

LEAD ARTICLE

Opening the Black Box: Understanding the Influence of Cropping Systems and Plant Communities on Bacterial and Fungal Population Dynamics

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ABSTRACT

Since the development of agriculture 10,000 years ago, crops and the soil they grow in have interacted and affected each other's existence. Soil microbial communities exert an important role in maintaining the health of soil and productivity of crops, and in turn, their population dynamics are influenced by agricultural systems. Researchers have attempted to assess community structure, as well as abundance, distribution, and function of species under different agricultural management systems. The effect of plants, soil, and agricultural management practices on the population dynamics of bacteria and fungi is reviewed, and methods to describe microbial communities are discussed.

Key words: agricultural practices, biochemical methods, microbial ecology, molecular methods, soils

INTRODUCTION

Soil microbial communities are major drivers of various biogeochemical cycles in the soil involving the processes of soil structure formation, organic matter decomposition, toxin removal, as well as the cycling of carbon, nitrogen, phosphorus, iron, and sulphur. In addition, soil microorganisms also influence plant health by suppressing plant pathogens, inducing systemic resistance in plants, and improving plant growth (Garbeva *et al.*, 2004^b; Jennifer *et al.*, 2004; O' Donnell *et al.*, 2001; Dodd *et al.*, 2000; Filion *et al.*, 1999; Molin and Molin, 1997). On the other hand, soil provides a habitat for plants, which in turn activates and sustains specific microbes through the selective release of exudates and leachates. The key functions and compositions can be influenced by different anthropologic activities including agricultural perturbation (Upchurch *et al.*, 2008; Gattinger *et al.*, 2002; Smalla *et al.*, 2001).

Cropping systems have been defined as temporal and spatial patterns of growing crops, in addition to any practices and technologies with which crops are produced (Okigbo, 1980; Andrews and Kassam, 1976). Different cropping systems lead to different impacts on the environment. The goal of cropping system research in the past was to maximize crop yields and to feed a growing population worldwide, but this resulted in intensive cropping systems that degraded the production base (soil, environment and water).

With ever increasing worldwide concern over the environment, crop systems may have to be altered to address the agricultural issues of contention.

The relationships among soil microbes, soil metrics and plant communities have not been fully elucidated due to their complexity and the limitation of analytical methods. However, the relative effect of different plants, soils and crop management practices that influence soil microbes can be evaluated. Accordingly, by learning how cropping systems influence the dynamic changes of microbes, a number of more sustainable agro-systems management strategies can be designed.

The effects of plants, soil types and crop management (crop rotation, tillage, pesticide, fumigation and fertilizer application) on bacterial and fungal population dynamics will be discussed in this paper.

The effect of plants on the dynamics of microbes

There is a hypothesis that the plant species cultivated is a major determinant of the soil microbial population since plants provide nutrients. Plant roots secrete a wide variety of compounds to attract microorganisms, including sugar, ethylene, amino acids, organic acids, vitamins and enzymes. Microorganisms respond differently to the compounds released by plants, therefore exudates of different plants select

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different microorganisms (Garbeva and Van Elsas 2008; Berg and Opelt, 2006; Berg and Roskot, 2002; Smalla *et al.*, 2001). In turn, microorganisms also affect the growth and development of plants, promoting the hypothesis that plant-microbial partnerships have successfully co-evolved (Sturz and Christie, 2003).

(a) The interaction between plants and microbes

Microbial communities have been grouped into deleterious, neutral and beneficial categories, based on the perceived relationship they have with plants (Dommergues, 1978). Microbes which adversely affected crop growth and development have been termed deleterious microorganisms (Schippers *et al.*, 1986^{a, b}; Suslow and Schroth, 1982). By contrast, beneficial microorganisms are able to increase crop growth and development in different ways. They can fix nitrogen available to plant uptake (Reinhold-Hurek and Hurek, 1998; James and Olivares, 1997), control phyto-pathogens, enhance the availability of minerals (Davison, 1988; Marschener and Pomheld, 1994), and alleviate plant stress in soils high in heavy metals (Burd *et al.*, 1998). Neutral microbial communities have no effect on plants.

