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A comparison of microbial communities in soil with and without a sugarcane cropping

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A COMPARISON OF MICROBIAL COMMUNITIES IN SOIL WITH AND WITHOUT A SUGARCANE CROPPING HISTORY

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DEDICATION

I dedicate this work to my family for their love, support, and for their understanding of the times that school and research took precedence over them.

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ABSTRACT

Sugarcane (inter-specific hybrids of Saccharum) is grown largely under long-term monoculture production in Louisiana. This can lead to a complex problem termed "yield decline" that results in poor root health and reduced productive capacity of sugarcane. This problem has been documented to be a limiting factor for sugarcane production in diverse regions, including Louisiana, Hawaii, Jamaica, and Australia. Previous work showed that biological factors affect root health and contribute to yield decline. The objectives of this study were to increase our understanding of microbial communities in sugarcane soils, to determine if there are differences in microbial communities associated with sugarcane roots in soil with and without a sugarcane cropping history, and to provide information on possible changes in the microbial communities resulting from monoculture that may contribute to yield decline.

To achieve these objectives, two approaches were used for comparing culturable organisms in soil microbial communities from soil with and without a sugarcane cropping history, and methods were adapted to reliably obtain DNA from soil microbial communities for molecular comparisons. In one approach, colonies grown on different types of culture media were quantified and characterized. In the other approach, sole carbon source utilization profiles (SCSUP) of soil communities grown in Biolog™ GN2 microplates were compared. Comparisons of the numbers and types of microorganisms that grew on various culture media demonstrated that differences exist between microbial communities associated with sugarcane roots in Louisiana soils with and without a recent sugarcane cropping history. The differences in community functional diversity detected by SCSUP supported the differences found in types of microorganisms isolated on selective media. The SCSUP results showed that differences in community functional diversity exist between sites in soils with a long-term sugarcane cropping history in common.

Methods for DNA extraction and polymerase chain reaction (PCR) amplification were optimized for sugarcane soil microbial community samples from Louisiana. This will allow molecular characterization of sugarcane rhizosphere microbial communities in the future.

CHAPTER 1. INTRODUCTION

Sugarcane (inter-specific hybrids of *Saccharum*) is grown largely under long-term monoculture production in Louisiana. Sugarcane is vegetatively propagated. Therefore, sugarcane cultivars are clones composed of genetically identical plants. Buds on planted stalk sections germinate and grow to produce the first "plant cane" crop. Following harvest of the stalks, buds on the stubble remaining in the soil can germinate to produce a ratoon crop. In Louisiana, sugarcane plants are typically in the field continuously for 3-5 years. The stubble is then plowed out during the spring, and, following a 4-6 month plant-free fallow period, the crop is replanted during late summer.

Monoculture can lead to poor root health and growth constraints in many crops, including sugarcane (Garside, et al., 1997; Magarey, 1996; Savory, 1966). A complex problem termed "yield decline" was defined by Garside, et al. in 1997, as "the loss of productive capacity of soils under sugarcane monoculture." This problem has been documented to be a important limiting factor for sugarcane production in diverse regions, including Louisiana, Hawaii, Jamaica, and Australia (Hoy, 1999; Magarey, 1996; Martin, et al., 1959; Metcalfe and Leather, 1963).

Soilborne pathogens, such as *Pythium* and nematode species, have been shown to adversely affect sugarcane growth and yield in Louisiana (Bond, et al., 2000; Cooper and Chilton, 1950; Edgerton, et al., 1929; Hoy and Schneider, 1988). However, evidence has suggested that additional biological factors may affect root health and contribute to yield decline in sugarcane. This possibility was suggested by results obtained by the following experimental methods: 1) biocide studies, in which selective fungicides, such as metalaxyl (selective for *Pythium*) or nematicides, did not completely alleviate poor root growth, whereas fumigants and dithiocarbamate broad-spectrum fungicides, mancozeb, maneb, and zineb, did, and 2) soil heat-treatments, in which soil solarization increased plant growth (Garside, et al., 1997; Magarey, 1996; Muchow, et al., 1994).

Soil fumigation studies using methyl bromide, chloropicrin-methyl bromide or metham sodium have resulted in increased sugarcane growth and yield in fumigated soil compared to non-fumigated soil (Croft, et al., 1984; Magarey, 1996; Martin, et al., 1959). Thus, fumigation has been used to benchmark potential yields and to reveal root health problems. The response to soil fumigation by many other crops, such as corn (Turco, et al., 1990), barley (Olsson and Gerhardson, 1992), apples (Mai and Abawi, 1981), strawberries (Wilhelm, et al., 1961), tomatoes (Jones, et al., 1966), and citrus (Sleeth, 1958) indicate that soil-based growth constraint can occur in many crops. In addition, growth increases on previously non-cropped lands by crops, such as sugarcane, corn, citrus, peaches, and apples, indicate that the occurrence of soil-based growth constraints are widespread (Magarey, 1999; Savory, 1966; Turco, et al., 1990; Wensley, 1956).

In the past, culture methods involving the use of culture media designed to enhance the growth of many or selected species were used to investigate soil and rhizosphere microorganism communities. It was later estimated that 0.01% to 10% of microorganisms in soil were capable of growth by cultivation (Olsen and Bakken, 1987; Richaume, et al.,

1993; Torsvik, et al., 1990). Therefore, a bias existed in soil microbial community studies because only microorganisms capable of growth under artificial conditions were being evaluated and not the total community. Count data obtained with fluorescence microscopy revealed that the majority of the non-colony-forming species in soil belonged to a group with very small cell size and high numbers and that the majority of the colony-forming species belonged to a group with large cell size and low numbers (Olsen and Bakken, 1987). It was suggested that the size of soil bacterial cells plays a role in their ability to grow in culture and that the ecological significance of colony-forming cells may be great despite their low numbers. The researchers further suggested that the small size and slow metabolism of the noncolony-forming species implied that their energy requirement represented a small fraction of the energy in the soil ecosystem (Olsen and Bakken, 1987). As long as researchers remain aware of the limitations, selective media methods can be used to evaluate culturable species as a component of soil microbial diversity (Dunbar, et al., 1999; Johnson and Curl, 1972; Olsen and Bakken, 1987; Sørheim, et al., 1989).

To study the soil microbial communities associated with sugarcane rhizosphere and non-rhizosphere soils with a long-term sugarcane cropping history (SCH) and no recent sugarcane cropping history (NSCH), it would be desirable to compare quantitative and qualitative aspects of microorganisms isolated on a non-selective medium and different media selective for groups of organisms. Soil dilution plating can establish countable colony numbers, and colony-forming units (cfu) per gram of soil can be calculated. A portion of the isolates can then be collected for identification to make qualitative comparisons of communities.

For total culturable bacterial enumeration, 10% tryptic soy agar (Difco, Becton-Dickinson, Sparks, MD) is widely used (Dunbar, et al., 1999; Johnson and Curl, 1972; Martin, 1975; Mazzola and Gu, 2000). Studies have shown that *Pseudomonas* species found in association with the rhizosphere can play either a beneficial or deleterious role in plant growth and crop yields. *Pseudomonas* species have played beneficial roles as biocontrol agents of rhizosphere associated phytopathogens, including Fusarium wilts on tomato (Khan and Khan, 2001), Rhizoctonia damping-off on cucumber roots (Kwok, et al., 1987), and *Gaeumannomyces graminis* take-all of wheat (Smiley, 1979). Some *Pseudomonas* spp. have been found to be plant pathogenic to lucerne, oats, and wheat (Ghosh, et al., 2001; Simpfendorfer, et al., 2001). Nehl, et al. (1997) determined that *Pseudomonas* spp. along with other rhizobacteria can fluctuate from being deleterious to plant growth promoting depending on environmental conditions, host genotype and mycorrhizal status. Therefore, total and fluorescent culturable bacterial populations in Louisiana sugarcane soils were evaluated with two selective media: King's B (King, et al., 1954) and Pseudomonas agar (Sigma, St. Louis, MO) were used.

It has been reported that phytopathogenic *Pseudomonas syringae* strains produce siderophores, which complex with ferric iron to provide themselves with iron under iron limited conditions and to gain a competitive advantage over other microorganisms lacking these siderophores (Bultreys, et al., 2001). Kloepper, et al. (1980) suggested that competition for iron is involved in biological control of plant diseases. In this case,

beneficial *Pseudomonas* spp. produce extracellular siderophores which place the phytopathogens at a disadvantage because they are unable to acquire iron. Therefore, chrome-azurol sulfate medium was used in this study to enumerate culturable total and siderophore producing bacteria (Schwyn and Neilands, 1987).

Actinomycetes produce antibiotics that are antagonistic to a number of different microorganisms, including some plant pathogens; however, some actinomycetes are phytopathogens themselves (Rouatt and Katznelson, 1961; Waksman, 1967). Therefore, total colonies developing on actinomycetes agar (Difco, Becton-Dickinson, Sparks, MD) were enumerated and compared for soil with and without a sugarcane cropping history.

Other phytopathogenic fungi besides *Pythium* have been demonstrated to be associated with sugarcane roots, including *Pachymetra* (Croft, et al., 1984), *Fusarium*, *Trichoderma*, *Rhizoctonia*, *Marasmius*, *Monilia*, *Collectotrichum*, *Phialophora*, *Humicola*, and *Thielaviopsis* (Watanabe, et al., 1974). Conversely, some fungal species can play beneficial roles against root phytopathogens. *Trichoderma* has been found to be antagonistic to *Pythium* spp. in sugarcane soils (LeBeau, 1938), and *Gliocladium virens* (Tu and Vaartaja, 1981) has been shown to be antagonistic to *Rhizoctonia solani* in white beans. Therefore, rose-bengal streptomycin medium selective for fungi was used for fungal enumeration (Martin, 1950).

Fusarium species are common in agricultural soils. Many crops are damaged by Fusarium wilts (Komada, 1975). It has been demonstrated that *Fusarium* spp. are pathogenic on sugarcane (Watanabe, et al., 1974). Therefore, Komada's medium selective for *Fusarium* (Komada, 1975) was used to enumerate and compare fusarial fungi in this study.

Phytopathogenic microorganisms can occur in root interiors (Dunfield and Germida, 2001; Marilley, et al., 1998; van Peer, et al., 1990). Therefore, to study the components of microbial communities colonizing sugarcane roots in soils with a long-term sugarcane cropping history and no recent sugarcane cropping history, it would be desirable to compare the quantitative and qualitative aspects of microorganisms isolated from root interiors.

The Biolog™ bacterial identification system (Biolog Inc., Hayward, CA) has been adapted to compare sole carbon substrate utilization profiles (SCSUP) of microbial communities. Many studies have used SCSUP to examine soil microbial communities associated with roots of plants, including hybrid larch (*Larix eurolepis* A. Henry), Sitka spruce (*Picea sitchensis* Bong. Carr.), wheat (*Triticum* spp.), and grass species (Bossio and Scow, 1995; Fang, et al., 2001; Gagliardi, et al., 2001; Garland and Mills, 1991; Grayston and Campbell, 1996; Lupwayi, et al., 1998). In this study SCSUP was used to determine if sugarcane soils with and without a sugarcane cropping history could be distinguished from each other and to identify any carbon substrates responsible for the differences.

Biolog GN and GP MicroPlates™ are 96 well microtitre plates that contain 95 different carbon substrates and one control well containing no carbon substrate. In the Biolog microplate, utilization of a carbon source is indicated as a colorless tetrazolium redox dye is reduced to a colored formazan product. Absorbance values of the color response in each microplate well are recorded with an automated microplate reader. For community comparisons, the SCSUP data are collected from the microplate and can be analyzed by multivariate techniques, including detrended correspondence analysis (DCA) (Garland and Mills, 1991), canonical variate analysis (CVA) (Grayston and Campbell, 1996), kinetic modeling (Lindstrom, et al., 1998) or principal component analysis (PCA) (Garland and Mills, 1991). These multivariate analyses discriminate communities based on their substrate utilization patterns and reduce the dimensionality of the data set generated from the 95 substrates (variables) measured by absorbance values, to help determine the factors (variables) that contribute to the differences between the communities studied. DCA uses weighted averages to transform data and is less sensitive to variation in average well cell densities in SCSUP (Garland and Mills, 1991). CVA requires *a priori* grouping of the variables in a study (Jeffers, 1967). Kinetic modeling requires frequent plate readings over several days and extra steps in manipulating data (Lindstrom, et al., 1998). PCA is the analysis of choice for most researchers using SCSUP because *a priori* grouping of the variables is not required (Jeffers, 1967), and it is less labor intensive. The SCSUP data also can be used to calculate different measures of microbial community functional diversity, such as substrate richness (the number of utilized carbon sources), substrate evenness (the equitability of activities across all substrates), substrate diversity (encompasses both substrate richness and substrate evenness) (Shannon index) (Zak, et al., 1994), and diversity and evenness (McIntosh index) (Staddon, et al., 1997).

SCSUP has limitations. The profiles only represent the activities of members of the community that grow rapidly under the conditions in the Biolog plates, and the ratio of numbers of replicates to the number of variables often used is not large enough to yield statistically stable results (Hurst, et al., 2002; Insam and Hitzl, 1999). However, Legendre and Legendre (1998) stated that the first few eigenvectors, those typically used in PCA, are little affected when the replicate-to-variable ratio is low; therefore, the problem of low replication with large numbers of variables should not lead to incorrect interpretation of ordination.

If one is concerned about the low replicate-to-variable ratio, a method that decreases the number of variables and increases the number of replications per sample (the EcoPlate™ system) is now commercially available from Biolog. The EcoPlate™ is a microtiter plate that is composed of 31 carbon substrates that are useful for soil community analysis, based on carbon sources previously requested by soil researchers. The 31 substrates are replicated three times on a single plate. However, carbon sources utilized in different soils vary; therefore, the extra substrate data obtained with the GN2 and GP2 MicroPlate™ system may be preferable to the EcoPlate™ in some applications (Biolog, Inc., Hayward, CA.).

Another point that should be given consideration is whether to use the GN2, GP2, or both microplates. It has been reported that the rhizosphere environment is dominated by fast-growing, nutritionally versatile gram-negative bacteria (Weller, et al., 2002), thus the GN2 MicroPlate™ would produce more information than the GP2 MicroPlate™ in rhizosphere studies. Also, 62 substrates are duplicated in the GN2 and GP2 plates with only 33 substrates unique to each plate. Therefore, the extra expense and work to gain information from just 33 extra GP2 substrates may not be justified.

Most recently, molecular research methodologies capable of characterizing microbial communities in soil have been developed. These methods depend on the efficient extraction of nucleic acids from the microorganisms of interest. There are numerous reports of different techniques to extract nucleic acids from soil microbial communities. The techniques lyse and extract nucleic acids either directly (Kuske, et al., 1997; Saano, et al., 1995; and FastDNA® SPIN® Kit (For Soil) Bio 101, Inc., Carlsbad, CA) or indirectly (Fægri, et al., 1977; Holben, et al., 1988; Lee, et al., 1996) from microorganisms. With direct lysis methods, microbial cells are lysed within the soil matrix. With indirect methods, the microbial cells are separated from the soil matrix by centrifugation and then lysed to release nucleic acids (Fægri, et al., 1977; Miller, et al., 1999; Saano, et al., 1995). There are advantages and disadvantages involved with each technique. Direct lysis methods yield higher DNA concentrations and are presumably less biased than indirect lysis methods. However, a disadvantage to direct lysis methods is that more PCR-inhibitory substances are co-extracted along with the DNA.

Numerous techniques are available to analyze extracted soil community nucleic acids. Most produce profiles of the soil community based on PCR amplified DNA using universal primers. Some profiling methods are described below.

Single-strand conformation polymorphism (SSCP) has been used to generate profiles of genes coding for 16S rRNA(DNA) based on minor differences in nucleotide sequence (Lee, et al., 1996; Schmalenberger and Tebbe, 2002). Schmalenberger and Tebbe (2002) used SSCP to compare bacterial communities in rhizospheres of transgenic maize and sugar beet.

PCR-based amplified ribosomal DNA restriction analysis (ARDRA) technique has been used to compare bacterial diversity and community composition from lake water samples (Bosshard, et al., 2000), from agricultural soils (Øvreås and Torsvik, 1998), and from rhizosphere soil of diseased wheat (McSpadden Gardener and Weller, 2001).

The PCR-based random amplified polymorphic DNA (RAPD) molecular marker technique has been used to estimate the similarity of microbial communities in termite mounds and soils (Harry, et al., 2001) and in rhizosphere soil of maize (Picard, et al., 2000).

It has been illustrated that PCR-based molecular techniques can be used to investigate microbial communities in soil and to provide information on changes in the soil microbial communities that occur with different agricultural treatments and disease (McSpadden

Gardener and Weller, 2001; Øvreås and Torsvik, 1998; Picard, et al., 2000; Schmalenberger and Tebbe, 2002). These techniques need to be evaluated for the potential to characterize microbial communities in sugarcane soils.

