Chapter 4
Plant Genetics for Study of the Roles of Root Exudates and Microbes in the Soil

Aparna Deshpande, Ana Clara Pontaroli, Srinivasa R. Chaluvadi, Fang Lu, and Jeffrey L. Bennetzen

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A. Deshpande
Department of Biological Sciences, Purdue University, West Lafayette, IN 47906, USA
and
Department of Biological Sciences, Houghton, MI 49931, USA

A.C. Pontaroli
Department of Genetics, University of Georgia, Athens, GA 30602, USA
and
Estación Experimental Agropecuaria Balcarce, Instituto Nacional de Tecnología Agropecuaria (INTA) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Balcarce CC 276 (7620), Argentina

S.R. Chaluvadi and F. Lu
Department of Genetics, University of Georgia, Athens, GA 30602, USA

J.L. Bennetzen (✉)
Department of Biological Sciences, Purdue University, West Lafayette, IN 47906, USA
and
Department of Genetics, University of Georgia, Athens, GA 30602, USA
and
Department of Biological Sciences, Houghton, MI 49931, USA
e-mail: maize@uga.edu

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4.1 Introduction

Although plants can be grown in sterile soil in aseptic growth chambers, their natural lives involve an intense and intimate interaction with a vast number of microbes, especially those found in soils. The number of different bacterial species in a single gram of soil has been estimated to be anywhere from a few thousand to many millions, depending on the soil source and the method of analysis (Foster 1988; Schloss and Handelsman 2006; Aislabie et al. 2008), with still-undescribed species making up a large share of the total. In addition to eubacteria and archaebacteria, many species of fungi, protists, and algae are also found in the soil, often in association with plant roots. The great majority of these soil microbes have not been studied to any significant degree, partly because conditions for their axenic culture have not been developed. For instance, only 26 of the approximately 52 identified major lineages, or phyla, within the domain Bacteria have cultured representatives. In fact, it is estimated that less than 1% of the bacterial species in the soil could be grown in culture with current approaches (Leadbetter 2003; Handelsman 2004; Leveau 2007), and this number is certain to be much lower if one considers that most rare microbial components of the soil are completely unknown.

Plants actively secrete very large quantities, and a great diversity, of organic compounds into the soil. Exudation of anywhere from 5 to 60% of total photoassimilate has been reported and found to be highly variable across environmental conditions (e.g., soil type, time of day, soil moisture, temperature) and plant genotype or growth stage (Bekkara et al. 1998; Groleau-Renaud et al. 1998; Hughes et al. 1999; Iijima et al. 2000; Aulakh et al. 2001; Garcia et al. 2001; Prosser et al. 2006). The roles of only a few of these compounds are known or guessed at (Merbach et al. 1999). Citrate is secreted, sometimes in very large quantities, to help acidify the soil and thereby promote root growth (Jones and Darrah 1994; Hinsinger et al. 2006), and this compound also helps bind aluminum in the soil, thereby decreasing its phytotoxic effects (Hoekenga et al. 2003). Some plants have been shown to exude phenolic compounds that exhibit allelopathic effects like the sorghum exudate sorgoleone that is an inhibitor of broadleaf and grass weeds at concentrations as low as 10 μM in hydroponic assays (Nimbal et al. 1996). Many other compounds, such as amino acids and sugars, are believed to be secreted by plant roots in order to promote rhizosphere microbial growth (Brimecombe et al. 2001), although the value to the plant of $\ll$1% of the rhizosphere microbes are not known in any system. Specific secreted phenolic compounds have been shown to be signal molecules that attract root colonization by useful microbes, nitrogen-fixing bacteria such as *Rhizobium*, and mycorrhizal fungi (reviewed in Bais et al. 2006).

