under gnotobiotic conditions. Nodules induced by these strains were all found to contain tetracycline-resistant rhizobia indicating that the plasmids were maintained in the cells during infection and nodulation. However, rhizopine was only detected in extracts of nodules induced by the positive control strain, 3169 (data not shown).

Discussion

The feasibility of creating rhizopine-producing transgenic plants was investigated. Previous work had indicated that the 5.3 kb fragment of pPM1093 containing the *mos* genes from *S. meliloti* L5-30 was sufficient for the production of the rhizopine MSI in nodules of *M. sativa* induced by an otherwise Mos$^-$ strain of *S. meliloti* (14). Here the same cosmid, and others containing the same *mos* locus, were found to be sufficient for the accumulation of rhizopine in nodules of *L. corniculatus* (see Figure 2.2). Additionally, no rhizopine specific catabolic activity was detected in extracts of roots or leaves of three different plant species. These data indicate that there is no *a priori* limitation to rhizopine accumulation *in planta*, and that it may be feasible to create transgenic plants that produce 3-*O*-MSI.

However, the goal of producing measurable quantities of rhizopine *in planta* was not achieved. The initial efforts used pPCV91-derived vectors in *Agrobacterium* GV3101CT. Contrary to expectations, the hairy roots and calli induced were generally hygromycin-sensitive, and tissues regenerated from them were not observed to accumulate rhizopines. It is possible that the *mos* sequences were not transferred into the genome of the infected plant tissues or that the *hpt* gene was not functional in these constructs. In a control test, hygromycin-resistant transformed plants were obtained with LBA4404(pBIB) but not GV3101(pPCV91). Also, attempts to transform *Arabidopsis* plants by vacuum infiltration with GV3101(pPCV91) failed to result in the generation of hygromycin

resistant seed. The presence of putative translational start sequences upstream of the predicted ORFs may also have prevented the proper expression of the Mos proteins. Because rhizopine accumulation was not observed, an alternative PCR-based molecular cloning strategy was pursued which also allowed for the use of an alternative selection strategy based on kanamycin resistance. While the *mos* sequences present on pMOS7B1 were clearly mobilized into the plant genome, as evidenced by hybridization signals observed in the Northern blot analyses (see Figure 2.10), they were not sufficient for rhizopine accumulation in any of the transformed lines of *A.thaliana* (see Figure 2.9).

There are several possible explanations for why rhizopine accumulation was not observed in the transformed *Arabidopsis* plants. First, the *mosA* gene did not appear to be properly expressed at the mRNA level and the MosA protein did not accumulate; this would preclude MSI synthesis, though it might still allow for SI accumulation. Second, the MosC protein may not have accumulated; if so, neither rhizopine would have been synthesized. It is also possible that significant point mutations were introduced during the PCR amplification of one or more of the *mos* ORFs. While it is known that occasional DNA sequence changes in PCR-amplified fragments do occur (5), several other functional genes were successfully cloned using the same amplification protocol (S. Rossbach, unpublished data). Both the expression of the MosA and MosB proteins of the expected sizes in *E. coli* (see Figure 2.6) and the positive cross-reaction of the anti-MosA and anti-MosB antisera with the wild-type proteins from nodules infected with *S. meliloti* L5-30 (see Figure 2.10) indicate that at least these two genes were cloned without major errors. Nonetheless, an alteration in the coding sequence of one or more of the amplified sequences may have occurred. Indeed, assays of the functionality of the reconstructed operon failed to verify the ability of the cloned *mos* genes to operate in concert to produce the rhizopines. It is also possible that the three deduced ORFs are necessary but not sufficient for the synthesis of rhizopines in plant cells or heterologous bacteria. Since rhizopine synthesis has only been reported in nodules infected with rhizobia containing *mos* sequences, other rhizobial genes and/or plant genes with nodule specific expression may be required for its synthesis. Lastly, the observed phenotypic changes in the transformed plants indicate that one or more of the *mos* genes can significantly affect plant development. If this is the case, the practical

production of rhizopine *in planta* may not be tenable using the known *mos* genes. I conclude that further investigation of *mos* gene expression in rhizobium will be required before any more fruitful attempts at creating biased rhizospheres based on rhizopine synthesis in transgenic plants can be made.

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Chapter 3

Evaluation of the Abundance of Culturable Bacteria Capable of Utilizing Potential Nutritional Mediators, and Investigation into the Impact of Glucosamine Amendment on Culturable Soil Bacteria

Abstract

The abundance of culturable soil microorganisms capable of growing on different organic substrates was examined. The numbers of CFUs were greatest on mixed media and were interpreted as a baseline estimate of the number of culturable heterotrophic bacteria in the soil. Of the tested substrates, *scyllo*-inosamine and *myo*-inositol were among the most selective, indicating that they are good candidates for nutritional mediators in a biased rhizosphere system. Additionally, the impact of glucosamine amendment on the relative abundance of different bacterial populations was investigated in the field. The nutrient amendment increased the numbers of inoculant and indigenous bacteria cultured as expected under conditions of nutrient limitation. However, the observed impact on the inoculant populations was small and transient when compared to the changes in the indigenous glucosamine-catabolizing bacterial populations. Amongst the glucosamine-utilizing bacteria that were isolated, a single morphotype was found to increase two- to three-fold in relative abundance over the course of the experiment. Partial sequencing of 16S rDNA amplified from this morphotype indicated that it is a strain of *Arthrobacter globiformus*. The implications of these results for the creation of biased rhizospheres are discussed.

Introduction

The ability of various microorganisms to utilize a specific organic nutrient will partially determine its effectiveness as a nutritional mediators in a biased rhizosphere system. The more commonly a compound can be used as a nutrient source, the less likely it is to directly impact the abundance and activities of the target microbial populations. Therefore, compounds that can be used by relatively few bacteria are good candidates for nutritional mediators (13). Opines and rhizopines have been hypothesized to be nutritional mediators in nature (3, 4, 10) and may be useful in creating biased rhizosphere systems (17, 18). Bacteria other than members of the genera *Agrobacterium* that can catabolize opines have been isolated (11, 12), but the enumeration of these organisms in the environment has only recently been reported (14, 15). A direct examination of the diversity and abundance of rhizopine-utilizing bacteria in the environment has not been previously reported (see

Chapter 4). Because the proposed biased rhizosphere systems based on these two organic nutrients depend on the use of inoculant strains, it was considered worthwhile to investigate the relative abundance of potential microbial competitors, *i.e.* indigenous microorganisms capable of utilizing different potential nutritional mediators.

A simplified model describing the impact of a nutrient amendment on microbial populations in an agricultural setting is diagrammed in Figure 3.1. For any compound used as an organic nutrient, there is likely to be a set of indigenous microorganisms capable of catabolizing it in the soil. The addition of a prescribed amount of an organic nutrient is predicted to increase the abundance and/or activities of those organisms to some degree (vector 1) for some period of time (vector 2). In the case of inoculant microorganisms, the population size is commonly observed to decline logarithmically after introduction into the soil (19). Nonetheless, one would expect that inoculants capable of utilizing that prescribed nutrient would also respond positively to its addition (vectors 3 and 4). While several reports have indicated the response of particular bacterial populations to nutrient amendment, none of these have compared the responses of inoculant versus indigenous microbial populations (5, 6, 16, 20).

The goals of the experiments described below were to (a) evaluate the relative specificity of several compounds that might be used as nutrient amendments in field plots, and (b) to determine the impact of a nutrient amendment in the field in terms of the model described above. The results of these experiments were instrumental in the design of the subsequent experiments (see Chapter 4, 5, and 6), and they provided some additional insight into the nature of indigenous and inoculated bacterial populations and the way in which they respond to organic nutrients.

Materials and Methods

Enumeration of soil bacteria

The soil used in these experiments was a Michigan sandy-loam that was obtained from the MSU Crop and Soil Sciences Research Farm, East Lansing, MI. Samples were diluted to 0.1 g per ml with sterile distilled water in 50 ml conical tubes. Bacteria were dislodged by alternating 10

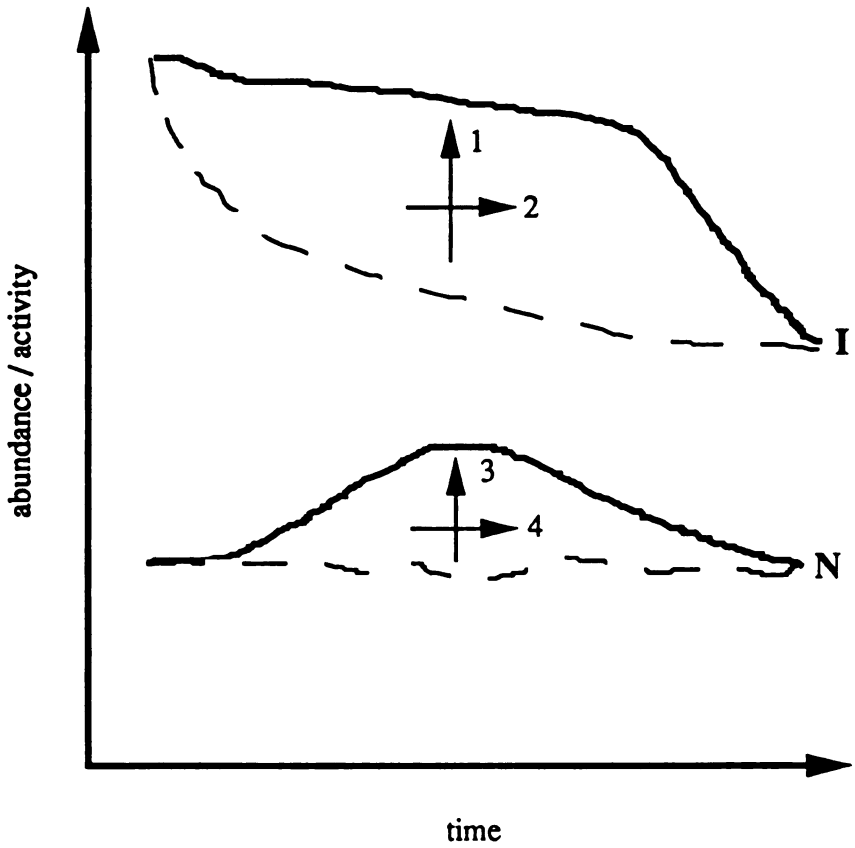

Figure 3.1: General model for the impact of nutritional mediators introduced into the soil and rhizosphere environment. Changes in inoculant (I) and indigenous (N) microbial populations capable of utilizing the nutrient are indicated in the presence (solid lines) and absence (dashed lines) of the amendment.

second treatments: vortex, sonication (tube placed in Ultra*sonik* cleaning bath, NEY Inc., Bloomfield, CT), vortex, sonication, and final vortex. Aliquots were then serially diluted in distilled water, and three 20 ul aliquots were spot inoculated onto defined media. Colonies were enumerated after 2 and 4 days of incubation at room temperature.

All of the defined media contained 1x BGTS mineral salts (25 mM KH_2PO_4 (pH 7.3), 10 mM NaCl, 25 ppm $MgSO_4 \cdot 7H_2O$, 1.25 ppm $CaCl_2$, 0.27 ppm $FeCl_3 \cdot 6\ H_2O$, 0.242 ppm $Na_2MoO_4 \cdot 2\ H_2O$, 3 ppm H_3BO_3, 1.83 ppm $NaSO_4 \cdot H_2O$, 0.287 ppm $ZnSO_4 \cdot 7\ H_2O$, 0.125 ppm $CuSO_4 \cdot 5\ H_2O$, 0.119 ppm $CoSO_4 \cdot 6\ H_2O$) and 1% SeaKem LE agarose (FMC, Rockland, ME). Individual organic nutrients were prepared as filter-sterilized stock solutions (50 mg/ml) and added as sole carbon and nitrogen source to a final concentration of 2 g/l. The media were named based on the nutrients tested: glucosamine (GN), glutamate (E), proline (P), glycine (G), threonine (T), mannopine, nopaline, octopine, *myo*-inositol (MI), or *scyllo*-inosamine (SI). In the case of *myo*-inositol-containing media, 20% w/v $(NH_4)_2SO_4$ was also added to the media to give a final concentration of 2 g/l. Two defined mixtures of carbon and nitrogen sources were also used: MCN 1 (0.35 g/l glucose, 0.35 g/l sodium succinate, 0.125 g/l pyruvate, 0.126 g/l glycerol, 0.05 g/l NaHCO3, 0.05 g/l $(NH_4)_2SO_4$, 0.1 g/l NH_4NO_3, 0.1 g/l $NaNO_3$, pH 7.2) and MCN 2 (0.4 g/l glucose, 0.3 g/l fructose, 0.1 g/l sodium citrate, 0.05 g/l leucine, 0.05 g/l glutamate, 0.05 g/l asparagine, 0.2 g/l $(NH_4)_2SO_4$, 0.25 g/l $NaNO_3$, 0.025 g/l glycerol, 0.025 g/l pyruvate, 0.025 g/l sodium acetate, 0.025 g/l sodium bicarbonate, 0.025 g/l sodium succinate, pH 7.2).

Glucosamine amendment experiment

The field experiment was conducted late in the summer of 1996. Microplots were defined by bottomless 50 ml plastic tubes inserted into unplanted plowed soil to a depth of ten centimeters one week prior to the start of the experiment. These plots were set up in a replicated block design over an area of one square meter. Each microplot was amended with 10 mls of sterile distilled water or 0.1% glucosamine $\pm$ inoculant bacteria.

Two strains, III and IV, were used individually as inoculants at two different concentrations. They are rifampicin-resistant variants of R2 and S3, two bacterial strains isolated from the same field plot in a screen for rhizopine-utilizing bacteria (see Chapter 4). These strains were grown on the mineral salts medium described above supplemented with glucosamine as sole carbon and nitrogen source. To prepare each inoculant, overnight cultures were diluted to a concentration of approximately 10^5 cells per ml, and 100 ul were spread onto solid media four hours prior to inoculation in the field. At the field site, cells were washed off of the plates with one ml of sterile distilled water and diluted 1: 2000 or 1:20000 in either sterile distilled water or 0.1% glucosamine.

Two replicate samples of each of the ten different treatments were taken after 1, 4 and 17 days of incubation in the field. Whole soil cores defined by the bottomless plastic tubes were removed from the field and returned to the laboratory for processing. For each sample, soil was removed from a tube, mixed, and weighed. Bacteria were enumerated as described above on four different media: 0.1xTSA and glucosamine-containing minimal media (GN) $\pm$ rifampicin (50 mg/l).

Results

Enumeration of culturable bacteria utilizing different substrates for growth

The abundance of culturable soil bacteria capable of utilizing various substrates as sole carbon and/or nitrogen source was investigated (see Table 3.1). Of the compounds examined, three classes were defined based on the number of CFUs appearing on the different media after two days and four days of incubation. Class A represents the least specific substrates (*i.e.* allowing the greatest number of bacteria to form colonies), while Class C represents the most specific substrates (*i.e.* allowing for the fewest number of bacteria to form colonies). In all cases, mixtures of nutrients gave rise to the greatest numbers of CFUs on the mineral salts media tested. Individual compounds, with the exception of glucosamine, were relatively more selective. Notably, *myo*-inositol and the rhizopines were among the most selective compounds. Mannopine, nopaline, and threonine were found to be equivalently selective substrates as the rhizopines in terms of the number of CFUs observed.

C and N source	days of incubation 2d	4d	class
MCN 1	7.2 - 7.7	7.7 - 8.7	A
MCN 2	7.5 - 8.3	7.5 - 8.3	A
glucosamine	6.7 - 7.7	7.5 - 8.7	A
glutamate	5.0 - 6.5	7.7 - 8.3	B
proline	5.0 - 6.5	7.7 - 8.3	B
glycine	4.0 - 5.0	7.3 - 7.7	B
threonine	< 4.0	6.3 - 7.0	C
mannopine	5.3 - 6.5	6.0 - 7.0	C
nopaline	4.0 - 5.5	6.0 - 7.5	C
octopine	4.0 - 5.0	6.7 - 7.7	BC
MI + NH_4	5.5 - 7.0	5.5 - 7.0	C
SI	4.5 - 6.0	5.5 - 7.3	C

Table 3.1: Log culturable counts per gram of soil on media containing different sources of carbon and nitrogen as substrates for growth. Class definitions are based on the log median CFUs: ≥ 7 after 2d (A), < 7 after 2d and >7 after 4 d (B), < 7 after 4d (C).

Impact of glucosamine amendment on indigenous and inoculant populations of soil bacteria

The two bacterial strains used as inoculants in this field experiment were rifampicin-resistant isolates of rhizopine-catabolizing bacteria that had been isolated from soil samples taken from the same research plot the previous autumn. Both of these strains were observed to be capable of utilizing glucosamine or *scyllo*-inosamine as sole carbon and nitrogen source for growth. The organisms were grown on glucosamine-containing media and introduced in aqueous solutions containing either distilled water or 1000 ppm of glucosamine. The number of CFUs growing on the rifampicin-containing media were approximately 1 to 3 percent of the numbers growing on GN media from the same sample (data not shown). The number of CFUs found on GN media numbered from roughly 10 to 30 percent of the numbers observed on the MCN media (data not shown). The impact of the glucosamine amendment on different populations of culturable bacteria is summarized in Table 3.2.

	MCN		GN		Rif	
sample day	2d	4d	2d	4d	2d	4d
1	1.3	1.3	0.9	0.9	**1.6**	**1.4**
4	**2.4**	**3.1**	**25.0**	**2.7**	2.1	2.0
17	**2.7**	**2.1**	**7.8**	**2.5**	1.7	0.7

Table 3.2: Relative increases in CFUs on various media due to glucosamine amendment. The ratio of ($CFUs_{amended\ soil}$ / $CFUs_{unamended\ soil}$) are tabulated after 2 and 4 days of incubation on various media. The media used consisted of 1x BGTS basal salt mixture supplemented with mixed C and N sources (MCN), glucosamine (GN), or rifampicin to select for inoculant strains (Rif). Rifampicin-containing media were found to specifically select for inoculant organisms. Significant differences ($P < 0.05$) are highlighted in bold.

sample day	unamended soil		glucosamine amended soil	
1	0.21	0.00 - 0.03	0.24	0.20 - 0.50
4	0.28	0.17 - 0.33	0.45	0.25 - 0.66
17	0.20	0.11 - 0.33	**0.60**	0.50 - 0.75

Table 3.3: The proportion of CFUs growing on GN media that displayed the white-convex (WC) morphotype. Medians and their associated interquartile range are tabulated. Significant differences due to the amendment ($P < 0.05$) are highlighted in bold.

A small but significant increase in the numbers of inoculant organisms due to the glucosamine amendment was detected after one day of incubation in the field, but the effect was small and not statistically significant in the later samplings. The impact on the indigenous catabolizers was much more striking. Large increases in the proportion of culturable catabolizers isolated from the glucosamine amended plots as compared to the unamended plots were noted at both 4 and 17 days post-inoculation. These increases were generally more pronounced after two days, as compared to four days, of incubation on the laboratory media. Additionally, there appeared to be an enrichment for a particular white convex (WC) morphotype over the time-course examined (see Table 3.3). Multiple isolates of this morphotype had very similar genomic fingerprint patterns, indicating that they are closely related phylogenetically (see Figure 3.2). Partial sequencing of the PCR-amplified 16S rRNA sequences indicated that the WC morphotypes were strains of *Arthrobacter globiformis* (data not shown).

Discussion

The effectiveness of a biased rhizosphere system will be partially dependent on the relative specificity of the organic nutrient used as a nutritional mediator. Here, several compounds were examined for their ability to promote the growth of culturable soil bacteria. Of these, *scyllo*-inosamine and *myo*-inositol were found to be among the most selective (*i.e.* allowed for the fewest number of organisms to be cultured). While the numbers of CFUs utilizing the most selective types of compounds (*i.e.* Class C compounds) were roughly equivalent, it is not known to what extent the diversity of the cultured isolates differ because the identity of the cultured organisms was not examined.

As expected, the use of glucosamine as an applied nutrient amendment resulted in increases in the numbers of indigenous and inoculant bacteria, as diagrammed in Figure 3.1. Under conditions of nutrient limitation, one would expect that an applied substrate would most significantly impact the populations of bacteria capable of utilizing it as a nutrient. The application of glucosamine resulted in significant increases in the numbers of CFUs growing on all three media, but the greatest differences

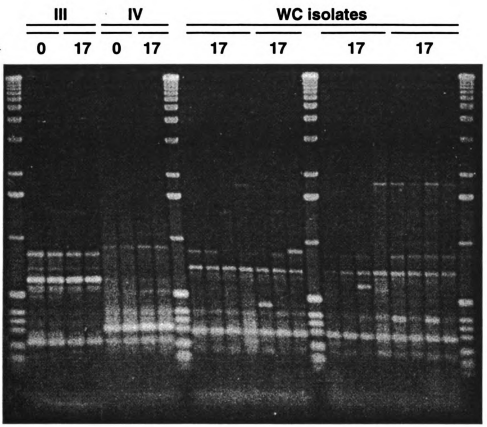

Figure 3.2: BOX genomic fingerprints of bacteria isolated from glucosamine amended soil samples. The recovered inoculant microorganisms (strains III and IV) retain their fingerprint pattern. A selection of fifteen isolates displaying the WC morphotype isolated in this experiment display similar genomic fingerprint patterns.

were observed on GN medium (see Table 3.2). The data indicate that the addition of the organic nutrient promoted the growth and culturability of both the inoculant and indigenous bacteria *in situ*. Similar responses of soil bacterial populations to nutrient amendments have been reported before in experiments using various organic substrates. In such studies, the impact of simple organic substrates has been noted to vary according to the concentration and identity of the applied substrate as well as to the identity of the targeted populations (1, 2, 6, 7, 8, 16, 20). However, comparisons between inoculant and indigenous bacteria are generally lacking.

In this study, the greatest change in relative abundance of CFUs resulting from the nutrient amendment occurred on the GN media. This is logical since one would expect that the glucosamine-utilizing bacteria have the first opportunity to acquire the nutritional benefit of the substrate. Increases were also observed on the MCN media, which might indicate that at least some of the carbon, nitrogen, and/or energy present in the glucosamine had filtered into other bacterial populations. The noted increases in the bacterial populations were long-lasting, indicating that nutrient amendment at planting could substantially impact indigenous microflora well into the growing season. In contrast, the observed increases in the relative abundance of the inoculant microorganisms were relatively small and transient compared to the changes on the indigenous soil bacterial populations. It is uncertain whether this is due to an inability of the inoculant to readapt to the soil environment from which it was first isolated or due to a decreased ability to effectively compete for the nutrient amendment. In any case, the application of the nutrient amendment preferentially benefited indigenous microorganisms that were preadapted to living in the soil environment and capable of utilizing the amendment as a nutrient source.

Because only a small percentage of soil bacteria are cultured on any prescribed media (9), it is not possible to comprehensively assess the utility of any particular substrate using the method described here. Thus, these results can only be interpreted as a first estimate of the potential effectiveness of a proposed nutritional mediator and its impact on soil and rhizosphere bacterial populations.

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Chapter 4

Detection and Isolation of Novel Rhizopine-Catabolizing Bacteria from the Environment

McSpadden Gardener B, de Bruijn FJ (1998) Detection and Isolation of Novel Rhizopine-catabolizing bacteria from the environment. Appl. Environ. Microbiol. *in preparation*

Abstract

Microbial rhizopine-catabolizing (Moc) activity was detected in serial dilutions of soil and rhizosphere washes. The activity observed generally ranged between 10^6 and 10^7 catabolic units (CUs) per gram, and the numbers of nonspecific culture-forming units (CFUs) were found to be approximately 10 times higher. A diverse set of thirty-seven isolates was obtained by enrichment on *scyllo*-inosamine (SI)-containing media. However, none of the bacteria that were isolated were found to contain DNA sequences homologous to the known *mocA*, *mocB*, and *mocC* genes of *Sinorhizobium meliloti*. Twenty-one of the isolates could utilize an SI preparation as sole carbon and nitrogen source for growth. Partial sequencing of 16S rDNA amplified from these strains indicated that five distinct bacterial genera (*Arthrobacter*, *Sinorhizobium*, *Pseudomonas*, *Aeromonas*, and *Alcaligenes*) were represented in this set. Only six of these twenty-one isolates could catabolize 3-O-methyl-*scyllo*-inosamine under standard assay conditions. Two of these, strains D1 and R3, were found to have 16S sequences very similar to *Sinorhizobium meliloti*. However, these strains are not symbiotically effective on *Medicago sativa*, and DNA sequences homologous to the *nodBC* genes were not detected in D1 and R3 by Southern hybridization analysis.