The objectives of this study are to increase our basic understanding of microbial communities in sugarcane soils, to determine if there are differences in microbial communities associated with sugarcane roots in soil with and without a sugarcane cropping history, and to provide information on possible changes in the soil microbial communities that occur with sugarcane monoculture that may contribute to sugarcane yield decline.

CHAPTER 2. EVALUATION OF CULTURABLE SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH SUGARCANE

2.1 Introduction

In Louisiana, sugarcane (inter-specific hybrids of *Saccharum*) is grown largely under long-term monoculture production with successive annual harvests obtained from an initial vegetative planting of stalks. Therefore, sugarcane cultivars are clones composed of genetically identical plants. Buds on planted stalk sections germinate and grow to produce the first "plant cane" crop. Following the first harvest of the stalks, buds on the stubble remaining in the soil can germinate to produce a ratoon crop. In Louisiana, sugarcane plants are typically in the field continuously for 3-5 years. The stubble is then plowed out during the spring, and following a 4-6 month plant-free fallow period, the crop is replanted during late summer.

Monoculture can lead to poor root health and growth constraints in many crops, including sugarcane (Garside, et al., 1997; Magarey, 1996). A complex problem termed "yield decline" was defined by Garside, et al. (1997) as "the loss of productive capacity of soils under sugarcane monoculture." Previous studies have provided evidence of yield decline in sugarcane. Yield responses to fumigation of 20% or greater have been obtained (Bell, 1935; Garside, et al., 1997; Magarey, 1999; Muchow, et al., 1994). Magarey (1999) demonstrated positive growth responses to soil solarization and soil incorporation of high rates of broad-spectrum dithiocarbamate fungicides. Increased plant growth in soils not previously cropped to sugarcane provides additional evidence of yield decline (Magarey, 1999).

Research has been conducted in Louisiana on the effects of soilborne pathogens, such as *Pythium* (Cooper and Chilton, 1950; Edgerton, et al., 1929; Hoy and Schneider, 1988; Johnson, 1954) and nematodes (Bond, et al., 2000) on sugarcane. In greenhouse experiments, soil incorporation of Mancozeb has improved root health and plant growth (J. W. Hoy, unpublished data), and organic material soil amendments reduced root rot severity and provided increases in plant growth not correlated with root colonization by *Pythium* (Dissanayake and Hoy, 1999). However, the effects of the total soil microbial community on sugarcane growth in Louisiana are not well understood.

One approach to study the total soil microbial communities associated with sugarcane roots growing in soils with a long-term sugarcane cropping history (SCH) and no recent sugarcane cropping history (NSCH) would be to compare the quantitative and qualitative aspects of culturable microorganisms. It has been determined that only 0.01% to 10% of microorganisms in soil could be isolated and grown in culture (Johnson and Curl, 1972; Olsen and Bakken, 1987; Richaume, et al., 1993; Torsvik, et al., 1990). Therefore, culturable microorganisms represent only a small portion of the total community. However, as long as researchers remain aware of its limitations, selective media methods can be used to identify and quantify culturable species as a means to characterize and compare soil microbial communities (Dunbar, et al., 1999; Johnson and Curl, 1972; Olsen and Bakken, 1987; Sørheim, et al., 1989).

Traditionally, culture methods involve soil dilution plating on artificial media. Medium components can be altered to selectively isolate and grow groups of microorganisms associated with plant roots. Culturable bacterial and fungal populations can be characterized to compare microbial communities. In addition, populations of selected organisms that can affect plant growth can be compared.

Pseudomonas species found in association with the rhizosphere can play either a deleterious or beneficial role in plant growth and crop yields (Ghosh, et al., 2001; Kwok, et al., 1987; Mazzola and Gu, 2000; Nehl, et al., 1997; Rouatt and Katznelson, 1961; Simpfendorfer, et al., 2001). Rouatt and Katznelson (1961) reported that there were high numbers of Pseudomonas spp. in the rhizosphere soil of six different crop plants tested, including red clover, flax, oats, maize, barley and wheat. They suggested that their fast rate of growth, ability to produce acidic substances, and production of fluorescent pigments that were inhibitory to other organisms gave them a competitive advantage. *Pseudomonas* spp. have played beneficial roles as biocontrol agents of rhizosphere associated phytopathogens, including Fusarium wilts on tomato (Khan and Khan, 2001) Rhizoctonia damping-off on cucumber roots (Kwok, et al., 1987) and *Gaeumannomyces graminis* take-all of wheat (Smiley, 1979). Some *Pseudomonas* spp. are pathogenic to lucerne, oats, and wheat (Ghosh, et al., 2001; Simpfendorfer, et al., 2001). Nehl, et al. (1997) found that *Pseudomonas* spp., along with other rhizobacteria, fluctuate from being deleterious to plant growth promoting, depending on environmental conditions, host genotype and mycorrhizal status.

It has been reported that phytopathogenic *Pseudomonas syringae* strains produce siderophores, which complex with ferric iron to provide themselves with iron under iron limited conditions and thereby gain a competitive advantage over other microorganisms (Bultreys, et al., 2001). In contrast, Kloepper, et al., (1980) suggested that competition for iron is involved in soils that suppress plant diseases. In these soils, *Pseudomonas* spp. produce extracellular siderophores which may place phytopathogens at a disadvantage.

Actinomycetes produce antibiotics that are antagonistic to a number of different microorganisms, including some plant pathogens; however, some actinomycetes are phytopathogens themselves (Rouatt and Katznelson, 1961; Waksman, 1967). Therefore, this group of organisms would be of potential interest in evaluating potential differences in soil microbial communities that might affect sugarcane growth.

Phytopathogenic fungi have been extensively studied for many important crops, including sugarcane (Alavi, et al., 1982; Garside, et al., 1997; Kuter, et al., 1983; Metcalfe and Leather, 1963; Schneider, 1982; Watanabe, et al., 1974). Other fungi can suppress plant disease (LeBeau, 1938; Tu and Vaartaja, 1981). Therefore, comparison of the total numbers and types of fungi associated with sugarcane roots in soils with and without a recent sugarcane cropping history would be desirable. *Fusarium* spp. are abundant in agricultural soils, and many crops are damaged by Fusarium wilts (Komada, 1975). It has been demonstrated that *Fusarium* spp. can be pathogenic on sugarcane (Watanabe, et al., 1974), so an evaluation of *Fusarium* populations could be of interest.

The Biolog™ bacterial identification system (Biolog Inc., Hayward, CA) was adapted to compare sole carbon substrate utilization profiles (SCSUP) of microbial communities (Garland and Mills, 1991). SCSUP has been used to study the potential functional diversity of the culturable component of soil microbial communities associated with roots of hybrid larch (*Larix eurolepis* A. Henry), Sitka spruce (*Picea sitchensis* Bong. Carr.), wheat (*Triticum* spp.), and grass species (Fang, et al., 2001; Gagliardi, et al., 2001; Grayston and Campbell, 1996; Lupwayi, et al., 1998). Biolog GN and GP MicroPlates™ contain 95 different carbon substrates and one control well containing no carbon substrate. In the Biolog microplate, a utilization pattern appears as a colorless tetrazolium redox dye is reduced to a colored formazan product when microorganisms utilize the substrates. For community comparisons, the SCSUP data is collected from the microplate and can be analyzed by a multivariate analysis technique, such as principal component analysis (PCA) (Grayston and Campbell, 1996). The SCSUP data also can be used to calculate different measures of microbial community functional diversity, such as substrate richness (the number of utilized carbon sources), substrate evenness (the equitability of activities across all substrates), substrate diversity (encompasses both substrate richness and substrate evenness) (Shannon index) (Zak, et al., 1994), and diversity and evenness (McIntosh index) (Staddon, et al., 1997). However, SCSUP has limitations. The profiles only represent the activities of members of the community that grow rapidly under the conditions in the Biolog plates, and the ratio of numbers of replicates to the number of variables used may not be large enough to yield statistically stable results (Hurst, et al., 2002; Insam and Hitzl, 1999). However, Legendre and Legendre (1998) stated that the first few eigenvectors, those typically used in PCA, are little affected when the replicate-to-variable ratio is low. An alternative microplate designed for microbial community analysis the EcoPlate™ system, is composed of 31 carbon substrates useful for community analysis that are replicated three times on a single plate.

The objectives of this study were to increase our basic understanding of microbial communities in sugarcane soils, to determine if there are differences in microbial communities associated with sugarcane roots in soil with and without a sugarcane cropping history, and to provide information on possible changes in the soil microbial community that occur with sugarcane monoculture that may contribute to sugarcane yield decline.

2.2 Materials and Methods

2.2.1 Study Sites and Soil Sample Collection Method

Three study sites were selected with SCH and NSCH fields either adjacent or closely situated to minimize variation in soil type, climate, and farm management. All sites were planted with the sugarcane cultivar LCP 85-384. All of the fields without a recent sugarcane cropping history were in the first season of sugarcane production. In contrast, all of the long-term sugarcane fields were in monoculture production (with a 4-6 month plant-free fallow period every fourth or fifth growing season) for 15 or more years.

The first site (Site 1) was on a sugarcane farm in St. Charles Parish, Louisiana. The NSCH field was converted from a mixed-species pasture. The NSCH field plants were in the first year of planting, and the directly adjacent SCH field plants were in the first ratoon crop. The samples were collected in May, 2001.

The second and third sites (Sites 2 and 3) were on sugarcane farms in Iberia Parish, Louisiana. The NSCH field was converted from mixed hardwood forest at Site 2 and from a mixed-species pasture at Site 3. At Site 2, the NSCH and SCH fields were on opposite sides of a waterway and separated by approximately 200 m. At Site 3, the NSCH and SCH fields were directly adjacent to each other. Samples were collected at Site 2 in June, 2002 and at Site 3 in August, 2002.

At all sites, 25 samples were collected in a Z pattern from each field. Roots and soil were removed from a zone approximately 2-25 cm below plants, and roots were shaken so that only tightly adhering soil would be collected for rhizosphere soil samples. The roots and adhering soil from each sample were placed in individual, sterile whirl-pak bags (Fisher Science, Pittsburgh, PA). The samples were placed in an ice chest with cold packs for transport to the lab. Non-rhizosphere (bulk) soil also was collected. Rhizosphere soil samples from all sites were used for comparisons of culturable microorganisms on general and selective media and sole carbon source utilization profiles. At Site 1, a composite of the 25 bulk soil samples was used for sole carbon source utilization profile and selective media analyses. Thoroughly mixed composites of bulk samples from both fields at each site were used for fertility profile and particle size analyses. Bulk soil samples were dried at 105^{o}C for 24 hr to measure gravimetric soil moisture content. Soil samples were stored at 4^{o}C overnight before initiating experiments.

Fertility profile analyses of NSCH and SCH fields included the following parameters: pH, content in parts per million (ppm) of salts, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), sulfur (S), zinc (Zn), copper (Cu), and content percentage of organic matter (OM). Content percentages of sand, silt, and clay from the particle size analyses were used to designate soil textures of the NSCH and SCH fields. Analyses were performed by the Louisiana State University Agricultural Center Soil Testing Laboratory.

2.2.2 Media for Culturable Organisms

Preliminary research was performed to discover the optimal growing conditions for culturable organisms in microbial communities from sugarcane field soils. Various inoculating solutions were evaluated, including sterile, distilled water, Winogradsky salt solution pH 7.0 (Holben, et al., 1988), phosphate buffer solution (0.01 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, and distilled H_2O, pH 7.0), and calcium chloride solution (0.01 M $CaCl_2$, pH 7.0). Various dilutions and volumes were evaluated.

Types of media used to characterize the microbial community were 10% tryptic soy agar (TSA) (Difco, Becton-Dickinson, Sparks, MD) for general bacterial colonies, two media selective for Pseudomonas species: King's B (KB) (King, et al., 1954) and Pseudomonas agar (PA) (Sigma, St. Louis, MO), actinomycete isolation agar (AA) (Difco) selective for

actinomycete colonies, and acidified potato dextrose agar (APDA) (PDA (Sigma) plus 2-ml of 25% lactic acid per liter) selective for fungal colonies.

Plate counts were expressed as colony forming units (cfu) per gram of dried soil (Johnson and Curl, 1972). TSA, PA, and KB plates were placed under ultra-violet light to visualize and count fluorescent bacterial colonies. The means of five replicate plates were calculated. Data were $\log_{10}$ transformed before analysis with the general linear model (GLM) and least significant difference (LSD) statistical methods (SAS Institute, Cary, NC).

The preliminary results revealed that soil serially diluted in 0.01 M $CaCl_2$ solution and 0.1 ml of inoculum per media plate was the optimal inoculation solution and volume for plating. The optimal dilutions to obtain countable colonies ranged from 10^{-2} to 10^{-4}. The two *Pseudomonas* selective media were comparable to each other in colony producing efficiency. For convenience, PA was used instead of King's B. For Site 1, air-dried soil samples were used for all media. For Sites 2 and 3, fresh or wet weight soil samples were used for all media except AA. Dried soil samples were used in addition to fresh with TSA and PA for comparison. Soil samples were aseptically air-dried for 2 wk as a pretreatment to inhibit growth of fungal species for actinomycetes cultivation (Pisano, et al., 1986).

Roots were shaken vigorously and rubbed against each other inside the whirl-pak bags to collect rhizosphere soil. The loose soil was collected from each of 25 bags and combined in another sterile bag to create a composite sample for each field. Five replicate sub-samples were then collected from the composite sample. Fresh (wet weight) soil was used within 24 hr from field collection. For future analysis, the remaining soil was poured into autoclaved, 60 ml glass jars with loosely sealed lids and placed in a covered box containing Drierite crystals (W. A. Hammond Drierite Co., Ltd., Xenia, OH) and allowed to dry in the dark.

Selective media used for Sites 2 and 3 were the same as for Site 1, except that rose-bengal (33 mg/L) streptomycin (30 µg/L) agar (RB) (Martin, 1950) was used instead of APDA for general fungal colonies, chrome-azurol sulfate agar (CAS) selective for siderophore producing bacterial colonies (Schwyn and Neilands, 1987), and Komada's agar (KO) (Komada, 1975) selective for fusarial fungal colonies were added to the experiment. The chrome-azurol sulfate medium was modified from Schwyn and Neilands (1987) in that protease peptone #3 was used instead of casamino acid to cultivate organisms that would not grow on minimal medium. Ten-fold serial dilutions of 1 ml of 0.01 M $CaCl_2$ were swirled into RB and KO media melted at 50^{o}C and allowed to cool and harden.

For Site 1, all plates were read after 6 days of incubation. For Sites 2 and 3, the CAS plates were read after 2 d of incubation, TSA and PA plates after 6 d, KO plates after 8 d, and AA plates after 12 d.

2.2.3 Culture of Microorganisms Colonizing Internal Root Tissues

To evaluate microorganisms associated with the interior of sugarcane roots from fields with and without a recent sugarcane cropping history, a modified method (Dunfield and Germida, 2001) was used. From Sites 2 and 3, four roots from each of 25 replicate samples per field for a total of 100 roots per sample were placed in a 1 L glass jar containing 250 ml of non-sterile dH_2O. This was replicated three times per field. To separate the rhizosphere soil from the roots, the roots were washed by shaking at 200 rpm for 20 min on a rotary shaker, and the soil/water suspension was discarded. The wash was repeated six times. Then, 5 g of washed roots from each replication were measured and cut with alcohol flamed scissors. The measured roots were placed in sterile, 1 L glass jars containing 200 ml of 1% (v/v) final concentration of sodium hypochlorite (NaOClO) solution. The roots and bleach solution were shaken at 200 rpm for 10 min. The roots were then rinsed four times for 5 min each in 100 ml of sterile, 0.01 M $CaCl_2$ solution in a 250 ml sterile glass beaker. The rinse water from the last rinse was saved, and 0.1 ml was used to spread plate on TSA to check for surface contamination. The roots were cut with flame-sterilized razor blades and tweezers, and the pieces were weighed into 250 mg amounts and transferred to sterile, 2 ml plastic centrifuge tubes with two tubes for each sample for a total of six tubes for each NSCH or SCH field. Each 2 ml tube contained a 5 mm glass bead and 1ml of 0.01 M $CaCl_2$. The tubes containing the roots were placed in a bead beater and shaken to macerate the root tissue for 30 sec. They were placed on ice for 2 min to cool and were shaken again for 30 sec. Then, the tubes were centrifuged at 7,000 g for 4-5 sec, and 1 ml of the supernatant was removed from tubes and mixed with 9 ml of $CaCl_2$ to make 10-fold serial dilutions of the samples. The dilutions (10^{-2} to 10^{-5}) were spread on 10% TSA. Colony forming units per ml of root extract on the plates were counted every two days for 12 d.