The question remains, what do most of these soil microbes do? The active secretion of so much of the fixed carbon produced by a plant suggests that these microbes are very important to the plants, but this idea is challenged by the observation that plants can grow efficiently in sterile soil. Of course, plants that are grown with fertilizers in a controlled environment do not need symbiotic relationships that yield limiting growth substances, like the fixed nitrogen provided

by rhizobia or the phosphate access provided by mycorrhizae. Perhaps, a more frequent value of rhizosphere microbial associations to a plant is exemplified in the "take-all" disease, where the *Gaeumannomyces graminis* var. *tritici* fungus that infects wheat roots is overcome in the soil by a beneficial bacterial competitor, a specific isolate of *Pseudomonas fluorescens* (Thomashow and Weller 1988; Capper and Higgins 2007). Unlike sterile soil, potential microbial pathogens in field soil may exist in staggering numbers and variety, and only attraction of beneficial or neutral microbial competitors of these pathogens to the rhizosphere would provide comprehensive protection to host plants.

In the absence of the ability to grow most soil microbes in pure culture, it is difficult to test their possible contributions to plant growth or plant disease. One cannot simply inoculate the soil with a single microbe and see its effects on a potential host plant if one cannot first grow that microbe. However, we have postulated that we can use our control over host plant genetics to accomplish the same goals of understanding the roles of microbes in the soil (Deshpande 2006). If one can find mutations in plants, or segregating natural variation, which determines the presence/absence or abundance of specific rhizosphere microbes, then this demonstrates a specific relationship between the product of the mutated or varying plant gene(s) and the biology of the affected microbe. For instance, if one finds a natural variation for a low level of sorgoleone production, and sees that this causes the root to no longer be colonized by mycorrhizae, then this indicates that sorgoleone is involved in mycorrhizal colonization (Akiyama et al. 2005).

We have been pursuing this approach to use plant host genetics to dissect plant–microbe interactions in the soil for the last 10 years. This research has proceeded very slowly because of the need to establish a foundation for the experiments, a very limited tool set, a challenging level of environmental variation in the experiments, a surprisingly low level of plant genetic variation for rhizosphere exudates (at least in *Arabidopsis thaliana*, see below), and the lack of funding for such research in the absence of compelling preliminary results. However, recent advances in DNA sequencing technology have offered the possibility that studies of plant genetic control of microbial interactions in the rhizosphere and root can be analyzed comprehensively. This chapter describes our initial results with the genetic and metagenomic analysis of these interactions.

4.2 Natural Variation and Mutagenesis in *Arabidopsis* to Identify Alterations in Root Exudate

We used a model dicot angiosperm, *Arabidopsis thaliana*, as a target for our initial studies of plant host genetic effects on rhizosphere microbial populations. Because high pressure liquid chromatography (HPLC) is such a powerful technique to separate and display low molecular weight organic compounds like phenolics, we decided to determine reproducible conditions for exudate production by the roots of *Arabidopsis* seedlings by scoring the production from seedlings grown under sterile

conditions. Seeds were first surface-sterilized by gently agitating them in a solution containing two volumes of 0.1% Triton X-100 and one volume bleach. Seedlings were grown on filter paper set atop moist glass beads for 15 days in Gamborg's B5 medium at a temperature of 24°C and an artificial light intensity of 100 μE m^{-2} s^{-1}. On day 15, fresh Gamborg's B5 medium diluted to 5% of its original concentration was added to the roots, and the medium (now with root exudates) was collected after 2 days of additional growth. Pools of ~100 seedlings were grown together in single vials for this analysis because smaller numbers of seedlings did not yield sufficient quantities of exudates for HPLC analysis. The liquid samples were frozen and dried in a Beckman lyophilizer and then resuspended in 98% methanol for reverse phase HPLC analysis. Under these conditions, a broad array of peaks representing different compounds were observed, and these were not produced by dead seeds or the growth media in the absence of growing seedlings (Fig. 4.1). Many of these peaks were found not to be reproducible from experiment to experiment, however, so a smaller range of peaks was chosen for specific focus. These six peaks gave qualitatively consistent profiles detected at 360 nm (Fig. 4.2). These peaks were both consistent across experiments and had the general properties of phenolics and related compounds that were good candidates as signal molecules.

Having established a reproducible assay system, we then looked at *A. thaliana* ecotypes Columbia, Landsberg *erecta*, Kashmir-1, Wassilewskeja, and Cape Verde Islands (CVI) for their root exudation of related compounds. Surprisingly, we saw no dependable variation for the compounds represented by these six peaks on the HPLC chromatogram. The ecotype CVI was included in this study because, at the level of DNA markers, it was the most different of any *Arabidopsis* ecotype available at that time. Hence, it was not possible to map genes responsible for variation in these compounds in any of the various mapping populations developed in *Arabidopsis* from crosses between these ecotypes.