Introduction

Rhizopines are inositol derivatives synthesized in legume nodules induced by specific members of the Rhizobiaceae (14, 31). The first rhizopine was isolated from alfalfa nodules infected with *Sinorhizobium meliloti* L5-30 (28). The structure of this compound was determined to be 3-O-methyl-*scyllo*-inosamine (MSI) (13). Genes on the large symbiotic plasmid of L5-30 were determined to be involved in the synthesis and catabolism of this compound (12). Transposon mutagenesis and subsequent DNA sequence analysis of the identified rhizopine catabolism (*moc*) locus from this strain revealed four open-reading frames (ORFs) involved in the catabolism of MSI (24). Three of these ORFs, *moc*A, *moc*B, and *moc*C, have been found to be sufficient to confer Moc activity onto otherwise Moc$^-$ strains of *Sinorhizobium meliloti*. (22). DNA sequences homologous to

these *moc* genes were not observed in a broad screen of known soil and rhizosphere bacteria (24), and only members of the Rhizobiaceae have been reported to be Moc^+ (14).

Some organic molecules may be used to specifically promote the growth and metabolic activities of soil and rhizosphere bacteria capable of utilizing them as growth substrates (5, 6, 13, 18, 23, 24, 25). Several recent reports have shown that these specific "nutritional mediators" can enrich for bacteria capable of utilizing them as growth substrates in soil and on plant leaves and roots (3, 4, 7, 10, 20, 27, 32, 33, 34). However, the potential of nutritional mediators to promote the activities of target microbial populations could be limited by a variety of factors, including the relative abundance of non-target microbes capable of catabolizing the compound. While several reports have indicated that other proposed nutritional mediators can be catabolized by a variety of indigenous bacteria (5, 15, 16, 19), no previous study has directly investigated rhizopine catabolism in the environment. Here we report the detection, isolation, and enumeration of previously unknown rhizopine-catabolizing bacteria from the environment and discuss the implications of their existence for the use of rhizopine as a selective nutritional mediator.

Materials and Methods

Soil and rhizosphere sampling

Samples of a Michigan sandy-loam were obtained from a fallowed plot on the MSU Crop and Soil Sciences Research Farm, East Lansing, MI. The plot had been left fallow for five years prior to these experiments and was covered by a variety of annual and perennial plants, including nodulated alfalfa. In September of 1995, approximately 50 kg was removed from the top 12 inches of the soil and transported to a research greenhouse for mixing and storage. Rocks and wooden debris were removed as the soil was homogenized in a rotary mixer. This soil was allowed to air dry and stored at room temperature until use.

For the initial detection and quantification of catabolic activities, aliquots of this soil were placed in plastic pots moistened, planted with *Medicago sativa* var. Cardinal, and incubated in a growth chamber (12:12 LD, 22°C). Soil and rhizosphere samples (< 100 mg each) were taken after 1

and 3 weeks of incubation. Samples were placed in 5 ml of distilled water in 15 ml Sarstedt tubes. Bacteria were dislodged by alternating 10 second treatments: vortex, sonication (tube placed in Ultra*sonik* cleaning bath, NEY, Inc., Bloomfield, CT), vortex, sonication, and final vortex. They were serially diluted in distilled water, and 50 ul aliquots were inoculated into one ml of the catabolism assay mixtures.

Preparation of nodule extracts and catabolism assays

Extracts containing 3-O-methyl-*scyllo*-inosamine (MSI) were obtained from alfalfa nodules induced by *Sinorhizobium meliloti* L5-30 under gnotobiotic conditions as described previously (23). Five grams of nodules were crushed with a mortar and pestle in 25 ml of distilled water. The suspension was centrifuged at 20,000 x*g* for 20 minutes to clarify the solution. The supernatant was filter-sterilized, aliquoted, and kept frozen at -20°C. The total solute concentration of these 1x extracts was determined gravimetrically to be 0.5% (w/v) and the concentration of MSI in the extracts was estimated to be 0.01% (w/v) by HVPE analysis (see below). Preparations containing synthetic *scyllo*-inosamine (SI) were provided by Dr. R. Hollingsworth (Michigan State University Department of Biochemistry). *Myo*-inositol was obtained commercially (Sigma Chemical Co., St. Louis, MO, USA).

The rhizopine catabolism assay used to define Moc activity was similar to that described previously (23). Assay mixtures contained 0.3x nodule extract in 1x BGTS (25 mM KH_2PO_4 (pH 7.3), 10 mM NaCl, 25 ppm $MgSO_4 \cdot 7H_2O$, 1.25 ppm $CaCl_2$, 0.27 ppm $FeCl_3 \cdot 6\ H_2O$, 0.242 ppm $Na_2MoO_4 \cdot 2\ H_2O$, 3 ppm H_3BO_3, 1.83 ppm $NaSO_4 \cdot H_2O$, 0.287 ppm $ZnSO_4 \cdot 7\ H_2O$, 0.125 ppm $CuSO_4 \cdot 5\ H_2O$, 0.119 ppm $CoSO_4 \cdot 6\ H_2O$). Assay mixtures were incubated for 5 days in a rotary incubator at 28°C and 200 RPM. The mixtures were centrifuged for 2 minutes at 13,000 x*g* to remove cell debris. The decanted supernatants were concentrated 10-fold by evaporation in a rotary Speed-Vac and resuspended in small volumes of sterile distilled water. Samples were stored at -20°C prior to analysis by high-voltage paper electrophoresis (HVPE). Ten microliters of each sample was loaded onto 3MM Whatman paper and air dried. Electrophoresis was performed at 3000 V in 1.1 M

acetic acid/0.7 M formic acid buffer. Visualization of α-diol-containing compounds, including MSI, resulted from staining with alkaline $AgNO_3$ (5). The rhizopine was detected as a dark spot running at a characteristic distance relative to the reference dye orange G ($R_{orange\ G}$ = - 0.9). Catabolism of SI and *myo*-inositol was scored as growth in similar assay mixtures, where either 0.2% SI or 0.2% *myo*-inositol + 0.1% $(NH_4)_2SO_4$ were added in lieu of the nodule extract. Growth was scored by visual inspection for turbidity.

Isolation and maintenance of bacteria

To increase the diversity of isolates obtained, bacteria were isolated from the soil described above in several different ways. For the liquid enrichment cultures, samples included: (a) air-dried soil (Soil D), (b) soil that had been saturated and kept at approximately field capacity for just over 5 months (Soil W), and (c) the crown rhizospheres of two separate 5 month-old alfalfa plants grown in this saturated soil (Rhizospheres R and S). Samples (≤ 5 g each) were placed in 25 ml of distilled water, vigorously shaken for 15 seconds, then diluted to the equivalent of 0.01 g/ml (w/v) in sterile distilled water. Seventy-five microliters of these mixtures were inoculated into 1 ml of SI-containing media (0.05% SI in 1x BGTS) and incubated at 28°C. After 3 days, the cultures were diluted and spread onto 0.1 x TSA for isolation of individual colonies. Seventeen isolates representing several distinct morphotypes from each of the samples were selected for further analysis. Isolates were also obtained by selection of organisms growing on solid media containing SI. Two soil samples (Soil W2a and W2b) were obtained from the same pots as described in (b) above two months later. Additionally, four soil samples (Soils Aa, Ab, Ba and Bb) were obtained in late April, 1996 from within five meters of the original sampling site in the field plot described above. Samples (≤ 4 g each) were diluted 0.1 g/ml in 1x BGTS, mixed vigorously, serially diluted, and plated onto solid media (1% agarose ± 0.2% SI ± 0.1% $(NH_4)_2SO_4$, or 0.1x TSA). Plate counts were determined after 2, 4, and 8 days of incubation at 28°C. At the four day time-point, twenty strains representing distinct morphotypes of the most abundant colonies were selected from each of the SI-containing

plates and purified on 0.1x TSA media. For all subsequent work, cultures were grown 28°C on 0.1x TSB or 0.1x TSA. Frozen stocks of purified isolates were kept in 15% glycerol at -70°C.

Characterization of bacterial isolates

MagnaGraph nylon membranes (MSI Scientific, Westboro, MA) and random-primed DIG-labeled probes prepared with the Genius I Kit (Boehringer Mannheim Biotechnologies, Indianapolis, IN) were used in all hybridization experiments according to the manufacturers' instructions. When screening the isolates for *moc*-like sequences, ten microliters of overnight cultures were spot-inoculated onto 0.1x TSA and incubated for 24 hours at 28°C prior to colony lifts. Probe templates consisted of the full-length ORFs of the *mocA*, *mocB*, and *mocC* genes from *S. meliloti* L5-30 (23). For the characterization of strains D1, R3, and L5-30, genomic DNA was isolated, digested, and transferred to nylon membranes following standard procedures (26). Hybridizations were performed overnight at 68°C and blots were subjected to two 10 minute high stringency washes (0.1x SSC, 0.1% SDS, $\geq$ 37°C). Genomic fingerprinting using the BOX primer was performed using whole cells as described elsewhere (21).

In the nodulation assays, large test tubes containing 15 ml of a nitrogen-free mineral salts media (1) and wicks of 3MM Whatman filter paper were autoclaved and allowed to cool to room temperature. Seeds of *Medicago sativa* var. Cardinal were surface sterilized by immersion in 3% hydrogen peroxide for 10 minutes followed by thorough rinsing in sterile distilled water. Two seeds were asceptically transferred onto the wick in each tube. Liquid cultures of the three bacterial strains were grown to saturation, pelleted by centrifugation, and washed twice with sterile-distilled water. 150 ul (containing $\geq 10^7$ cells) of these washed cultures was used to inoculate each tube. Four replicates tubes were prepared for each strain and for the uninoculated control.

Statistics

The nonparametric sign test (17) was used for all comparisons. Differences in relative population sizes were conservatively estimated by finding the maximum factor which still yielded

significant results in each of the comparisons. Tests were performed using SchoolStat software (David Darby, WhiteAnt Occasional Publishing, Victoria, Australia, ddarby@ariel.ucs.unimelb.edu.au). All P-values less than or equal to 0.15 are reported.

Results

Detection and quantification of Moc activity in the environment

Rhizopine-catabolizing (Moc) activity was detected in serial dilutions of soil and rhizosphere washes after five days of incubation in standard assay mixtures (see Figure 4.1). Since it could not be assumed that the breakdown of 3-*O*-methyl-*scyllo*-inosamine (MSI) in these assays was due to the activities of individual organisms, the enumeration of Moc activity in these assays is referred to in terms of catabolic units (CUs) analogous to culture-forming units (CFUs). The median Moc activity was observed to be 10^6 to 10^7 CUs per gram ($n = 6$) in both soil and rhizosphere samples. The median *myo*-inositol-catabolizing (Mic) activity was observed to be approximately five-fold higher, but this difference was not statistically significant. These data contrast with the median bacterial growth in these assays ($P < 0.04$) which was observed to be approximately 10^8 CFUs per gram. Bacterial growth in the assay mixtures increased with time and correlated well with the disappearance of uncharged organic compounds from the nodule extract as visualized by HVPE analysis. Catabolism of the uncharged compounds generally preceded catabolism of MSI (data not shown).

The number of bacteria growing on the crude nodule extract was estimated to be just under 10 times that growing on *myo*-inositol (MI) as sole carbon source ($P < 0.11$). Likewise, the number of CFUs was approximately 10 times the number of units of Moc activity present in the assay mixtures ($P < 0.11$), though this ratio was measured to be ≥ 100 in half of the assays. Similarly, the number of colonies growing on *scyllo*-inosamine (SI) containing medium was less than the number growing on 0.1x TSA ($P < 0.005$). When the SI medium was supplemented with 0.1% (w/v) $(NH_4)_2SO_4$, the number of colonies observed was intermediate between the numbers observed on SI medium and 0.1x TSA medium ($P < 0.04$). Again, the number of bacteria growing on SI-

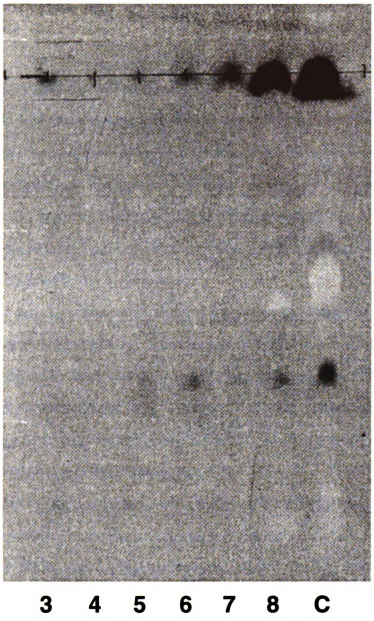

Figure 4.1: Detection of Moc activity in the environment. MSI in assay mixtures is detected as a positively-staining spot (*) with a defined electrophoretic mobility relative to orange G. Uncharged a-diol-containing organic compounds remain at the origin (**). Moc activity is scored as the absence of a detectable signal co-migrating with MSI after 5 days of incubation in the standard assay mix. To quantitate the observed activity, 10-fold serial dilutions (3 - 8) of soil and rhizosphere samples were assayed by comparison to an uninoculated control (C). In this example, 104 catabolic units (CUs) per gram of Moc activity were detected.

containing media was estimated to be approximately 10-fold less than the number growing on 0.1xTSA ($P < 0.11$).

Isolation and characterization of Moc⁺ bacteria

Liquid and solid media containing SI were used to enrich for Moc⁺ bacteria from soil and rhizosphere samples. A total of thirty-seven isolates were selected for further investigation based on the type of enrichment used, the sample source, and colony morphology of the isolate. All of these isolates were screened by colony hybridization for the presence of DNA sequences homologous to three of the known *moc* genes from *Sinorhizobium meliloti* L5-30 (see Figure 4.2). However, no hybridization was observed for any of the isolates.

Twenty-one of the isolates were found to be capable of growing on minimal medium containing the SI preparation as sole carbon and nitrogen source (data not shown). Each of these strains was characterized by partial 16S rDNA sequencing. DNA sequence analysis revealed that several distinct phylogenetic groups were represented, including Gram-negative α, β, and γ Proteobacteria and a Gram-positive phylum (see Table 4.1). Amplified rDNA restriction analysis (ARDRA) of representatives of each of these groups,
using *Msp*I and *Rsa*I in single restriction digests, confirmed these designations (data not shown). The 16S rDNA sequences of strains that were determined to belong to the same genus were found to be ≥ 98% identical over the region analyzed. However, rep-PCR genomic fingerprinting of these strains using the BOX primer revealed that most of the isolates were genotypically distinct (See Figure 4.3). Seventeen distinct patterns were observed in the set of twenty-one genomic fingerprints. Seven strains, all belonging to the γ proteobacteria based on their 16S sequence, were found to fall into three distinct groups based on their BOX-PCR generated genomic fingerprints: (D2, W2), (2,6), and (7,14,18). Additionally, some similarities were noted in the patterns generated from two sets of putative *Arthrobacter* strains (*i.e.* (R4,S3) and (1,1N)). Interestingly, the putative *S. meliloti* strains D1 and R3 had distinct BOX-genomic fingerprints from *S. meliloti* L5-30 (L).

Figure 4.2: Colony hybridization of bacterial isolates with *moc* gene probes. DIG-labeled probes corresponding to the *moc*A, *moc*B, and *moc*C genes of *S. meliloti* L5-30 were hybridized to blots of colonies lifted from replica plates such as those shown. All thirty-seven of the isolates obtained from liquid (a) and solid (b) media containing SI were examined. L5-30 (L) was included twice on all of the plates as a positive control.

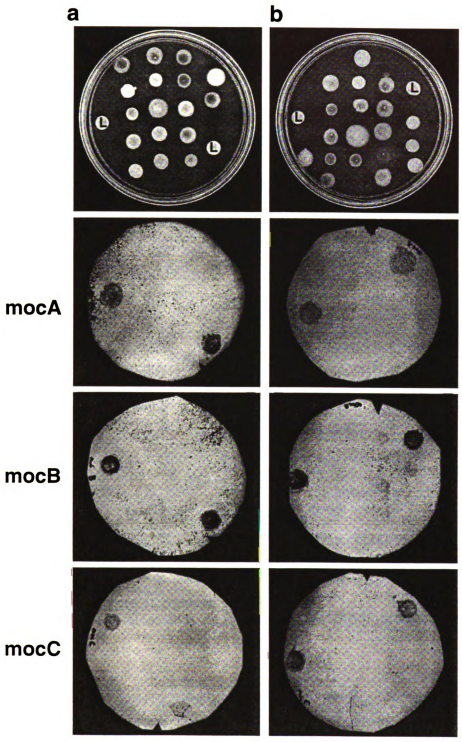

Figure 4.2

enrichment	source[a]	strain	bp	highest sequence match[b]	S_{ab}
Liquid medium	Soil D	**D1**	332	***Rhizobium meliloti***	0.855
		D2	381	*Azospirillum* sp.	0.921
	Soil W	W1	345	*Azospirillum* sp.	0.924
		W2	337	*Azospirillum* sp.	0.914
		W4	316	*Pseudomonas putida* mt-2	0.956
		W5	382	*Alcaligenes eutrophus* 335	0.890
	Rhizosphere R	R1	394	*Azospirillum* sp.	0.939
		R2	314	***Arthrobacter globiformus***[c]	0.538
		R3	333	***Rhizobium meliloti***	0.922
		R4	346	***Arthrobacter globiformus***	0.812
	Rhizosphere S	S1	335	*Azospirillum* sp.	0.904
		S3	317	***Arthrobacter globiformus***	0.886
Solid medium	Soil W2a	1	315	***Arthrobacter globiformus***	0.990
		1 N	273	***Arthrobacter globiformus***	0.982
	Soil W2b	2	372	*Azospirillum* sp.	0.962
	Soil Bb	6	380	*Pseudomonas putida* mt-2	0.916
		7	382	*Azospirillum* sp.	0.948
	Soil Ba	11	388	*Aeromonas media*	0.923
	Soil Ab	14	384	*Azospirillum* sp.	0.924
		16	350	*Azospirillum* sp.	0.940
	Soil Aa	18	372	*Azospirillum* sp.	0.763
		20	383	*Azospirillum* sp.	0.943

[a]See Materials and Methods of description.
[b]Highest match using SIM_RANK software.
[c]The low similarity value of this strain is due to a large number of ambiguous bases in the obtained sequence.

Table 4.1: Characterization of isolates capable of growing on the SI mix as sole carbon and nitrogen source by partial sequencing of their 16 rRNA genes. Strains found capable of catabolizing MSI are highlighted in bold. Sequence differences, not counting ambiguous base calls, were < 2% over 300 bp of aligned sequence for isolates with the same phylogenetic match.

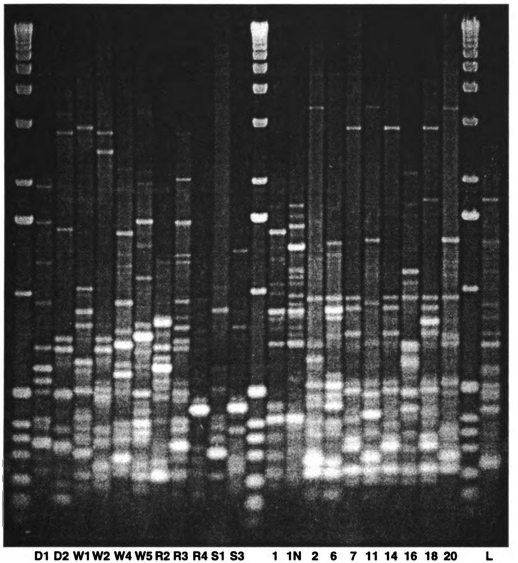

Figure 4.3: BOX-PCR generated genomic fingerprints of isolates capable of growing on SI as sole carbon and nitrogen source. Seventeen distinct patterns can be observed in the set of twenty-one genomic fingerprint obtained from the rhizopine-catabolizing isolates. Three sets of strains with very similar patterns were observed: (D2, W2), (2,6), and (7,14,18). Additionally, strains D1 and R3 had distinct BOX-genomic fingerprints from *S. meliloti* L5-30 (L).

Of the twenty-one isolates, only six were designated Moc$^+$ based on their performance in the standard catabolism assay (see Figure 4.4). Four of these isolates, R4, S3, 1, and 1N, appear to belong to the genus *Arthrobacter*, based on their 16S rDNA sequences. The other two strains, D1 and R3, appear to be most closely related to *S. meliloti*, the species from which the *moc* genes were initially isolated (12, 23).

Because the *moc* gene probes did not hybridize to genomic DNA from strains D1 and R3, we further analyzed these two putative *S. meliloti* strains. Attempts to amplify portions of the *nif HDK* and *nodBC* loci using conserved primers were unsuccessful despite amplification of appropriately sized fragments from *S. meliloti* L5-30 (data not shown). Additionally, no hybridization was observed to a *nodBC* probe generated from the L5-30 sequence (see Figure 4.5). Strians D1 and R3 also failed to nodulate alfalfa under gnotobiotic conditions.

Discussion

We have detected and characterized rhizopine-catabolizing activity in soil and rhizosphere environments, and we isolated a collection of novel rhizopine-catabolizing bacteria. Twenty-one bacterial strains capable of growing on synthetic SI were identified, six of which were found to be able to catabolize MSI under standard assay conditions. The abundance of two of these Moc$^+$ strains, 1 and 1N, approximated the total amount of rhizopine-catabolizing activity in the soil. Thus, while it is likely that we only isolated a subset of the entire diversity of rhizopine-catabolizing microbes from these samples, we have identified at least some of the more abundant strains.

DNA sequences similar to the known *moc* genes were not detected in any of the isolated organisms. This was particularly surprising with regards to isolates D1 and R3 which were identified as *S. meliloti* based on the sequence of their 16S rRNA genes. While it has been shown that the known *moc* genes are located on the large symbiotic plasmid in *S. meliloti* (12), D1 and R3 may be lacking that plasmid, as indicated by their non-symbiotic phenotype. Additionally, previous reports have indicated that Moc$^+$ *S. meliloti* strains contained *moc* genes that were very similar to those from

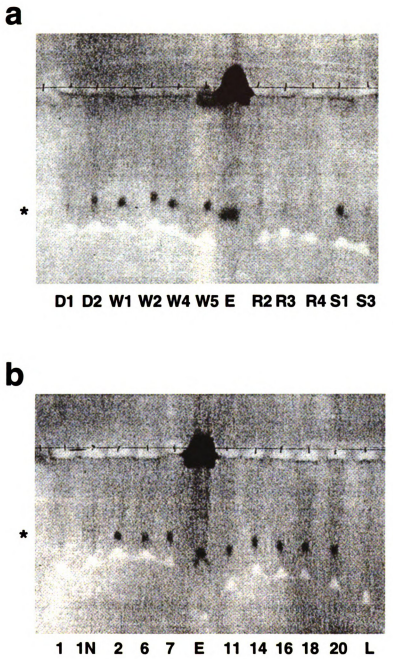

Figure 4.4: Determination of the Moc phenotype of isolates. Twenty-one isolates capable of growth on SI were obtained from liquid (a) and solid (b) media and assayed for their ability to catabolize MSI (*) in nodule extracts. L5-30 (L) was included as a positive control.

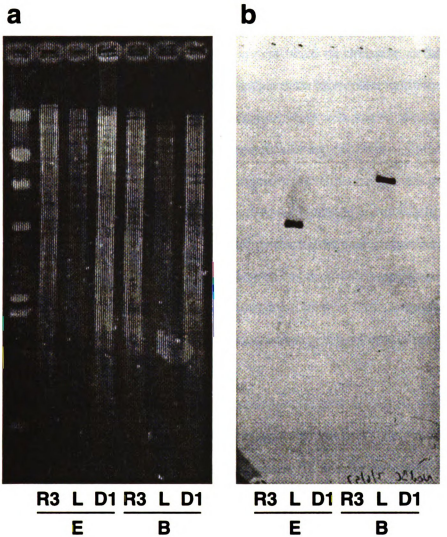

Figure 4.5: Southern blot analysis of D1, R3, and L5-30 for the presence of *nod* gene sequences. (a) Ethidium-stained agarose gel containing 1 mg of genomic DNA from each of the three strains digested with either EcoRI (E) or BamHI (B). (b) Southern blot of the same gel hybridized with a DIG-labeled probe specific to 1 kb spanning the *nodBC* genes of *S. meliloti.*

L5-30 (24, 30, 31). However, our colony hybridization results with D1 and R3 lead us to suggest that alternative genes responsible for MSI catabolism may be present in some strains of rhizobia.