2.2.4 Collection and Storage of Isolates

To gain qualitative information from the culture media experiments, bacterial colonies on TSA from all sites were isolated and stored at -20°C for future identification. For Site 1, colonies were not chosen at random. Colonies that differed by morphology from the NSCH and SCH field soil types were isolated and stored. However, from Sites 2 and 3, 40 colonies from each NSCH or SCH field soil were randomly picked and isolated from the culture plates and stored. After the final counts were taken, plates were placed over a 100 square grid (6 mm/square), and colonies from every other square were picked. If the edge of a colony was touching other colonies, that one was skipped until a single colony surrounded by empty space was found. Three to four culture plates were required to obtain 40 colonies for each field. The chosen colonies were then sub-cultured on TSA plates three times by dilution streaking to insure purity and placed on TSA slants. Preservation of the chosen colonies was conducted by modifying the method of Perkins (1962). The colonies were washed off of the slants with 200 µl of a sterile skim milk solution (7 g skim milk/100 ml dH_2O plus 15 ml glycerol/85 ml dH_2O) pipetted into (13 x 100 mm) test tubes half-filled with 6-12 mesh, grade 40 silica gel (Aldrich, Milwaukee, WI). The test tubes were placed in ice to decrease the high temperature produced from

the dehydration of the solution and finally placed in storage at -20°C. Bacteria from the internal root colonizing microorganism experiment for site 2 were isolated and stored at -20°C for future identification using the same storage method as for the selective media cultured isolates.

2.2.5 Determination of Sole Carbon Source Utilization Profiles

Extraction of microbial cells for Site 1 soil samples was conducted by modifying the method of Grayston and Campbell (1996). For rhizosphere samples, one root from each of 25 replicate samples per field for a total of 25 roots per sample was placed in a twice-autoclaved, 1L glass jar containing 250 ml of sterile phosphate buffer (0.01 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, and distilled H_2O, pH 7.0). This was replicated three times per field. The phosphate buffer and roots were shaken at 100 rpm for 1.5 h on a rotary shaker to separate the rhizosphere soil from the roots. The rhizosphere soil suspensions were poured into 250 ml sterile polypropylene bottles (Nalgene, Rochester, NY), and ten-fold serial dilutions (3 ml of solution into 27 ml of 0.01 M $CaCl_2$) were made from the suspensions. Likewise, ten-fold serial dilutions of bulk soil suspension were made. For each soil sample, 30 ml of 10^{-3} dilution was placed in an autoclaved, 50 ml centrifuge tube and centrifuged at 750 g for 10 min at 4°C to pellet any soil particles that could interfere with optical density determination. Aliquots of 150 µl were then added to each of 96 wells of Biolog gram-negative (GN2) MicroPlates (Biolog, Hayward, CA). There were three replicate plates per field. The plates were incubated at 25°C in the dark. Each well in a plate was measured for color absorbance at 600 nm every 24 h for 5 d with a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT) (Campbell, et al., 1997).

For NSCH and SCH soil samples from Sites 2 and 3, the inoculum was extracted and standardized by modified methods (Goodfriend, 1998; Lupwayi, et al., 1998). Four roots from each of 25 sub-samples per field for a total of 100 roots per sample were placed into each of five replicate twice-autoclaved, glass, 1 liter jars with 250 ml of phosphate buffer. The phosphate buffer and roots were shaken as for Site 1. Instead of using ten-fold serial dilutions, the solutions were centrifuged at 2,000 x g for 20 min at 4°C to pellet any soil. The supernatants were then poured into 250 ml polypropylene copolymer centrifuge bottles and centrifuged at 10,000 x g for 10 min at 4°C to collect bacterial pellets. The supernatants were discarded, and the pellets were vortex-mixed in 25 ml of sterile, 0.85% sodium chloride. The five sub-samples were then combined to create a composite sample. Three replicate samples were then made from the composite sample. Any remaining soil particles were allowed to settle for 5 min, and 1 ml from each replicate was used to read optical density at 600 nm. To standardize each replicate, absorbance values were adjusted to 0.08-0.10 O.D. The adjusted samples were shaken for 24 hr at room temperature to allow time for the bacteria to utilize any extraneous carbon in the suspension. Aliquots of 150 µl of the standardized samples were then added to each well in Biolog GN2™ plates. The plates were incubated at 28°C in the dark. Each well in the Biolog plates was then measured for absorbance as for Site 1.

Preliminary research was performed with the Biolog plate absorbance data to determine the analysis method that would reveal the most useful information. The plates were

analyzed using the following data sets: means of individual well data of the three replicate plates for each day compared separately and means for individual wells over days 2, 3, and 4, for each plate. The fifth day data was not used because of excessive growth and fungal contamination. The averaged data whether for single or multiple dates were dissimilar to visually determined results in single plates on single dates. Therefore, single day, individual plate data were analyzed by principal component analysis (PCA) (SAS Institute, Cary, NC) to compare SCSUP for each site. Data used for PCA were from the single day reading with the maximum amount of positive wells before contamination by fungi occurred. The data recorded at 72 hr were used for Site 1, and at 24 hr for Sites 2 and 3. The first principal component was related to overall plate responses, and the second and third principal component were related to individual carbon substrates. A covariate matrix was used because the units of measurement were the same.

Microbial communities from individual plate wells were preserved for future molecular profiling of samples from Sites 2 and 3. Microbial communities in Biolog plates were pipetted out of individual wells that differed between NSCH and SCH field soil types and stored at -20°C in 500 µl of sterile, dH_2O in 1.5 ml micro-centrifuge tubes.

2.3 Results

2.3.1 Soil Fertility Profile and Particle Size Analyses

For all three sites, pH, organic matter, and fertility parameters were generally numerically higher (no statistical analysis was performed) in NSCH fields, except for Site 3 where pH, salt, and K were higher in SCH fields. The soil textures of each field at Sites 1 and 2 were silt loam. The soil textures of each field at Site 3 were different from each other and from Sites 1 and 2. At Site 3, the NSCH field soil texture was sandy clay loam and the SCH field was loam (Table 1).

2.3.2 Comparison of Culturable Microorganisms

For Site 1, total bacteria cfu/g of soil in bulk soil was higher in NSCH than SCH on TSA, PA, and AA media (Fig. 1). However, there was no difference between SCH and NSCH rhizosphere soils for total bacteria cfu/g of soil on TSA, PA, and AA media. On TSA, fluorescent bacteria were isolated from rhizosphere soil of NSCH but not SCH samples, with a mean cfu/g of soil of $4.0 \times 10^4 \pm 7.1 \times 10^3$ (standard error), and none were isolated from bulk soils. Fluorescent bacterial colonies were isolated from both NSCH and SCH samples on PA. Fluorescent colonies were more numerous on PA plated with diluted rhizosphere soil from NSCH samples with a mean cfu/g of soil of $1.5 \times 10^6 \pm 3.2 \times 10^5$ compared to $1.7 \times 10^4 \pm 3.2 \times 10^5$ for SCH samples, and none were detected in bulk soil platings as with TSA. Each APDA plate produced fewer than five colonies; therefore, fungal cfu/g could not be determined and compared.

For Site 1, cfu/g of soil of microbial organisms was compared on different media for bulk and rhizosphere soil samples from NSCH and SCH fields (Table 2). On TSA, PA, and AA, the total cfu/g of soil was higher in rhizosphere than bulk soil, except on AA from NSCH samples.

TABLE 1. Soil fertility profiles and particle size analyses of experimental sugarcane fields.

Site	Cropping history [x]	pH	Content (ppm)										Content (%)			
			Salts	P	K	Ca	Mg	Fe	Mn	S	Zn	Cu	OM[y]	Sand	Silt	Clay
1	NSCH	6.6	336	232	274	2,958	617	84	14	9	6	6	2.1	12	73	15
1	SCH	6.1	112	207	174	1,606	325	64	14	5	2	3	1.5	10	68	22
2	NSCH	7.0	1,493	199	200	2,491	441	48	34	26	4	1	2.1	5	75	20
2	SCH	6.8	898	180	115	2,904	383	34	27	43	2	1	1.6	5	78	17
3	NSCH	5.8	297	99	158	1,776	325	114	36	18	3	2	3.0	52	15	33
3	SCH	6.1	328	78	219	1,241	189	65	32	17	1	1	1.8	44	31	25

[x]No recent sugarcane cropping history (NSCH), and long-term sugarcane cropping history (SCH).
[y]Organic matter.

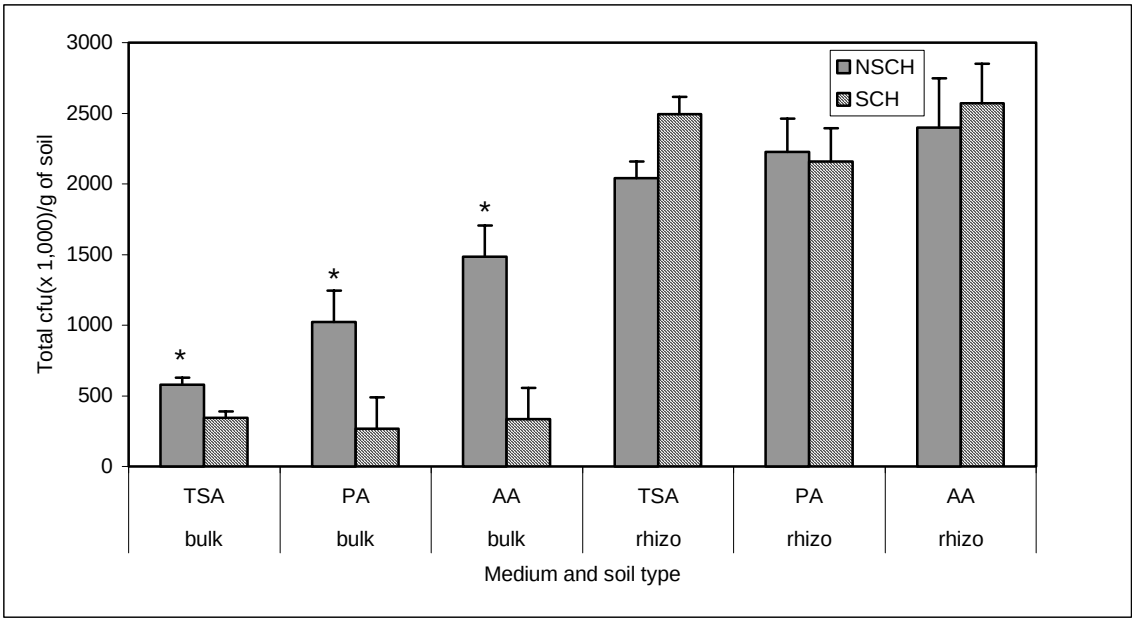

Figure 1. Total microbial cfu/g of soil on tryptic soy agar (TSA), Pseudomonas agar (PA), and actinomycetes agar (AA) from bulk and rhizosphere (rhizo) soils from fields with no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) at Site 1. Bars represent standard error of the mean, and an asterisk denotes a significant difference between NSCH and SCH for a certain medium type (P=0.05).

TABLE 2. Comparison of total bacterial cfu/g of soil in bulk and rhizosphere soil samples collected from no recent sugarcane cropping history (NSCH) and sugarcane cropping history (SCH) fields at Site 1.

	Field type and culture medium[x]		
Soil type	NSCH		
	TSA	PA	AA
Rhizosphere	$2.0x10^6 \pm 9.0x10^4$ a	$2.2x10^6 \pm 1.8x10^5$ a	$2.4x10^6 \pm 4.3x10^5$ a
Bulk	$5.8x10^5 \pm 7.8x10^4$ b	$1.0x10^6 \pm 1.8x10^5$ b	$1.5x10^6 \pm 2.7x10^5$ a
Soil type	SCH		
	TSA	PA	AA
Rhizosphere	$2.5x10^6 \pm 8.6x10^4$ a	$2.2x10^6 \pm 2.0x10^5$ a	$2.5x10^6 \pm 8.7x10^4$ a
Bulk	$3.4x10^5 \pm 7.4x10^4$ b	$3.6x10^6 \pm 2.0x10^5$ b	$3.3x10^5 \pm 1.1x10^5$ b

[x]Media used were tryptic soy agar (TSA), Pseudomonas agar (PA), and actinomycete agar (AA). Values are mean cfu/g of soil and standard error from five replicates. Means for rhizosphere and bulk soil within a medium type followed by the same letter are not significantly different (P=0.05).

For Site 2, only rhizosphere soil was used as inoculum for all media. Total cfu/g of soil was higher in SCH soil on TSA with fresh inoculum, while there was no difference between NSCH and SCH with the air-dried soil inoculum on TSA (Fig. 2). In contrast, there was no difference in total bacterial cfu/g of soil on PA with fresh soil inoculum; however, with air-dried soil inoculum, total cfu/g of soil was higher in NSCH soil. There was no difference in cfu/g of soil between the soil types on AA medium.

Fluorescent bacterial colonies did not appear on TSA with fresh or air-dried soil inoculum in either SCH or NSCH soils. Fluorescent bacterial cfu/g of soil was higher in NSCH soil with air-dried but not fresh soil inoculum on PA (Fig. 3). There was no difference in cfu/g of soil between SCH and NSCH soils for total or siderophore producing bacteria on CAS. Total fungal cfu/g of soil was higher in NSCH soil with fresh soil inoculum for fungi on RB. On KO medium, more fungi were detected in NSCH than SCH soil. The mean cfu/g of soil was $1.3 \times 10^3 \pm 5.3 \times 10^1$ in NSCH soil and $7.1 \times 10^1 \pm 5.3 \times 10^1$ in SCH soil.

For Site 3, only rhizosphere soil was used for inoculum for plating on all media. Total bacterial cfu/g of soil was higher in NSCH than SCH soil on TSA and AA with air-dried soil inoculum. There was no difference in total bacterial cfu/g of soil on TSA with fresh inoculum and on PA with fresh or air-dried soil inoculum (Fig. 4). Fluorescent bacteria did not appear on TSA with fresh or air-dried soil inoculum from either field. For Site 3, there was no difference between NSCH and SCH in fluorescent bacteria cfu/g of soil on PA with air-dried or fresh soil inoculum (Fig. 5). Total and siderophore producing bacteria cfu/g of soil were higher in SCH soil on CAS medium. There was no difference in total fungal cfu/g of soil detected on RB in either soil. On KO medium, the fungal mean cfu/g of soil was higher in NSCH ($1.0 \times 10^3 \pm 8.2 \times 10^1$) than SCH ($4.8 \times 10^2 \pm 9.2 \times 10^1$).

A comparison of microbial cfu/g of soil detected on different media considered by experimental sites revealed differences among Sites 2 and 3 for NSCH and SCH field types (Table 3). Site 1 was not compared because the samples were processed differently. The mean cfu/g of soil of fluorescent bacteria on PA, siderophore producing bacteria on CAS, and fungi on KO were not different between Sites 2 and 3 for NSCH soil, and fluorescent bacterial counts were not different in SCH. For all other media types, the numbers of microorganisms detected were higher at Site 3 for both field types.

2.3.3 Bacteria in Internal Root Tissues

For Site 2, the numbers of total bacterial cfu/ml of root extract from samples on 10% TSA medium were either greater than 300 or less than 30 with all dilutions. Therefore, the colonies were not countable for statistical evaluation. However, 40 colonies each from NSCH and SCH soils were collected at random from the TSA plates and saved for future identification and comparison.

For Site 3, the detection of bacterial colonies from all samples on TSA plates inoculated with the last rinse solution indicated that microorganisms on the root exterior may not

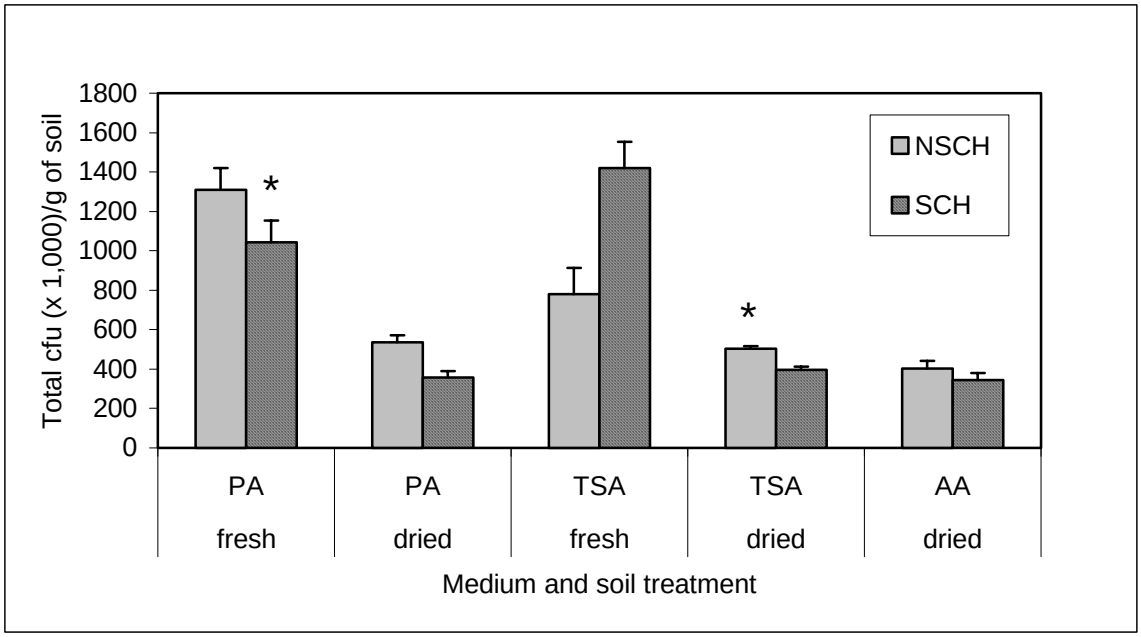

Figure 2. Total microbial cfu/g of soil on tryptic soy agar (TSA), Pseudomonas agar (PA), and actinomycetes agar (AA) using fresh and air-dried soil inocula from fields with no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) at Site 2. Bars represent standard error of the mean, and an asterisk denotes a significant difference between NSCH and SCH soils for a certain medium (P=0.05).