(b) The influence of plants on bacteria and fungi

Many studies have shown that plant species strongly influence the diversity of bacterial communities residing in the soil and rhizosphere. Most of them focus on the bilateral interactions between plants and deleterious microorganisms. In this paper, current studies on the role of host species in beneficial plant-microbe interactions are investigated. It is thought that the characterization of the interaction between plants and beneficial microbes could improve overall plant health and productivity.

Thousands of plant pathogens, including bacteria, fungi and viruses have been studied to elucidate mechanisms of pathogenesis and host resistance. In contrast, much less is known about the interaction of plants with neutral and beneficial microbes. Several invasive beneficial plant-microbes such as rhizobia and mycorrhizae have been described.

The most intensively studied plant-microbe system is legumes and rhizobia (Denarie *et al.*, 1992; Fisher and Long, 1992; Caetabi-Abiikes and Gresshoff, 1991). It was found that there is variability within a plant species to interact with rhizobia. The Gene-for Gene hypothesis narrows

the symbiotic relationship between legume plants and rhizobia by stating that the interaction between a bacterial strain and a plant cultivar or genotype is either compatible or incompatible. Researchers have shown that pea cultivar 'Afghanistan' could not be nodulated by many rhizobia strains that were able to nodulate other pea cultivars. They also found that pea cultivars presumed to be from the centre of origin of peas, failed to be nodulated by European strains of *Rhizobium* (Young and Matthew, 1982; Lie, 1978; Gorovo, 1928). In soybean and clover, genotype determines nodulation (Lewis-Henderson and Djordjevic, 1991; Cregan *et al.*, 1989; Sadowsky *et al.*, 1988; Miller and Sirois, 1982). Rosas and his colleagues (1998) investigated host contributions to nodulation by genetically enhanced *Rhizobium* strains. They screened a large collection of bean germplasm for accessions that were preferentially nodulated by the mutant strain KIM5 (a *Fix⁻* mutant of the wild-type) when planted into soil with rhizobia indigenous to Honduras. They identified 50 genotypes from 820 diverse bean accessions that had either very low or very high preferential nodulation by KIM5. Most of the cultivars with very high preferential nodulation by KIM5 were from Middle American origin suggesting that the isolated breeding and development of bean cultivars in different regions may have led to selection for nodulation by specific strains. It was found that preferential nodulation by KIM5 is controlled primarily by two Loci. The genetic mapping of the quantitative trait loci (QTL) conditioning preferential nodulation variation by KIM5 is currently under way. Interestingly, one of these QTL is mapped to a location that has a QTL conditioning resistance to common bacterial blight caused by *Xanthomonas campestris* (Nodari *et al.*, 1993). It is interesting to observe genetic proximity between host genes that affect pathogenic and beneficial plant-microbe interactions. However, the practical application of biological nitrogen fixation can also be improved through the breeding of plants and should not just focus on nitrogen fixing bacteria such as rhizobia.

Arbuscular mycorrhizal (AM) fungi mobilize phosphorus and other minerals from the soil and exchange these nutrients for carbon with their plant hosts. Unlike rhizobium, AM fungi have very little specificity in terms of host range and most families of vascular plants are able to support an AM symbiosis (Newman and Reddell, 1987). Significant cultivar variability in the response to AM fungi has been measured in faba bean, pea, alfalfa, corn, wheat, peach palm and pearl millet (Clement and Habte, 1995;

Bradbury *et al.*, 1991; Duc and Trouvelot, 1989; Krishna *et al.*, 1985; Hall, 1978). Plant breeding can affect AM fungal associations with plants. Hetrick *et al.* (1992, 1993) found that modern wheat cultivars are less responsive to mycorrhizal symbiosis. However other work in corn and soybean has produced opposite results, showing that modern cultivars are more responsive to AM fungal colonization. Host resistance to fungal pathogens has been shown to affect the interaction of plants with AM fungi; corn with more resistance to a variety of fungal pathogens contained fewer AM arbuscules in their roots (Toth *et al.*, 1990). Both the plant response to the AM fungus and the behaviour of fungus on the host can be affected by host variability. Therefore, breeding and crop improvement should focus on the host genes supporting interactions with AM fungi and enhancing the benefits of this symbiotic relationship.