One of the motivations for performing this study was to explore the potential impact of indigenous catabolizers on a "biased rhizosphere" system based on rhizopine as the nutritional mediator. Rhizopines were hypothesized to be good candidates for rhizosphere nutritional mediation because only beneficial soil bacteria (*i.e.* nitrogen-fixing rhizobia) were known to catabolize these compounds (13, 23, 25). Additionally, some evidence indicated that catabolism of MSI may play a role in competitiveness of certain strains for nodule occupancy (9). Other compounds present in the root exudate of legumes may also act as nutritional mediators stimulating nodulation by *Rhizobium* (8, 11). That nutritional mediators can stimulate other beneficial plant-microbe interactions has also been shown. The biological control of plant pathogens has been enhanced by the concurrent application of salicylate and bacteria capable of catabolising it (2, 35). Thus, it seems possible to promote the beneficial activities of plant-associated microbes capable of catabolizing nutritional mediators.

It is reasonable to assume that nontarget microbial populations capable of catabolizing any given nutrient exist, and their response to that nutrient may complicate the predicted outcome of efforts to bias microbial communities with nutritional mediators. Ideally, the abundance and diversity of microbes capable of using a selected nutritional mediator should be minimal. Some evidence indicates that opine-catabolizing bacteria represent a relatively small fraction of the total number of culturable bacterial heterotrophs in soil and rhizosphere environments (20). However, we have observed that the numbers of soil bacteria capable of growing on mannopine, nopaline, or octopine as sole carbon and nitrogen source was similar to those reported here for SI and *myo*-inositol + $(NH_4)_2SO_4$ (data not shown). This discrepancy may reflect differences in media composition and/or differences in the abundance of indigenous catabolizers in different soils. Diverse types of bacteria have been reported to catabolize mannityl opines, including members of *Arthrobacter* and *Pseudomonas* (15, 16, 19). The coincidental isolation of rhizopine catabolizers from these same genera likely reflects the metabolic diversity that is known to be present in these two genera. Of

course, plant-pathogenic *Agrobacterium* can also catabolize opines (5, 6). Because of this, opine-based nutritional mediators may not be suitable for biasing rhizosphere microbial populations in agricultural settings (19).

Other factors may also play significant roles in determining the effectiveness of nutritional mediators in the complex milieu of the rhizosphere environment. The relative number of target and nontarget catabolizers, their relative efficiency in utilizing the nutritional mediator, and the consequences of their enrichment on microbial ecology in and around the plant roots may all be factors. In our catabolism assays, we have noticed that the catabolism of MSI appears to follow catabolism of other α-diol-containing compounds present in the nodule extracts, both in the serial dilutions of environmental samples and in the cases of the Moc$^+$ isolates (data not shown). This may indicate that MSI is not a preferred metabolic substrate for the microorganisms capable of catabolizing it. Additionally, the growth rate of the Moc$^+$ isolates generally exceeded that of L5-30 both in the nodule extracts and in 0.1x TSB. To what extent these observations would relate to a rhizopine-based "biased rhizosphere" in the field remains an open question.

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Chapter 5

Examination of Soil and Rhizosphere Bacterial Communities Using a Modified T-RFLP Analysis of Amplified 16S rDNA

McSpadden Gardener B, de Bruijn FJ (1998) Examination of soil and rhizosphere bacterial communities using a modified T-RFLP analysis of amplified 16S rDNA. Appl. Environ. Microbiol. *in preparation*

Abstract

Soil and rhizosphere bacterial populations are described using a rapid PCR-based technique which, when combined with culture-based methods, provided unique insights into the structure of these microbial communities and their responses to environmental changes. Serial dilutions of soil and rhizosphere washes were used directly for PCR amplification of 16S rDNA using the conserved eubacterial primers 8F-HEX and 1492R. Subsequent restriction analysis of the fluorescently labelled products (FT-ARDRA) provided a reproducible baseline community profile that was used to monitor changes in the dominant bacterial members of microbial communities and to tentatively identify them. When applied to model communities of phylogenetically diverse environmental isolates, differences in the absolute abundance of unique organisms was found to be correlated with signal intensity, but comparisons between the relative abundance of different organisms could not be made quantitatively. Distinct differences between soil and rhizosphere communities were detected. When serial dilutions of soil and rhizosphere washes were subsequently incubated in liquid media, the FT-ARDRA profiles indicated that some, but not all, of the numerically dominant populations were cultured. The combination of PCR- and culture-based techniques also revealed that certain bacterial populations respond differentially to nutrient amendment depending on their localization in the soil or rhizosphere.

Introduction

The soil environment harbors diverse microbial communities (3, 17, 30, 32). To understand the nature of microorganisms *in situ* , information on the identity, abundance, and activities of these organisms is required. Much effort has been applied to describing these microbial communities as a whole, and a variety of useful techniques have been developed (1). Profiles based on carbon-source utilization (10), extractable lipids (*e.g.* PLFA, FAME)(4, 11), extractable nucleic acids (*e.g.* GC-content of DNA)(13), and PCR-amplified genes (*e.g.* ribotyping, DGGE, TGGE)(12, 23, 26) have been used to display differences in the structure of soil and rhizosphere communities (*e.g.* 3, 4, 6, 11, 12, 16, 36). While limited, each in its own way, these methods have proved useful complements to culture-based approaches in the study of microbial communities from a variety of habitats (21).

The data obtained by these various methods often allow for the identification of differences in microbial communities, and correlations with associated environmental and empirical variables can be made. However, the ability to identify and quantify those differences in terms of specific bacterial populations is essential to dissecting the mechanisms responsible for the different patterns. In this regard, molecular methods based on the amplification of 16S ribosomal RNA genes by PCR have greatly expanded our knowledge of microbial diversity (24, 33). Data useful for the elucidation of bacterial phylogeny have been obtained through the (optional) cloning and sequencing of amplified products and then compared to established databases (*e.g.* GenBank, RDP) to make inferences about the microbial populations present in different environmental samples (31). Alternatively, restriction fragment length polymorphisms of amplified 16S rDNA (ARDRA) have been used to distinguish bacterial communities and identify novel community components (14, 18, 20, 34).

Here we describe soil and rhizosphere bacterial populations using a high throughput method involving multiple replicate profiles of the terminal restriction fragment length polymorphisms (T-RFLPs) of amplified 16S rDNA sequences. We call this approach fluorescent-tag amplified rDNA restriction analysis (FT-ARDRA). This methodology was applied to serial dilutions of soil and rhizosphere washes as well as to cultures of those samples to reveal new information about bacterial populations in these environments and their response to nutrient amendment.

Materials and Methods

Model bacterial communities

Five of the strains (numbered 1 - 5) used for the creation of model communities were isolated from bulk soil and the rhizospheres of alfalfa plants grown in a Michigan sandy-loam (MSU Crop and Soil Sciences Research Farm, East Lansing, MI). Full-length 16S rRNA genes from each strain were amplified using the conserved eubacterial primers 8F and 1492R (35). The identities of these strains, presented in Table 5.1, was based on the direct partial sequencing of these amplified products. Greater than 250 bp of 16S rDNA sequence was generated from each sample using the primer 519R (15) and compared to the RDP data base using the SIM_RANK program (19).

Additional strains (numbered 6 - 9) were obtained from a laboratory collection of rhizosphere isolates (J. Stolzfus and F.J. de Bruijn, unpublished data). For preparation of the model community mixtures, individual strains were grown on 0.1x TSA media at 28°C. Individual colonies were removed from the plates and resuspended in 1 ml distilled water. Cells were pelleted by centrifugation (30 sec, 6000 xg) and resuspended in 0.3 ml of distilled water. The cell density was measured and adjusted so that all cultures had a final absorbance, at 600 nm, of 0.1 per ml. Subsequent direct microscopic counts of these cultures indicated that this absorbance corresponded to approximately 1.5×10^8 cells per ml for most cultures. Notably, strain 5 was characterized by much larger cells and the adjusted culture contained only 0.2×10^8 cells per ml. Mixture G (containing all 10 strains) was prepared by mixing 10 ul from each individual culture. Mixtures A, B, C, D, E, and F contained only five strains (1 -5) and were prepared in a similar manner except that one strain was added in excess. For mixtures A, C, and E, an additional 80 ul of strain 1,2, and 4 were used, respectively. For mixtures B, D, and F, an additional 30 ul of strain 1,2, and 4 were used, respectively. Two sets of each mixture were prepared. Samples were frozen at -20°C overnight and subsequently thawed at room temperature to be used as templates for PCR amplification.

Soil and rhizosphere samples

Several kilograms of a Michigan sandy loam were air dried, mixed, and aliquoted into separate potting units containing approximately 50 g. Each unit was moistened with either 5 ml of 2% *myo*-inositol (+) or an equal volume of distilled water (-). Three to five alfalfa seeds (NitraginGold pre-inoculated, PH1B10277, Idaho Crop Improvement Association, Boise, ID) were spread on top of each unit of soil and imbibed with 100 ul of distilled water to promote rapid germination. Soil and plants were incubated in a growth chamber (12:12 LD, 22°C) for one week.

Samples were processed sequentially, alternating (+) and (-) treatments. Soil samples (approximately 150 mg each) were taken from the surface at least 1 cm away from alfalfa shoots with a clean spatula. Rhizosphere samples (approximately 25 mg of root tissue + approximately 30 mg of attached soil) were obtained by gently separating the roots from surrounding soil and cutting off the

attendant shoot. Samples were promptly placed in 5 ml of distilled water in 15 ml Sarstedt tubes. Bacteria were dislodged by alternating 15 second treatments: vortex, sonication (tube placed in Ultra*sonik* cleaning bath, NEY, Inc.), vortex, sonication, and final vortex. A 50 ul aliquot was transferred to a prefilled (200 ul distilled water per well) 96-well microtiter plate for serial dilution. Twenty microliters of each well-mixed dilution was then inoculated into replicate microtiter plates containing 200 ul per well of either 0.1x TSB medium or MA medium (25 mM KH_2PO_4 (pH 7.3), 10 mM NaCl, 25 ppm $MgSO_4 \cdot 7H_2O$, 1.25 ppm $CaCl_2$, 0.27 ppm $FeCl_3 \cdot 6\ H_2O$, 0.242 ppm $Na_2MoO_4 \cdot 2\ H_2O$, 3 ppm H_3BO_3, 1.83 ppm $NaSO_4 \cdot H_2O$, 0.287 ppm $ZnSO_4 \cdot 7\ H_2O$, 0.125 ppm $CuSO_4 \cdot 5\ H_2O$, 0.119 ppm $CoSO_4 \cdot 6\ H_2O$, 1 g/l *myo*-inositol, 1 g/l $(NH_4)_2SO_4$). This procedure was repeated until all soil and rhizosphere samples had been processed. Roots were then rinsed in distilled water and transferred to a 1.5 ul tube containing 750 ul of distilled water to be crushed with a sterile pestle. A 50 ul aliquot from each was transferred to a microtiter plate for serial dilution as described above. Dilution plates were stored at -20°C. Culture plates were incubated at room temperature in the dark for 18 hours before sampling for PCR.

Fluorescent-tag amplified ribosomal DNA restriction analysis (FT-ARDRA)

HPLC-purified primers 8F-HEX (5'- *AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- ACG GCT ACC TTG TTA CGA CTT -3') were designed according Weisburg *et al.* (35) and were synthesized by *PE*Applied Biosystems (Foster City, CA). The polymerase chain reaction was performed according to conditions similar to those that have been described before for whole cell rep-PCR (25). Master mixes were prepared while template samples were thawed at room temperature. The master mix contents for 30 reactions was: 150 ul of 5x Gitschier buffer (83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl, pH 8.8, 33.5 mM $MgCl_2$, 33.5 uM EDTA, 150 mM β-mercaptoethanol), 6 ul BSA (20 mg/ml), 75 ul DMSO, 256 ul sterile distilled water, 37.5 ul dNTPs (2.5 mM, mixed 1:1:1:1), 30 ul each primer (25 pmol per ul), 3.75 ul RNAse (2.5 mg/ml), 10 ul *Taq* DNA polymerase. Twenty microliter aliquots of the master mix were distributed into 8-strip PCR tubes. Five microliters of thawed template (containing 10^4 to 10^6 bacteria) were mixed in thoroughly by micropipetting.

Negative control reactions were performed for each amplified set. Reactions were processed in either PTC100 or PTC200 cyclers (MJ Research, Inc., Watertown, MA). Cycling conditions: 95°C for 7 min, then 35 x (94°C 1 min, 50°C 1 min, 65°C 8 min), then 65 °C for 16 min, followed by 4 °C storage.

Restriction digestions were performed in 96-well microtiter plates. Each reaction consisted of 7 ul of a PCR reaction in a total reaction volume of 40 ul and 8 U of either *Rsa*I or *Msp*I (Boehringer-Mannheim, Indianapolis, IN). Reactions were incubated at 37°C for two to four hours. Reactions were then stored at -20°C. For standard ARDRA, 8 ul aliquots were loaded onto 1.5% agarose gels and electrophoresed in 0.5x TAE buffer. For FT-ARDRA, 10 ul of each sample were submitted to the Michigan State University DNA Sequencing Facility (East Lansing, MI) for electrophoresis and imaging. Briefly, 2 ul of each sample were mixed with 2 ul of G2500-TAMRA and loaded onto 6% urea-containing polyacrylamide gels (24 WTR plates). Electrophoresis was performed on an ABI373 sequencer (*PE*Applied Biosystems, Foster City, CA) and run for 14 hr at 1680 V. Data was collected automatically using the B-filter and analyzed using GeneScan 2.1 Software (*PE*Applied Biosystems, Foster City, CA).

Results

Analysis of model bacterial communities

Phylogenetically diverse strains of soil and rhizosphere bacteria were examined using an improved T-RFLP analysis of amplified 16S rRNA gene sequences (FT-ARDRA). The 16S rDNA gene was amplified from all of the tested strains using the conserved eubacterial primers 8F-HEX and 1492R. Digestion and electrophoretic separation of the amplified products gave rise to distinct banding patterns that were generally in good agreement with the patterns predicted by the RDP database sequences of phylogenetically related strains (see Table 5.1).

Examination of the digested products on ethidium bromide-stained gels revealed that the 16S rDNA of some strains was amplified better than others (data not shown). This qualitative observation

was confirmed quantitatively by measuring the amount of fluorescence of the HEX-labeled 5' fragment (see Table 5.1). When averaging four measurements, the amount of fluorescence,

		5'*Rsa*I fragment[b] (bp)			5'*Msp*I fragment[b] (bp)		
Strain	Putative Identity[a]	Expected	Observed	Area[c]	Expected	Observed	Area[c]
1	*Pseudomonas fluorescencs*	860	878	118	490	497	153
2	*Arthrobacter macroides*	478	458	103	68	68, 70	78
3	*Rhizobium meliloti*	711(+148)[d]	832	78	406	402	99
4	*Bacillus macroides*	455	458	45	150	154	23
5	*Alcaligenes eutrophus*	470	476	76	440	433	92

[a] As determined by partial 16S rDNA sequencing.
[b] Mean size of major peak(s), n = 4.
[c] Mean area of major fluorescent peak, units of fluorescence / 1000, n = 4.
[d] The deduced sizes of the first and second fragments are listed.

Table 1: FT-ARDRA data of isolated strains. The 16S rDNA sequences were amplified from each of the five strains, digested with either *Rsa*I or *Msp*I, and the resulting fragments were separated by PAGE. The observed sizes of the 5'-HEX-labelled fragments were determined by GeneScan software. The expected sizes were deduced from corresponding 16S rDNA sequences present in the RDP database. The fluorescent signals of different digests of the same strain varied by less than two-fold. The fluorescent signals of different strains varied by up to 13-fold, indicating differential amplification efficiency.

measured as the total area of the major peaks, varied from strain to strain by up to 6.7-fold. This is significantly greater than the variation between replicates of the same strain, which differed by less than 2-fold. For individual reactions, the amount of fluorescence varied from strain to strain by up to 10-fold and the variation between replicates of the same strain differed by no more than 4-fold.

Mixtures of cultured strains were used to evaluate the ability of the FT-ARDRA technique to detect the phylogenetically distinct components of complex communities known to inhabit the soil and rhizosphere environments (see Figure 5.1). In mixtures where five distinct strains were present in equal proportions, all of the strains could be readily detected. However, as expected, the fluorescent signals ascribed to each of the strains were not equally intense. In more complex mixtures containing 10 strains in roughly equal proportions, most but not all of the strains could be unequivocally

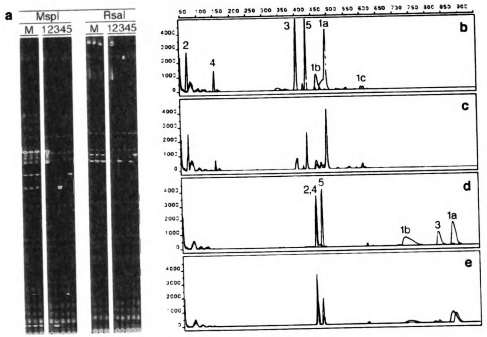

Figure 5.1: Fluorescent gel and corresponding FT-ARDRA profiles of model communities containing five phylogenetically diverse strains. Fluorescent gel data (a) is analyzed with GeneScan software to generate the corresponding profiles (b - d). Composites of individual strains (b & d) are compared to mixtures (M) of all five strains amplified together (c & e). Peaks corresponding to individual strains are numbered according to Table 1.

detected (see Figure 5.2). Independently amplified replicates of these mixtures showed that the FT-ARDRA profiles were very reproducable.

Serial dilution of the model communities prior to amplification showed that the general character of the profile was maintained while the total signal decreased as a function of template abundance (see Figure 5.2). Template abundance was correlated with total fluorescent signal of the profile, and the overall pattern was generally maintained over the dilution series. The rank order of peak intensities did not change significantly over the range of 10^6 to 8 x10^3 templates. Thus, the technique is robust with regards to sample size where reactions containing 10^4 to 10^6 bacteria are used. In mixtures where one strain represented a dominant fraction of the whole (69% or 43%), the fluorescent signal for that strain was increased, though not proportionately (see Figure 5.3). The amount of increase in signal intensity was dependent on the identity of the dominant strain. For example, there was a 150% increase in signal intensity for a 350% increase in the total number of cells of strain 1 (mixture B). In contrast the signal corresponding to strain 5 increased by over 1000% (Mixture D) when it was present as the single numerically dominant strain in the model community. In all cases the overall amplification efficiency was not greatly affected when one strain was present in greater abundance than the others.

Examination of soil and rhizosphere bacterial communities

FT-ARDRA was used to examine the bacterial communities of soil and rhizosphere environments and their response to nutrient amendment (Figure 5.4). Each profile was found to contain approximately ten major fluorescent signal bands, most of which were observed in both soil and rhizosphere samples. Overall, both soil and rhizosphere profiles could be readily distinguished from one another. Most strikingly, the profiles of the rhizosphere samples contain major signals absent or much reduced in the profiles of the nearby bulk soil. These rhizosphere-enhanced signals are most easily observed at 350 bp and between 490 and 510 bp in the *Msp*I profiles and at 475 bp, around 600 bp and between 820 and 900 bp in the *Rsa*I profiles (see Figure 5.4). The major signal peaks from both the soil and rhizosphere samples occurred in size ranges expected for known soil

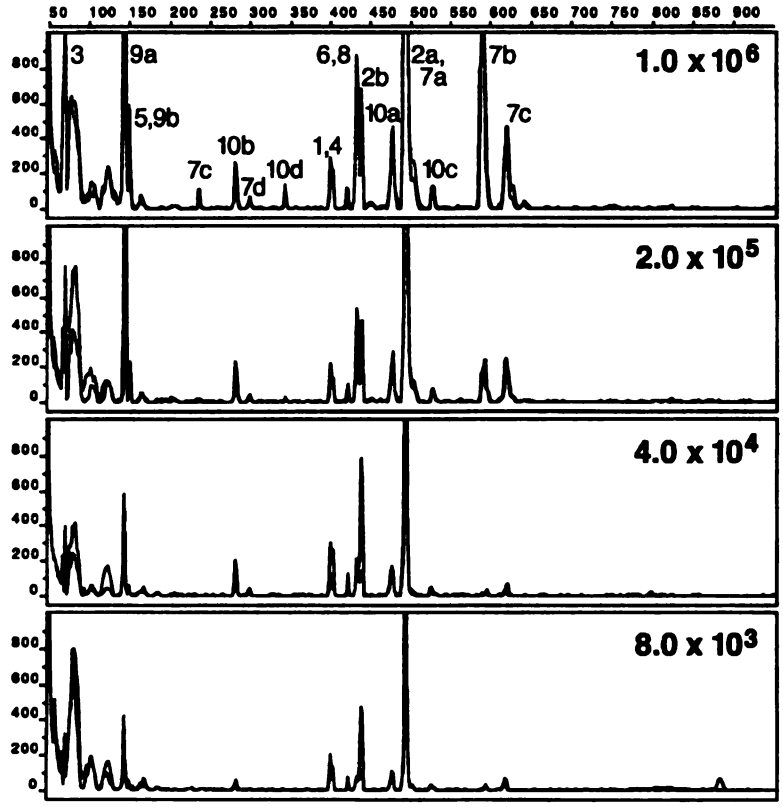

Figure 5.2: Dilution extinction of FT-ARDRA profiles using different quantities of model community templates. Ten strains were mixed together in equal proportions, and serial dilutions of this mixture were amplified separately and digested with *Msp*I. Note that template abundance is correlated with total fluorescent signal of the profile, and that the overall pattern is generally maintained over the dilution series.

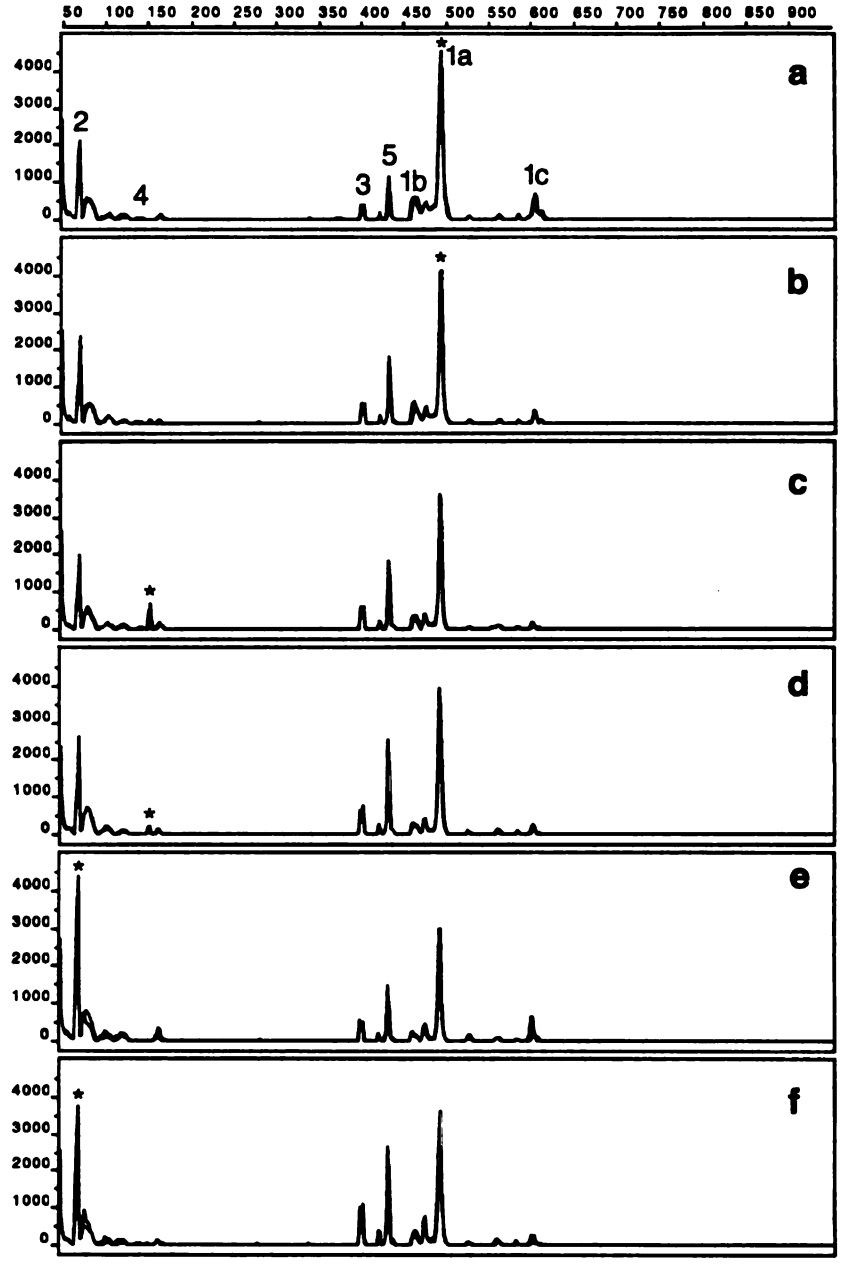

Figure 5.3: Changes in the FT-ARDRA profiles due to changes in the relative abundance of component bacteria. All mixtures contain strains 1, 2, 3, 4, and 5. Dominant strains represent 69% (a, c, e) or 43% (b, d, e) of the total number of bacteria present in the template mixture. Peaks are labeled by strain number and dominant strains are indicated by an (*) in the profile of each mixture. Profiles are those generated by *Msp*I digestion.

and rhizosphere bacterial genera (compare to Table 5.1). Occasionally, a distinctly novel band appeared in one of the replicated profiles (*e.g.* the 270 bp band in one of the *Msp*I digests of the soil sample, see Figure 5.4). Because such bands were not repeatedly observed, little can be said conclusively about their significance (see Discussion).