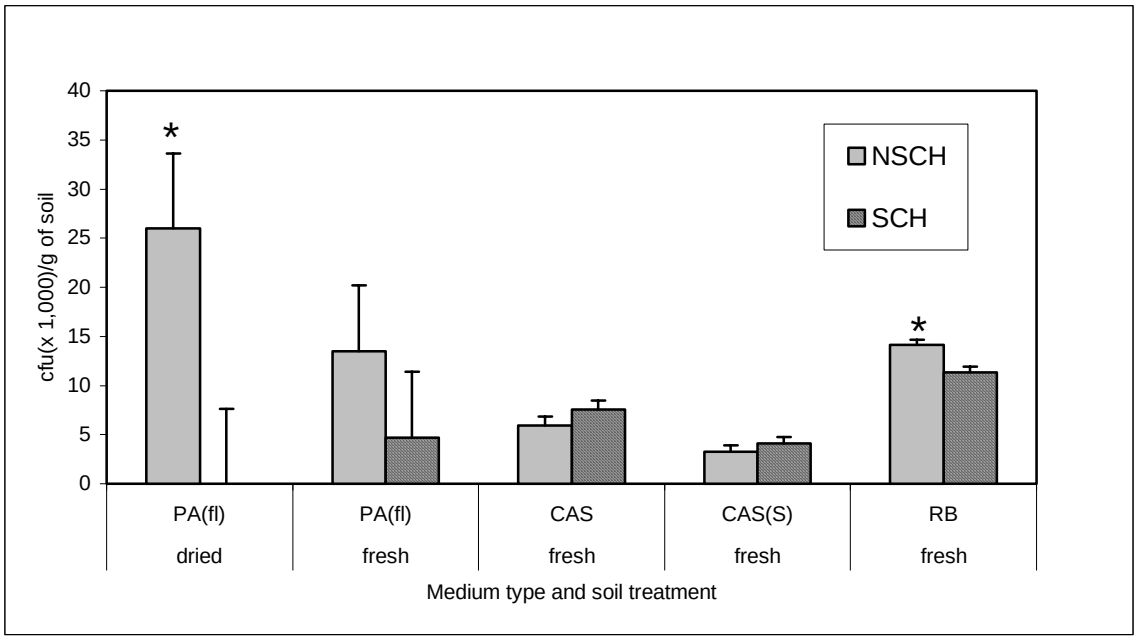

Figure 3. Microbial cfu/g of soil on Pseudomonas agar (PA) for fluorescent (fl) bacteria, on chrome-azurol sulfate medium (CAS) for total and siderophore producing (S) bacteria, and on rose-bengal streptomycin (RB) developing from fresh or air-dried (dried) soil inocula from fields with no recent sugarcane cropping history (NSCH) and sugarcane cropping history (SCH) at Site 2. Bars represent standard error of the mean, and an asterisk denotes a significant difference between NSCH and SCH soils for a certain medium (P=0.05).

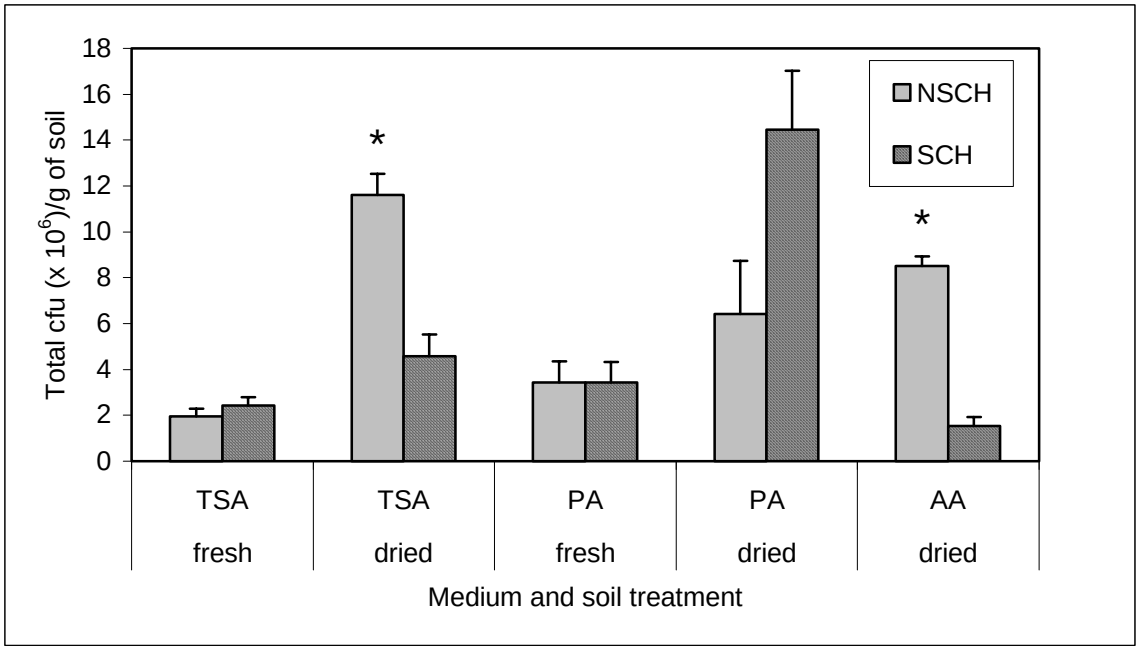

Figure 4. Total microbial cfu/g of soil on tryptic soy agar (TSA), Pseudomonas agar (PA), and actinomycetes agar (AA) plated with fresh and air-dried (dried) soil inocula from fields with no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) at Site 3. Bars represent standard error of the mean, and an asterisk denotes a significant difference between NSCH and SCH soils for a certain medium (P=0.05).

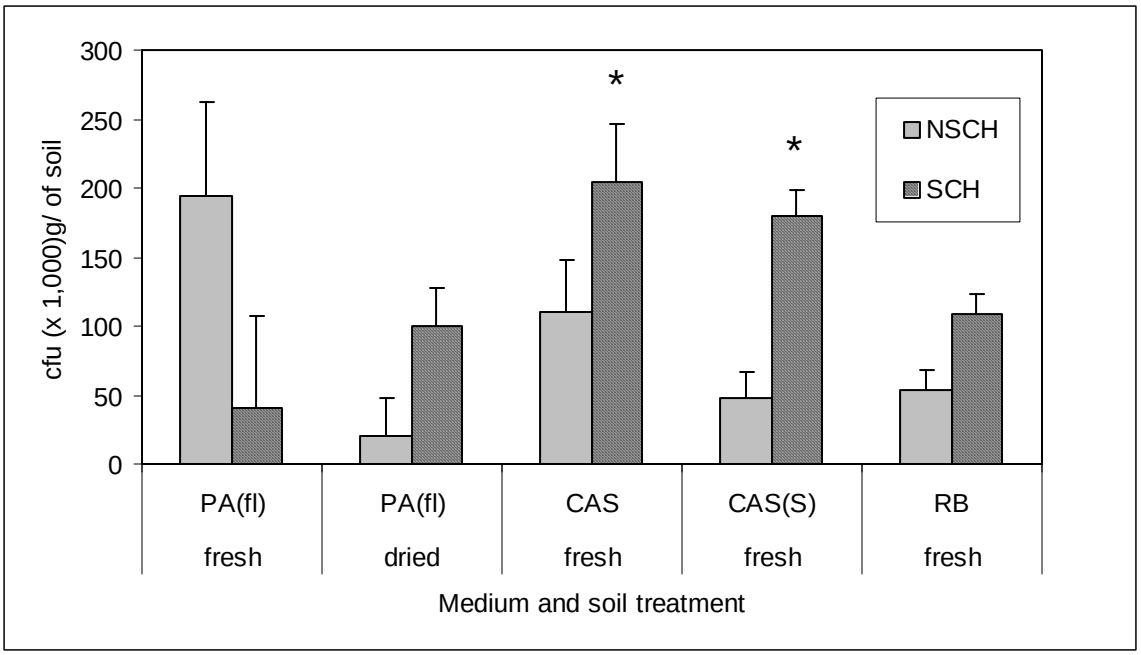

Figure 5. Total microbial cfu/g of soil on Pseudomonas agar (PA) for fluorescent (fl) bacterial counts using freshly collected and air-dried soil, chrome-azurol sulfate medium (CAS) for total and siderophore (S) bacterial counts, and rose-bengal streptomycin (RB) medium plated with fresh soil inocula from no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) fields at Site 3. Bars represent standard error of the mean, and an asterisk denotes a significant difference between NSCH and SCH soils for a certain medium (P=0.05).

TABLE 3. Comparison of populations (cfu/g of soil) of microbial organisms isolated on different selective media from sugarcane rhizosphere soil samples collected from fields with no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH).

	Field[x]			
	NSCH		SCH	
	TSA (fresh)	p value	TSA (fresh)	p value
Site 2	$7.8 \times 10^5 \pm 2.2 \times 10^5$ b	0.0015	$1.4 \times 10^6 \pm 2.6 \times 10^5$ b	0.0424
Site 3	$1.9 \times 10^6 \pm 2.2 \times 10^5$ a		$2.4 \times 10^6 \pm 3.0 \times 10^5$ a	
	TSA (dried)		TSA (dried)	
Site 2	$5.0 \times 10^5 \pm 8.8 \times 10^5$ b	<0.0001	$4.0 \times 10^5 \pm 3.0 \times 10^5$ b	<0.0001
Site 3	$1.2 \times 10^7 \pm 9.3 \times 10^5$ a		$4.6 \times 10^6 \pm 3.0 \times 10^5$ a	
	PA (tot)		PA (tot)	
Site 2	$5.0 \times 10^5 \pm 5.9 \times 10^5$ b	<0.0001	$3.6 \times 10^5 \pm 2.2 \times 10^6$ b	<0.0001
Site 3	$6.4 \times 10^6 \pm 5.9 \times 10^5$ a		$1.4 \times 10^7 \pm 2.5 \times 10^6$ a	
	PA (fl)		PA (fl)	
Site 2	$2.6 \times 10^4 \pm 1.6 \times 10^4$ b	0.0059	$0.0 \pm 3.4 \times 10^4$ b	<0.0001
Site 3	$2.0 \times 10^4 \pm 1.6 \times 10^4$ a		$1.0 \times 10^5 \pm 3.8 \times 10^4$ a	
	AA		AA	
Site 2	$4.0 \times 10^5 \pm 3.8 \times 10^5$ b	<0.0001	$3.4 \times 10^5 \pm 9.5 \times 10^4$ b	<0.0001
Site 3	$8.5 \times 10^6 \pm 3.8 \times 10^5$ a		$1.5 \times 10^6 \pm 9.5 \times 10^4$ a	
	CAS (tot)		CAS (tot)	
Site 2	$6.0 \times 10^4 \pm 9.1 \times 10^2$ b	0.0160	$7.5 \times 10^4 \pm 9.1 \times 10^3$ b	0.0002
Site 3	$1.1 \times 10^5 \pm 1.9 \times 10^4$ a		$2.0 \times 10^5 \pm 1.9 \times 10^4$ a	
	CAS (S)		CAS (S)	
Site 2	$3.3 \times 10^4 \pm 6.1 \times 10^3$ a	0.0643	$4.1 \times 10^4 \pm 6.1 \times 10^3$ b	0.0003
Site 3	$4.8 \times 10^4 \pm 1.5 \times 10^4$ a		$1.8 \times 10^5 \pm 1.5 \times 10^4$ a	
	RB		RB	
Site 2	$1.4 \times 10^4 \pm 5.7 \times 10^2$ b	0.0002	$1.1 \times 10^4 \pm 5.7 \times 10^2$ b	<0.0001
Site 3	$5.3 \times 10^4 \pm 2.7 \times 10^4$ a		$1.1 \times 10^5 \pm 2.7 \times 10^4$ a	
	KOM		KOM	
Site 2	$1.3 \times 10^3 \pm 5.3 \times 10^1$ a	0.0901	$7.1 \times 10^1 \pm 5.3 \times 10^1$ b	<0.0001
Site 3	$1.0 \times 10^3 \pm 8.2 \times 10^1$ a		$4.8 \times 10^2 \pm 9.2 \times 10^1$ a	

[x]Media types used were tryptic soy agar (TSA), Pseudomonas agar (PA) for total (tot) and fluorescent (fl) bacterial cfu/g of soil, actinomycetes agar (AA), chrome-azurol sulfate medium (CAS) for total and siderophore (S) producing bacteria, rose-bengal streptomycin medium (RB) and Komada's medium (KO). Values are mean cfu/g of soil and standard error from five replicates. Data were $\log_{10}$ transformed before general linear model analysis. Means within a medium and field type followed by the same letter are not significantly different (P=0.05).

have been completely eliminated. Therefore, no colonies were saved from the Site 3 samples.

2.3.4 Visual Characterization of Isolates Obtained from Different Soil Types

For all sites, field types, rhizosphere, and root interiors, the most common type of bacterial colony on all media was white to off-white, circular shaped, and 3-4 mm in diameter (dia.) after 6 d of growth. For Site 1, colonies were not chosen at random. Instead, ones that differed in morphology were isolated from the NSCH and SCH field soil types and stored for future identification. In general, yellow colony types appeared following plating of the rhizosphere soil but not of the bulk soil, regardless of NSCH and SCH soil type.

For Site 2, NSCH rhizosphere soil plating yielded 25 different colony types among the 40 randomly isolated colonies. Most of the different colony types represented single colonies, but four were white and 5 mm in dia., another four were white and 1 mm in dia., and five were yellow and 3 mm in dia. Plating SCH soil yielded 22 different colony types among the 40 isolated colonies. Again, most of the different colony types were represented by single colonies. Three were yellow and 4 mm in dia., and four were white and 1 mm in dia.

For Site 3, plating of NSCH rhizosphere soil yielded 17 different colony types among the 40 randomly isolated colonies. Three were yellow and 5 mm in dia, and nine were yellow and 4 mm in dia. Sixteen different colony types were isolated from SCH soil. Most types were represented by a single colony, and three were yellow and 4 mm in dia.

For Site 2, plating of NSCH root extracts yielded 10 different colony types among the 40 randomly isolated colonies. Two were yellow and 3 mm in dia. Eight different colony types were isolated from SCH root extracts. Most types were represented by a single colony, and three were white with leathery surfaces and 3 mm in dia.

2.3.5 Comparison of NSCH and SCH Sole Carbon Source Utilization Profiles

At Site 1, rhizosphere microbial communities in soil with or without a recent sugarcane cropping history could be distinguished by PCA of sole carbon source utilization profiles (Fig. 6). However, there was high variability among the NSCH replicates, and one replicate showed similiarity to the SCH samples. The PCA of rhizosphere soil samples indicated that the first (overall plate reaction) and second (individual well reactions) principal components explained 55% of the standardized variance, and the first three principal components explained 72%.