Having failed to detect useful natural variation for exudate production, we next investigated the production of the compounds represented by these six peaks in EMS mutagenized Columbia and Landsberg *erecta* backgrounds, with M3 seed provided by Lehle Seeds. Most surprisingly, out of 2,000 M3 populations analyzed, not a single reproducible variation in any of these peaks was identified. Given the mutation rate in these EMS populations, we expected that 2,000 M3 would have provided an average of 1–2 homozygous and 3–4 segregating knockout mutations per gene for every gene in the *Arabidopsis* genome. Hence, for the first time in the history of genetics, we apparently identified a series of biological processes to produce numerous compounds that are not affected by mutational inactivation of any single gene. This astounding result remains unexplained.

Because it was expected that many of the compounds in the studied six peaks were phenolics, we also looked at known mutations in phenolic pathways, including knockout mutations in *fad1-2*, *fae2-1*, *gsm1-1*, *gsm1-2*, *hy5-1*, *mur2-1*, *mur4-1*, *mur5-1*, and *rhd1-1*, plus the double mutants *fah1-7*/*tt3-1*, *tt3-1*/ *tt7-1*, and *tt4-1*/*tt5-1* (Koornneef 1990; Lemieux et al. 1990; Haughn et al. 1991; Miquel and Browse 1992, Reiter et al. 1997) obtained from the ABRC. In addition, a line exhibiting transgenic *F5H* overexpression, generously provided by the laboratory of Dr. Clint