Application of a nutrient amendment (*i.e. myo*-inositol) to the soil caused the community profiles of both the soil and rhizosphere to change dramatically. Comparison of multiple profiles indicated that the most apparent change could be ascribed to organisms with a unique 16S rDNA restriction pattern (indicated by an "*" in Figure 5.5). This signal consisted of an ~670 bp 5'*Rsa*I fragment and an ~500 bp *Msp*I fragment and appeared in both soil and rhizosphere samples after application of the nutrient amendment. This signal is referred to as the 670/500 ribotype. Comparison of this pattern to theoretical digests of known sequences present in the RDP database indicated that this ribotype may correspond to a strain of *Pseudomonas putida*. Additional quantitative changes in certain signal peaks were also noted in the chromatograms, indicating that several additional ribotypes responded to nutrient amendment.

FT-ARDRA of cultures inoculated from soil and rhizosphere washes was used to further investigate the nature of the bacterial communities (see Figure 5.6). After 18 hours of incubation in liquid media, the banding patterns of the amplified samples were generally similar to those derived from the direct washes. Because the samples were diluted for culturing, fluorescent bands indicated bacterial growth in the media. Major signals representative of the 670/500 ribotype (*) appeared in the culture profiles of the amended (+) as well as the unamended (-) rhizosphere samples. Thus, cells represented by the 670/500 ribotype could be easily cultured from the rhizosphere regardless of the nutrient treatment. Because the amount of fluorescent signal in the rhizosphere cultures was similar, no increase in the abundance of this ribotype due to the nutrient amendment was evident. This contrasted directly with the rhizosphere washes where a dramatic increase was observed in the ~670 bp fluorescent signal due to the nutrient amendment. This indicates that the nutrient amendment affected the physiological status as well as the abundance of the bacterial population represented by the 670/500 ribotypes.

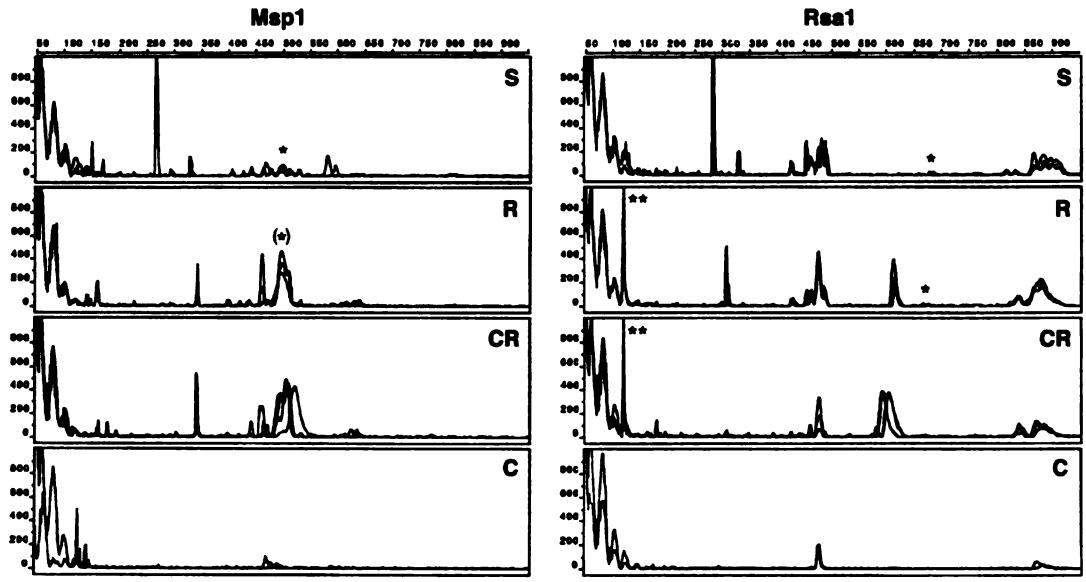

Figure 5.4: FT-ARDRA profiles of natural communities. Profiles were generated by digesting amplified products with *Msp*I or *Rsa*I. Chromatographic traces from four independent samples were used for each sample to display the reproducibility of the technique. S, soil; R, rhizosphere; CR, crushed rhizosphere.

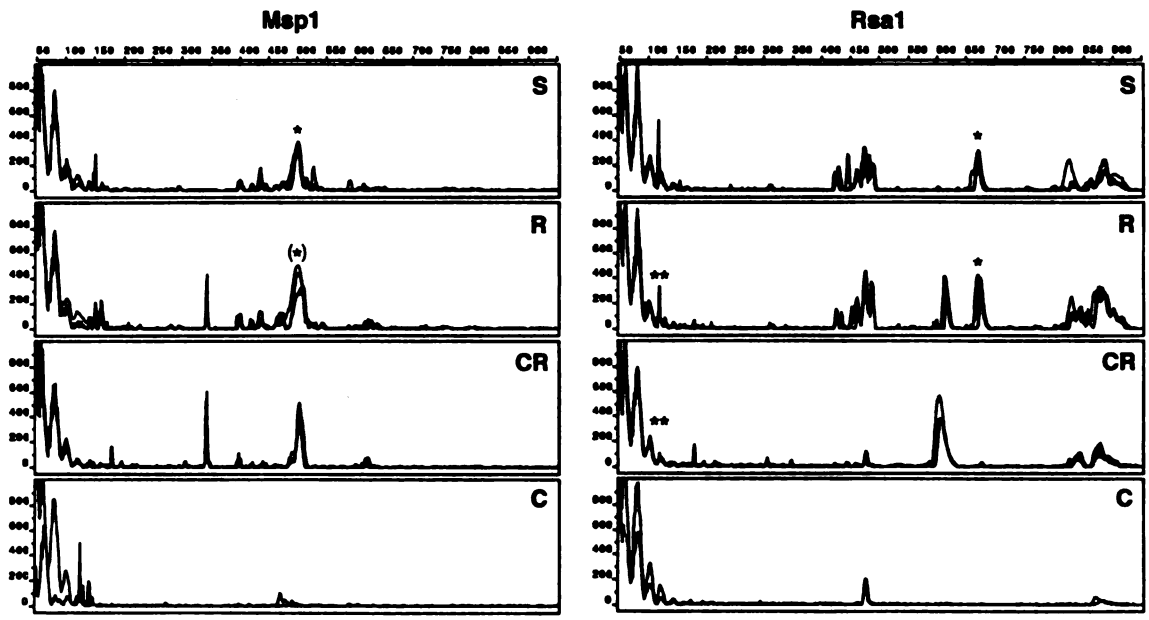

Figure 5.5: Changes in FT-ARDRA profiles of natural communities due to *myo*-inositol amendment. Profiles are analogous to those in Figure 4. Soil (S), rhizosphere (R), and crushed rhizosphere (CR) samples were taken from four separate pots that had been saturated once with a 2% solution of *myo*-inositol.

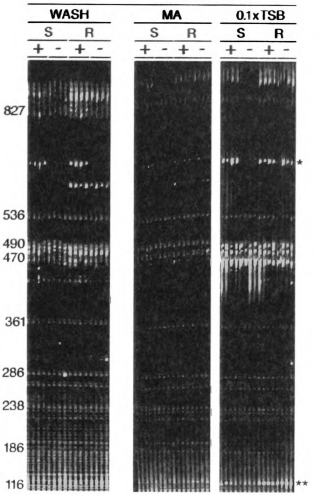

Figure 5.6: Comparison of washes and cultures of the same samples. Fluorescent gels of direct sample washes (W) and cultures of the same samples incubated for 18 hr at room temperature in two different media (MA or 0.1x TSB). Multiple soil (S) and rhizosphere (R) samples are shown from both amended (+) and unamended (-) treatments. Amplified products were digested with *Rsa*I.

These data support the hypothesis that the nutrient amendment causes a physiological change in the 670/500 ribotype that results in metabolic activity and growth. This change results in a dramatic increase in the observed fluorescent peak and, by inference, in the apparent numerical abundance of the ribotype in the washes. However, analysis of the cultured communities suggests that very similar numbers of the ribotype are present, because the signal intensities are similar in both (+) and (-) treatments. The best explanation for these results is that some large proportion of 670/500 type organisms are inaccessible to detection by PCR (see Discussion). Similarly, changes in the abundance of a 119 base pair band (**) also indicate this type of physiological switching. However, in this case, *myo*-inositol appears to repress the growth and activity of this ribotype in the rhizosphere. The application of *myo*-inositol reduced the abundance of this signal in the rhizosphere washes, but this had little effect on the amount of 120 bp fluorescent signal observed in the cultures of those washes.

Discussion

The utility of PCR to examine mixed microbial populations has become widely recognized (17, 24, 31, 33). Methods based on amplification of the 16S ribosomal RNA and DNA have figured prominently in the recent explosion of data on bacterial communities (24). However, many of the published protocols are complicated by the isolation of DNA from environmental samples prior to amplification, purification of amplified products, and/or the analysis of clone banks from the amplified products (31). The costs involved in these steps can quickly make comprehensive studies prohibitively expensive. Additionally, studies which rely on the analysis of clone banks may be subject to unintended bias if a non-representative amplification event occurs (see Figure 5.4). In contrast, the FT-ARDRA method described in this paper allows for high throughput of multiple independent samples by direct amplification of small samples and direct digestion of amplification products prior to electrophoresis.

Valid concerns have been raised regarding potential artifacts associated with amplification of 16S rDNA from mixed templates (27, 34). To address these concerns, we evaluated our technique

using mixtures of phylogenetically distinct bacteria isolated from soil and rhizosphere environments. Representatives from both Gram(+) and Gram(-) genera were used. 16S rRNA gene products were amplified from all of the individually tested strains, indicating that the combined stresses of the freeze-thaw treatment and PCR cycling were sufficient for cell lysis (see Figure 5.1). There was some variation in the efficiency of amplification from strain to strain, but whether this was due to incomplete cell lysis or differences in priming efficiency is not known. Furthermore, one might predict that only a single fluorescent band should be observed for each strain. In fact, more than one major fluorescent band was observed in a few instances (see Figure 5.2). Additionally, several minor fluorescent bands were also observed in some cases. These bands most likely represent intragenomic variation of the rDNA genes (8). Given the reproducibility of the distinct band sizes from independent applications of the technique, it is unlikely that these multiple signals are due to stochastic production of chimeras or sequence mutations that result in altered digestion patterns. One of the advantages of performing FT-ARDRA as described is that the complexity of the template is likely reduced by serial dilution, reducing the chances for chimeric artifacts to occur. This is, of course, also a limitation because only the most abundant bacteria that can be recognized by the primer set will be detected. The remainder of the bacterial diversity is thereby ignored.

In applying this technique, we recommend that comparisons be based on full profiles (spanning 50 to 950 base pairs) using two different restriction enzymes. We routinely use *Msp*I and *Rsa*I because they efficiently resolve different sets of known soil genera (see Figure 5.1). The use of two different enzymes is essential for making inferences about the abundance and identity of individual ribotypes. Correlations in the peak areas of complementary band sizes (*e.g.* in the case of the 670/500 ribotype, see Figure 5.5) can be used to make inferences about increases and decreases in the abundance of individual ribotypes. Visualization of bands smaller than 50 base pairs is not generally useful because of the presence of excess labeled primers and associated artifacts. Sometimes these artifacts spilled over into the range of 50 to 150 base pairs and interfered with the detection and analysis of signals in that region of the profile (*e.g.* compare Figures 5.1 and 5.2).

While detection of bands greater than 950 base pairs in size is possible, we have not generally observed significant signals above this size limit.

When applying this technique to complex environmental samples, one needs to be cautious about evaluating the large amount of quantitative and qualitative information generated by T-RFLP analysis (18). In general, different phylogenetic groups will have different restriction patterns of their amplified 16S rDNA (18, 22). It has been estimated that ARDRA using three well chosen restriction enzymes is sufficient to identify organisms at the genus level (22). Because FT-ARDRA provides additional information regarding the identity of the 5' restriction fragment, one would expect that fewer digests would be required to make identifications with the same degree of accuracy. From our experience, combining the information on the fluorescent gels and ethidium stained gels was sufficient to confirm the classification of the isolated strains that had been previously established by partial DNA sequencing (B. McSpadden Gardener, J. Stolzfus, and F. J. de Bruijn, unpublished data). However, any identification made in the absence of culturing must be viewed as preliminary (9). With this in mind, we note that the major signals observed in the FT-ARDRA profiles of both soil and rhizosphere samples fall into size classes similar to those expected for genera known to inhabit these environments. This observation leads us to suggest that the majority of bacteria present in these samples are similar to known bacterial genera based on the similarity of their 16S rRNA sequences.

Differences in experimental samples were detected using FT-ARDRA as changes in the areas of fluorescent peaks. Here, we report the occurrence of distinct differences between soil and rhizosphere samples as indicated by comparing replicate FT-ARDRA profiles generated using two different restriction enzymes. These differences generally correspond to rhizosphere-specific increases in the signal intensity of several different size classes of 5' terminal restriction fragments (see Figures 5.4 and 5.5). This observation is consistent with the "rhizosphere effect" ascribed to the relatively nutrient-rich below-ground niche surrounding plant roots (29).

Because different strains amplify with different efficiencies, especially when present in mixtures, the relative abundance of different organisms cannot always be directly compared (see

Figure 5.1). Nonetheless, changes in peak area of a designated size class can be clearly correlated with monotonic changes in the abundance for individual strains which produce bands of that size class in the absence of comigrating signals from other organisms. For example, an increase in the relative abundance of strain 5 can be easily observed using *Msp*I but not *Rsa*I because in the latter digest the 5' fragment comigrates with the 5' fragment of strain 3 which amplifies much more efficiently (see Figure 5.1). Despite these limitations, we think that this method can provide valuable new information about bacterial communities in the environment, especially when sample washes and subsequent cultures are analyzed in concert with each other.

FT-ARDRA of cultures complements the analysis of samples used to inoculate them. Because physiologically active and numerically dominant bacterial populations will have a reproductive advantage in liquid media, we hypothesize that their relative abundance will be enhanced in short term enrichment cultures as those described here. From this we deduce that dominant signals, present in both the liquid cultures and direct washes, represent abundant and physiologically active bacterial populations. We observed that a carbon nutrient amendment, *myo*-inositol, promoted a prolific rise in a 670/500 ribotype in both the soil and rhizosphere environments (compare Figures 5.4 and 5.5). This ribotype was detected in the washes of the amended samples only; however, it was also detected in the cultures of the unamended rhizospheres (see Figure 5.6). This indicates that the rhizosphere environment (and by analogy the nutrient amendment) alters the physiological state of this ribotype allowing it to grow readily in the liquid media. We hypothesize that some large proportion of 670/500 type organisms are present in the unamended rhizospheres but escape detection. This may be due to responses of this ribotype to the nutrient starved environment of the soil (7). In bacteria, starvation may result in physiological conditions that make bacteria more resistant to lysis (2, 28) or minimize chromosomal copy number (5, 8). However, when these bacteria are presented with favorable conditions, modeled by the liquid media used here, they quickly respond in such a way that they become detectable by this PCR-based approach. The absence of the 670/500 signal in both the washes and cultures of the unamended soil samples leads us to suggest that the nutrient amendment stimulates the activation and subsequent growth of specific bacterial

populations capable of catabolizing it. This is consistent with the idea that soil bacteria represent a microbial "seed bank" of metabolic potential that "germinates" in response to favorable environmental conditions.

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Chapter 6

Impact of *myo*-Inositol Amendment on *Sinorhizobium meliloti* and Other Bacterial Populations in the Rhizosphere of *Medicago sativa*

McSpadden Gardener B, Cotton C, de Bruijn FJ (1998) Impact of *myo*-inositol amendment on *Sinorhizobium meliloti* and other bacterial populations in the rhizosphere of *Medicago sativa*. Appl. Environ. Microbiol. *in preparation*

Abstract

Application of the nutrient amendment *myo*-inositol stimulated specific changes in bacterial populations in the rhizosphere of *Medicago sativa*. Under gnotobiotic conditions, a strain of *Sinorhizobium meliloti* capable of catabolizing *myo*-inositol (Mic^+) outcompeted a near-isogenic non-catabolizing (Mic^-) strain for rhizosphere colonization when *myo*-inositol was added to the growth medium. For plants grown in non-sterile soil, the median number of Mic^+ culture forming units (CFUs) was observed to be higher from samples amended with *myo*-inositol at planting. ARDRA and FT-ARDRA of the most abundant Mic^+ CFUs indicated quantitative shifts in the composition of the bacterial populations reaching saturating growth after 5 days of incubation. FT-ARDRA of soil and rhizosphere washes also detected quantitative differences between amended and unamended rhizospheres. In both laboratory and field experiments, T-RFLPs of the amplified 16 rDNA corresponding to *S. meliloti* were more abundant in samples that had been amended with *myo*-inositol. Nodulation of *M. sativa* was greatest in the amended field plots, and increases in nodulation correlated with increases in the amount of *myo*-inositol applied at planting. These data indicate that the nutrient amendment *myo*-inositol can promote the growth of bacteria capable of catabolizing it in the rhizosphere environment and that it promotes the growth and activity of *S. meliloti* in the rhizosphere of *M. sativa*.

Introduction

Biological nitrogen fixation plays a major role in the productivity and sustainability of agricultural systems (11, 28). The intimate symbiosis between leguminous plants and rhizobia provides the largest proportion of fixed nitrogen in agricultural systems world-wide (11, 28). Efforts to increase agricultural yields of legumes and subsequent crops in rotations has led to the widespread application of rhizobial inoculants with a high capacity for nitrogen fixation (1, 22). However, the effectiveness of these treatments are often undermined by the presence of indigenous rhizobia which outcompete the inoculant strains for nodule occupancy (37, 44).

Several alternative approaches have been pursued to improve the effectiveness of this nitrogen-fixing symbiosis (43). Increasing inoculum levels has shown limited success, primarily in soils with low indigenous populations of rhizobia (22). Laboratory selection of strains with traits that improve their competitiveness and/or enhance nitrogen fixation capabilities has also led to improved effectiveness of inoculant strains (13, 22). Some studies have also examined the possibility of promoting the activity of rhizobia by supplementing the soil and/or inoculant formulation with nutrients (9, 12, 27, 45). More recently, efforts to improve the symbiosis have included genetic engineering of rhizobia and/or their corresponding plant host (2, 25, 33, 36). In this regard, it has been proposed that the rhizosphere environment may be specifically managed so as to promote the activities of beneficial microorganisms (23, 24).

Nutritional mediators can be defined as organic substrates that alter ecological relationships between microorganisms so as to promote the growth of catabolizers over non-catabolizers. In the diverse milieu of soil microbial communities (39), it is likely that numerous organisms will have the ability to catabolize any given substrate. So, if the goal is to promote a particular subpopulation of bacteria, the ability to catabolize a nutritional mediator should relatively rare (21, 33). Several recent reports indicate that nutritional mediation can occur in the soil and rhizosphere environments (4, 5, 6, 9, 15, 24, 35). In some instances, the application of the nutritional mediator has been shown to promote plant health by promoting the growth and/or activity of beneficial microorganisms (4, 9, 46).

In a screen of potential nutritional mediators, we have observed that a relatively small proportion of soil and rhizosphere bacteria are capable of utilizing *myo*-inositol as sole carbon source to support growth *in vitro* (see Chapter 3). Thus, *myo*-inositol may be useful as a selective nutritional mediator of competition between catabolizing (Mic^+) and non-catabolizing (Mic^-) bacteria. Because *S. meliloti* are capable of utilizing this relatively specific substrate, we hypothesized that the application of *myo*-inositol would stimulate the growth and/or activity of *S. meliloti* in the rhizosphere of *M. sativa*. Here we present the results of several experiments which consistently support these hypotheses and provide additional information on the impact of *myo*-inositol amendment on other bacterial populations in the soil and rhizosphere.

Materials and Methods

In vitro competition experiments

Two strains of *S. meliloti* differing only in their ability to catabolize *myo*-inositol were used; strain 1021 (Sm^r, Mic^+) and its near-isogenic relative (1021 (*idh*::*Tn5*)) called IM56 ($Strep^r$, Kan^r, Mic^-) (10). Fresh single colonies were obtained from streaks on YMA media supplemented with either 200 ug/ml of streptomycin (for 1021) or 100 ug/ml of kanamycin (for IM56), inoculated separately into 3 ml of YMB media, and incubated in a rotary shaker overnight at 28°C and 200 rpm. The following morning, seeds of *Medicago sativa* were surface sterilized by incubation in 3% hydrogen peroxide for 10 minutes and then washed repeatedly in sterile distilled water. The seeds were placed onto large petri plates containing B&D medium (3) or B&D amended with 500 PPM of *myo*-inositol. The cells in one milliliter of each *S. meliloti* culture were pelleted by centrifugation, washed in sterile distilled water, pelleted again, and resuspended in sterile distilled water. The cell density was measured and adjusted so that the cultures had a final absorbance, at 600 nm, equal to 0.1 per ml. The two cultures were then mixed in a 1:1 ratio. Four microliters of the *S. meliloti* mixture was pipetted onto each seed. Plates were then sealed and placed into a growth chamber (12 hr light: 12 hr dark, 22°C) for incubation. Eight replicates of each condition were used.

The relative abundance of Mic^+ and Mic^- rhizobia was determined as follows. From each plate, samples of 5 seeds were collected after 1 day of incubation. Subsequently, rhizospheres from 5 seedlings were sampled after 0.5, 1, and 2 weeks of incubation. Root samples were promptly placed in 5 mls of distilled water in 15 ml Sarstedt tubes (Numbrecht, Germany). Bacteria were dislodged by alternating 15 second treatments: vortexing, sonication (tube placed in Ultra*sonik* cleaning bath, NEY, Inc.), vortexing, sonication, and a final vortexing. A 50 ul aliquot was transferred to a 96-well microtiter plate, prefilled with 200 ul distilled water per well, for serial dilution. For the 0 timepoint, an aliquot of the *S. meliloti* mixture was serially diluted directly. Twenty microliters of each well-mixed dilution was then spot-inoculated, in triplicate, onto YMB + streptomycin (200 ug/ml) for total rhizobial counts and onto YMB + kanamycin (100 ug/ml) for Mic^-

mutant rhizobial counts. Plates were incubated at 28°C for three days prior to counting. The value of (1 - [(counts on YMB + Kan) / (counts on YMB + Strep)]) was used to calculate the proportion of Mic^+ rhizobia.

Growth chamber experiments

The soil used was a sandy loam obtained from the same MSU research plot used in the field experiments described below. Several kilograms of this Michigan sandy loam were air dried, mixed, and aliquoted into separate potting units containing approximately 50 g of soil. Each unit was moistened with 5 ml of distilled water or a solution of *myo*-inositol at a concentration of 0.002%, 0.02 %, 0.2%, or 2% (w/v). Several seeds of *Medicago sativa* (NitraginGold pre-inoculated, PH1B10277, Idaho Crop Improvement Association, Boise, ID) were planted in each unit. Soil and plants were incubated in a growth chamber (12:12 LD, 22°C).