Non-pathogenic plant-associated microbes such as biocontrol agents and plant growth-promoting rhizobacteria (PGPR) can suppress disease or enhance plant growth. Unlike *Rhizobium* and AM fungi, most non-pathogenic plant microbes do not invade plant tissue. Many studies have shown that plants differ in the level of disease suppression obtained from a particular biocontrol agent (Smith *et al.*, 1997; Liu *et al.*, 1995; King and Parke, 1993). The host variation for disease suppression by the biocontrol agent *Bacillus cereus* UW85 was studied using a tomato recombinant inbred line (RIL) mapping population and provided the first description of the interaction of plants with disease-suppressive bacteria (Smith *et al.*, 1999).

Characterizing the host effects on biocontrol mechanisms is a method used to study the role of host differentiation in disease suppression by a biocontrol agent (Smith *et al.*, 1999). One of the mechanisms by which PGPR and other biocontrol agents function is by inducing host resistance. Liu *et al.* (1995) demonstrated cultivar specificity in the induced resistance to anthracnose of cucumber by PGPR strains.

From another biocontrol mechanism perspective, plants can have a strong effect on microbial communities through antibiosis. For example, the production of 2, 4-diacetylphloroglucinol (2,4-DAPG) by biocontrol strains is influenced by plants. 2,4-DAPG producers occur with lower frequency in non-rhizosphere soil than in the corresponding rhizosphere soil and are strongly affected by the cultivation of maize (Picard, 2000). It was noted that the expression of 2, 4-

DAPG in strain CHA0 was significantly lower in the rhizosphere of dicots (bean and cucumber) than in the rhizosphere of monocots (maize and wheat). It has been extensively reported in studies that the plant type can influence the genotypic and phenotypic diversity of bacterial strains producing secondary metabolites (Bergsma-Vlami *et al.*, 2005; Garbeva *et al.*, 2004³; Linda, 2002). Lenoardo *et al.* (2006) reported that the host crop plays a key role in modulating rhizosphere colonization by different genotypes of 2,4-DAPG producing *Pseudomonas fluorescens*. Population densities of indigenous 2, 4-DAPG producers differed in the rhizosphere of alfalfa, barley, beans, flax, lentils, lupines, oats, peas and wheat. Oats and lentils were measured to have the lowest and highest densities of 2, 4-DAPG producers, respectively. It was found that permanent grassland and grassland-derived plots supported the highest densities of microbes producing the antifungal antibiotic pyrrolnitrin, while the prevalence of this pyrrolnitrin biosynthetic locus was found to be lower in the arable land plots by using real-time PCR assays (Garbeva *et al.*, 2004⁴).

It is believed that rapid and extensive colonization of biocontrol agents on plant surfaces, particularly seed, plays an important role in disease suppression (Thomasshow and Weller, 1996). There are however some inconsistent results from several studies. For example, colonization by the biological agent did not correlate with disease suppression in studies with *Arabidopsis* (Liu *et al.*, 1995) and pea (King and Parke, 1993). Zhang (2004) found that the biocontrol agent *Pseudomonas chlororaphis* PA-23 showed successful disease suppression of *Sclerotinia sclerotiorum* on canola, even though the population of bacteria colonizing the plant was quite low. In contrast, it was found that the capability of maize lines to support colonization by the biocontrol strain *P. cepacia* 526 was correlated to differences in resistance to stalk rot (Hebbar *et al.*, 1992). Similarly, Smith *et al.* (1999) found disease suppression was correlated to population growth of the biocontrol agent on the seed in a study conducted with the tomato RIL. Therefore, disease suppression might be enhanced by identifying the host genotypes that support high populations of the biocontrol agent.