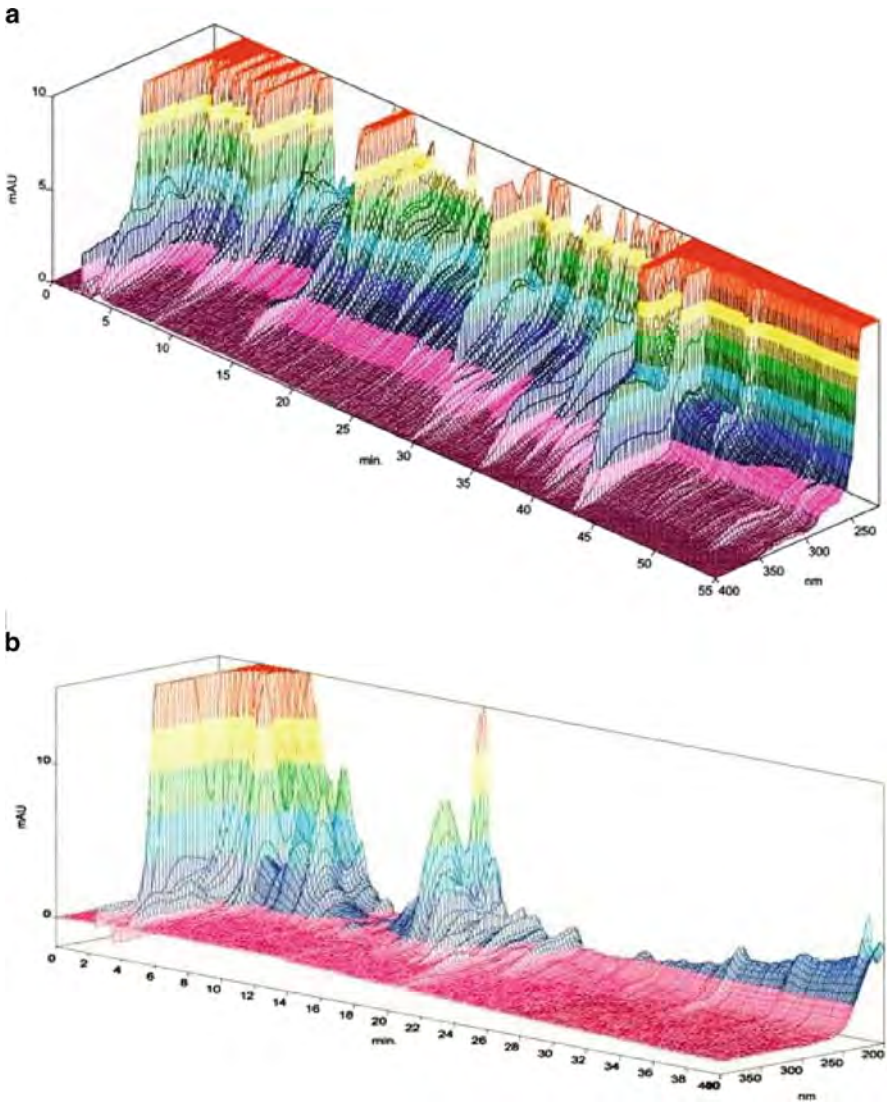

Fig. 4.1 A three-dimensional metabolite profile of root exudates showing the retention time (*X*-axis), peak intensity (*Y*-axis), and the UV range of 200–400 nm (*Z*-axis). (**a**) Root exudates of wild-type *Arabidopsis thaliana* plants, ecotype Columbia; (**b**) negative control (growth media processed as exudate)

Chapple (Purdue University), was also investigated. *Arabidopsis* lines that were mutant in these genes were found to not exhibit any qualitative changes in the six putative phenolic peaks that we focused on throughout this project.

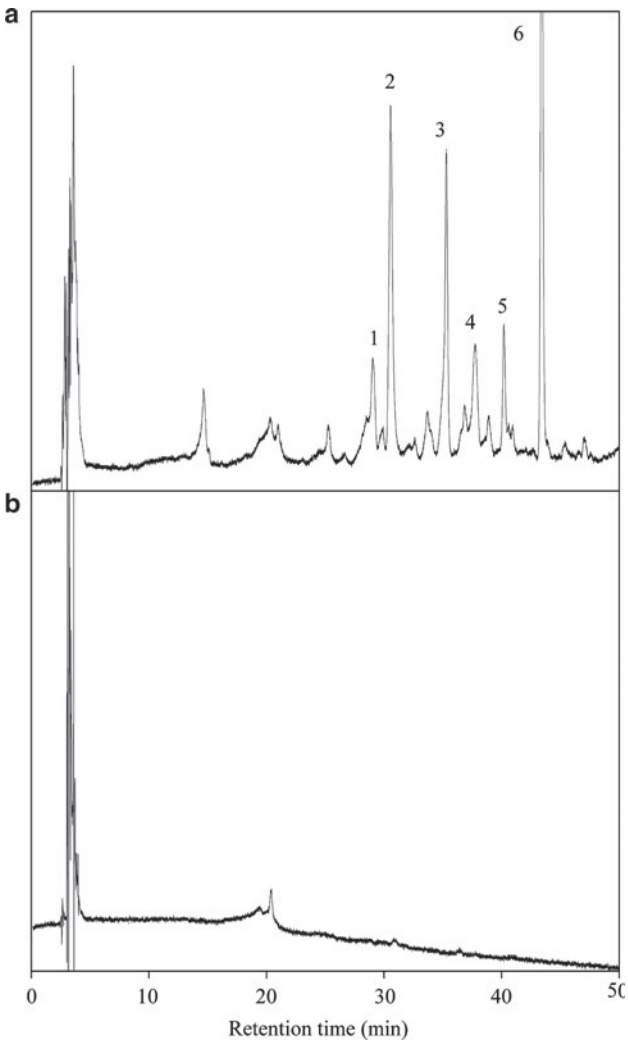

Fig. 4.2 Chromatograms of wild-type *Arabidopsis thaliana* root exudates showing the six major peaks detected at 360 nm. (**a**) Ecotype Columbia; (**b**) negative control

The rationale of the *Arabidopsis* studies had been to identify genetically determined exudate variation and then to follow this up with the characterization of both the exudate compound(s) affected and the degree to which this variation altered soil microbial populations associated with the *Arabidopsis* root. In the absence of identified genetic variation, such follow-up studies were not performed.