Soil samples (approximately 150 mg each) were taken from the surface away from alfalfa shoots with a clean spatula at 1, 4, and 7 days post-germination. Rhizosphere samples (approximately 25 mg f.w. root + 30 mg soil) were defined by gently separating the roots of three plants from surrounding soil and cutting off the attendant shoots. Samples were promptly placed in 5 mls of distilled water in 15 ml, and bacteria were dislodged as described above. A 50 ul aliquot was transferred to a 96-well microtiter plate, prefilled with 200 ul distilled water per well, for serial dilution. For the one week time point, twenty microliters of each well-mixed dilution was then inoculated into replicate microtiter plates containing 200 ul per well of either MA (a modified GTS mineral salts medium containing *myo*-inositol as sole carbon source) (20) or 0.1x TSB medium. This procedure was repeated, alternating samples from the different treatments, until all soil and rhizosphere samples had been processed. Dilution plates were stored at -20°C. Culture plates were incubated at room temperature in the dark. After five days of incubation, a 100 ul aliquot of the terminal serial dilution of each sample which displayed saturating growth was saved for further investigation by mixing in 100 ul 35% sterile glycerol and freezing at -70°C.

Field experiments

Two field studies were undertaken in the summer of 1997 at a research plot at Michigan State University. The soil was tested by the MSU Soil Testing Laboratory using standard procedures, and the analysis is presented in Table 6.1. After tillage and raking, the soil was planted to *Medicago sativa* in 6 x 150 foot strips running north to south. The eastern strip (designated A) contained pre-inoculated seed (NitraginGold pre-inoculated, Apron coated, PH1B10277, Idaho Crop Improvement Association, Boise, ID) and the western strip (designated B) was planted with seed from the same lot that had been washed, surface sterilized, and air dried in the laboratory the day before planting. After planting the field was marked and treated as shown in Figure 6.1. In field test I, treatments were applied as surface sprays of either distilled water or solutions of 1000 mg/l *myo*-inositol at a rate of 1.6 l/m^2. In field test II, treatments were similarly applied as solutions of 0, 50, 100, 200, 500, and 1000 mg/l *myo*-inositol at a rate of 3.2 l/m^2. Germination followed the first significant rain, one week post-planting. Ten days post-planting, germination rate was measured by counting the number of *M. sativa* seedlings proximal to replicate 1.5 m transects of each treatment block.

Sampling of the field plots for was conducted 1, 2, 4, and 5 weeks post-germination. Soil and air temperature were taken at the time of each sampling. The sampling procedure for the first three time points was as follows: Samples were placed individually into pre-labeled Ziploc plastic bags, partially sealed to prevent desiccation, and carefully transported to the laboratory for immediate processing. For field test I A, two plants per treatment block were carefully dug up with the surrounding soil so as to minimally disturb the structure of the rhizosphere sample. For field test II A and II B, two sets of five closely spaced plants were sampled from each treatment block. In the laboratory, rhizosphere samples were processed sequentially, alternating treatments. Either one (field test I) or five (field test II) rhizospheres per sample were gently removed from surrounding soil and placed into 25 mls of sterile distilled water. Bacteria were dislodged from the rhizospheres and serially diluted as described above. Cultures were inoculated as described above for field test I samples only and incubated up to three week at room temperature in the dark. Bacterial growth was scored by visual inspection of turbidity. Nodules were counted using a dissecting microscope. At

five weeks post-germination, an additional sampling was undertaken to adequately determine the extent of nodulation in test I A and I B ($n \geq 50$ plants per treatment).

Fluorescently-tagged amplified ribosomal DNA restriction analysis (FT-ARDRA)

The analysis of terminal restriction fragment length polymorphisms (T-RFLPs (18)) of amplified 16S rRNA genes was performed as described previously (20). Briefly, conserved eubacterial primers 8F-HEX (5'- *AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- ACG GCT ACC TTG TTA CGA CTT -3') were used to directly amplify 16S rDNA sequences from a broad cross-section of the microbial communities present in soil and rhizosphere washes. Master mixes were prepared and aliquoted to 8-strip PCR tubes while template samples were thawed at room temperature. The first or second dilution (containing roughly 10^4 to 10^6 bacteria) were mixed thoroughly by micropipetting. Negative control reactions were performed for each amplified set. Reactions were processed in either PTC100 or PTC200 cyclers (MJ Research, Inc., Watertown, MA). Cycling conditions: 95°C for 7 min, then 35 x (94°C 1 min, 52°C 1 min, 65°C 8 min), then 65 °C for 16 min, followed by 4 °C storage.

Restriction digests were performed in 96-well microtiter plates. Each reaction consisted of 7 ul of a PCR reaction in a total reaction volume of 40 ul and 8 U of either *RsaI* or *MspI* (Boehringer-Mannheim, Indianapolis, IN). Reactions were incubated at 37°C for at least 2 hours. Reactions were then stored at -20°C. For standard ARDRA, 8 ul aliquots were loaded onto 1.5% agarose gels and electrophoresed following published protocols (34). For FT-ARDRA, 10 ul of each sample were submitted to the Michigan State University DNA Sequencing Facility (East Lansing, MI) for electrophoresis and imaging. Briefly, 2 ul of each sample were mixed with 2 ul of G2500-TAMRA and loaded onto 6% urea-containing polyacrylamide gels (24 WTR plates). Electrophoresis was performed on an ABI373 sequencer (*PE*Applied Biosystems, Foster City, CA) run for 14 hrs at 1680 V. Data was collected automatically using the B filter and analyzed using GeneScan 2.1 Software (*PE*Applied Biosystems, Foster City, CA).

Statistics

Because non-normal distributions of the sample data were observed, nonparametric statistics were applied. Mann-Whitney U statistics were calculated using SchoolStat software (David Darby, WhiteAnt Occasional Publishing, Victoria, Australia, ddarby@ariel.ucs. unimelb.edu.au) for making comparisons between treatments. Kendall's correlation coefficient was calculated following the method described by Fisher (7) when determining the relationship between the amount of *myo*-inositol added and fluorescent peak area or the numbers of nodules. All P-values less than or equal to 0.15 are reported.

Results

Impact on Sinorhizobium meliloti under gnotobiotic conditions

We hypothesized that *myo*-inositol amendment could positively impact the growth and activities of *myo*-inositol catabolizing (Mic^+) bacteria in the rhizosphere. This hypothesis was tested by examining the impact of *myo*-inositol on *S. meliloti* populations under gnotobiotic conditions. Two strains, *S. meliloti* 1021 (Mic^+) and IM56 (Mic^-), differing only in their ability to catabolize *myo*-inositol were co-inoculated in a 1:1 proportion onto seeds of *Medicago sativa* and the abundance of each was followed over time. The combined results of eight independent tests are shown in Figure 6.1. Over a two week time course, the Mic^+ and Mic^- populations remained in approximately equal proportions in the absence of the *myo*-inositol amendment. In contrast, the addition of *myo*-inositol to the medium resulted in an increase in the relative abundance of Mic^+ strain (see Figure 6.1). This difference in the relative amount of Mic^+ and Mic^- bacteria was noticeable after one day of incubation and persisted throughout the course of the experiment ($P < 0.10$ for all pair-wise comparisons except for day 7). Thus, the addition of *myo*-inositol can mediate a proportional increase in Mic^+ over Mic^- bacteria in the rhizosphere.

The only observed phenotypic difference in the plants observed under these gnotobiotic conditions was an increase in nodulation on the amended plates. The fraction of nodulated plants on

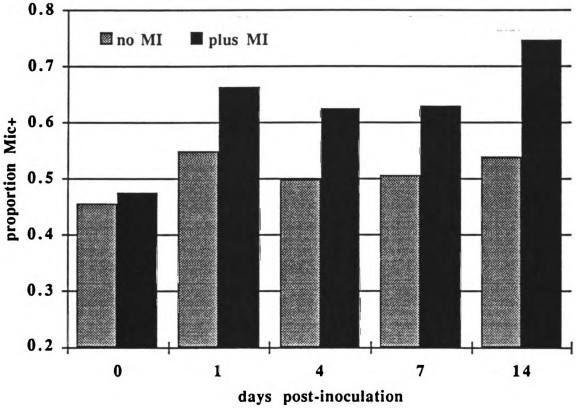

Figure 6.1: Impact of *myo*-inositol amendment on the relative abundance of Mic$^+$ *S. meliloti* in the rhizosphere of *M. sativa*. Surface-sterilized seeds were placed onto B&D agarose supplemented with a 500 PPM *myo*-inositol solution (plus MI) or an equal volume of water (no MI). Wild-type strain 1021 (Mic$^+$) and its near isogenic mutant IM56 (Mic$^-$) were inoculated onto the seeds in equal proportions.

each plate was significantly increased in samples that had been amended with *myo*-inositol (37% vs. 13%; $P < 0.10$). We subsequently determined that *myo*-inositol was not specifically promoting this increase since similar results were obtained when sucrose was used as the amendment in the medium (data not shown).

Impact on culturable rhizosphere bacteria in field soil

If the rhizosphere of plants grown in natural soil is a carbon-limited environment, one would expect that the number of culturable bacteria would increase in response to carbon amendment. Furthermore, the application of a particular carbon source (*e.g.* *myo*-inositol) should primarily benefit bacteria which can directly catabolize it (*e.g.* Mic$^+$ bacteria). Indeed, the application of *myo*-inositol to a natural soil at planting resulted in distinct increases in the number of bacteria cultured from rhizospheres of *M. sativa* (see Table 6.1). The observed increases were most pronounced for

			field test I A (16)			g.c. test
media	time[a]	cond.[b]	1 wk[c]	2 wk[c]	4 wk[c]	1 wk[c]
MA	0.5	-	**1.3 x 10^5**	**2.7 x 10^5**	7.0 x 10^5	**1.7 x 10^5**
		+	**1.8 x 10^5**	**3.8 x 10^5**	5.7 x 10^5	**3.9 x 10^5**
	3	-	**5.5 x 10^5**	**7.4 x 10^5**	2.2 x 10^6	n.d.
		+	**6.9 x 10^5**	**8.8 x 10^5**	1.8 x 10^6	n.d.
0.1x TSB	0.5	-	**1.9 x 10^6**	**1.9 x 10^6**	**3.0 x 10^6**	1.3 x 10^6
		+	**2.2 x 10^6**	**3.0 x 10^6**	**3.3 x 10^6**	8.7 x 10^5
	3	-	6.7 x 10^6	1.5 x 10^7	9.2 x 10^6	n.d.
		+	5.0 x 10^6	7.4 x 10^6	6.3 x 10^6	n.d.

[a]Weeks of incubation in liquid media.
[b]Presence (+) or absence (-) of *myo*-inositol amendment at planting.
[c]Sampling time point in weeks (wk) post-germination.

Table 6.1: The median number of culture forming units (CFUs) from rhizospheres of *Medicago sativa*.

Increases correlated with *myo*-inositol amendment are highlighted in bold. cultures grown on MA media, where *myo*-inositol was provided as sole-carbon source, one week after germination. The numbers of fast growing (0.5 week incubation) and slow growing (3 week incubation) Mic^+ bacteria from the inositol-amended rhizospheres were higher in both growth chamber and field studies. Similar increases were noted for a field test involving *myo*-inositol amendment to plots planted to soybean (data not shown). In contrast, only the fast growing fraction of bacteria cultured on 0.1x TSB increased following *myo*-inositol amendment in the field, and no such increase was observed in the growth chamber.

Since the abundance of culturable bacteria increased in response to the nutrient amendment, we hypothesized that the diversity of these populations were altered as well. To determine if *myo*-inositol amendment changed the composition of the cultured fraction of bacteria at one week post-germination, samples containing the most abundant, fast growing, culturable bacteria were analyzed using ARDRA and FT-ARDRA. In the growth chamber experiments, distinct changes in both types of profiles were associated with *myo*-inositol amendment in both the soil and rhizosphere samples, indicating that these cultures are qualitatively different to some degree (see Figures 6.2 and 6.3). The changes were most obvious in the soil samples, where the *myo*-inositol amendment consistently resulted in the appearance of several major bands in the digests of the MA cultures. Notably, there was an increase in the number of times that the *S. meliloti*-like signals (occurring at ~400 bp in the *Msp*I profiles and ~830 bp in the *Rsa*I profiles) were observed in the MA cultures of samples that had been amended with *myo*-inositol (see Figure 6.3). In the field experiment, the changes in the ARDRA profiles appeared to be somewhat different depending on the treatment, however, no discernible pattern could be recognized (data not shown). These differences appeared as occasional novel peaks and quantitative differences in some common peaks in FT-ARDRA profiles generated using *Msp*I and *Rsa*I (see Figure 6.4). These data indicate that the application of *myo*-inositol in the field promoted the growth of different culturable bacteria.

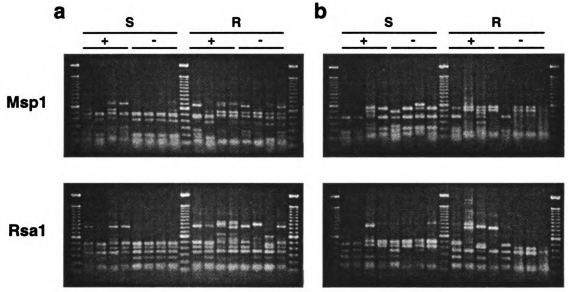

Figure 6.2: ARDRA gels displaying RFLPs in the amplified 16S rRNA sequences from cultures obtained in the growth chamber experiment. Aliquots of the terminal dilution cultures in MA media (a) or 0.1x TSB media (b) displaying saturating growth were used as templates for PCR. Ethidium-stained agarose gels display the *Msp*I and *Rsa*I restriction digests of amplified sequences from cultures of four independent samples take from soil (S) or rhizosphere (R) that had been amended with *myo*-inositol (+) or an equal volume of distilled water (-).

Figure 6.3: FT-ARDRA profiles of the terminal MA cultures obtained in the growth chamber experiment. The size of fluorescent signals is given in base pairs on the x-axis. Fluorescence intensity is diplayed on the y-axis. Overlaid chromatographic traces from the four independent samples are displayed for each condition. These profiles were generated using the samples displayed in Figure 6.1(a).

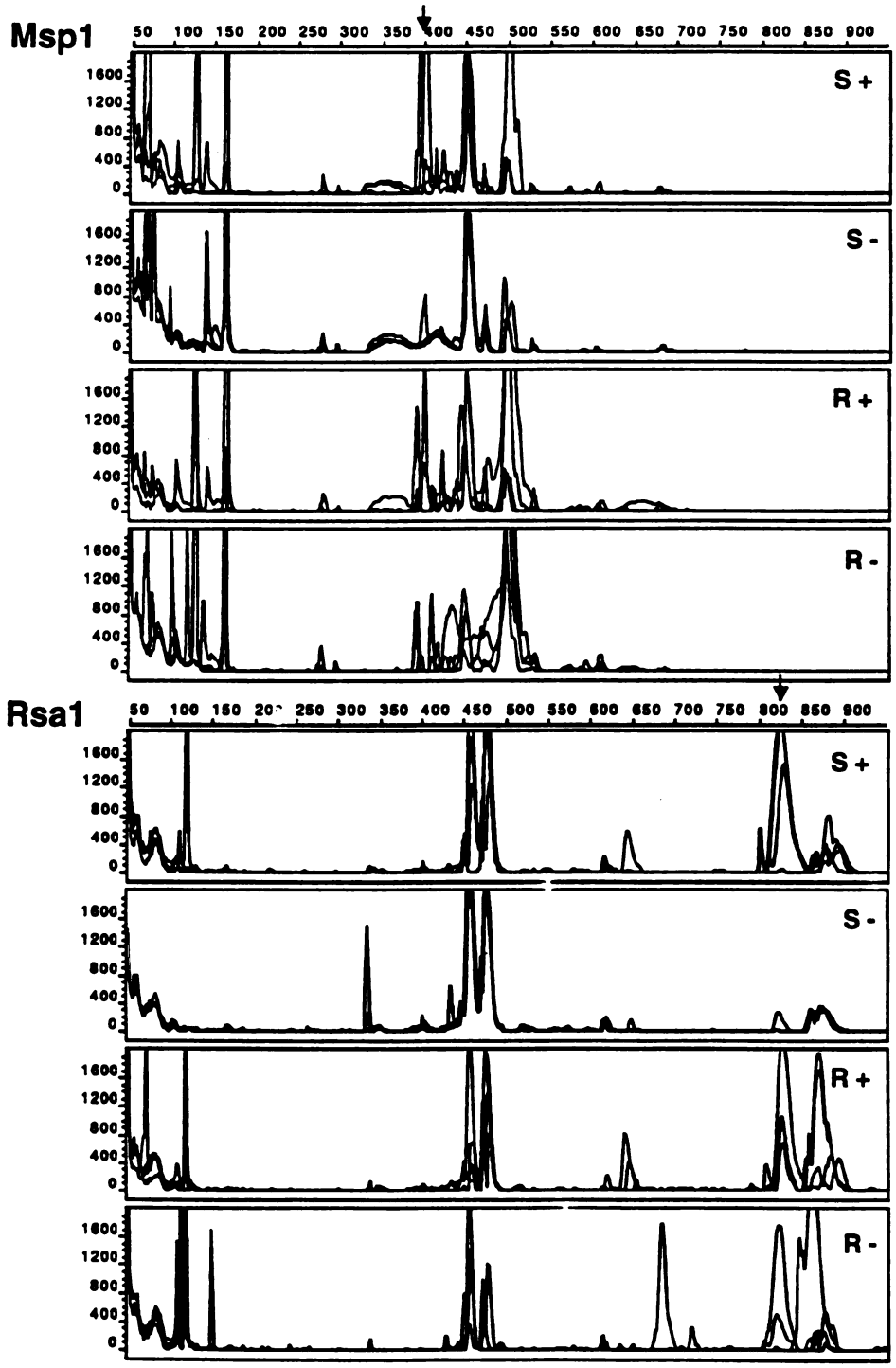
Msp1
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
1600
1200
800
400
0
S +
S -
R +
R -
Rsa1
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
1600
1200
800
400
0
S +
S -
R +
R -

Figure 6.3

Figure 6.4: FT-ARDRA profiles of rhizosphere cultures obtained from field test I. Aliquots of the terminal MA and 0.1x TSB cultures of amended (+) and unamended (-) rhizosphere samples were used for PCR amplification. Overlaid chromatographic traces are presented as in Figure 2 except that data from eight independent samples are displayed for each condition.

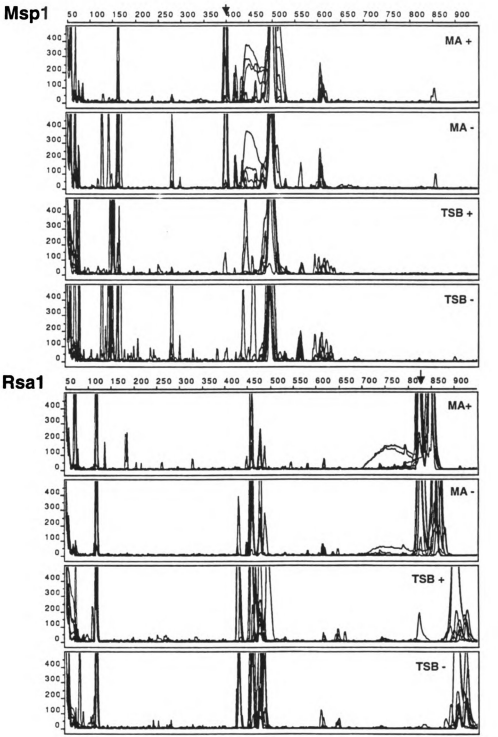
Msp1
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
400
300
200
100
0
MA +
MA -
TSB +
TSB -
Rsa1
MA+
MA -
TSB +
TSB -

Figure 6.4

Impact on bacterial populations recovered in soil and rhizosphere washes

FT-ARDRA was used to directly examine the impact of *myo*-inositol amendment on bacterial populations in the soil and rhizosphere without prior culturing. Previously, we reported that the profiles of these "wash" samples differed markedly in a growth chamber experiment (20). In contrast, the profiles of unamended and amended rhizosphere samples from the field experiments were remarkably similar over the entire time-course (see Figure 6.5). Nonetheless, quantitative changes in several signals were observed repeatedly in both field experiments at 1, 2, and 4 weeks post-inoculation (see Table 6.2). These changes may represent a lasting impact of *myo*-inositol amendment on the corresponding rhizosphere bacterial populations.

Since wild-type *S. meliloti* can utilize *myo*-inositol as a growth substrate, we hypothesized that the application of *myo*-inositol would stimulate the growth and/or activity of these beneficial bacteria. In the growth chamber experiment, distinct quantitative increases in signals corresponding to *S. meliloti* (an ~400 bp *Msp*I signal and an ~830 bp *Rsa*I signal) were observed in amended samples (see Figure 6.6). The fluorescence of the ~400 bp *Msp*I signal is significantly increased in soil and rhizosphere samples amended with *myo*-inositol ($P < 0.10$ and $P < 0.05$, respectively). The ~830 bp *Rsa*I signal did not generally appear to be unique and unobstructed by nearby signals, so the quantification of the peak area was not possible. Nonetheless, comparison of the amended and unamended samples indicate a distinct increase the intensity of this signal (see Figure 6.6). In the field experiments, quantitative increases in the *S. meliloti*-like *Msp*I signal were also observed, particularly at one and two weeks post-germination (see Figure 6.5). In field test II, where a titration of *myo*-inositol from 0 to 32 kg per ha was applied, a significant positive correlation was found to exist between the amount of the nutrient amendment and the intensity of the *S. meliloti*-like *Msp*I signal ($\tau = 0.600$, $P < 0.10$). At two weeks post-germination, this correlation was weaker ($\tau = 0.467$, $P < 0.15$), and it was absent four weeks after germination ($\tau = 0.067$, NS). These data indicate that the number of amplifiable *S. meliloti*-like 16S rDNA targets had increased in response to *myo*-inositol amendment.

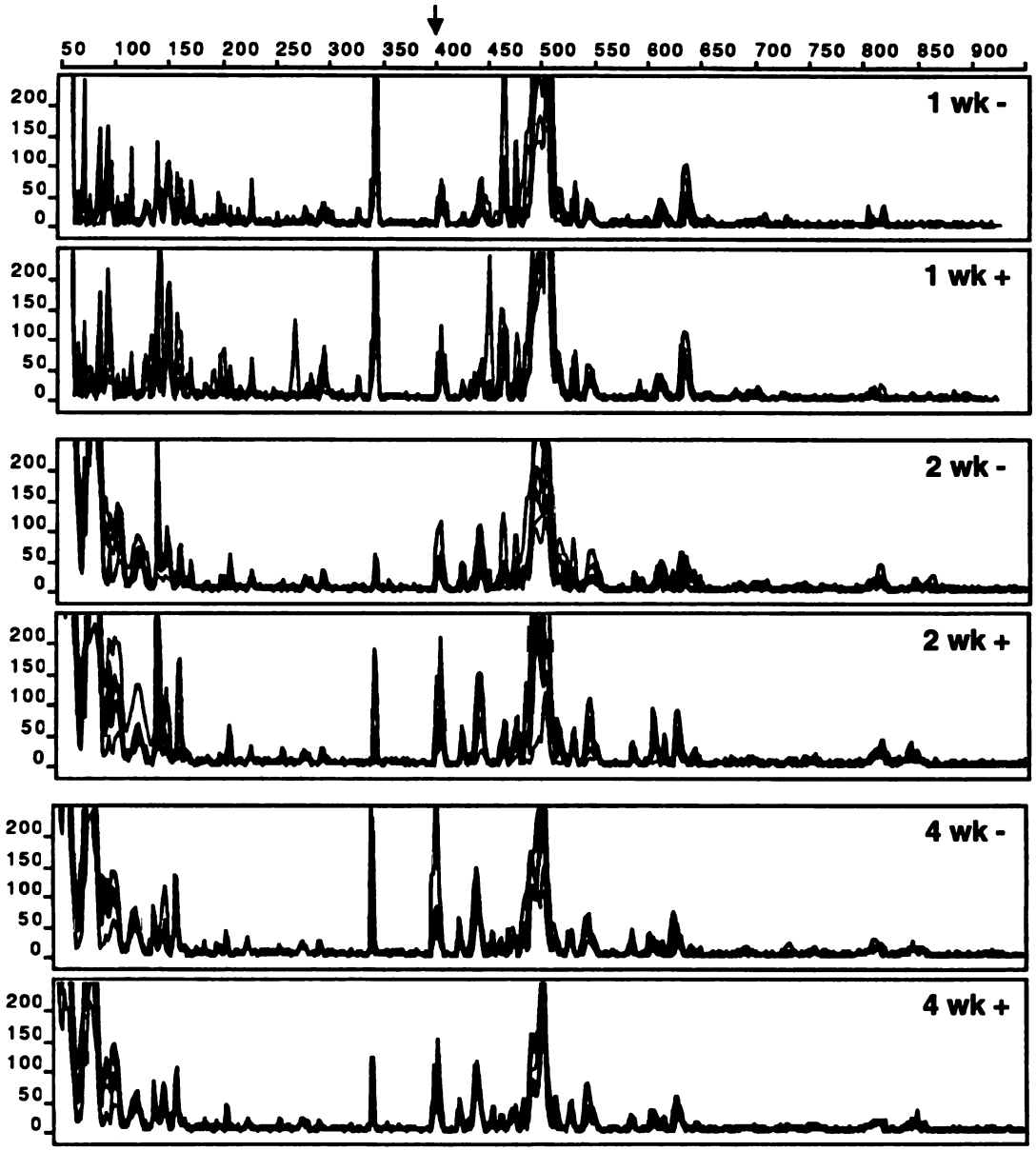

Figure 6.5: FT-ARDRA profiles of rhizosphere washes generated with *Msp*I. The profiles of eight independent rhizosphere samples from each condition are overlaid for each time point (1, 2, and 4 weeks post-germination) in these results from field test I. Plots were amended with 16 kg/ha of *myo*-inositol (+) or an equal volume of water (-).

	fluorescent signals (bp) showing a change in intensity		
enzyme digest	increase	[a]inconsistent change	decrease
*Msp*I	400, 404, 487 **495, 505**, 545, 635	75, 96, 141, 150 153, 300, 345, 400, 404, 408, 424, 440, 465, 475, **500**, 518 615, 800, 820	
*Rsa*I	**476, 479**	121, 173, 308, 312, 317, **427**, **456, 464**, 470, **485**, 490, 494, 610, 640, 830 840, **860, 880**	**119**

[a]Band sizes that change in only one field experiment and only at one time point are included here, along with those that were not observed to be affected by the amendment in both field experiments.