Garbeva *et al.* (2004³) explored the rhizosphere effect of maize, oats, barley and commercial grass on the abundance of bacterial antagonists of the potato pathogen *Rhizoctonia solani*, AG3. They found barley and oat rhizospheres

supported the highest antagonistic *Pseudomonas* populations, compared to the rhizospheres of maize and grass. The proportion of isolates with antagonistic activity towards the soil-borne pathogen *Verticillium dahliae* was highest for the strawberry rhizosphere, followed by oilseed rape and potato rhizospheres. A rather high proportion of *Pseudomonas putida* B was identified from the strawberry rhizosphere, while Enterobacteriaceae (*Serratia* spp., *Pantoea agglomerans*) and *Stenotrophomonas* were mainly isolated from the rhizosphere of oilseed rape (Berg and Opelt, 2006; Berg and Roskot, 2002).

With the help of advanced culture-independent techniques, the relationship in plant-bacterial communities' interactions was revealed in more detail. The rhizosphere of canola was dominated by the *Cytophaga-Flavobacterium-Bacteroides* division and the α -Proteobacteria subdivision (Kaiser *et al.*, 2001). There were four bacterial divisions in the maize roots, in which a new bacterial genus and species in the *Flexibacter* group, *Dyadobacter fermentans* were found (Chelius and Triplett, 2001). Haichar *et al.* (2008) reported plant root exudates shape soil bacterial community structure. Bacteria related to Sphingobacteriales were specific to monocotyledons such as wheat and maize, whereas bacteria of *Enterobacter*, Rhizobiales can colonize both monocotyledons and dicotyledons. A 2001 study found that old wheat cultivars were colonized by phylogenetically diverse rhizobacteria, whereas the rhizosphere of modern cultivars were dominated by fast-growing Proteobacteria (Germida and Siciliano, 2001). In a pyrosequence-based study, Nacke *et al.* (2011) found that different tree species had statistically significant effects on soil bacterial diversity, richness and community composition in forests.

More and more evidence has shown that plants influence not only the structure of bacterial communities, but also bacterial function. Bremer *et al.* (2007) reported that species of non-leguminous plants directly influenced the composition of *nirk*-type denitrifier in soil. It was also found that different rice cultivars shaped the composition and activity of ammonia-oxidizing bacterial populations (Briones *et al.*, 2002).

The effect of soil

Soil is a major determinant of the composition of microbial communities. Several soil traits such as texture, structure, organic matter, microaggregate stability, pH, salinity and the

presence of nutrients determine the microbial habitable niches.

It is difficult to characterize the effect of soil on the changes in microbial communities by using culture dependent methods. It has been said that a 'black box' was opened when microbial communities in soil were analyzed. However, with the advancement of modern technology and the development of culture independent methods, the microbial diversity in the soil under different environmental conditions has been described. By amplifying small subunit ribosomal DNA (SSU rDNA) from fractions with 63 and 35% G+C contents, Nusslein and Tiedje (1999) reported that the soil bacterial community composition differs in forest and pasture soil. *Fibrobacter* and *Syntrophomonas* are the most dominant members in forest soil while *Burkholderia* and *Rhizobium-Agrobacterium* assemblages dominate pasture soil.

Several related studies have provided evidence that soil type is an important determinant of the structure of residing microbes. By analyzing the grouping of DGGE fingerprints obtained from different soils from different geographical locations, Gelsomino *et al.* (1999) found similar soil types tend to select similar communities. Chiarini *et al.* (1998) reported that soil type had the greatest effect on density and community structure of bacteria associated with the roots of field-grown maize, whereas different maize cultivars had no significant effect. Da Silva *et al.* (2003) analyzed the diversity of *Paenibacillus* populations in maize plants grown in two different soils and obtained similar results showing that soil type rather than maize cultivar type was the determining factor in community structure.

In a study of Wisconsin pasture soil, three bacterial divisions: the Proteobacteria, the *Fibrobacter* and the low G+C gram-positive bacteria were represented in nearly 60% of the 16S rDNA clones (Borneman *et al.*, 1996). In Siberian tundra soil, it was found that over 60% of the 16S rDNA clones belonged to the Proteobacteria and 16% to the *Fibrobacter* division (Zhou *et al.*, 1997). It was also found that the soil type was the dominant factor influencing the diversity of the population of culturable fluorescent *Pseudomonas* spp. (Latour *et al.*, 1996). 2,4 -DAPG producers of the D genotype have been isolated from monoculture wheat fields throughout the United States, but have not been reported in European soils. In the Dutch soils studied to date, only genotypes such

as F and M have been detected (De Souza and Raaijmakers, unpublished). Similar studies in Manitoba, Canada have revealed similar findings (Fernando and Li Ru, unpublished).