4.3 Plant Genetic Determination of Natural Variation in Rhizosphere and Root-Associated Microbes in the Grasses

After arriving at the University of Georgia (UGA) in 2003, our lab decided to look at several grass species as targets for the study of root–microbe interactions. These studies have not yet involved exudates analysis but went directly to a metagenomic analysis of soil microbes. The soil used was from different UGA fields, but each experiment involved mixing one field soil source with a uniform potting mixture (to make roots easier to subsequently extract) and then placing equal amounts of this mixture in each large pot used in the experimental study. Seeds for host plants were germinated in these soils, and seedlings were then grown in the greenhouse under the same conditions for each duplicated or triplicated plant in the experiment. The assay system has been to sequence either total DNA or 16s ribosomal DNA amplicons prepared from the soil that clings to an extracted root ("rhizosphere" or Rh), the microbes firmly attached to a root washed with water ("root-external microbes" or REM), and the microbes remaining after the root is treated with chitinase, lysozyme, and various levels of hydrogen peroxide ("root-internal microbes" or RIM). Of course, the sample termed REM contains both root-internal and root-external microbes, while the Rh sample is certainly contaminated by broken root fragments that would yield some root-internal and root-external microbes.

In order to guarantee that the DNA analyzed would provide a comprehensive description of the microbes that were present, a vigorous DNA extraction protocol (http://fgp.bio.psu.edu/methods/ctab.html) was followed. Hence, the DNA extraction procedure for Rh, REM, and RIM samples yields not just the microbial DNA but also DNA from any other organisms or tissue fragments that were present in the sample. Especially in the case of the REM and RIM samples, this meant that there was a tremendous amount of host plant DNA present. Hence, random shotgun sequencing of all root-associated samples was mostly an exercise in sequencing the host plant genome, with yields of 10–20% of cloned DNA (Table 4.1) that was verified as nonplant. At the time of these analyses, neither the sorghum nor maize genomes had been fully sequenced, so many of the sequences labeled unknown could be screened for homology to these genomes once the ongoing sequencing projects are completed. Regardless, it was clear that this was an expensive route to pursue for metagenomic discovery.

Because the majority of maize nuclear DNA is methylated at the cytosines in 5′-CG-3′ and 5′-CNG-3′ sequences, we decided in one experiment to transform all of our soil DNA into DH5-α because cytosine methylated DNA such as that seen in maize and other grasses is often destroyed by this *Escherichia coli* strain (Palmer et al. 2003). Sequences of the resulting clones provided a significant decrease in maize DNA, and a significant increase in the percent of bacterial sequences recovered (Table 4.1) but decreased the amount of mycorrhizal DNA that was observed (data not shown). Hence, this potential metagenomic enrichment technology

Table 4.1 Shotgun sequence analysis of DNA from the plant–soil interface

Host plant species	Microbial fraction targeted[a]	Seq, type[b]	Number of sequences	Percent of sequences with highest homology to DNA from the listed organisms									Unknown
				Host plant sequences	Moss	Eubact.	Archaea	Fungi	Protist	Diatom	Animal	Phage	
Zea mays	Rh+REM+RIM	RS	732	88.3	–	3.7	–	2.2	–	0.1	0.4	–	5.3
		MF	218	30.2	–	48.6	0.5	6.9	–	0.5	–	–	13.3
	REM+RIM	RS	259	89.2	–	1.9	–	4.6	–	–	0.8	–	3.5
		MF	249	34.1	–	44.6	–	6.0	–	–	0.8	–	14.5
	RIM	RS	382	89.3	–	1.1	–	1.3	0.3	–	–	–	8.1
		MF	176	43.7	–	46.0	–	2.8	–	0.6	1.7	0.6	4.6
Sorghum bicolor	Rh+REM+RIM	RS	366	88.3	–	4.6	–	0.5	–	–	–	–	6.6
		MF	95	48.4	2.1	36.8	–	4.2	–	–	–	–	8.4
	REM+RIM	MF	84	76.2	–	13.1	–	3.6	–	–	–	–	7.1
	RIM	MF	83	44.5	–	30.1	–	1.2	–	–	1.2	–	22.9
Sorghum propinquum	Rh+REM+RIM	RS	352	80.7	–	5.1	–	1.2	–	–	–	–	13.1
		MF	89	58.4	–	12.4	–	3.4	–	–	–	–	25.9
	REM+RIM	MF	83	60.2	1.2	24.1	–	–	–	–	–	–	14.5
	RIM	MF	85	51.8	–	21.2	–	1.2	–	–	–	–	25.9