Also at Site 1, SCH and NSCH bulk soil microbial communities could be distinguished by PCA of sole carbon source utilization profiles (Fig. 7). However, one of the SCH replicates was different than the other two. The PCA of bulk soil samples indicated that

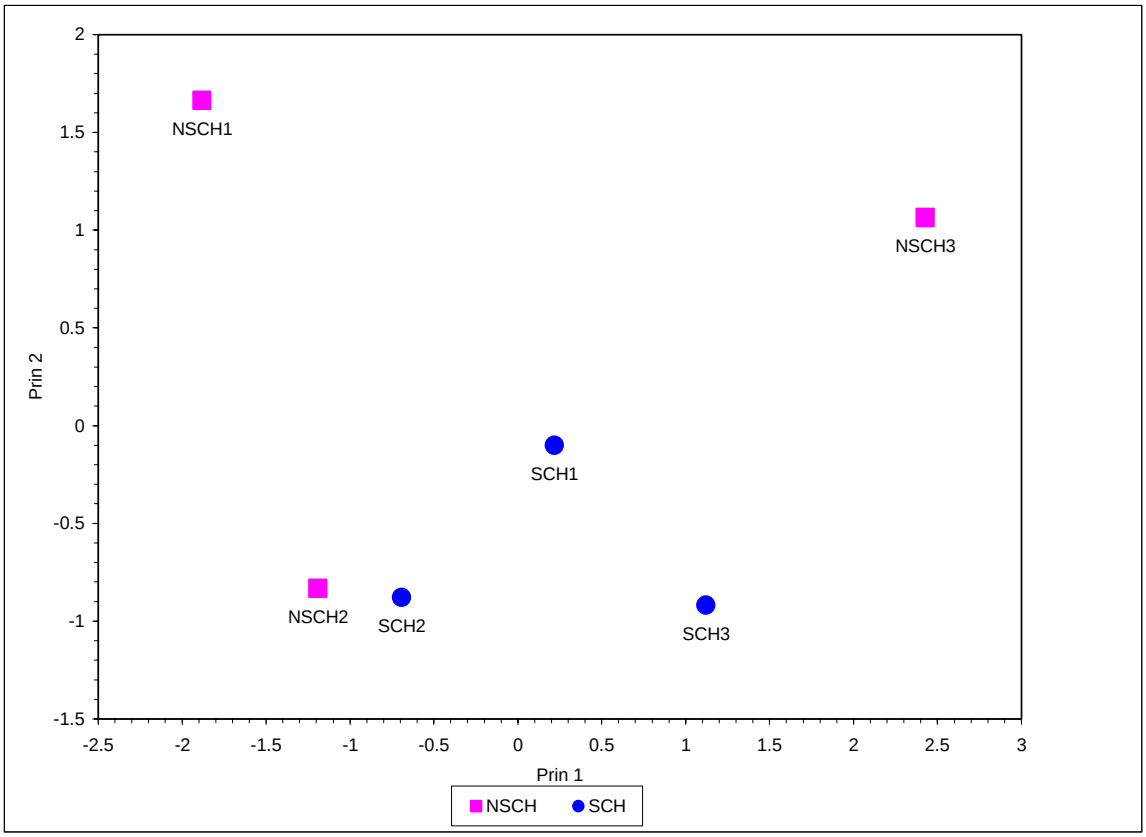

Figure 6. Principal component analysis of Biolog GN2 plate data for rhizosphere soil samples from no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) fields at Site 1.

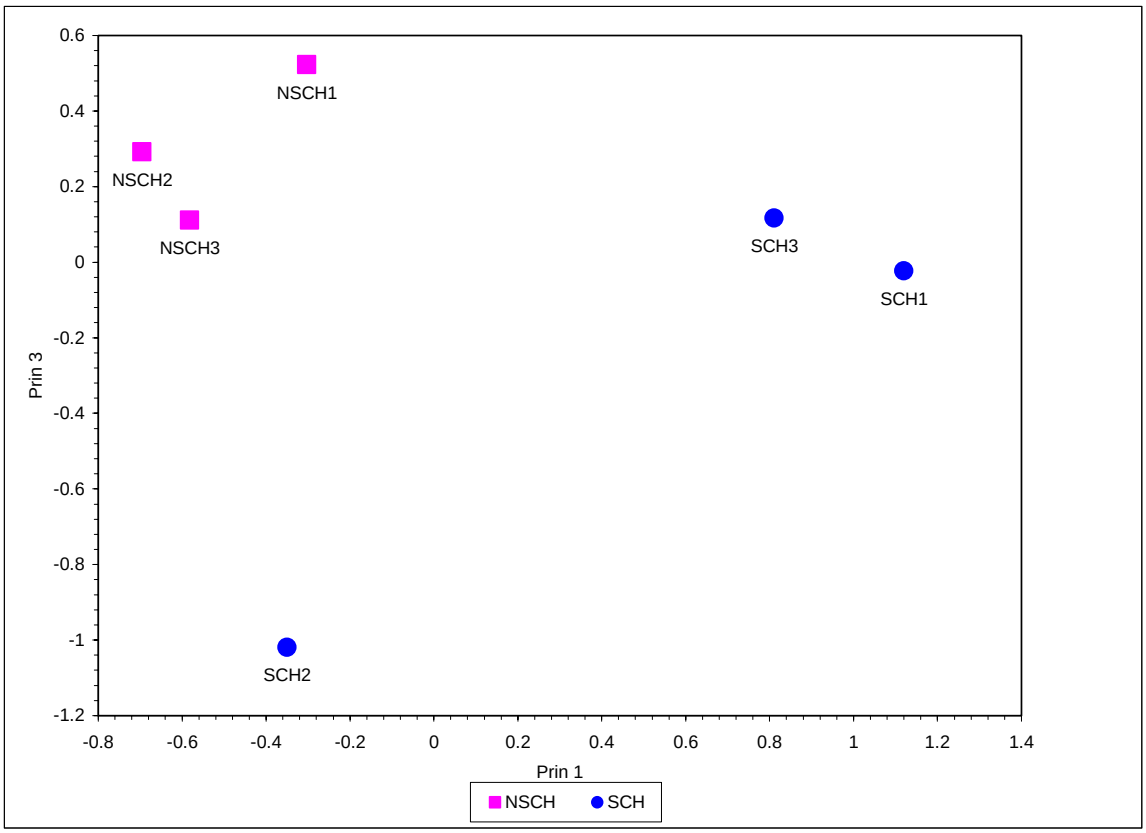

Figure 7. Principal component analysis of Biolog GN2 plate data for bulk soil samples from no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) fields at Site 1.

the first and second principal components explained 64% of the standardized variance, and the first three principal components explained 84%.

Only 12 substrates were utilized overall in the bulk soil samples at Site 1. Therefore, the absorbance values of the other 83 substrates were discarded to rid the PCA of redundant data. For bulk soils, the carbon substrates utilized in SCH soil but not NSCH were D-mannose, D-galacturonic acid, D-saccharic acid and D, L-carnitine, and substrates utilized in NSCH but not SCH were D-gluconic acid, β-hydroxybutyric acid, L-histidine, and L-leucine, L-pyroglutamic acid, and 2-amino ethanol. Microbial communities from both types of bulk soil utilized the carbon substrates D,L-lactic acid and L-glutamic acid.

At Site 1, SCH and NSCH bulk and rhizosphere soil microbial communities could not be clearly distinguished by PCA of sole carbon source utilization profiles (Fig. 8). One of the replicate SCH bulk soil was separated from the other two SCH bulk samples by principal component 1. The replicates of SCH rhizosphere soil did not cluster tightly, and the NSCH rhizosphere replicates did not cluster at all. The GN2 plate data PCA indicated that the first and second principal components explained 62% of the standardized variance, and the first three principal components explained 71%.

At Site 2, SCH and NSCH rhizosphere soil microbial communities could be distinguished by PCA of sole carbon source utilization profiles (Fig. 9). However, one replicate plate from each of the soil types was separated from the other two of its type by principal component 2. The GN2 plate data PCA indicated that the first and second principal components explained 90% of the standardized variance, and the first three principal components explained 94%.

For Site 2 rhizosphere soil, six carbon substrates were utilized by the rhizosphere community in SCH samples but not NSCH samples, including glycogen, tween 40, propionic acid, succinamic acid, 2-aminoethanol, and glycerol. Three substrates were utilized in rhizosphere NSCH samples but not SCH samples: gentiobiose, acetic acid, and malonic acid. Microbial communities in both types of rhizosphere samples utilized numerous carbon substrates in common, including N-acetyl-D- glucosamine, adonitol, L-arabinose, cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, sucrose, D-trehalose, methyl pyruvate, mono-methyl succinate, cis-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, p-hydroxyphenylacetic acid, D, L-lactic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-serine, D, L-carnitine, urocanic acid, inosine, uridine, thymidine, D, L-α-glycerol phosphate, and glucose-6-phosphate.

At Site 3, SCH and NSCH rhizosphere soil microbial communities were again separated by PCA of sole carbon source utilization profiles (Fig.10). Variability was detected in one SCH sample for overall plate reaction (principal component one) and among the NSCH sample replicates for some substrates utilized (principal component two). The

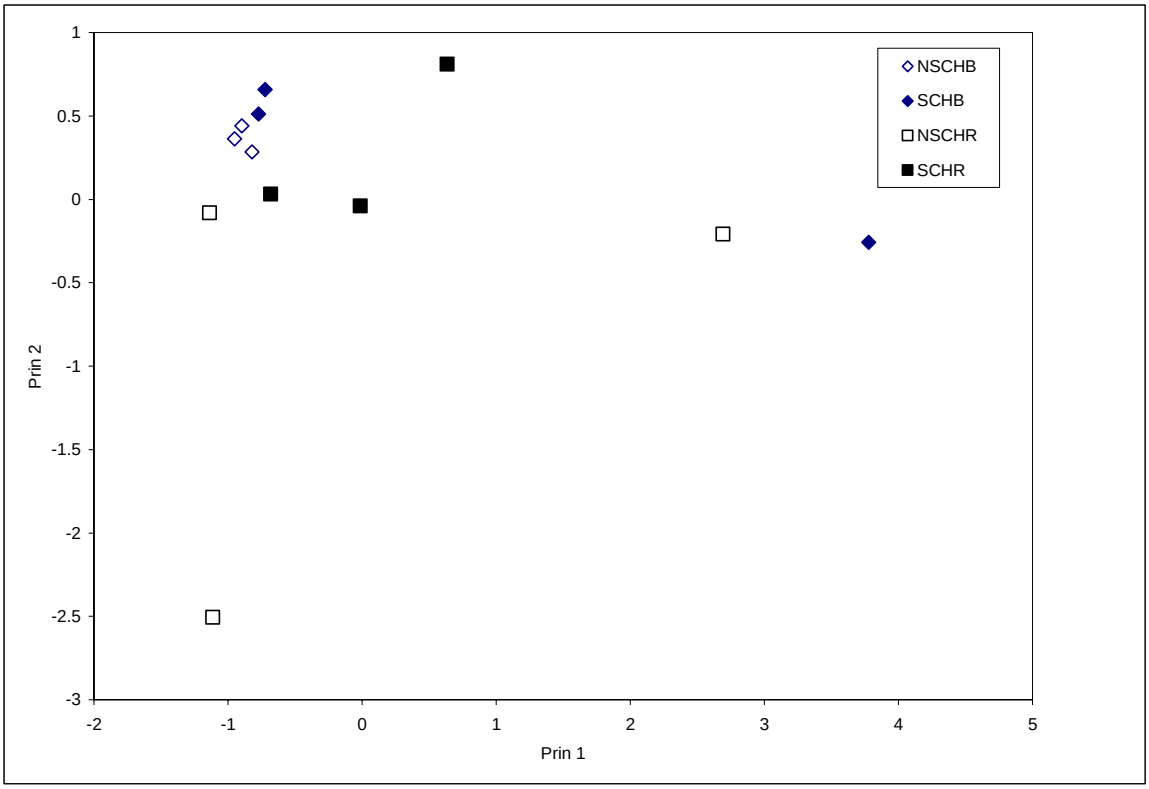

Figure 8. Principal component analysis of Biolog GN2 plate data for bulk soil samples from a field with no recent sugarcane cropping history (NSCHB), bulk soil samples from a field with a long-term sugarcane cropping history (SCHB), rhizosphere soil samples from a field with no recent sugarcane cropping history (NSCHR), and rhizosphere soil samples from a field with a long-term sugarcane cropping history (SCHR) at Site 1.

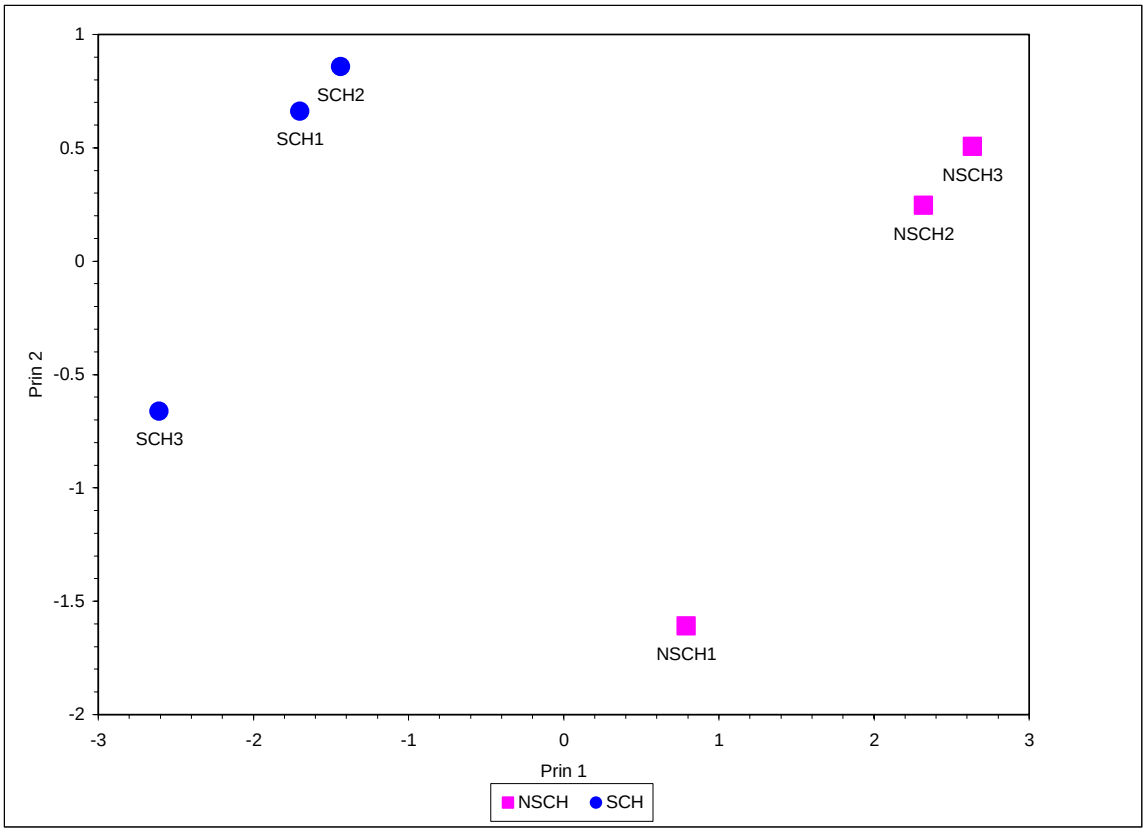

Figure 9. Principal component analysis of Biolog GN2 plate data for rhizosphere soil samples from fields with no recent sugarcane cropping history (NSCH) and long-term sugarcane cropping history (SCH) at Site 2.

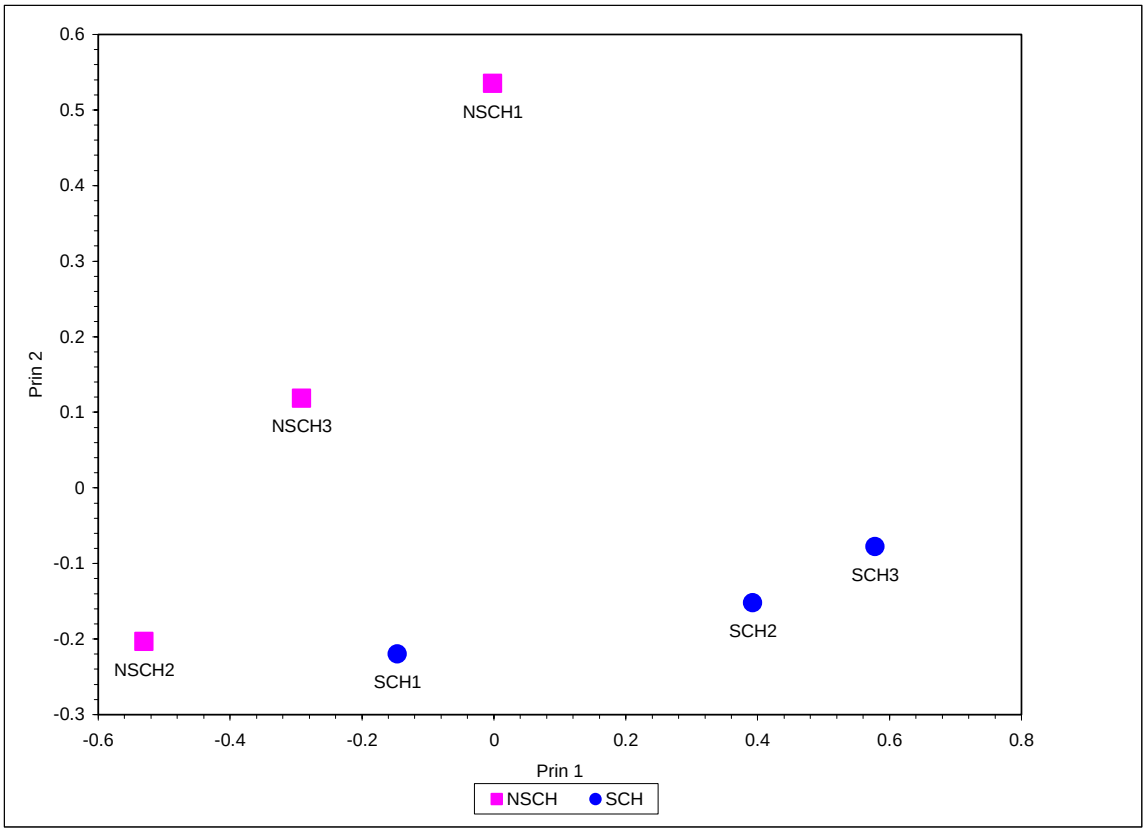

Figure 10. Principal component analysis of Biolog GN2 plate data for rhizosphere soil samples from from fields with no recent sugarcane cropping history (NSCH) and long-term sugrcane cropping history (SCH) at Site 3.