There is a presumption that spermosphere-bacterial communities originate from soil. Buyer *et al.* (1999) provided the evidence that soil type exerts a greater effect on the spermosphere microbial community structure than seed type. The frequency and relative proportion of specific clay minerals in soils may influence the activity of microbes. For example, the presence of illite inhibited the antagonistic activity of fluorescent *Pseudomonas* spp. isolates against the pathogen *Thielaviopsis basicola*, whereas, this activity was stimulated by vermiculite (Stutz *et al.*, 1986^{a, b}; Stotzky and Post, 1967). This phenomenon seems to be due to the interference of clay with the iron uptake of the microbe. FeIII concentration is limited in alkaline and neutral soils and increases with increasing soil acidity. In this regard, siderophore-producing bacteria, with high affinities for iron, have been found to inhibit some iron requiring pathogens in alkaline and neutral soils (Dowling *et al.*, 1996; Schroth and Hancock, 1982).

Fierer and Jackson (2006) found that the diversity and richness of soil bacterial communities were highly correlated to soil pH with the highest bacterial diversity in neutral soils and lower diversity in acidic soils in a comparison of 98 soil samples from across North and South America. Salinity was found to be the major environmental determinant of microbial community composition, based on comprehensive analysis of 21,752 16S rRNA sequences compiled from 111 studies of diverse physical environments (Lozupone and Knight, 2007). In a field study carried out in Scotland, it was found that bacterial community structure was mainly driven by sample field location and moisture had a comparatively higher impact on bacterial communities compared to soil nitrogen or carbon (Singh *et al.*, 2009).

Soil particle size also affects the diversity of microbes. Ranjard *et al.* (2000) first demonstrated that microbial diversity varies with soil particle size by using ribosomal intergenic spacer analysis (RISA). Sessitsch *et al.* (2001) found that microbial diversity increases with decreasing particle size. α -Proteobacteria dominated in larger particles, while the Holophaga /*Acidobacterium* was common in clay particles. In addition, soil with organic matter supported a greater microbial biomass.

The influence of cropping systems in the variation of microbes

Cropping systems include plant community structure, tillage, rotation, fertilizers, fumigants, and irrigation practices. Compared to non-agriculture soils, such as forests and grasslands, bacterial communities can change in response to agricultural management practices (Acostamartinez *et al.*, 2008; Jangid *et al.*, 2008; Lauber *et al.*, 2008; McCaig *et al.*, 1999). Buckley and Schmidt (2001) explored the dynamic changes of microbial structure in response to different agricultural management techniques. They found significant differences between a field used for long-term studies of agricultural management historically cultivated succession field (HCS) and a never cultivated succession field (NCS). Compared to HCS, alpha Proteobacteria, beta Proteobacteria and Actinobacteria were significantly higher in the NCS field. In the western Amazon soil survey, it was found that changes in land use altered the structure of bacteria. Bacteroidetes and *Alpha* Proteobacteria were mostly found in crop and pasture soil, whereas Firmicutes and Actinobacteria were mostly found in the primary forest and old secondary forest (da C Jesus *et al.*, 2009).

Comparative studies have reported that microbial communities can change in response to cropping systems (Gunapala and Scow, 1998; Baath *et al.*, 1995; Bossio and Scow, 1995; Wander *et al.*, 1995; Bloem and Ruiters, 1992; Hassink *et al.*, 1991).

Crop rotation and tillage drive changes in soil microbes

Crop rotation implies a temporal sequence in which different crops are grown on the same land (Las, 2003). Crop rotation, which is now the most important cultural practice used to control plant pathogens worldwide, is believed to improve yield and to reduce disease because it encourages the development of niche heterogeneity in the rhizosphere and rotates with non-host crops. In contrast, monoculture, the repetitive growing of the same crop in the same land (Andrews and Kassam, 1976) reduces microbial competition in the rhizosphere by lowering biodiversity among root-associated bacteria and fungi, thus enabling the pathogen population to develop (Andrews and Harris, 2000; Knops and Tilman, 1999). By using T-RFLP and Pyrosequencing, it was found that a wheat-oat-canola-pea crop rotation increases Proteobacteria significantly, compared to wheat monoculture (Li *et al.*, 2010^{a, b}).