[a]*Rh + REM + RIM* rhizospheric + root-external + root-internal microbes, *REM + RIM* root-external + root-internal microbes, *RIM* root-internal microbes

[b]*RS* random shotgun, *MF* methyl-filtered

was abandoned because it was not likely to yield a representative description of the microbes present in the soil, rhizosphere, or root samples. We also abandoned the hydrogen peroxide treatment in our RIM purification process because the level of treatment that we employed (2 min in 3% H_2O_2) appeared to lead to degradation of some DNA inside roots (data not shown). Moreover, although hydrogen peroxide treatment greatly lowered the number of sequences that were recovered from the extracted DNA, it did not show any obvious effect upon the relative abundances of classes of eubacteria that were recovered (data not shown). Hence, further investigation of hydrogen peroxide treatment, to identify an appropriate level of exposure for removing external microbes without damaging root-internal DNA, is warranted but may not be necessary.

Our first experiments were on the plant species *Zea mays* (maize), *Sorghum bicolor* (sorghum), and *S. propinquum* (a wild and interfertile relative of sorghum). The results with random shotgun sequencing of Rh, REM, and RIM microbes (Table 4.2) indicated that sequences representing many different kingdoms and phyla of microbes (archaebacteria, eubacteria, fungi, protists), small animals (e.g., nematodes and insects), mosses, and even a bacteriophage were present in the data, although most of the sequences were either from the host plant or of unknown origin. Interestingly, the organisms in the RIM sample (presumed root-internal microbes) included protists like *Cercozoa* (a flagellate protozoan that consumes bacteria) and the diatom *Thalassiosira*. These DNA sequences were annotated in early 2009, when internal funding for this project was exhausted, so reannotation at this date would be much more informative because additional plant sequences could be identified, and more of the unknown sequences would be attributed to many of the additional microbes that have been sequenced since that time.

For reasons of cost effectiveness, we decided to primarily switch to the standard process for amplification of rRNA genes (Weisburg et al. 1991; Tringe and Hugenholtz 2008) for microbe identification. This has the disadvantage of a potential for differential degrees of amplification of different sequences (thus providing a skewed quantitative description of the microbes present) and the possible lack of amplification of highly diverged microbes. For cost reasons with the maize and sorghum samples, only a few eubacterial rRNA sequences were investigated, providing between 173 and 191 eubacterial reads per duplicated data set. Even with this limited amount of data, certain patterns were clear. The most abundant eubacteria both outside and inside roots were from the class betaproteobacteria, although the deltaproteobacteria were about equally abundant in the REM (root external) samples for both *S. bicolor* and *S. propinquum* (data not shown).

Table 4.2 Analysis of soil and root-associated organisms with 16s, 17s, and 18s rRNA sequences in switchgrass cultivar "Alamo"

Species	Treatment	Eubact. phylotypes	Archaea phylotypes	Fungal phylotypes	Protist phylotypes	Animal phylotypes
Switchgrass	Rh	668	13	37	19	46
Switchgrass	REM	409	3	53	6	5
Switchgrass	RIM	284	2	50	8	8

The alphaproteobacteria and gammaproteobacteria were also relatively abundant in both REM and RIM samples. Such species as the acidobacteria, bacilli, chloroflexi, clostridia, and deinococci were found both in REM and RIM samples but at low abundances. The sphingobacteria were of moderate abundance in the REM samples, but much rarer in the root-internal samples (RIM). Most dramatic, the *Sorghum* samples (especially *S. propinquum*) had a >2X lower percentage of eubacteria from known classes compared to maize, suggesting that a greater number/variety of exotic microbes associate with the roots of plants in the genus *Sorghum* than with maize.