Table 6.2: Changes in FT-ARDRA profiles of rhizosphere washes following *myo*-inositol amendment in the field. The fluorescent peak areas of eight independent samples from each treatment were compared at 1, 2, and 4 weeks post-germination in two different field experiments. Major signal peaks are highlighted in bold.

Impact on nodulation of Medicago sativa

The impact of *myo*-inositol amendment on nodulation of *M. sativa* was also investigated. In both field tests, nodulation was significantly increased (see Table 6.3). The observed increases were between 12 and 36 percent in plots amended with 16 kg of *myo*-inositol per ha. It is interesting to note that the observed increases in nodulation were greatest for uninoculated seed, generally greater than five times the increase observed for plants grown from seed preinoculated with *S. meliloti*. In field test II, the correlation between nodulation (nodules/mg rhizosphere) and *myo*-inositol amendment (kg/ha) was higher for the uninoculated plots ($\tau = 0.87$, $P < 0.01$) than the inoculated plots ($\tau = 0.47$, $P < 0.15$). In the growth chamber studies this correlation between nodulation and the nutrient amendment was highly significant ($\tau = 0.91$, $P < 0.005$). These results provide further evidence for the amendment-induced stimulation of *S. meliloti* in the rhizosphere of *M. sativa*.

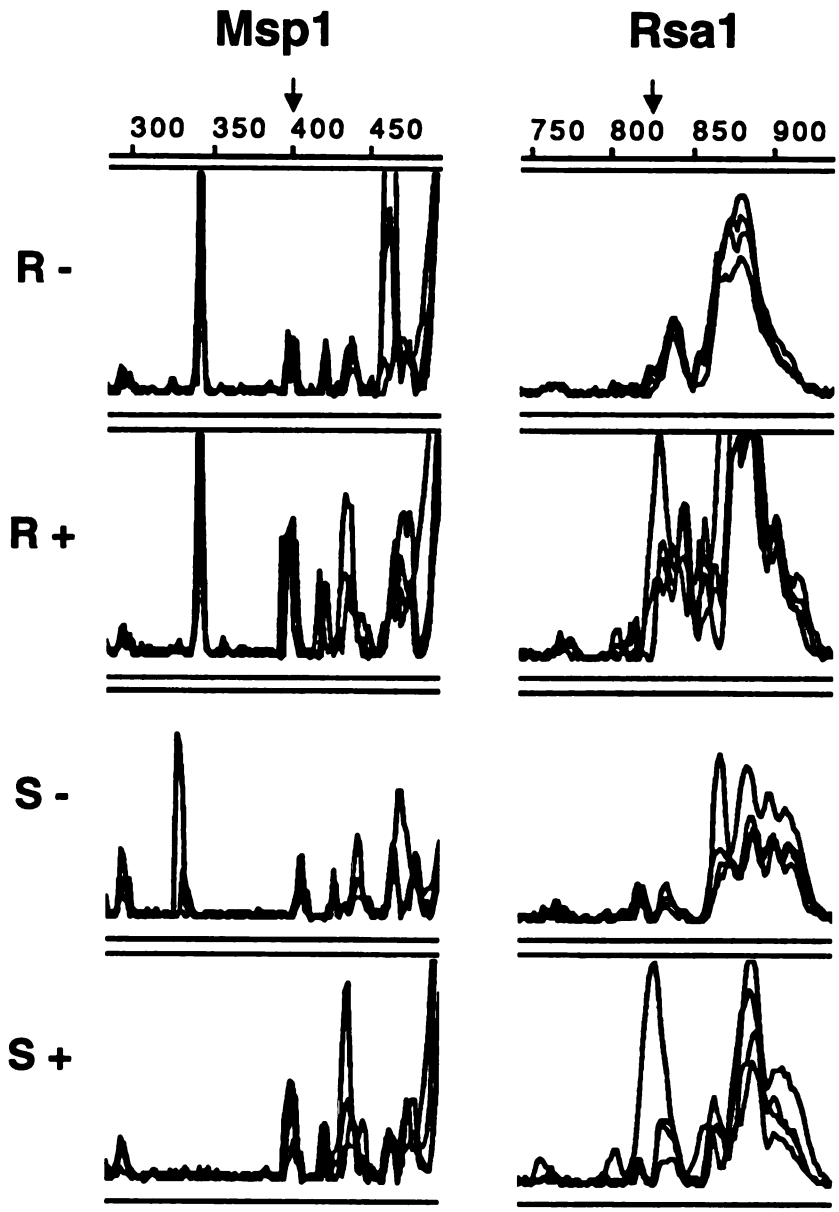

Figure 6.6: Increases in *S. meliloti*-like signals due to *myo*-inositol amendment in the growth chamber experiment. Portions of the FT-ARDRA profiles of soil (S) and rhizosphere (R) washes are displayed. Changes in signals corresponding to those expected for *S. meliloti* (MspI 404 bp, RsaI 830 bp) are discussed in the text.

field test[a]	% change[b]	P[c]
I A (16)	2.2	NS
I B (16)	14	NS
I all data (16)	12	0.166
II A (16)	18	NS
II B (16)	99	NS
II all data (16)	36	0.074
II A (32)	12	NS
II B (32)	106	NS
II all data (32)	30	0.092

[a] Field tests are distinguished based on the use of seed pre-inoculated with *S. meliloti* (A) or uninoculated seed (B). The number in parentheses refers to the amount of *myo*-inositol applied (kg/ha).
[b] Change in the median number of nodules per mg of rhizoshpere.
[c] P-value obtained from comparing amended and unamended samples using the Mann-Whitney test.

Table 6.3: Increases in nodulation of *M. sativa* following *myo*-inositol amendment in the field.

Discussion

In our laboratory, we have been examining the potential of using certain compounds to promote the growth of specific bacterial populations able to utilize them in the soil and rhizosphere environments (31, 32, 33). In the course of these investigations, we discovered that glucosamine amendment significantly increased the number of culturable glucosamine-utilizing bacteria in the field (see Chapter 3). While this amendment increased the number of inoculant bacteria *in situ*, a more significant and longer lasting affect was noted for the indigenous soil bacteria (see Chapter 3). Furthermore, analysis of the colonies cultured on glucosamine-containing medium indicated that a single morphotype was enriched *in situ* over the course of two and a half weeks after the amendment was applied (see Chapter 3). These observations led us to hypothesize that a nutrient amendment may be found which could specifically increase the numbers and activities of beneficial soil microorganisms when applied at planting.

We undertook this study to examine whether *myo*-inositol could serve as a nutritional mediator promoting the growth and activities of the nitrogen-fixing symbiont of *Medicago sativa*, *S. meliloti*. *Myo*-inositol was chosen for several reasons. First of all, members of the Rhizobiaceae, including *S. meliloti*, can utilize it as a carbon source for growth in culture (16, 26, 40). Since a relatively small number of other soil and rhizosphere bacteria living in the tested soil can utilize the compound as sole-carbon source in culture (19), we expected that a greater proportion of the nutritional benefit would be directed to the target populations of *S. meliloti* with this compound. Also, large quantities of pure *myo*-inositol can be obtained commercially, making amendment experiments feasible. Finally, a near-isogenic mutant unable to catabolize this compound was available for doing competition studies (10).

There is some evidence that *myo*-inositol may act as a nutritional mediator of rhizobial activity in nature. Inositol substantially accumulates in the nodules of several legume species; however, little is known about its role in the nitrogen-fixing symbiosis (8, 17, 38, 41, 42). The compound is structurally similar to 3-*O*-methyl-*scyllo*-inosamine, a compound implicated in promoting competitiveness of some *S. meliloti* strains for nodule occupancy (14, 21). While the catabolic pathway for *myo*-inositol is inducible, it does not seem to be active in the nodule (29, 30). Thus, if the accumulated inositol is not metabolized while the nodule is functioning, it may play a role in maintaining rhizobia after nodule senescence.

In this work, we have shown that *myo*-inositol amendment can promote the growth of culturable Mic$^+$ bacteria in the rhizosphere of *Medicago sativa*. Under gnotobiotic conditions, *myo*-inositol amendment was found to promote the growth and activity of strain 1021, a wild-type Mic$^+$ *S. meliloti*, over IM59, its near-isogenic Mic$^-$ competitor, in the rhizosphere of *Medicago sativa* (see Figure 6.1). In the absence of the amendment, no difference in the ability of the wild-type and mutant to colonize the rhizosphere was observed; the ratio of wild-type to mutant remained approximately 1:1. In contrast, the proportion of Mic$^+$ bacteria was significantly greater in the presence of the amendment; the ratio of Mic$^+$ to Mic$^-$ strains was approximately 2:1 for the first week

of the experiment and approximately 3:1 after two weeks. Thus, the amendment contributed to a proportional increase of Mic^+ over Mic^- bacteria in the rhizosphere.

Because wild-type *S. meliloti* can utilize *myo*-inositol as a growth substrate, we hypothesized that this nutrient amendment would result in a measurable increase in the number and activity of indigenous populations of this organism. FT-ARDRA was used to monitor changes in *S. meliloti* populations in soil and rhizosphere washes and cultures inoculated from them. In the growth chamber experiments, signals indicative of *S. meliloti* increased significantly following the addition of *myo*-inositol (see Figure 6.6). These *S. meliloti*-like signals were also more abundant in MA cultures of *myo*-inositol amended samples (see Figures 6.3 and 6.4). In the field experiments, increases in the *S. meliloti*-like signals of the *Msp*I profiles were observed up to four weeks after germination. Additionally, increases in the amplitude of this ~400 bp signal correlated with increases in the concentration of the applied *myo*-inositol amendment in field test II. These increases may be due to an increase in the number of *S. meliloti*, their chromosome copy number, or a reflection of increased susceptibility to lysis and amplification; all indications of a larger and/or more active population. We also observed an increase in nodulation (see Table 6.3) due to *myo*-inositol amendment. This increase in nodulation was significantly correlated with the amount of *myo*-inositol applied in the field. These data indicate that the mutualistic symbiosis of *M. sativa* and *S. meliloti* is promoted by *myo*-inositol amendment.

This work describes some intriguing correlations between the application of a nutrient amendment, subsequent increases in a beneficial rhizosphere microorganism, and a stimulation of a beneficial symbiosis. Unfortunately, we cannot causally relate the increases in nodulation with the apparent increases in *S. meliloti*, nor can we conclusively state that the nutrient amendment directly stimulated the growth and activity of *S. meliloti* in the natural environment. This is partly because other bacteria respond to the amendment and may alter the relationships between the symbiotic partners (see Table 6.2). Thus, it may be that the observed changes in the rhizosphere bacterial communities indirectly stimulate one or both of the symbionts so as to produce the observed results. The complexity of microbial communities greatly complicates the search for mechanistic

understanding of below-ground ecological relationships. However, by examining these systems with a variety of complementary tests, we may slowly accumulate a preponderance of evidence supporting the mechanistic explanation provided by the biased rhizosphere hypothesis.

Acknowledgements

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Appendix

Analysis of Microbial Field Release Data

McSpadden Gardener B, de Bruijn FJ (1997) Analysis of Microbial Field release data. Chapter 7.X.X. *In* Molecular Microbial Ecology Manual. Kluwer: Amesterdam. *submitted*

Introduction

Recent advances in microbial ecology promise to contribute significantly to the development of many useful technologies, such as bioremediation of polluted sites and microbe-mediated plant growth promotion. These technologies often depend on the use of large scale releases of microbes into the environment. Because of this, much interest has been generated over the safety and efficacy of such releases. To properly assess the impact of field releases, precise quantitative descriptions of microbial populations, biomass, and activity are required.

Numerous methods have been developed to measure ecologically relevant quantities, many of which are described in this volume and elsewhere (*e.g.* 26). Each has certain strengths and weaknesses with regards to addressing the broader questions of safety and efficacy of microbial inoculants. Because of the complex nature of the field environment, these critical questions require the use of complementary methods and suitable statistical analyses to draw valid conclusions from field studies.

In this chapter we describe an experimental approach that will provide a basic statistical assessment of microbial field release data. Because of the diversity of potential inoculants, it is impossible to list which sets of methods are most appropriate for all applications. Therefore, we will provide an empirical framework that is independent of the nature of the microbial inoculum or its intended application. The prerequisites for and presentation of basic statistical analyses will be described. Because of the nature of the data obtained from the field release of microbes, nonparametric statistical methods will be detailed. At the end of the chapter, we will present examples to assist the reader in understanding the types of questions that can be easily addressed with each of the described methods. An appendix containing all of the necessary statistical tables is also included.

Overview (Figure A.1)

Thorough methodological and statistical analyses are essential to interpret field data. In the complex external environment, multiple techniques are required to quantify the relationships between inoculant populations, activity, and environmental impact. However, all such relationships are confounded by natural variation in the quantities measured. Characterization of both the inoculant organism and the site of release is prerequisite to the rational design of an experimental study. Determining the extent of variation present in an experimental system will indicate the degree of replication required for statistically significant results to be obtained. Because the extent of such variation is often unknown prior to a field release, small scale pilot studies can be effective in refining the experimental design. The experimental objectives and the structure of the data obtained will determine the extent of sampling and the type of statistical analysis required.

Inoculant characterization

The qualitative characteristics of the microbe's physiology and ecology should be relatively well understood prior to large scale field releases (24). The identity of the organism should be reasonably stable with regards to the assay used to monitor its presence in the environment. Some attention must also be paid to the relative strengths and weaknesses of various molecular markers used to track microbes in the environment (8). The survival characteristics of the organism need to be investigated (*e.g.* whether or not it forms resting spores). Combined, this qualitative knowledge will allow for more accurate quantification of inoculant abundance and activity.

Site characterization

To assess the environmental impact of an inoculant, a site must be characterized prior to inoculation. A variety of standardized tests can be used to describe a site (23). Some of these tests are listed in Table A.1. While it may not be possible to perform all of

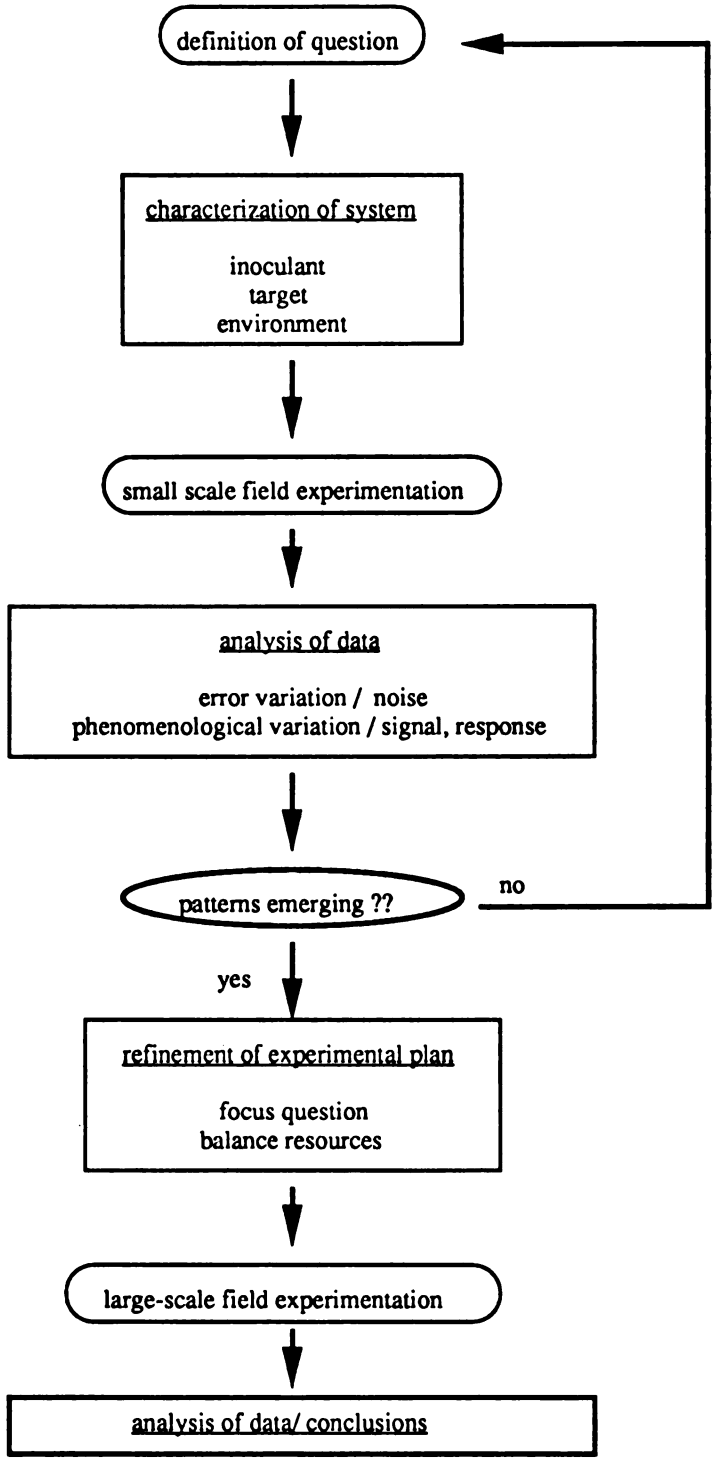

Figure A.1: General methodology for microbial field release experiments.

Table A.1: Information used in site descriptions.

General description
Location and topography
Climatic data for the experimental period
Vegetation
Management history
Soil composition
Structure (sand: silt: clay)
Soil moisture and pH
Cation exchange capacity
Soil organic matter
Nutrient status (concentrations of N, P, K, and micronutrients)
Study-specific data
Biota relevant to expected impact of inoculant, *e.g.* pathogen abundance
Chemical constituents relevant to inoculant activity, *e.g.* organic pollutants

these tests, more complete characterizations will allow for more thorough comparisons with other studies.

Proper sampling of the test site is required to determine the extent of variation of the study area. Several studies have attempted to describe the appropriate scale for studying various microbiological processes (*e.g.* 11, 17, 20). However, the extent of sampling required will depend on the scale of the field study and the degree of heterogeneity at the site. Consequently, sample size and sampling intensity must be established empirically for each application.

Preliminary assessment of data variation

The sources of data variation should be identified and characterized in preliminary control experiments prior to performing a full scale field study. Variation can arise from many sources, natural and methodological. The degree of natural variation can be measured by performing controlled replicates of homogeneous, independent units. For any given method, perform control experiments to determine the effects of sample handling and

processing time on data variation. This information is needed to describe the quantitative precision of the method and to determine a practical sample size. A measure of dispersion, such as the standard deviation, can be used to describe the degree of variation present in the data.

For any given experiment, the reproducibility of data is dependent on the degree of natural variation, the precision of the quantitative method, and the number of times the method is applied. Imprecise measurement or inadequate replication will lead to irreproducible results and a bias against detecting differences in the system being studied. Alternatively, excessive precision or replication will lead to the detection of differences which are of questionable importance. The challenge for researchers is to identify phenomenologically significant patterns. Statistics, if properly applied, can help characterize those patterns.

Experimental design considerations

Once preliminary studies have been completed, a careful review of the data is necessary. The assumptions for performing statistical analyses should be tested. In particular, the sampling scheme should be random with regard to the measured variable. Evidence of systematic bias will appear in a test of randomness on the data. One may characterize the distribution of the measured variable to determine the type of statistical test(s) that can be properly applied. Because the distribution may change over time and space, differences should be viewed cautiously. Care should be taken not to extrapolate beyond the scope of the tested experimental conditions. Finally, the time, labor, and material expense required to expand the study should guide the refinement of the scientific questions that can be fruitfully addressed given the constraints of the experimental system.

Statistical analysis of the data

Statistical analysis will complement the experimental approaches used by summarizing the numerical relationships present in the data. Consideration of which statistics will be used in the data analysis will help determine the experimental design. A core set of tests useful for making comparisons and describing functional relationships is detailed below. Readers familiar with parametric statistics (*e.g.* Student's *t* and Pearson's *r*) might wonder why these common statistical tools are not described. The reason is that nonparametric statistics will generally offer greater versatility when dealing with microbial field release data.

The patchiness of the field environment, both spatially and temporally, is likely to give rise to complex distributions of measured variables. Additionally, the apparent distributions of microbial counts and activity (or any other variable) will depend on the scale at which they are sampled (3,19). With such complexities, statistics dependent on well-defined distribution curves are unlikely to be applicable in as many situations as analogous nonparametric statistics which have no such assumption. Since microbial numbers and activity do not remain constant over time (*e.g.* 1), researchers must be cautious in making any assumptions regarding the underlying distributions of sample data.

Because nonparametric methods are not always taught in introductory statistics courses, a brief introduction follows. More detailed discussion of nonparametric statistical tests can be found elsewhere (3, 15, 21, 25).

Nonparametric statistics

Statistics summarize sample data by reducing the information into a single number. Measures of central tendency (*e.g.* mean, median) and dispersion (*e.g.* standard deviation, variance) calculated from the sample data are used to describe the populations from which they are obtained. However, to make quantitative comparisons between data sets or to describe functional relationships between measured variables, statistical tests are required.

The nature of the underlying distributions will determine the type of statistical tests needed to analyze the data. In this regard, nonparametric tests and their parametric analogs differ. Nonparametric tests do not require the underlying distribution of data values to be precisely defined. In contrast, parametric tests assume that the distribution is known and can be described mathematically. In general, the assumption is that the data follow a "normal" (*i.e.* Gaussian) distribution, or that they can be made to conform to this distribution by transformation of the raw data. If parametric tests are applied to data sets whose distributions are not normal, then their validity is undermined. While several reports have indicated that microbial counts and their biochemical functions can be described by a log-normal distribution (5, 13, 16), exceptions to this pattern have been noted in the literature (10, 11). Therefore, one cannot assume *a priori* that field data from microbial releases will conform to a definable distribution. While one may assess the distribution of an experimental variable, such a study requires a relatively large data set (*e.g.* $\geq$ 30 independent samples) which may not be practical. In general, nonparametric statistics should be used if the distribution of the sampled variable is not, or cannot be, ascertained with reasonable certainty.

Additionally, nonparametric statistics have other useful properties. For instance, they can be used to analyze ordinal data. This type of data differs from interval data (*e.g.* microbial counts, rates of nitrogen fixation) in that the units do not follow a uniformly divisible unit scale. As a practical example, data relating to disease severity induced by plant pathogens is commonly reported on ordinal scales. These ratings often progress from low (few symptoms) to high (plant death) on an arbitrarily defined scale. Such data can best be analyzed by nonparametric tests. Nonparametric statistical tests are also easy to perform, since they do not require sophisticated calculation. While most statistical packages will perform a basic battery of nonparametric tests, none perform all of the methods described here. Computer spreadsheet programs that can order and sum may speed up some of the initial data handling but are not essential. In this regard, most

graphing programs have such options and so will nicely complement the requirements for data presentation discussed below.