GN2 plate data PCA indicated that the first and second principal components explained 72% of the standardized variance, and the first three principal components explained 90%.

For Site 3 rhizosphere soil, five carbon substrates were utilized in rhizosphere SCH samples but not NSCH samples: glycogen, p-hydroxyphenylacetic acid, succinamic acid, D-serine, and phenylethylamine, while four substrates were utilized in rhizosphere NSCH samples but not SCH samples, including D-mannitol, D-glucuronic acid, β-hydroxybutyric acid, and glucose-6-phosphate. Both types of rhizosphere samples utilized numerous carbon substrates in common, including dextrin, tween 80, N-acetyl-D-glucosamine, sucrose, D-trehalose, methyl pyruvate, cis-aconitic acid, citric acid, D-galacturonic acid lactone, D-gluconic acid, α-ketoglutaric acid, D, L-lactic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-hisitdine, hydroxy-L-proline, L-proline, L-pyroglutamic acid, L-serine, γ-aminobutryic acid, urocanic acid, inosine, putrescine, glycerol, and glucose-6-phosphate.

Comparing SCH rhizosphere soil at all three sites, the soil microbial communities were separated by PCA of sole carbon source utilization profiles (Fig. 11). Variability was detected in one Site 1 sample for overall plate reaction (principal component one). The GN2 plate data PCA indicated that the first and second principal components explained 54% of the standardized variance, and the first three principal components explained 88%.

Comparing both NSCH and SCH rhizosphere soil at all three sites, the soil microbial communities for the different sites were separated by PCA of sole carbon source utilization profiles rather than by NSCH and SCH soil types (Fig.12). The GN2 plate data PCA indicated that the first and second principal components explained 59% of the standardized variance, and the first three principal components explained 76%.

2.4 Discussion

Differences were detected using multiple methods to compare culturable microorganisms between soil microbial communities associated with sugarcane roots in soils in Louisiana with and without a recent sugarcane cropping history. Previous research in Australia has indicated that long-term cultivation of sugarcane can cause changes in the soil microbial community (Garside, et al., 1997; Magarey, 1996; Magarey, et al., 1997a; Pankhurst, et al., 2000). Magarey et al. (1997a) reported that total culturable bacterial populations of bulk soil were similar between paired fields from 17 sites. However, there was a trend for higher numbers of fluorescent *Pseudomonas* spp. and actinomycetes in the soils with no sugarcane cropping history. Pankhurst et al. (2000) found higher populations of *Pseudomonas* spp. in the rhizosphere of sugarcane plants growing in fields without a continuous sugarcane cropping history. Total bacteria were similar, but additional

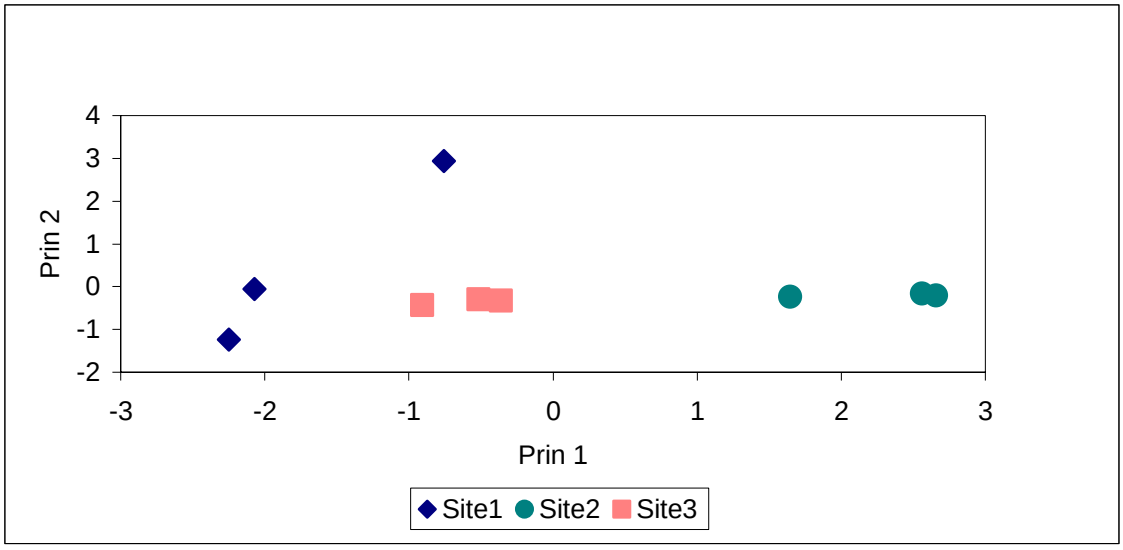

Figure 11. Principal component analysis of Biolog GN2 plate data for rhizosphere soil samples from long-term sugarcane cropping history (SCH) fields from three experimental sites.

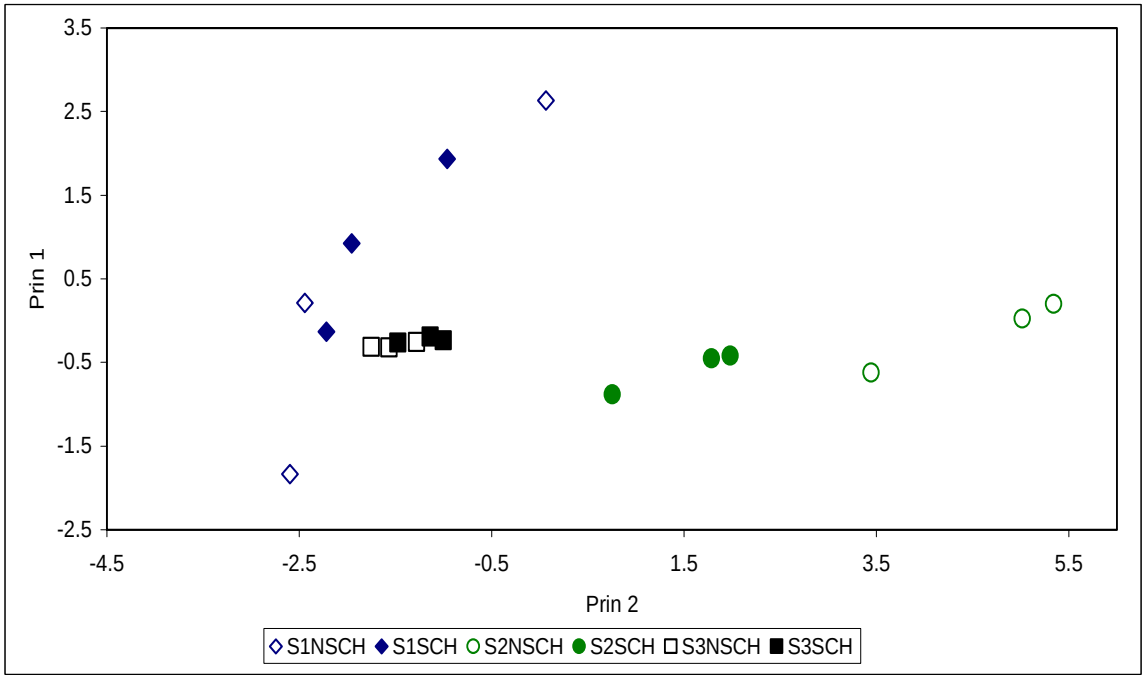

Figure 12. Principal component analysis of Biolog GN2 plate data for rhizosphere soil samples from Site 1fields with no sugarcane cropping history (S1NSCH) and sugarcane cropping history (S1SCH), Site 2 paired fields (S2NSCH and S2SCH), and Site 3 paired fields (S3NSCH and S3SCH).

qualitative differences were detected between rhizosphere soil communities from continuous sugarcane fields and those previously in pasture (Pankhurst, et al., 2000). The diversity of bacterial genera was greatest in continuous sugarcane soil, but genera in addition to *Pseudomonas*, such as *Bacillus*, capable of inhibiting the growth of *Pythium*, were detected in the soil previously in pasture.

Similar results were obtained in the Louisiana study. No consistent differences were detected in total bacterial populations in rhizosphere soils from fields with and without a sugarcane cropping history. However, qualitative differences between communities were detected with soil dilution plating on culture media and with sole carbon source utilization profiling. Higher populations of *Pseudomonas* spp. were found in NSCH soils at two locations, and higher numbers of actinomycetes were detected in NSCH soil at the third site. These findings could be significant, since members of these groups of microorganisms can inhibit the growth of root pathogens, including *Pythium*. One finding from Louisiana with uncertain implication was that the number of siderophore producing bacteria differed between NSCH and SCH at one of two sites. Additional qualitative differences in the make-up of microbial communities in NSCH and SCH soils may be revealed once the collected isolates have been identified.

The SCSUP results provide another line of evidence that qualitative differences exist between NSCH and SCH soils. However, this technique does not provide any information concerning the nature of any differences that are detected.

Evidence obtained with other plants besides sugarcane support the idea that plant type can influence the make-up of the rhizosphere microbial community. A dilution plate culture method study conducted by Rouatt and Katznelson (1961), compared microbial communities of rhizosphere, rhizoplane, and non-rhizosphere soil of six different plant species. They found that rhizosphere soil microbial communities from six different crop plants tested were affected by plant type rather than soil type. These results were supported by SCSUP differences detected for rhizsosphere soils from wheat, potato, soybean, and sweetpotato (Garland, 1996).

Research in Australia also suggested that changes in the fungal community are associated with yield decline in sugarcane (Magarey, et al., 1997a). Application of the broad-spectrum fungicide, mancozeb, decreased root colonization by a dematiaceous, sterile fungus and improved root health and plant growth. It was suggested that the role of dematiaceous, sterile fungi in yield decline of sugarcane should be investigated further. In other research, Kao and Hsieh (1986) found that in monocultured sugarcane soil *Fusarium* and *Trichoderma* predominated. However, Watanabe, et al. (1974) found that *Fusarium* was frequently isolated from both healthy and unhealthy sugarcane.

At both sites in Louisiana where the fungal community associated with the rhizosphere of sugarcane was evaluated, differences were detected between NSCH and SCH soils. The population of total fungi was higher in NSCH soil at Site 2, and the number of fusarial fungi was higher in NSCH soil at Sites 2 and 3.

The study results comparing bulk and rhizosphere soil bacterial communities were similar to the findings with sugarcane from Australia (Magarey, et al., 1997a; Pankhurst, et al., 2000). More bacteria were present in bulk NSCH soil compared to SCH soil, but the total population levels in rhizosphere NSCH and SCH soils were similar. In Louisiana, bacterial populations were higher in rhizosphere compared to bulk soil. This finding supports previous reports that numbers of microorganisms are higher in rhizosphere than bulk soils (Cooper and Chilton, 1950; Hurst, et al., 2002; Rouatt and Katznelson, 1957). The microbial community comparison was therefore focused on the rhizosphere of sugarcane roots, since this is the community most likely to affect plant growth.

Chemical properties can affect the numbers and types of organisms in a soil microbial community. Organic matter was numerically lower in Louisiana SCH soils. Lower pH was the attribute most consistently associated with yield decline soils in Australia (Bramley, et al., 1996), but there was no consistent difference in pH between NSCH and SCH soils in Louisiana.

In this study, sole carbon source utilization profiling was able to detect differences in soil microbial communities associated with a certain plant type. The difference between this study and previous SCSUP research showing that plants can influence the make-up of the microbial community in soil (Garland, 1996; Rouatt and Katznelson, 1961) is that, in this case, the rhizosphere microbial community associated with a crop plant, sugarcane, was shown to be affected by prior cultivation of that plant.

Different types of microplates are available from Biolog. The GN2 and GP2 microplates are designed for identification of gram negative and gram positive bacteria, respectively. However 62 of the substrates are common to the GN2 and GP2 plates, while each has 33 unique substrates. Since many bacteria associated with rhizosphere environments are gram negative (Hurst, et al., 2002), it was decided that the 33 extra carbon substrates in GP2 plates would not provide enough additional information to justify its use in this study. Biolog now offers the EcoPlate™ system that is composed of 31 carbon substrates useful for soil community analysis that are replicated three times on a single plate. However, experiments with GN2 and GP2 microplates revealed that most of the substrates utilized by microbial communities from sugarcane rhizosphere or bulk soils in Louisiana were not available in the EcoPlate™.

PCA was used in this study to analyze SCSUP data instead of DCA, CVA, and kinetic modeling because *a priori* grouping of the variables was not required and it was less time consuming. Microbial community functional diversity estimates for substrate richness and evenness evaluated with Shannon or McIntosh indices were not used in this study because more information concerning diversity can be obtained with culturing on different media and with molecular techniques (Garland and Mills, 1999).

An important question partially addressed by this study was: Does sugarcane monoculture create a rhizosphere microbial community that is similar at different locations and times? Furthermore, does long-term cultivation of the sugarcane plant result

in the establishment of a community with consistent characteristics, and do members of that community that may not be recognized as pathogens contribute to yield decline? The SCSUP results showed that differences in community functional diversity exist between sites in soils with a long-term sugarcane cropping history in common. The sites were sampled at different times (Site 1, May 2001; Site 2 June 2002; Site 3, August, 2002), so differences in environmental and edaphic conditions existed between sites at the time of sampling. Differences in functional diversity might be expected to occur under varied conditions. In addition, one study (Srinivasan, 1968) found that sugarcane genotype affected the make-up of the rhizosphere microbial community. However, there still could be a commonality in types of organisms making up the rhizosphere microbial community of sugarcane growing in any soil previously cropped for an extended period with sugarcane. The results from this study and Australia concerning the types of culturable organisms in the rhizosphere suggest that similarities do exist. However, the evidence is incomplete, and the potential effects of any one feature, such as reduced levels of fluorescent Pseudomonads, are uncertain. Molecular approaches for characterizing and identifying organisms in soil microbial communities may eventually be able to identify any consistent features of the rhizosphere community associated with sugarcane monoculture, but elaborating explanations for yield decline will still remain as a considerable challenge. Even if our understanding of this complex problem remains incomplete, improvements in understanding of community changes induced by monoculture might lead to new ideas on how to influence the system to improve plant growth.

CHAPTER 3. DEVELOPMENT OF METHODS TO OBTAIN DNA SUITABLE FOR MOLECULAR COMPARISON OF SOIL MICROBIAL COMMUNITIES

3.1 Introduction

In Louisiana, sugarcane (inter-specific hybrids of *Saccharum*) is grown largely under long-term monoculture production. The crop is vegetatively propagated by planting of stalks. Therefore, sugarcane cultivars represent clones composed of genetically identical plants. Buds on planted stalk sections germinate and grow to produce the first "plant cane" crop. Following the first harvest of stalks, buds on the stubble remaining in the soil can germinate to produce a ratoon crop. In Louisiana, sugarcane plants typically produce three to five successive crops. The stubble is then plowed out during the spring, and following a 4-6 month plant-free fallow period, the crop is replanted during late summer.

Monoculture can lead to poor root health and growth constraints in many crops, including sugarcane (Garside, et al., 1997; Magarey, 1996; Savory, 1966). A complex problem termed "yield decline" was defined by Garside, et al. (1997) as "the loss of productive capacity of soils under sugarcane monoculture." Previous studies have provided evidence of a biological component to yield decline in sugarcane, such as 20% and larger yield responses to fumigation (Bell, 1935; Garside, et al., 1997; Magarey, 1999; Muchow, et al., 1994) and positive growth responses to soil solarization and soil incorporation of the broad-spectrum fungicide mancozeb (Croft, et al., 1984; Magarey, 1996; Magarey, et al., 1997b). The evidence suggests that soil microbial factors in addition to recognized pathogens, such as *Pythium* (Edgerton, et al., 1929; Hoy, 1999; Magarey, 1996) and nematodes (Bond, et al., 2000; Spaull and Cadet, 1990; Stirling, et al., 1999) are involved with yield decline.

Techniques are now available to analyze soil microbial community nucleic acids. Most produce profiles of the community based on polymerase chain reaction (PCR) amplified DNA using primers universal for bacteria. Several molecular techniques have provided information on changes in the soil microbial communities that occur with different agricultural treatments and disease incidences (McSpadden Gardener and Weller, 2001; Øvreås and Torsvik, 1998; Picard, et al., 2000; Schmalenberger and Tebbe, 2002).

Single-strand conformation polymorphism (SSCP) has been used to generate profiles using a 250-400 bp fragment of the gene coding for 16S rRNA(DNA). The technique can separate DNA fragments with minor differences in nucleotide sequence (Lee, et al., 1996; Schmalenberger and Tebbe, 2002). Schmalenberger and Tebbe (2002) used SSCP to compare bacterial communities in rhizospheres of transgenic maize and sugar beet.

The PCR-based amplified ribosomal DNA restriction analysis (ARDRA) technique has been used to compare bacterial diversity and community composition from lake water samples (Bosshard, et al., 2000), from agricultural soils (Øvreås and Torsvik, 1998), and from rhizosphere soil of diseased wheat (McSpadden Gardener and Weller, 2001). Restriction enzyme digestion of rDNA can produce distinctive banding patterns

following electrophoresis. However, ARDRA works best once individual organism rDNA is separated from the community rDNA pool by cloning.