Recently, we have begun studies of the microbial populations associated with the candidate biofuel crop called switchgrass (*Panicum virgatum*). In our first experiments, we have observed that the Rh, REM, and RIM populations for switchgrass are quite distinct (Table 4.2). For instance, archaebacteria were very abundant in the soil sample employed and frequent in the Rh populations but were very rare in the REM and RIM samples. As seen with maize and sorghum previously, mycorrhizal DNA was greatly enriched within the roots (the RIM samples). In general, bacterial, archaebacterial, protist, and animal diversity dropped off dramatically on and inside the roots compared to the rhizosphere, but detected fungi were actually more diverse both on and inside roots compared to the rhizosphere (Table 4.2). Preliminary results indicate that different switchgrass cultivars yield very different abundances for some microbial species (data not shown), suggesting that host genetics might be used to characterize the factors that determine the specific host–microbe associations involved.

4.4 Implications and Perspectives

The relationship between plant growth and soil microbes remains one of the great mysteries in the life sciences. Other than nitrogen fixation by root-internal or root-associated bacteria (Elbeltagy et al. 2001), only a few cases are known where a soil microbe provides some benefit to an associated plant (Thomashow and Weller 1988; Bais et al. 2006; Capper and Higgins 2007; Javot et al. 2007; Evelin et al. 2009). However, the tremendous contribution of photosynthate and a great variety of apparent signaling compounds that are actively released into the soil by roots indicate that most rhizosphere microbes are intentionally attracted by the plant. The simplest model for the role(s) of these microbes is protection from disease caused by that subset of microbes or animals in the soil that can pathogenize or parasitize plants via their roots. It is striking that the species diversity of microbes in the soil is orders of magnitude greater than that available to the aerial parts of the plants, yet soil-vectored/root-targeted pathogens of plants are relatively rare compared to those that infect above the ground. In one very preliminary experiment, we observed that greenhouse-grown maize, sorghum, and sunflower were slightly less vigorous if grown on field-derived soil than they were on sterilized field soil. Least healthy of all were plants grown on the same field soil that had been treated

with erythromycin, a broad-spectrum antibiotic that should have killed many of the eubacteria, suggesting that these bacteria provide some nutrients or protection from other microbes in the soil.

The most surprising results in this study were that no *Arabidopsis* mutants were identified for exudate production. There exists the very trivial explanation that the stocks that we obtained were not actually mutagenized. It is also possible (however unlikely) that every one of these exudates compounds is synthesized by enzymes and regulated by proteins that are encoded by redundant genetic pathways. The lack of natural variation in exudate production by *Arabidopsis* was also a surprise, and it reinforces the idea that these compounds are so important that their composition and approximate levels are fixed within the species. However, a recent study has found that two *Arabidopsis* ecotypes in our study (CVI and Landsberg *erecta*) were quite different in their exudates profile, and that this strongly affected rhizosphere microbial composition (Micallef et al. 2009). We have no explanation for the dramatic difference in conclusions about exudate variability between our results and those of Micallef and coworkers, other than the differences in the exudate assay systems employed. It has also been recently observed that some ATP-binding cassette (ABC) transporter mutants of *Arabidopsis* lead to altered root secretion of phytochemicals and significantly altered fungal and bacterial communities in the rhizosphere (Badri et al. 2009). It is puzzling that such mutations were not detected in our experiments.

The much-greater diversity of microbes outside the root compared to on the root (REM) and inside the root (RIM) suggests that there is a much greater diversity of environments and niches to fill in the soil than within a plant. The absence of archaebacteria from inside the roots makes sense, given the facts that the great majority of archaebacteria are extremophiles and that plants (like all other organisms) attempt to maintain a consistently moderate internal environment that is necessary for the physiology associated with efficient growth and development.

The most promising results to date are the differences observed in microbial populations associated with different cultivars of switchgrass. The tetraploidy and near-obligate outcrossing nature of this grass species makes it ideally unsuited for genetic dissection of any trait, including plant determination of soil microbial populations. Nonetheless, a perennial plant like switchgrass is particularly dependent on a durable and very efficient root system, so studies in the switchgrass rhizosphere are important. However, if funding were available, such studies would probably move much more rapidly if performed in diploid grasses with excellent genetics, such as maize, rice, or the close switchgrass relative called foxtail millet (*Setaria italica*) (Doust et al. 2009).

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