The properties of nonparametric statistics make them uniquely suited to analyzing field data of microbial releases. In this chapter a core set of these statistical tools will be described. Detailed examples of each method will be provided as a model for better understanding the types of research questions to which these methods can be applied.

Presentation of results

Because statistics condense data into more easily understood bits of information, it is critical that the mathematical tools used be fully described. The sample size, number of replicates, test performed, and level of significance of the test statistic form the backbone of any statistical analysis; they should be presented together for clarity. In graphical presentations, error bars should indicate a specified confidence interval for the plotted points. All of these quantities are defined in the next section. Often, presentation of the raw data can be a valuable aid in the judicious evaluation of results; however, this may be impractical if a study is particularly large. The goals of clarity and completeness must be balanced with, but not subservient to, conciseness.

The quantitative characteristics of data analyzed using statistics may be confounded by several variables. Sometimes it is relatively straightforward to control for these differences. For instance, in the case of immunofluorescence assays, cross-reactivity to non-target cells can be tested in uninoculated controls. However, other potential variables may not be easily identified. For example, the amount of antigen binding to inoculum cells recovered may change because of differential adaptation to the soil, treatment, and/or sampling procedure. Therefore, the assumption that such potential confounding variables are of little consequence must either be directly tested or clearly stated in the discussion of the results.

Procedures

Getting started

The following tests are presented in their most basic format and require the use of the statistical tables included as appendices at the end of this chapter. Althought there are a number of ways to organize the calculations of the test statistics, the methodology described below is designed for a manual or computer-assisted approach.

The basic procedure for all statistical tests begins with the statement of a hypothesis to be tested. This null hypothesis is generally that there are no quantifiable differences between sample populations, though, other null hypotheses can be specified (see below (4) Quantifying differences). The raw data for a given empirical test (*i.e.* the measured variable) are then tabulated for processing. Generally, nonparametric statistical procedures require that each raw data value be ranked with respect to all others in the data to be analyzed. Ranks proceed from 1 (lowest value) to N (highest value) and are tabulated in an adjacent column. Equal sample values are assigned a corresponding average rank value. The sum of the ranks for each treatment group is then used in a formula specific to each test. The calculated test statistic is then compared to one of the tables included at the end of this chapter to determine the probability, P, that the test statistic would be as extreme as the calculated value if the null hypothesis were true. The P value is equivalent to the level of significance, α, which corresponds to the probability of unjustly disproving the null hypothesis. It is this value that one uses in making statements about the statistical significance of a particular result. Note that failure to disprove the null hypothesis is *not* equivalent to proof of it. A relatively high P value simply indicates that there is insufficient evidence to disprove the null hypothesis.

The algebraic conventions used in the procedures described below are as follows:

$H_0\{\ \}$ = the null hypothesis being tested
$H_1\{\ \}$ = the alternative hypothesis accepted if there is a sufficiently low P value for the calculated test statistic
x = sample value
n = sample size for an individual sample population
$N = \sum_{i=1}^{k} n_i$, the total sample size
k = the number of sample populations, *i.e.* qualitatively different treatments or conditions
$r(x)$ = the rank of a sample value
$R_i = \sum_{i=1}^{n} r_i(x)$, the rank sum for an individual sample population
P = probability that the data support the null hypothesis (equivalent to α)
df = degrees of freedom with which the test statistic may be calculated

Most of the statistical tests described below assume that the measured variable is randomly sampled. Failure to validate this assumption can cause bias in the conclusions drawn from the statistical analysis. Validation is generally inherent in the experimental design. Hence, the experimental setup may involve random assignment of treatments to a field, or conversely, random sampling within the field. Tests of the randomness assumption (*e.g.* a runs test, see 25) are generally not reported in the literature, but they should be performed when the data sets are sufficiently large (*e.g.* $n \geq 12$).

Summary statistics

Complex data sets are often simplified by summarizing many observations with statistics that describe the central tendency and dispersion of the data (see Table A.2). Measures of central tendency are the most commonly reported. However, such numbers are not particularly meaningful without a corresponding measure of dispersion (*e.g.* the standard deviation). Generally speaking, the most informative descriptions are made using the mean or median and their corresponding confidence intervals. A confidence interval describes the range of values within which the central measure of the population from which the sample was drawn truly exists, given a specified probability. Sample means

Table A.2: Commonly used summary statistics of sample populations.

Central tendency of the data		
Mean:		$\bar{x} = (\sum_{i=1}^{n} x_i) / n$
Median:	for odd n	Value of ranked observation numbered: $(n+1) / 2$
	for even n	The mean of the ranked values numbered: $n / 2$ and $(n+2) / 2$
Dispersion of the data		
Variance		$s^2 = \sum_{i=1}^{n} (x_i - \bar{x})^2 / (n-1)$
Standard deviation		$s = \sqrt{s^2}$
Coefficient of variation		$V_s = s / \bar{x}$

with nonoverlapping confidence intervals can, at first glance, be considered significantly different. However, one should note that sample means with overlapping confidence intervals may still be significantly different by the criteria of comparison tests such as those described in the next section. The mean is perhaps the most commonly used measure of central tendency; however, it is sensitive to outlying values. Nevertheless, a relatively conservative nonparametric confidence interval for the mean can be calculated. The median is a better descriptor of data that come from unknown distributions, but it is only useful if the sample size is sufficiently large (*e.g.* $n \geq 7$).

(1) Describing the confidence interval (CI) for:

a) A sample mean (using Chebyshev's inequality (3)):
   i) Specify a P value (generally 0.05 or 0.1).
   ii) Calculate: $CI_{mean} = \bar{x} \pm (w)(s)$ where $1/w^2 = P$

b) A sample median (using the sign test(15)):
   i) List the sample values of a single experimental treatment in column 1.
   ii) Rank the samples values in column 2.
   iii) Go to Table A.3. Find the value, V, approximating a chosen P

(*e.g.* 0.05 or 0.1) and n of the sample.

iv) The CI_{median} is the range described by the sample values with $r(x) = (V)$ or $r(x) = (n\text{-}V)$.

Making Comparisons

Because the confidence intervals described above are large for unknown distributions, they are not likely to detect significant differences between data sets with small sample sizes. In such instances, statistical tests using ranked data are much more powerful. The following procedures allow comparisons to be made between data sets without relying on summary statistics such as the mean or median. They test for differences in location on the scale of the measured variable. Following this section, a method to quantitate such differences between sample populations will be presented.

One must choose a procedure that is specific to the number of conditions tested and the independence of the sample measurements. Dependent measurements from the same experimental unit must be analyzed differently than measurements taken from replicate independent units. The investigator decides what constitutes an independent sample unit (*e.g.* a single leaf, multiple leaves from a single plant, or multiple leaves from multiple plants).

(1) Comparing two sample populations based on:

a) Independent observations (using the Mann-Whitney test (14)): Samples sizes may be different, although the test is somewhat more powerful when the two samples sizes are approximately equal for any given N. H_0\{the two populations are similar\}, H_1\{the two populations differ\}.

i) List values for the two samples, A and B, in two different columns.
ii) Considering the entire data set, rank the data from low to high. If two or more values are the same, assign an average rank value to all equivalent values.
iii) Sum the ranks for each group: $\sum_{i=1}^{n_A} r_i = R_A$ and $\sum_{i=1}^{n_B} r_i = R_B$. One can verify the math with the following identity: $R_A + R_B = (n_A + n_B)(n_A + n_B + 1) / 2$
iv) Calculate: $U_A = R_A - [n_A(n_A+1) / 2]$ and $U_B = R_B - [n_B(n_B+1) / 2]$
v) Identify U which is the greater of U_A and U_B.
vi) Compare U to Table A.4 to determine its P value.
vii) If for the given sample sizes U is $\leq$ the tabled value, then reject H_0.

b) Dependent (paired) observations from the same experimental units (using the Wilcoxon-signed rank test (27)): H_0\{the two populations are similar\}, H_1\{the two populations differ\}.

i) List sample values by group in two different columns.
ii) Tabulate the row by row differences of the two columns in column 3.
iii) Rank the absolute value of each difference in column 4.
iv) Sign the ranks in column 5, following the designations in column 3.
v) Sum the ranks of the (+) and (-) differences respectively:

$$\sum_{i=1}^{n_{(+)}} r_i = R_{(+)} \text{ and } \sum_{i=1}^{n_{(-)}} r_i = R_{(-)}$$

One can verify the math with the following identity: $R_{(+)} + R_{(-)} = (n)(n+1)/2$
v) T is the lesser of $R_{(+)}$ and $R_{(-)}$.
vi) Compare T to Table A.5 to determine its P value.
vii) If T is $\leq$ the tabled value using the given value of n, then reject H_0.

(2) Comparing three or more sample populations, based on:

a) Independent observations (using the Kruskal-Wallis test (12)): This test performs well even for small samples ($N \geq 9$), but special tables are required for determining the level of significance. These can be found in many statistical texts but are too lengthy to include here. However, the test statistic can be fairly evaluated using the χ^2 table presented in the appendix, whenever $N > 20$ and $n_i \geq 4$ for all samples. H_0\{all k populations are similar\}, H_1\{one or more populations differ\}.

i) List all data values in a k (columns) by n (rows) matrix.
ii) Considering the entire data set, rank the data from low to high. If two or more values are the same, assign an average rank value to all equivalent values.
iii) Calculate: $H = \{12/[N(N+1)]\sum_{i=1}^{k}(R_i^2/n_i)\} - 3(N+1)$
iv) Compare H to Table A.6 using $(k-1)$ df to determine its P value.
v) If H is $\geq$ the tabled value, then reject H_0.

To subsequently identify which sample population(s) are significantly different, perform Dunn's procedure (2) for multiple pair-wise comparisons as follows:

i) Calculate the mean ranks for each group: $\overline{R}_i = R_i / n_i$
ii) For each comparison, calculate: $z = |\overline{R}_i - \overline{R}_j| / [N(N+1)(1/n_i + 1/n_j)/12]^{1/2}$
iii) Read the one-sided P value for the calculated z using Table A.7. Call it P_1.
iv) Calculate: $P = P_1[k(k-1)]$
v) Optional: Tabulate all the P values in a k by $(k-1)$ matrix. Highlight significant differences (*i.e.* low P values).

b) Dependent (blocked) observations from the same experimental units (using Friedman's test (4)): As for the H statistic, special tables are required for determining the precise level of significance for Friedman's S statistic. In the absence of more lengthy tables, the test statistic can be reasonably assessed using the χ^2 table in the appendix when $k \geq 4$. H_0\{all k populations are similar\}, H_1\{one or more populations differ\}. The formulas here assume equal sample sizes (*i.e.* all n are equal).

i) List all data values in a k (columns) by n (rows) matrix.

ii) Considering the data row by row, rank the data from low to high. If two or more values in the same row have the same value, assign an average rank value to all equivalent values.

iii) Calculate: $S = \{12/[nk(k+1)]\sum_{i=1}^{k} R_i^2\} - 3n(k+1)$

iv) Compare S to Table A.6 with $(k-1)$ df to determine its P value.

v) If S is $\geq$ the critical value reject H_0.

To subsequently identify which sample population(s) are significantly different, perform Dunn's procedure (2) for multiple pair-wise comparisons as follows:

i) Calculate the rank sum, R_i, for each group.

ii) For each comparison, calculate: $z = |R_i - R_j| / [nk(k+1)/6]^{1/2}$

iii) Read the one-sided P value for the calculated z using Table A.7. Call it P_1.

iv) Calculate: $P = P_1[k(k-1)]$

v) Optional: Tabulate all the P values in a k by $(k-1)$ matrix. Highlight significant differences (*i.e.* low P values).

Note: Multiple pair-wise comparisons should be performed only if a significant difference between sample populations has been detected using the H or S statistic.

Quantifying differences

The identification of population differences and the calculation of the statistical significance of those differences is relatively straightforward. However, it is most often desirable to quantify those differences. One nonparametric method has been described by Hodges and Lehmann (6), but it requires numerous calculations and specialized tables. Another involves modifying the null hypothesis in terms of some quantitative factors. Both methods provide good, though not necessarily identical, estimates of quantitative differences between treatments.

(1) To calculate the factor by which two sample groups differ:

i) Calculate the sample means for each k (sample population).

ii) Estimate the factor, C_1, by which two treatments differ significantly. As a first best estimate use the ratio coefficient: $C_1 = (0.75)(\bar{x}_A / \bar{x}_B)$ where $\bar{x}_A > \bar{x}_B$.

iii) Multiply the raw data for the treatment with a *lower* mean value by C_1.

iv) Rerun the nonparametric statistical test appropriate to the data set (U, T, H, *or* S).

v) Repeat ii) through iv) iteratively to find the highest coefficient that still yields a statistically significant result (*e.g.* with a chosen $P \leq 0.10$).

vi) Estimate the factor, C_2, by which the treatments differ significantly. As a first best estimate use the ratio coefficient: $C_2 = (0.75)(\bar{x}_A / \bar{x}_B)$ where $\bar{x}_A > \bar{x}_B$.

vii) Multiply the raw data for the treatment with a *upper* mean value by $(1 / C_2)$.

viii) Rerun the nonparametric statistical test appropriate to the data set as in step iv).

ix)Repeat vi) through viii) iteratively to find the highest coefficient that still yields a statistically significant result.
x) The estimated ratio of the two population means lies between C_1 and C_2.

Note: In cases where three or more samples are compared, rigorous estimates (*i.e.* with an easily defined P value) can only be made when treatment groups separate into two distinct classes as determined by Dunn's multiple range test. In such instances, the data from all samples not considered significantly different should be multiplied by the factors C_1 and C_2 when required in steps iii) and vii).

Describing functional relationships

A central goal of field studies is to establish whether or not the application of an inoculant is related to a particular measured response. While correlation does not establish causality, it can provide the basis for establishing a mechanistic explanation for the phenomena being investigated. To determine the relationship between variables, the formulas for two rank-based correlation coefficients are presented below along with a graphical method for describing the monotonic relationship in a data set. In a monotonic relationship, the values of two measured variables (*e.g.* cell counts and disease resistance) will generally increase or decrease together. The values of the correlation coefficients described below will range from -1 to 1, where higher absolute values indicate stronger relationships between the measured variables.

(1) Monotonic correlation:

a) For discrete data, use Kendall's test (9) as follows: H_0\{the two variables are not correlated\}, H_1\{the two variables are correlated\}. Note that the correlation coefficient τ = $\{[4K / n(n-1)] - 1\}$ and that Table A.8 is given in terms of K.

i) List the data values for the first variable (x) from low to high in Column 1.
ii) List the corresponding values for the second variable (y) in Column 2.
iii) Place the rank of each y value next to it in Column 3. The next two calculations are made from the rankings in this column.
iv) Calculate Q_1 by taking each ranking in turn and counting how many values listed below it have a higher ranking. Thus, Q_1 equals the number of values in rows 2 through n which have a higher value than the ranking in row 1, *plus* the number of values in rows 3 through n which have a higher value than the ranking in row 2, *plus* . . . . . *plus* 1 if the value in row n is higher value than the ranking in row $(n-1)$.
v) In a similar manner, calculate Q_2 by taking each ranking in turn and counting how many values listed below it have a lower ranking.

vi) Considering the raw data, calculate: $N_t = \sum_{i=1}^{m} (t_i - 1)/2$

where t is the number of tied observations for each x or y value, m, that has more than one observation.

vii) Calculate: $K = [2|(Q_1 - Q_2)| + n(n-1)] / 4 + N_t$

viii) Compare this value to Table A.8 to determine its P value.

ix) If $K \geq$ the tabled value, then reject H_0.

x) If K is significant, calculate τ.

b) For data from continuous distributions, use Spearman's coefficient (22) as follows: H_0 {the two variables are not correlated}, H_1 {the two variables are correlated}.

i) For the first variable, tabulate the x values in column 1, and $r(x)$ in column 2.

ii) For the second variable, tabulate the corresponding y values in column 3 and the $r(y)$ in column 4.

v) Calculate the differences: $d_i = r(x_i) - r(y_i)$ and tabulate in column 5.

vi) Calculate: $r_s = 1 - [(6 \sum_{i=1}^{n} d_i^2)/(n^3 - n)]$

vi) Compare this value to Table A.9 to determine its P value.

vii) If $r_s \geq$ the tabled value, then reject H_0.

(2) Monotonic regression:

a) Graph the observed monotonic relationship between variables (using the method of Iman and Conover (7)): This procedure results in a jagged regression line on a scatter plot of the raw data. This line can be used as a starting point to describe the mathematical relationship (*e.g.* linear, logarithmic, discontinuous) between two variables. If the first variable, x, is controlled by the investigator, one needs only to plot a single regression line. If neither variable is controlled, the procedure should be repeated, switching the designation of the variables, to give both regression lines.

i) In a table, list x, $r(x)$, y, and $r(y)$ in four separate columns.

ii) Graph the points of $r(y)$ versus y. Connect all data points sequentially.

iii) Calculate Spearman's correlation coefficient, r_s (see above).

iv) Calculate and tabulate in Column 5 the expected rank of each y value using the equation: $E[r(y)] = r_s\, r(x) + (n+1)(1 - r_s)/2$

v) Using ii) and iv), calculate an expected value of y, $E(y)$, for each rank value of x. Place these values in Column 6.

vi) Plot $E(y)$ versus x and connect the points.

vii) On the same graph, plot y versus x for a visual reference.

Examples

The following examples use hypothetical data to illustrate the proper application of statistical tests to various research topics of current interest to microbial ecologists. They are provided solely as a training guide to the statistical procedures described above.

Making comparisons

(1) Comparing two groups of independent measurements:

A bacterial suspension is inoculated into two qualitatively different field soils. Soil A is a loamy sand and soil B is a silt loam. Approximately ten independent soil cores of equal size are collected randomly from each field. The cores are placed in plastic bags, stored on ice, and brought to the laboratory for analysis. The inoculated bacteria can be selectively enumerated by virtue of a fluorescence-based assay. The following data represent direct counts of bacteria obtained from the same dilution (10^{-3} per gram).

Soil A: 148, 98, 123, 45, 80, 96, 32, 165, 117, 130
Soil B: 186, 208, 95, 152, 48, 174, 214, 230, 215

Because the two soils are qualitatively different, the data are compared using the Mann-Whitney U statistics. Here, $n_A = 10$ and $n_B = 9$, giving $R_A = 71$ and $R_B = 119$. Using the procedure outlined above $U_A = 16$ and $U_B = 74$. Using the latter value, the level of significance is found in Table A.4 to equal 0.02. In other words, the probability that the population size of the inoculant in the two soils is the same given the above data is quite small. The observed ratio of the mean counts is 1.6, but this overestimates the ratio to which we can subscribe a firm P value. By iteratively rerunning the analysis using the method described above, the counts were found to differ by a factor of about 1.25 at the chosen level of significance ($\alpha = 0.10$). Qualitatively, the number of cells detected differed in the two soils ($P \leq 0.02$). Quantitatively, the counts of the inoculum in soil B were about 25 percent higher than in soil A ($P \leq 0.10$). The underlying assumptions are that the population distributions, sampling, and assay conditions are the same.

(2) Comparing two groups of dependent/paired measurements:

A fungicide is applied to legume seed and researchers are interested in examining its effects on nodulation. While the viable counts of rhizobia recovered from seed treated with fungicide do not differ from that of the control, data is lacking on the impact on nodulation.

Therefore an experiment is set up using ten plants each of seven different cultivars. These seven cultivars represent distinct genetic lines selected for growth in different geographic locations. The seeds are sown into fourteen separate flats and grown in the greenhouse. After six weeks, the plants are uprooted and brought to the lab. From each set of plants, one hundred nodules are independently removed, surface sterilized, crushed, and plated on YMA agar with and without antibiotics selective for the inoculant. After incubation, plates are scored for growth. The number of nodules occupied by the inoculant rhizobia are presented below.

cultivar:	1	2	3	4	5	6	7
- fungicide:	98	98	98	97	93	90	95
+ fungicide:	96	98	64	94	97	93	94

The two treatments are compared using the Wilcoxon signed rank test because the data are likely to be cultivar dependent. For these data, $R_{(+)} = 10.5$ and $R_{(-)} = 17.5$. Using the lesser of these, the test statistic, *T*, is compared to Table A.5. The value is not significant ($P >> 0.10$) so the null hypothesis cannot be rejected. Thus, there is insufficient evidence that the application of the fungicide significantly affects nodule occupancy of this species by the inoculant. Nonetheless, nodulation of cultivar 3 appears to be greatly affected by the fungicide treatment, and further investigation of this observation is warranted.

(3) Comparing three or more groups of independent observations:

Four different bacterial strains are compared for their colonization of the roots of a single cultivar of wheat. Inoculants are mixed into the soil of four separate pots immediately prior to sowing the seeds. Three seeds are sown in each pot, and after two weeks seedlings are thinned to one plant per pot. After four weeks, plants are uprooted and rhizosphere samples are prepared by sonication of whole root systems in 50 mls of

distilled water. This wash is then serially diluted and spot plated onto selective media. The colony forming units (CFU) of the inoculant were counted after three days of incubation.

inoculant:	A	B	C	D
plant 1	86	147	225	222
plant 2	52	128	192	203
plant 3	38	77	184	157
plant 4	24	33	147	53

Here there are four qualitatively different treatments (*i.e.* $k = 4$). Because each plant can be considered an independent unit, the Kruskal-Wallis procedure is appropriate. The rank sums ($R_A = 15$, $R_B = 25.5$, $R_C = 50.5$, $R_D = 45$) suggest that there are differences between treatment groups. More formally, one calculates $H = 9.1$ which is significant, ($P < 0.05$). This means that one or more of the inoculant strains differs from the others in rhizosphere abundance. To discover which strains differ significantly, Dunn's multiple comparison procedure is performed. Strain A is found to differ from strain C ($P \leq 0.05$) and, arguably, strain D ($P \leq 0.16$). If one performs multiple comparisons using Dunn's procedure *after* a significant H value has been established for a given data set, one can reasonably accept P values up to 0.20. No other combination of strains was found to differ significantly. Because strain B does not differ significantly from any other strain, no rigorous estimate of the quantitative differences can be made using the method given above.

At this point, we run into the problem of defining the experiment. If strain B is dropped from the analysis, a quantitative estimate of population differences can be made. However, one must be careful in making such *post hoc* decisions, because they are bound to have repercussions on the estimated P values of any calculated statistic. In the spirit of exploratory data analysis, the practice is fine as a starting point when the treatments are qualitatively different, since the patterns are suggestive of significant differences. However, if the treatments are qualitatively the same (*e.g.* different concentrations of cells of a single strain), one cannot honestly omit treatments if the research questions focuses on the purely quantitative differences of the treatments. To reiterate the recommendation

above, all experimental comparisons should be repeated independently to verify the observed patterns. The calculated test statistics for each replicate should be reported with their corresponding levels of significance.

Multiple comparison tests should only be performed *after* a significant H (or S) statistic has been established for a given data set. For example, if one were to remove strain A from the analysis above, no significant differences would be detected using the procedure of Kruskall and Wallis. Despite this, Dunn's procedure would still detect differences in the counts of strains B and C ($P \leq 0.12$). This latter result, however, would not be acceptable since the original null hypothesis (that the counts of strains B, C, and D are equal) could not be rejected.

(4) Comparison of three or more groups of dependent/blocked observations:

In preparing for commercialization of a new product, a company is attempting to determine which formulation will give the most consistent yield increases. Ten sites, spread throughout the company's market area, are tested. At each site, four treatments are used: three formulations and one uninoculated control. Two of the formulations contain a single strain (A or B), while the third contains both strains. Before the exact yield tonnage was determined, an ordering of lowest (1) to highest (4) yield of the four treatments at each of the sites was made.

formulation:	A	B	A&B	no inoculant
1	3.5	2	3.5	1
2	3	1.5	4	1.5
3	4	2	3	1
4	2	4	3	1
5	3	1	4	2
6	3	2	4	1
7	3.5	2	3.5	1
8	3	2	4	1
9	4	1	3	2
10	3.5	1.5	3.5	1.5
R_i	32.5	19	35.5	13

Since the yields are likely to be dependent on the site at which the plants are grown, Friedman's test is the appropriate choice. As the yields are already ranked on a per site basis, no further ordering is required. The rank sums are included in the above table for reference. Calculation of the test statistic shows that S = 20.8 which has, when compared to Table A.6, a corresponding $P < 0.001$. Dunn's procedure is used to identify which of the treatments differ. Formulation A is significantly different from B ($P \leq 0.12$) and the control ($P \leq 0.005$), but it is not significantly different from the mixed A&B formulation. The mixed formulation is also different from B ($P \leq 0.026$) and the uninoculated control ($P \leq 0.001$). Formulation B is not significantly different from the control. Formulations A and A&B give consistently higher yields across the entire market area.