The PCR-based random amplified polymorphic DNA (RAPD) technique has been used to compare microbial communities in various termite mounds and soils (Harry, et al., 2001) and in rhizosphere soil of maize (Picard, et al., 2000). The RAPD method was able to estimate the similarity of communities, but it will not readily lead to the identification of organisms accounting for differences in the communities.

Molecular microbial community analysis methods depend on the efficient extraction of nucleic acids from the microorganisms of interest. There are numerous reports of many different techniques to extract nucleic acids from soil microbial communities. These techniques lyse and extract nucleic acids directly or indirectly from microorganisms in soil (Fægri, et al., 1977; Miller, et al., 1999; Picard, et al., 1992; Saano, et al., 1995). There are advantages and disadvantages involved with each technique. Direct methods that lyse organisms in the soil matrix yield higher DNA concentrations and are presumably less biased than indirect lysis methods (Saano, et al., 1995). However, a disadvantage to direct lysis methods is that PCR-inhibitory substances are coextracted along with the DNA (Picard, et al., 1992). With indirect methods, the microbial cells are separated from the soil matrix by centrifugation and then lysed to release nucleic acids (Fægri, et al., 1977; Holben, et al., 1988).

The goals of this study were to increase our basic understanding of microbial communities in sugarcane soils, to determine if there are differences in microbial communities in soils with a sugarcane cropping history (SCH) and with no sugarcane cropping history (NSCH), and to provide information on changes in the soil microbial communities that occur with sugarcane monoculture that may contribute to sugarcane yield decline. To accomplish this, the first objective was to develop methods to reliably isolate DNA from soil and then amplify rDNA suitable for use in molecular techniques for comparisons of soil microbial communities.

3.2 Materials and Methods

3.2.1 Study Sites and Soil Sample Collection Method

Study sites were selected with SCH and NSCH fields either adjacent or closely situated to minimize differences in soil type, climate, and farm management. All sites were planted with the sugarcane variety LCP 85-384. All of the fields without a recent sugarcane cropping history were in the first season of sugarcane production. In contrast, all of the long-term sugarcane fields were in monoculture production (with a plant-free fallow period every fourth or fifth growing season) for 15 or more years.

At all sites, 25 samples were collected in a Z pattern from each field. Roots and tightly adhering soil were collected for rhizosphere soil samples. The roots and adhering soil were removed from a zone approximately 2-25 cm below plants and placed in individual sterile whirl-pak bags (Fisher Science, Pittsburgh, PA). Non-rhizosphere, bulk soil

samples also were collected. The samples were placed in an ice chest with cold packs for transport to the lab.

3.2.2 Nucleic Acid Extraction Methods for PCR

Research was performed to determine the optimal method to extract DNA from sugarcane field soils. The methods selected included two modified indirect DNA extraction methods, Holben, et al. (1988) combined with the DNA extraction method of Lee, et al. (1996) and Kuske, et al. (1997), and two direct DNA extraction methods, Saano, et al. (1995) and the commercially available FastDNA® SPIN Kit® (For Soil) (Bio 101, Inc., Carlsbad, CA). Finally, a Mini-Beadbeater-8™ cell disrupter (BioSpec Products, Inc. Bartlesville, OK) was used with the indirect extraction methods that converted them to direct extraction methods. The extracted DNA was then assessed for suitability for PCR amplification.

For the first indirect extraction method (Holben, et al., 1988), bacteria were separated from the soil matrix and lysed as follows: 50 g of wet-weight bulk soil from a sugarcane field was combined with 200 ml of homogenization solution (Winogradsky salt stock solution composed of 1.43 mM K_2HPO_4, 1.01 mM $MgSO_4 \cdot 7H_2O$, 2.14 mM NaCl, 4.75 µM $Fe_2(SO_4)_3 \cdot 7H_2O$, and 14.8 µM $MnSO_4 \cdot 4H_2O$ diluted 1:20 for a working solution, with sodium ascorbate added just before use to a final concentration of 0.2 M, pH 7.2, along with 10 g of acid-washed poly(vinylpolypyrrolidone) (PVPP) (Sigma Chemical Co., St. Louis, MO). The soil sample in homogenization solution and acid-washed PVPP was homogenized in a Waring blender for three, 1 min intervals with cooling in an ice water bath for 1 min between homogenization intervals. The homogenate was transferred into 250 ml centrifuge bottles and centrifuged at 1,000 x g for 15 min at 4°C to pellet the soil debris. The supernatant was saved. The pellet was resuspended in fresh homogenization solution blended and centrifuged for two more rounds, and the supernatants from all three homgenization rounds were collected and combined. The combined supernatant was then centrifuged at 23,000 x g for 20 min at 4°C to collect the bacterial fraction. Bacterial pellets were resuspended in 200 ml of 2% (wt/vol) of sodium hexametaphosphate, pH 8.0, and re-centrifuged at 23,000 x g for 20 min at 4°C to collect pellets. Then the pellets were given two washes by resuspending in 1 ml of TE buffer (33 mM Tris, pH 8.0, and 1 mM EDTA) and centrifuging at 23,000 x g for 20 min at 4°C.

The DNA was then extracted from the bacterial pellets by the method of Lee, et al., (1996). After the second wash, the pellet was suspended in 300µl of lysozyme solution (0.15 M NaCl, 0.1 M EDTA, (pH 8.0), and 15 mg of lysozyme/ml) and incubated in a water bath at 37°C for 1 hr, with mixing by inversion every 15 min. After incubation, the sample was cooled on ice. Then, 300 µl of SDS buffer (0.1 M NaCl, 0.5 M Tris-HCl, pH 8.0, 4% (wt/vol) sodium dodecyl sulfate (SDS)) was added, and the sample was incubated in an ice bath for 10 min. The sample was then placed at 55°C for 10 min. This freeze-thaw process was repeated three times. The bacterial genomic DNA was extracted and purified from cell lysates by two sequential phenol-chloroform, isoamyl alcohol (24:1) extractions (equal volumes to DNA sample ~600 µl). The upper aqueous phase was collected and mixed with phenol-chloroform, isoamyl alcohol (24:1) to repeat the

process. The aqueous supernatant was mixed with two volumes (~600 µl) of 70% (vol/vol) ethanol at room temperature and vortexed. Then the mixture was allowed to stand for 2 min at room temperature then centrifuged in a microfuge at 16,000 x g for 5 min at 4°C to precipitate and pellet DNA. The DNA pellet was resuspended in 300 µl of 70% ethanol, vortexed, and again centrifuged at 16,000 x g at 4°C for 5 min to wash it. The ethanol supernatant was then discarded, and the DNA pellet was allowed to dry in the tube by standing in a positive-flow laminar flow hood for 10 min. After drying, the DNA pellet was resuspended in 50 µl of sterile TE buffer.

Soil bacterial DNA also was indirectly extracted from soil by a modified procedure of Kuske, et al. (1997), in which bacterial cells were separated from the soil matrix by placing 30 g of wet-weight bulk soil in 120 ml of TENS buffer (50 mM Tris, pH 8.0, 20 mM disodium EDTA, 100 mM NaCl, and 1% (wt/vol) SDS) in a sterile 250 ml centrifuge bottle. This soil suspension was mixed well by vortexing 1 min then placed on ice for 10 min. The process was repeated twice. The suspension was then incubated at 70°C for 1 hr with vortex mixing at 15 min intervals. After incubation, the solution was again vortexed 1 min and placed on ice 10 min three times. Then, the sample was centrifuged at 6,000 x g at 4°C for 10 min. The supernatant was collected in a sterile, 250 ml centrifuge bottle, and the soil pellet was resuspended in 30 ml of TEN buffer (TENS without SDS, pH 8.0). The sample was centrifuged as before, and the supernatant was pooled with the first supernatant. The soil pellet was then resuspended in 45 ml of TEN buffer and exposed to three rounds of thermal shock by placing the samples in a -70ºC freezer and 70ºC hot water bath for 2 min each. The sample was then centrifuged two times as before, and the supernatants were pooled with the original supernatants. The pellet was discarded, and the pooled supernatant was centrifuged at 23,000 x g at 4ºC for 20 min. Then, the pellet containing cell lysates was given two washes by resuspending in 1 ml of 10 mM Tris, pH 8.0 and 1 mM EDTA and centrifuging at 23,000 x g for 20 min at 4°C.

The bacterial genomic DNA was separated and purified from cell lysates by two sequential phenol-chloroform, isoamyl alcohol (24:1) extractions (equal volume to DNA sample ~1 ml). Following centrifugation at 9000 x g at 4°C for 15 min, the upper aqueous phase containing DNA was collected and again mixed with phenol-chloroform isoamyl alcohol (24:1) (equal volume to DNA sample ~1 ml) in a sterile, 15 ml centrifuge tube. Then, it was centrifuged at 9000 x g at 4°C for 15 min. The supernatant containing the aqueous phase with the DNA was transferred to a sterile, 2.5 ml microtube. The supernatant was mixed with two volumes (~2 ml) of 70% (vol/vol) ethanol at room temperature and vortexed. Then, the mixture was allowed to stand for 2 min at room temperature and centrifuged in a microfuge at 16,000 x g for 5 min at 4°C to precipitate and pellet DNA. The DNA pellet was resuspended in 300 µl of 70% ethanol, vortexed, and again centrifuged at 16,000 x g at 4°C for 5 min to wash it. The ethanol supernatant was then discarded, and the DNA pellet was allowed to dry in the tube by standing in a positive-flow laminar flow hood for 10 min. After drying, the DNA pellet was resuspended in 50 µl of sterile TE buffer.

Soil bacterial DNA was directly extracted by a modified procedure of Saano, et al. (1995). Cells were lysed by adding 1 g of air-dried bulk soil sample in 2.5 ml of buffer (120 mM Na_2HPO_4, pH 8.0, 1% (wt/vol) SDS, and 100 µg/ml proteinase K) in a 15 ml polypropylene tube and mixed well by vortexing. It was then incubated 1 hr at 37^{o}C with occasional shaking. Then, 450 µl of sterile, 5 M NaCl solution was added and vortexed. The NaCl concentration was raised to prevent nucleic acids from dissolving in cetyltrimethyl-ammonium bromide (CTAB), which was added next as 375 µl of 10% (wt/vol) CTAB in 0.7 M NaCl. This solution was incubated at 65°C for 20 min to denature proteins and polysaccharides. The bacterial genomic DNA was separated and purified from cell lysates by two sequential phenol-chloroform, isoamyl alcohol (24:1) extractions (equal volume to DNA sample ~3.5 ml) as follows. It was centrifuged at 9000 x g at 4°C for 15 min. The upper aqueous phase containing DNA was collected and again mixed with phenol-chloroform isoamyl alcohol (24:1) (equal volume to DNA sample ~3.5 ml) in a sterile, 15 ml centrifuge tube. Then it was centrifuged at 9000 x g at 4°C for 15 min. The supernatant containing the aqueous phase with the DNA was transferred to a sterile, 2.5 ml microtube. An equal volume (~ 800 µl) of isopropanol was added, mixed well, and incubated for 1 hr at -20^{o}C to precipitate the nucleic acids. This was centrifuged for 15 min at 4°C at 10,000 x g. The isopropanol was poured off, and the DNA pellet was allowed to dry in the tube by standing in a positive-flow laminar flow hood for 10 min. After drying, the DNA pellet was resuspended in 200 µl of sterile, distilled water.

Soil bacterial DNA was extracted directly by the FastDNA® SPIN Kit® (For Soil). All reagents were supplied in the kit, except ethanol to add to the salt/ethanol/ wash solution (SEWS-M). DNA was extracted according to the manufacturers instructions as follows: 500 mg of air-dried sugarcane bulk or rhizosphere soil was added to a multimix 2 Tissue Matrix Tube designed to lyse all microorganisms and a homogenization solution containing ceramic and silica particles, 978 µl sodium phosphate buffer, and 122 µl of MT Buffer. The sample was homongenized in the multimix 2 tissue matrix tube for 30 sec at the homogenization speed (3200 rpm) of the Mini-Beadbeater-8™ cell disrupter. Then it was centrifuged at 14,000 x g at room temperature for 15 min. The pellet was discarded. The supernatant was transferred to a sterile, 2 ml tube. Then, 250 µl protein precipitation solution (PPS) reagent was added and mixed by inverting the tube ten times. This mixture was centrifuged at 14,000 x g at room temperature for 5 min to pellet the protein precipitant. The supernatant was transferred to a sterile, 2 ml tube. Then, 1 ml of binding matrix suspension (silica matrix) was added to the supernatant. It was shaken for 2 min by placing tubes in a rack on a shaker at 500 rpm. This allowed the DNA to bind to the matrix. The tubes were allowed to stand in a rack for 3 min for the silica matrix to settle. Then, 500 µl of the supernatant was transferred to another tube just in case the DNA did not bind to the matrix. The remaining supernatant and the binding matrix were resuspended, and a portion of it (600 µl) was transferred to a spin filter and centrifuged at 14,000 x g at room temperature for 1 min. The liquid in the catch tube of the spin filter was discarded, and the remaining supernatant from the binding matrix tube was added to the spin filter and centrifuged as before. The liquid in the catch tube was discarded. Then, 500 µl of SEWS-M (containing ethanol) was added to the spin filter and centrifuged at 14,000 x g at room temperature for 1 min. The spin filter was removed to decant the liquid in the catch tube and then the filter was placed into the catch tube again. The spin

filter was centrifuged at 14,000 x g at room temperature for 2 min to "dry" the matrix by removing the residual SEWS-M wash solution. The spin filter was removed and placed in a new kit-supplied catch tube and air-dried under a positive flow laminar hood with the cap open for 5 min at room temperature. Then, 50 µl of DES (Dnase/Pyrogen Free Water) was added, and the silica pellet was resuspended by brief vortexing or finger-flicking the spin-filter to elute DNA. Then, the suspension was centrifuged at 14,000 x g at room temperature for 1 min to transfer 50 µl of the eluted DNA to the catch tube.

The FastDNA® SPIN Kit® (For Soil) lysed cells mechanically by use of a silica matrix and a bead-beater homogenizer. Therefore, to determine if lysing cells by bead- beater homogenization would enhance DNA extraction of the other methods evaluated, the methods were modified to include cell lysis by bead-beater homogenization without enzymatic or physical lysis by freeze-thawing. Three different sizes of sterile glass beads (0.1 mm, 0.5 mm, and 1.0 mm dia.) in the amount of 0.66 g each combined for a total of 2 g were placed in a sterile, 2 ml plastic screw cap centrifuge tube. Then 0.5 g of bulk or rhizosphere soil from sugarcane fields was added to the tube with the appropriate lysis buffer for each extraction method tested. The positive control sample was cultured soil bacteria from the same soil samples dilution plated on 10% tryptic soy agar. A negative control with no soil was used throughout the entire protocol. All protocols were compared to the FastDNA® SPIN Kit® (For Soil) protocol.

With the Holben, et al. (1988) protocol, a soil sample was placed in a sterile, 2 ml centrifuge tube with 2 g of glass beads and covered with 1.25 ml of Winogradsky salt solution prepared the same as in the first extraction protocol and 1.2 mg of acid-washed PVPP. Then, the sample was homogenized at 3200 rpm for 30 sec and centrifuged at 12,000 x g at room temperature for 3 min. One milliliter of the supernatant was transferred to a sterile, 2 ml centrifuge tube and centrifuged at 1,000 x g at 4°C for 15 min to pellet bacterial cell debris. The supernatant was collected and placed in a sterile, 15 ml centrifuge tube. The bacterial cell debris pellet was washed in 1.25 ml of Winogradsky's salt solution and centrifuged at 1,000 x g at 4°C for 15 min. The wash was repeated twice, and the second and third supernatants were pooled with the first. The pooled supernatants were centrifuged at 23,000 x g at 4°C for 20 min to pellet DNA. The pellet was washed and centrifuged at 10,000 x g at 4°C for 30 min in 2% sodium hexametaphosphate (wt/vol), pH 8.5. This wash was repeated once. The pellet was resuspended, washed, and centrifuged at 7,000 x g at 4°C for 30 min in 2 ml of TE buffer (10 mM Tris, pH 8.0, and 1 mM disodium EDTA). This wash was repeated once. The supernatant was discarded. The DNA pellet was allowed to dry in the open centrifuge tube under a positive flow laminar hood for 10 min before final purification.

With the Kuske, et al. (1997) DNA extraction protocol, a soil sample was placed in a sterile, 2 ml centrifuge tube with 2 g of glass beads and covered with 1.25 ml of TENS buffer (50 mM Tris, pH 8.0, 20 mM disodium EDTA, 100 mM NaCl, and 1% (wt/vol) SDS). Then, the sample was homogenized with at 3200 rpm for 30 sec and centrifuged at 12,000 x g at room temperature for 3 min. One milliliter of the supernatant was transferred to a sterile, 15 ml centrifuge tube, and the soil pellet was washed in 1.25 ml of TEN buffer, pH 8.0 (TENS buffer without SDS). The washed pellet was centrifuged at

6,000 x g at 4°C for 10 min. This wash was repeated once. The second supernatant was pooled with the first. The pooled supernatants were centrifuged at 23,000 x g at 4°C for 20 min to pellet DNA. The supernatant was discarded. The DNA pellet was allowed to dry in the open centrifuge tube under a positive flow laminar hood for 10 min before final purification.