The decisions to be made based on these data depend on the marketing strategy and production costs. Without doubt, Formulation A&B is the most effective product. However, the differences in A and A&B may not warrant the extra costs of producing the mixed formulation. To make such a decision, the precise yield data (*e.g.* in tons per hectare) must be evaluated. The interval scale data may also be useful in determining if there are significant site to site yield variations (as assumed above). To perform such an analysis, the raw data would have to be ranked by column instead of by row, with k =10 and $n = 4$.

Describing functional relationships

(1) Monotonic correlation:

The relationship between the phyllosphere population size of a biological control strain and observed disease resistance is investigated. Population sizes were determined by colony counts coming from washes of healthy leaf material harvested late in the season from different plants in a single field. Each plant was scored for disease control on an ordinal scale of 1 (no protection / most severe symptoms) to 5 (greatest protection / no disease symptoms).

CFU per g	disease control	CFU per g	disease control
9.7e3	1	4.2e4	4
3.8e5	1	2.3e6	4
7.4e4	2	7.4e6	4
2.3e4	2	9.5e5	5
3.7e6	2	2.5e6	5
9.2e5	3	3.8e6	5
4.2e5	3	3.9e6	5
1.4e5	4	4.2e6	5

Since the disease control scale is discrete, the use of the K statistic based on Kendall's correlation coefficient is the appropriate method. The value of $Q_1 = 77$ and $Q_2 = 22$. There are no tied values in the CFU data, but the disease control data has numerous ties, and $N_t = 5.5$. Using these values, it can be shown that $K = 93$. By comparing this value to Table A.8, the correlation was found to be highly significant ($P < 0.005$). The corresponding coefficient $\tau = 0.55$, a value which indicates a moderate monotonic correlation. Thus, these data strongly suggest that a positive functional relationship exists between inoculant population size and disease control. However, that relationship is most likely confounded by other variables, a conclusion arising from the moderate coefficient value ($\tau < 0.7$).

2) and 3) Monotonic regression:

A researcher wishes to investigate the effect of nutrient addition on biodegradation of a pollutant in a contaminated soil. The nutrient solution and inoculant strain are mixed into soil microcosms containing 200 PPM of the pollutant. After two weeks of incubation the remaining pollutant is extracted from the soil and quantified. The addition of the nutrient solution alone indicated that no degradation of the pollutant occurred in absence of the inoculant (data not shown). The experimental data for microcosms inoclulated with a standard concentration of inoculant (10^6 cells per gram of soil) are tabulated below.

PPM nutrient	PPM pollutant	E(y)
1	192	199
2	184	194
5	196	189
7	198	187
10	167	177
20	140	162
50	115	138
70	181	115
100	64	105
200	104	71
500	52	59
700	14	54
1000	57	40
2000	20	26
3000	37	18

A scatter plot of the data shows a "noisy" downward trend, with the data becoming highly variable in the region where most of the decrease occurs (see Figure A.2). It would be improper to define the relationship *a priori*, because no single relationship seems to describe the entire data set. Since the relationship between nutrient amendment and degradation may not be constant over the range of applied nutrient concentrations, a monotonic regression procedure would be useful in identifying a range of values within which particular relationships (*e.g.* linear, logarithmic) exist. Thus, the procedure of Iman and Conover is an exploratory tool used to identify and describe monotonic patterns in data. In this instance, Spearman's correlation coefficient is $r_S = -0.91$, which is highly significant ($P < 0.01$). This indicates that there is, in fact, a strong negative trend in the data. Using this r_S value and a plot of $r(y)$ versus y, the expected y values are calculated and tabulated next to the observed values (see above). These values are then plotted against the nutrient concentration to generate the regression line in Figure A.2. The curve appears multiphasic over the range of nutrient concentrations tested, but it is observed to be roughly linear above 5 PPM of added nutrient. This result could be used to justify a range of values that should be examined in further investigations.

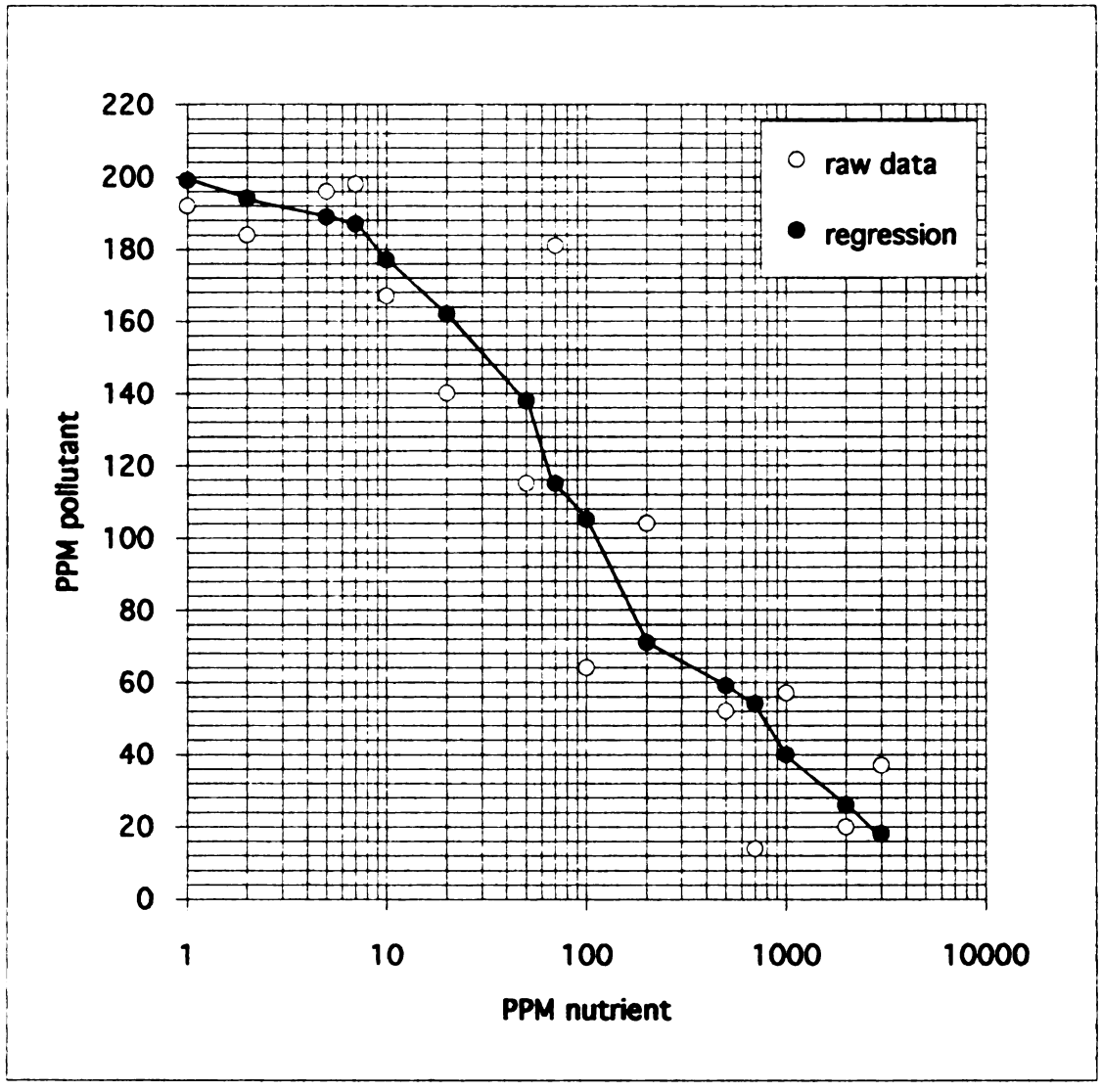

Figure A.2: Example monotonic regression curve.

Statistical Tables

The following tables were assembled following those presented in *Biostatistics: A Methodology for the Health Sciences* by L.D. Fisher and G. van Belle (3). More comprehensive tables for the tests described in this chapter can be found in other statistical texts (*e.g.* 25) or as separate volumes (*e.g.* 18).

Table A.3: Binomial tail probabilities. A P value is chosen to specify the confidence interval for the median of a sample population using the binomial sign test.

n	ν	P	n	ν	P	n	ν	P	n	ν	P
2	0	0.2500	8	3	0.3633	12	0	0.0002		13	0.0009
	1	>>>>		4	>>>>		1	0.0032		14	0.0001
	2	0.2500		5	0.3633		2	0.0193			
				6	0.1445		3	0.0730	15	0	0.0000
3	0	0.1250		7	0.0352		4	0.1938		1	0.0005
	1	>>>>		8	0.0039		5	0.3872		2	0.0037
	2	>>>>					6	>>>>		3	0.0176
	3	0.1250	9	0	0.0020		7	0.3872		4	0.0592
				1	0.0195		8	0.1938		5	0.1509
4	0	0.0625		2	0.0898		9	0.0730		6	0.3036
	1	0.3125		3	0.2539		10	0.0193		7	>>>>
	2	>>>>		4	>>>>		11	0.0032		8	>>>>
	3	0.3125		5	>>>>		12	0.0002		9	0.3036
	4	0.0625		6	0.2539					10	0.1509
				7	0.0898	13	0	0.0001		11	0.0592
5	0	0.0313		8	0.0195		1	0.0017		12	0.0176
	1	0.1875		9	0.0020		2	0.0112		13	0.0037
	2	>>>>					3	0.0461		14	0.0005
	3	>>>>	10	0	0.0010		4	0.1334		15	0.0000
	4	0.1875		1	0.0107		5	0.2905			
	5	0.0313		2	0.0547		6	>>>>	16	0	0.0000
				3	0.1719		7	>>>>		1	0.0003
6	0	0.0156		4	0.3770		8	0.2905		2	0.0021
	1	0.1094		5	>>>>		9	0.1334		3	0.0106
	2	0.3438		6	0.3770		10	0.0461		4	0.0384
	3	>>>>		7	0.1719		11	0.0112		5	0.1051
	4	0.3438		8	0.0547		12	0.0017		6	0.2272
	5	0.1094		9	0.0107		13	0.0001		7	0.4018
	6	0.0156		10	0.0010					8	>>>>
						14	0	0.0001		9	0.4018
7	0	0.0078	11	0	0.0005		1	0.0009		10	0.2272
	1	0.0625		1	0.0059		2	0.0065		11	0.1051
	2	0.2266		2	0.0327		3	0.0287		12	0.0384
	3	>>>>		3	0.1133		4	0.0898		13	0.0106
	4	>>>>		4	0.2744		5	0.2120		14	0.0021
	5	0.2266		5	>>>>		6	0.3953		15	0.0003
	6	0.0625		6	>>>>		7	>>>>		16	0.0000
	7	0.0078		7	0.2744		8	0.3953			
				8	0.1133		9	0.2120			
	0	0.0039		9	0.0327		10	0.0898			
	1	0.0352		10	0.0059		11	0.0287			
	2	0.1445		11	0.0005		12	0.0065			

Table A.4: Critical values for the Mann-Whitney *U* statistic.

		P							P				
na	nb	0.20	0.10	0.05	0.02	0.01	na	nb	0.20	0.10	0.05	0.02	0.01
3	2	6					10	2	17	19	20		
3	3	8	9				10	3	24	26	27	29	30
							10	4	30	33	35	37	38
4	2	8					10	5	37	39	42	44	46
4	3	11	12				10	6	43	46	49	52	54
4	4	13	15	16			10	7	49	53	56	59	61
							10	8	56	60	63	67	69
5	2	9	10				10	9	62	66	70	74	77
5	3	13	14	15			10	10	68	73	77	81	84
5	4	16	18	19	20								
5	5	20	21	23	24	25	11	2	19	21	22		
							11	3	26	28	30	32	33
6	2	11	12				11	4	33	36	38	40	42
6	3	15	16	17			11	5	40	43	46	48	50
6	4	19	21	22	23	24	11	6	47	50	53	57	59
6	5	23	25	27	28	29	11	7	54	58	61	65	67
6	6	27	29	31	33	34	11	8	61	65	69	73	76
							11	9	68	72	76	81	83
7	2	13	14				11	10	74	79	84	88	92
7	3	17	19	20	21		11	11	81	87	91	96	100
7	4	22	24	25	27	28							
7	5	27	29	30	32	24	12	2	20	22	23		
7	6	31	34	36	38	39	12	3	28	31	32	34	35
7	7	36	38	41	43	45	12	4	36	39	41	43	45
							12	5	43	47	49	52	54
8	2	14	15	16			12	6	51	55	58	61	63
8	3	19	21	22	24		12	7	58	63	66	70	72
8	4	25	27	28	30	31	12	8	66	70	74	79	81
8	5	30	32	34	36	38	12	9	73	78	82	87	90
8	6	35	38	40	42	44	12	10	81	86	91	96	99
8	7	40	43	46	49	50	12	11	88	94	99	104	108
8	8	45	49	51	55	57	12	12	95	102	107	113	117
9	2	16	17	18			13	2	22	24	25	26	
9	3	22	23	25	26	27	13	3	30	33	35	37	38
9	4	27	30	32	33	35	13	4	39	42	44	47	49
9	5	33	36	38	40	42	13	5	47	50	53	56	58
9	6	39	42	44	47	49	13	6	55	59	62	66	68
9	7	45	48	51	54	56	13	7	63	67	71	75	78
9	8	50	54	57	61	63	13	8	71	76	80	84	87
9	9	56	60	64	67	70	13	9	79	84	89	94	97
							13	10	87	93	97	103	106

Table A.4 (continued)

		P							P				
na	nb	0.20	0.10	0.05	0.02	0.01	na	nb	0.20	0.10	0.05	0.02	0.01
13	11	95	101	106	112	116	16	11	115	122	129	135	140
13	12	103	109	115	121	125	16	12	125	132	139	146	151
13	13	111	118	124	130	135	16	13	134	143	149	157	163
							16	14	144	153	160	168	174
14	2	23	25	27	28		16	15	154	163	170	179	185
14	3	32	35	37	40	41	16	16	163	173	181	190	196
14	4	41	45	47	50	52							
14	5	50	54	57	60	63	17	2	28	31	32	34	
14	6	59	63	67	71	73	17	3	39	42	45	47	49
14	7	67	72	76	81	83	17	4	50	53	57	60	62
14	8	76	81	86	90	94	17	5	60	65	68	72	76
14	9	85	90	95	100	104	17	6	71	76	80	84	87
14	10	93	99	104	110	114	17	7	81	86	91	96	100
14	11	102	108	114	120	124	17	8	91	97	102	108	112
14	12	110	117	123	130	134	17	9	101	108	114	120	124
14	13	119	126	132	139	144	17	10	112	119	125	132	136
14	14	127	135	141	149	154	17	11	122	130	136	143	148
							17	12	132	140	147	155	160
15	2	25	27	29	30		17	13	142	151	158	166	172
15	3	35	38	40	42	43	17	14	153	161	169	178	184
15	4	44	48	50	53	55	17	15	163	172	180	189	196
15	5	53	57	61	64	67	17	16	173	183	191	201	207
15	6	63	67	71	75	78	17	17	183	193	202	212	219
15	7	72	77	81	86	89							
15	8	81	87	91	96	100	18	2	20	32	34	36	
15	9	90	96	101	107	111	18	3	41	45	47	50	52
15	10	99	106	111	117	121	18	4	52	56	60	63	66
15	11	108	115	121	128	132	18	5	63	68	72	76	79
15	12	117	125	131	138	143	18	6	74	80	84	89	92
15	13	127	134	141	148	153	18	7	85	91	96	102	105
15	14	136	144	151	159	164	18	8	96	103	108	114	118
15	15	145	153	161	169	174	18	9	107	114	120	126	131
							18	10	118	125	132	139	143
16	2	27	29	31	32		18	11	129	137	143	151	156
16	3	37	40	42	45	46	18	12	139	148	155	163	169
16	4	47	50	53	57	59	18	13	150	159	167	175	181
16	5	57	61	65	68	71	18	14	161	170	178	187	194
16	6	67	71	75	80	83	18	15	172	182	190	200	206
16	7	76	82	86	91	94	18	16	182	193	202	212	218
16	8	86	92	97	102	105	18	17	193	204	213	224	231
16	9	96	102	107	113	117	18	18	204	215	225	236	243
16	10	106	112	118	124	129							

Table A.5: Critical values for the signed rank test using the Wilcoxon *T* statistic.

	P					P			
n	0.10	0.05	0.02	0.01	n	0.10	0.05	0.02	0.01
5	1				30	152	137	120	109
6	2	1			31	163	148	130	118
7	4	2	0		32	175	159	141	128
8	6	4	2	0	33	188	171	151	138
9	8	6	3	2	34	201	183	162	149
10	11	8	5	3	35	214	195	174	160
11	14	11	7	5	36	228	208	186	171
12	17	14	10	7	37	242	222	198	183
13	21	17	13	10	38	256	235	211	195
14	26	21	16	13	39	271	250	224	208
15	30	25	20	16	40	287	264	238	221
16	36	30	24	19	41	303	279	252	234
17	41	35	28	23	42	319	295	267	248
18	47	40	33	28	43	336	311	281	262
19	54	46	38	32	44	353	327	297	277
20	60	52	43	37	45	371	344	313	292
21	68	59	49	43	46	389	361	329	307
22	75	66	56	49	47	408	379	345	323
23	83	73	62	55	48	427	397	362	339
24	92	81	69	61	49	446	415	380	356
25	101	90	77	68	50	466	434	398	373
26	110	98	85	76					
27	120	107	93	84					
28	130	117	102	92					
29	141	127	111	100					

Table A.6: Critical values for the χ^2 distribution, which approximates the critical values for the *H* and *S* statistics.

	P						P				
df	0.10	0.05	0.025	0.010	0.001	df	0.10	0.05	0.025	0.010	0.001
1	2.71	3.84	5.02	6.63	10.83	26	35.56	38.89	41.92	45.64	54.05
2	4.61	5.99	7.38	9.21	13.82	27	36.74	40.11	43.19	46.96	55.45
3	6.25	7.82	9.35	11.34	16.27	28	37.92	41.34	44.46	48.28	56.89
4	7.78	9.49	11.14	13.28	18.47	29	39.09	42.56	45.72	49.59	58.30
5	9.24	11.1	12.83	15.09	20.52	30	40.26	43.77	46.98	50.89	59.70
6	10.64	12.59	14.45	16.81	22.46	35	46.06	49.80	53.20	57.34	66.62
7	12.02	14.07	16.01	18.47	24.32	40	51.81	55.76	59.34	63.69	73.40
8	13.36	15.51	17.53	20.09	26.12	45	57.61	61.66	65.41	69.96	80.08
9	14.58	16.92	19.02	21.67	27.88	50	63.17	67.50	71.42	76.15	86.66
10	15.99	18.31	20.48	23.21	29.59	55	68.80	73.31	77.38	82.29	93.17
11	17.27	19.68	21.92	24.72	31.26	60	74.40	79.08	83.30	88.38	99.61
12	18.55	21.03	23.34	26.22	32.91	65	79.97	84.82	89.18	94.42	105.99
13	19.81	22.36	24.74	27.69	34.53	70	85.53	90.53	95.02	100.43	112.32
14	21.06	23.68	26.12	29.14	36.12	75	91.06	96.22	100.84	106.39	118.60
15	22.31	25.00	27.49	30.58	37.70	80	96.58	101.88	106.63	112.33	124.84
16	23.54	26.30	28.85	32.00	39.25	85	102.08	107.52	112.39	118.24	131.04
17	24.77	27.59	30.19	33.41	40.79	90	107.57	113.15	118.14	124.12	137.21
18	25.99	28.87	31.53	34.81	42.31	95	113.04	118.75	123.86	129.97	143.34
19	27.20	30.14	32.85	36.19	43.82	100	118.50	124.34	129.56	135.81	149.45
20	28.41	31.41	34.17	37.57	45.31						
21	29.62	32.67	35.48	38.93	46.80						
22	30.81	33.92	36.78	40.29	48.27						
23	32.01	35.17	38.08	41.64	49.73						
24	33.20	36.42	39.36	42.98	51.18						
25	34.38	36.65	40.65	44.31	52.62						

Table A.7: Standard normal distribution tail probabilities, to be used in Dunn's procedure for multiple pair-wise comparisons.

	P			P	
z	two-sided	one-sided	z	two-sided	one-sided
0.60	0.5485	0.2743	2.10	0.0357	0.0179
0.65	0.5157	0.2578	2.15	0.0316	0.0158
0.70	0.4839	0.2420	2.20	0.0278	0.0139
0.75	0.4543	0.2266	2.25	0.0244	0.0122
0.80	0.4237	0.2119	2.30	0.0214	0.0107
0.85	0.3953	0.1977	2.35	0.0188	0.0094
0.90	0.3681	0.1841	2.40	0.0164	0.0082
0.95	0.3421	0.1711	2.45	0.0143	0.0071
1.00	0.3173	0.1587	2.50	0.0124	0.0062
1.05	0.2937	0.1469	2.55	0.0108	0.0054
1.10	0.2713	0.1537	2.60	0.0093	0.0047
1.15	0.2501	0.1251	2.65	0.0080	0.0040
1.20	0.2301	0.1151	2.70	0.0069	0.0035
1.25	0.2113	0.1056	2.75	0.0060	0.0030
1.30	0.1936	0.0968	2.80	0.0051	0.0026
1.35	0.1770	0.0885	2.85	0.0044	0.0022
1.40	0.1615	0.0808	2.90	0.0037	0.0019
1.45	0.1471	0.0735	2.95	0.0032	0.0016
1.50	0.1336	0.0668	3.00	0.0027	0.0013
1.55	0.1211	0.0606	3.05	0.0023	0.0011
1.60	0.1096	0.0548	3.10	0.0019	0.0010
1.65	0.0989	0.0495	3.15	0.0016	0.0008
1.70	0.0891	0.0446	3.20	0.0014	0.0007
1.75	0.0801	0.0401	3.25	0.0012	0.0006
1.80	0.0719	0.0359	3.30	0.0010	0.0005
1.85	0.0643	0.0322	3.35	0.0008	0.0004
1.90	0.0574	0.0287	3.40	0.0007	0.0003
1.95	0.0512	0.0256	3.50	0.0005	0.0002
2.00	0.0455	0.0228	3.60	0.0003	0.0002
2.05	0.0404	0.0202	3.70	0.0002	0.0001

Table A.8: Critical values for the *K* statistic based on Kendall's rank correlation coefficient.

	P						
n	0.20	0.15	0.10	0.05	0.01	0.005	0.001
3	3						
4	5	6	6	6			
5	8	8	9	9	10		
6	11	11	12	13	14	15	
7	14	15	16	17	18	20	21
8	18	19	20	22	23	25	26
9	23	24	25	27	28	31	33
10	28	29	31	33	34	37	40
11	34	35	37	39	41	44	47
12	40	42	43	46	48	52	55
13	47	49	51	53	56	61	64
14	54	56	58	62	64	69	73
15	62	64	67	70	73	79	83
16	70	73	75	79	83	89	94
17	79	82	85	89	93	100	105
18	89	91	95	99	103	111	117
19	99	101	105	110	114	123	129
20	109	112	116	121	126	135	142
21	120	123	127	133	138	148	156
22	132	135	139	146	151	161	170
23	144	147	152	159	164	176	184
24	156	160	165	172	178	190	200
25	169	173	179	186	193	205	216

Table A.9: Critical Table A.9: Critical values for Spearman's rank correlation coefficient, r_s.

	P				P		
n	0.10	0.05	0.01	n	0.10	0.05	0.01
5	0.900			20	0.377	0.450	0.570
6	0.829	0.886		21	0.368	0.438	0.556
7	0.714	0.786	0.929	22	0.359	0.428	0.544
8	0.643	0.738	0.881	23	0.351	0.418	0.532
9	0.600	0.700	0.833	24	0.343	0.409	0.521
10	0.564	0.648	0.794	25	0.336	0.400	0.511
11	0.536	0.618	0.755	26	0.329	0.392	0.501
12	0.497	0.591	0.727	27	0.323	0.385	0.491
13	0.475	0.566	0.703	28	0.317	0.377	0.483
14	0.457	0.545	0.675	29	0.311	0.370	0.475
15	0.441	0.525	0.654	30	0.305	0.364	0.467
16	0.425	0.507	0.635				
17	0.412	0.490	0.615				
18	0.399	0.476	0.600				
19	0.388	0.462	0.584				

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