With the Saano, et al. (1995) DNA extraction protocol, a soil sample was placed in a sterile, 2 ml centrifuge tube with 2 g of glass beads and covered with 1.25 ml of lysis buffer (120 mM sodium phosphate, pH 8.0, 1% (wt/v) SDS), vortexed briefly, and incubated for 1 hr at 37°C. Then the sample was homogenized at 3200 rpm for 30 sec and centrifuged at 12,000 x g at room temperature for 3 min. One milliliter of the supernatant was transferred to a sterile, 15 ml centrifuge tube and mixed with 225 µl of 0.5 M NaCl. This solution was mixed with 188 µl of 10% (v/v) (CTAB) and 0.7 M NaCl and incubated at 60°C for 20 min. The bacterial genomic DNA was separated and purified from cell lysates by two sequential phenol-chloroform, isoamyl alcohol (24:1) extractions (equal volume to DNA sample ~1.5 ml). This mixture was centrifuged at 9000 x g at 4°C for 15 min. The upper aqueous phase containing DNA was collected and again mixed with phenol-chloroform isoamyl alcohol (24:1) (equal volume to DNA sample ~1.5 ml) in a sterile, 15 ml centrifuge tube. Then it was centrifuged at 9000 x g at 4°C for 15 min. The supernatant containing the aqueous phase with the DNA was transferred to a sterile, 2.5 ml microtube. The solution was mixed with an equal volume (600 µl) of isopropanol and incubated for 1 hr at -20°C. To concentrate precipitated DNA, this solution was centrifuged at 10,000 x g at 4°C for 15 min. The isopropanol was poured off, and the DNA pellet was allowed to dry in the open centrifuge tube under a positive flow laminar hood for 10 min before final purification.

Final DNA purification for all methods was performed with the Prep-A-Gene® DNA Purification Kit (Bio-Rad Laboratories, Hercules, CA). At the end of purification, all DNA pellets were resuspended in 50 µl of sterile, distilled H_2O.

After purification, the DNA template from the combined Holben, et al. (1988) and Lee, et al. (1996) extraction protocol was amplified with the Lee, et al. (1996) PCR protocol. Briefly, a 250 bp 16S rDNA fragment was amplified from genomic DNA with the oligonucleotide primers SRV3-1, (5´-CGG YCC AGA CTC CTA CGG G-3´), and SRV3-2, (5´-TTA CCG CGG CTG CTG GCA C-3´) (Y is C or T). The amplification mixture contained 1.5 mM $MgCl_2$, 10.0 mM Tris-HCl, pH 8.8, at 25°C, 50.0 mM KCl, 0.1% (vol/vol) Triton X-100, 200 µM each deoxy-nucleoside triphosphate dNTP, 2.0 U (units) *Taq* polymerase (Sigma), 0.1 µM each oligonucleotide primer, and 1:100, 1:50, 1:10, and 1:2 dilutions of DNA template solution in a final volume of 100 µl. The sample was amplified using an Amplitron II (Barnstead|Thermolyne, Corp., Dubuque, IA) thermal cycler under the following conditions: 94°C 5 min initial denaturation, 30 cycles of 94°C for 1.5 min, 62°C for 1.5 min, and 72°C for 2.0 min, and a final extension at 72°C for 2 min.

The extracted soil bacterial DNA templates from all other extraction protocols were PCR amplified using the primers and a modified protocol of Bosshard, et al. (2000). Briefly, a

1.5 kb 16S rDNA fragment was PCR amplified from genomic DNA with the oligonucleotide primers S-D-Bact-0008-a-S-20 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and S-D-Bact-1492-a-A-19 (5′-GGT TAC CTT GTT ACG ACT T-3′). The amplification reaction mixture contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 1 U of *Taq* polymerase, (Promega, Madison, WI), 0.2 µM each primer, 0.14 µl of bovine serum albumin fraction V (BSA) (100 µg/µl) (Sigma, St.Louis, MO) with undiluted and a 1:25 dilution of 5 µl of DNA template solution in a final volume of 25 µl. The PCR was conducted as follows: 3 min initial denaturation at 95°C, followed by 25 cycles of 1 min at 95°C, 1 min at 50°C , and 2 min at 72°C, with a final extension step of 10 min at 72°C after cycling was complete.

In addition to evaluating the DNA extraction protocols, two amplification protocol modifications were evaluated. Samples extracted with the method of Kuske, et al. (1997) were amplified with 0.14 µl of BSA (100 µg/µl) per 25 µl of amplification mix and without BSA in the amplification mix. The FastDNA® SPIN Kit® (For Soil) extracted samples were optimized for DNA template concentration with undiluted and 1:25 diluted samples in the amplification mix.

All PCR products were electrophoresed in an agarose gel (1.5%) in 1X Tris-borate-EDTA buffer, stained with EtBr (0.5 µg/ml), and visualized with a ultra-violet illuminator. With the FastDNA ® SPIN Kit® (For Soil) protocol, bands at the 1.5 kb region were excised and purified with the Gene Clean® II Kit, (Bio 101, Inc., Carlsbad, CA) and reamplified with the Bosshard, et al. (2000) PCR primers and protocol.

3.3 Results

PCR amplification of DNA from the first extraction protocol (Holben, et al. 1988 and Lee, et al. 1996) resulted in weak bands corresponding to the targeted 250 bp region with the 1:50 and 1:100 template dilutions, whereas amplicons were not detected with the 1:2 and 1:10 dilutions or the negative control.

Amplification of DNA from the second extraction protocol (Kuske, et al. 1997) yielded bands for all template dilutions in the targeted 1.5 kb region from the cultured positive control bacteria but not from the soil matrix. These bands were amplified with and without BSA in the amplification mix. The cultured positive control bacteria samples without BSA did not amplify. Products were not amplified with the negative control.

During comparisons of the direct DNA extraction protocols, bands were not amplified with the Saano, et al. (1995) protocol, whereas amplification of bands in the 1.5 kb targeted region was obtained with the soil samples extracted by the FastDNA® SPIN® Kit (For Soil). The inclusion of homogenization with the bead-beater did not improve PCR amplification of rDNA for any method except for one sample of the Saano, et al. (1995) protocol that yielded a 1.5 kb band.

Undiluted and 1:25 diluted genomic DNA templates obtained with the FastDNA ® SPIN Kit® (For Soil) were amplified for NSCH and SCH bulk soil samples (Fig. 13).

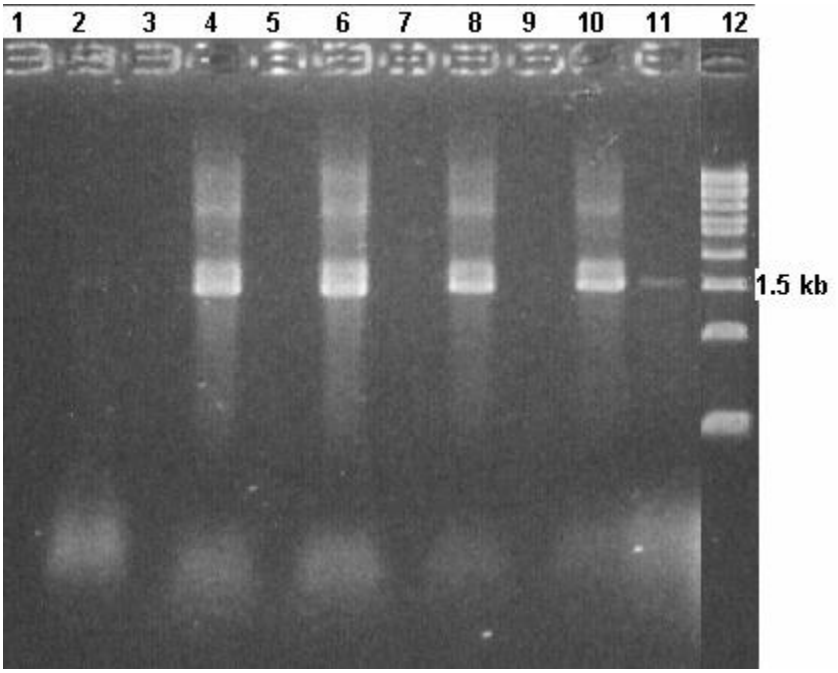

Figure 13. Agarose gel electrophoresis (1.5%) of PCR-amplified 16S rDNA products from soil bacteria. Targeted bands (1.5 kb) were amplified with S-D-Bact-0008-a-S-20/S-D-Bact-1492-a-A-19 primers. Lanes 1, 3, 5, 7, 9, empty, Lane 2 undiluted no recent sugarcane cropping history (NSCH) soil sample, Lanes 4 and 6 1:25 diluted NSCH soil samples reps 1 and 2, respectively, Lanes 8 and 10 1:25 diluted long-term sugarcane cropping history (SCH) soil samples reps 1 and 2, respectively, Lane 11 undiluted SCH soil sample, Lane 12 1 kb DNA ladder (Sigma).

PCR-amplified 16S rDNA bands in the 1.5 kb region were detected from 1:25 diluted NSCH soil samples but not from an undiluted NSCH soil sample. Similarly, amplified product bands were detected for 1:25 diluted SCH soil DNA samples, while a weak band was amplified from an undiluted SCH soil sample. FastDNA ® SPIN Kit® (For Soil) extracted NSCH and SCH rhizosphere soil bacteria DNA, amplified with BSA and 1:25 dilution consistently produced amplified 16S rDNA bands in the 1.5 kb region (Fig 14).

3.4 Discussion

Variable results were obtained with different protocols evaluated for extraction of bacterial DNA from sugarcane soils. Only one method consistently extracted bacterial DNA from the soils, the FastDNA ® SPIN Kit® (For Soil). This method utilized physical disruption of microbial cells by bead beater homogenization. Therefore, the different experimental extraction procedures were modified to include direct extraction of nucleic acids from bacterial cells by bead beater homogenization. However, extraction of DNA was still unsuccessful or inconsistent (as measured by successful PCR amplification) suggesting homogenization was not a major factor in nucleic acid extraction with the sugarcane soil samples.

PCR amplification of DNA from the one consistent method, the FastDNA ® SPIN Kit® (For Soil), was optimized to produce a high yield of soil bacterial rDNA suitable for use in molecular techniques for comparisons of soil microbial communities. It was found that, for optimal amplification of sugarcane soil bacterial DNA, the addition of BSA fraction V and 1:25 fold dilutions of DNA templates were necessary. The addition of BSA to the amplification mixture stabilizes enzymes used during PCR amplification, thereby enhancing the amount of DNA product (Ausubel, et al., 1998; Ogram, 1998). Dilution of the sample DNA template can improve results by decreasing the amount of amplification inhibitors (i.e. phenols, polysaccharides, and purification reagents) present in the sample (Ogram, 1998), and it may decrease the viscosity of the DNA preparation which can lead to shearing of genomic DNA (Gerstein, 2001).

DNA was further purified and increased by excising the amplified product from the electrophoresis gel and conducting a second round of amplification and electrophoresis. This step separated the target DNA from unamplified DNA of other soil organisms and organic compounds. The DNA from the second amplification was then excised from the agarose gel for final purification with the Gene Clean® II kit (Young, et al., 1993).

It has been reported that no single procedure is currently available that will extract nucleic acid equally well with all applications and all samples (Ogram, 1998; Saano, et al., 1995). Some factors that affect the composition and quality of nucleic acid extraction include the efficiency of lysis of target organisms, the efficient separation of target organisms or nucleic acid (depending on whether direct or indirect methods were used) from particulates in the sample, and the separation of nucleic acid from organic compounds (Ogram, 1998). It was suggested that one will need to modify existing procedures to overcome problems with nucleic acid extraction of environmental samples.

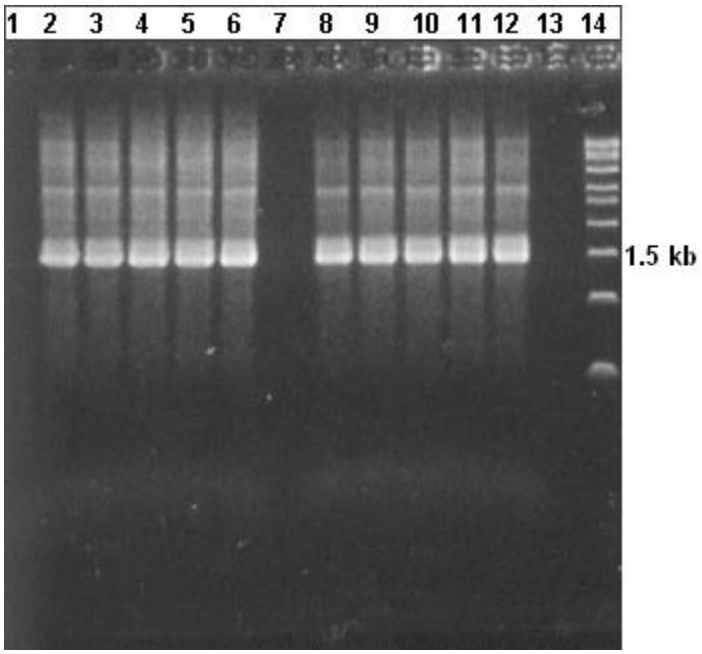

Figure 14. Agarose gel electrophoresis (1.5%) of PCR-amplified 16S rDNA products from soil bacteria. Targeted bands (1.5 kb) were amplified with S-D-Bact-0008-a-S-20/S-D-Bact-1492-a-A-19 primers. DNA templates were diluted to 1:25 final concentration and bovine serum albumin fraction V (BSA) included in the amplification mix. Lanes 1, 7 and 13 empty, Lanes 2-6 five replications of no recent sugarcane cropping history (NSCH) rhizosphere soil samples, Lanes 8-12 five replications of long-term sugarcane cropping history (SCH) rhizosphere soil samples, and Lane 14 1 kb DNA ladder (Sigma).

To further study the components of soil microbial communities associated with sugarcane roots growing in soils with SCH and NSCH, it would be desirable to compare DNA banding patterns by SSCP, ARDRA, or RAPD methods to acquire molecular information from the purified total community DNA and cultured isolates obtained in this study. Cloning and sequencing would lead to the identification of organisms in the NSCH and SCH rhizosphere microbial communities. These studies should lead to a better understanding of the biotic factor(s) involved in yield decline of sugarcane in Louisiana.

CHAPTER 4. SUMMARY AND FUTURE RESEARCH NEEDED

Yield decline in sugarcane fields has been reported world-wide for more than 40 years, yet it still remains unexplained in many respects. A microbial etiology of yield decline has been suggested but no specific microorganism(s) have been implicated. The objectives of this study were: 1) to increase our basic understanding of microbial communities in Louisiana sugarcane soils, 2) to determine if there are consistent differences in microbial communities in soil with and without a sugarcane cropping history, and 3) to provide information on possible changes in the soil microbial communities that occur with sugarcane monoculture that may contribute to sugarcane yield decline.

Results were obtained from two approaches for comparing culturable organisms in soil microbial communities. Comparisons of the numbers and types of microorganisms that grew on various culture media demonstrated that differences exist between microbial communities associated with sugarcane roots in Louisiana soils with and without a recent sugarcane cropping history. The differences in community functional diversity detected by SCSUP support the differences found in types of microorganisms isolated on selective media from the rhizosphere of sugarcane roots growing in soils with different cropping histories. The SCSUP results showed that differences in community functional diversity exist between sites in soils with a long-term sugarcane cropping history in common. The sites were sampled during different months (Site 1, May 2001; Site 2, June 2002; and Site 3, August 2002), so differences in environmental and edaphic conditions existed between sites at the time of sampling. Differences in functional diversity might be expected to occur under varied conditions. However, there still could be a commonality in types of organisms making up the rhizosphere microbial community of sugarcane growing in any soil previously cropped for an extended period with sugarcane. The results from this study concerning the types of culturable organisms in the rhizosphere suggest that similarities do exist. However, the evidence is incomplete, and the potential effects of any one feature, such as reduced levels of fluorescent Pseudomonads, are uncertain.

Through this research project, a DNA extraction and PCR amplification method has been optimized for sugarcane soil samples from Louisiana. Molecular approaches for characterizing and identifying organisms in soil microbial communities may eventually be able to identify any consistent features of the rhizosphere community associated with sugarcane monoculture. Therefore, it is desirable to conduct further research into this problem by identifying the cultures isolated using culture media, and by conducting PCR based nucleic acid analyses on microbial communities collected from wells in Biolog plates and on DNA extracted from total communities from the experimental soil samples.

Although our understanding of this complex problem remains incomplete, improvements in understanding of community changes induced by monoculture might lead to new ideas on how to influence the system to improve plant growth.

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