Tillage is the mechanical manipulation of the soil and the plant residue to prepare a seedbed where crop seeds can be planted. It directly influences the physical and chemical properties of the soil, affects soil temperature and moisture, enhances the release of soil nutrients for crop growth, controls weeds and is the major tool in soil sanitation by accelerating the decomposition of pathogens in crop residue. Intensive tillage can cause soil erosion, accelerate carbon loss, increase greenhouse gas emissions and create unsuitable conditions for beneficial microbes that control plant pathogens. In response farmers have shifted from conventional tillage to conservation tillage systems in many parts of the world.

According to the definition provided by Conservation Technology Information Centre conventional tillage is 'the tillage methods that leave less than 15% residue uncovered after planting, or less than 560 kg ha⁻¹ of small grain residue equivalent throughout the critical wind erosion period', while conservation tillage is 'tillage methods that leave more than 15% crop residue (trash), or more than 560 kg ha⁻¹ of small grain residue on the surface of the soil (including minimum tillage methods)' (CTIC, 1995).

Different combinations of crop rotation and tillage practices lead to various changes in the structure of microbial communities. Lupwayi *et al.* (1998) investigated the effect of tillage and crop rotation on the diversity and community structure of bacteria by using the BIOLOG system. They found that conservation tillage and legume-based crop rotations support higher diversity of soil bacterial communities.

Disease incidence in monoculture corn in the central United States was studied under various types of reduced tillage and conservation tillage systems. Several foliar pathogens of corn, such as *Helminthosporium turcium*, *H. maydis*, *Phyllosticta maydis* and *Cercospora zeaemaydis* were more severe in reduced tillage than in conservation tillage (Boosalis *et al.*, 1981; Hilty *et al.*, 1979; Burns and Shurtleff, 1973; Arny *et al.*, 1970), while *Colletotrichum graminicola* could not be controlled under both tillage practices (Phillips *et al.*, 1980). Conservation tillage decreases soil temperature in the spring and early summer, and may be conducive to damping-off and diseases caused by soil-borne pathogens favoured by low temperature.

When the frequency of potato cropping increases, potato yields decrease due to the

deleterious microbial factor (Schippers *et al.*, 1986^{a, b}). The deleterious effect is likely due to microbial metabolites secreted by pathogens, which affect physiological processes in root cells. There is evidence that accumulating cyanide inhibits the energy metabolism of potato root cells in high-frequency potato cropping, possibly owing to the accumulation of the HCN precursor in soil. The plant cultivars may differ in their sensitivity to the deleterious effect, therefore, cultivar rotation could be considered as a means to control pathogen and reduce yield loss. It has also been noted that for wheat, barley, bean and maize, yields decrease with an increase in the frequency of cropping (Schippers *et al.*, 1986^{a, b}).

However, there is a fairly well established phenomenon where the disease incidence decreases in long-term monoculture soil. For instance, it was found that take-all disease, caused by *Gaeumannomyces graminis* var. *tritici*, decreased naturally in wheat monoculture fields, while the population of beneficial *Pseudomonas* spp. increased (Raaijmakers *et al.*, 1997, 1998, 1999). This type of soil is called a disease-suppressive soil. Many studies have discussed how disease-suppressive soils occur and the mechanism involved (Parke and Gurian-Sherman, 2001; Smith and Goodman, 1999; Mazzola, 1999).

Conservation tillage and conventional tillage practices may increase, decrease, or have no effect on plant diseases. Ibekwe *et al.* (2002) found biomass amounts were significantly higher and ammonia oxidizers more diverse in no-till (NT) soil than in conventional-till (CT) soil. However, by using phospholipid fatty acid analysis (PLFA) and denaturing gradient gel electrophoresis (DGGE), Helgason *et al.* (2010) investigated long-term no-till management effects on microbial communities in the Canadian prairie agro-ecosystems. They found that differences in bacterial communities were related to depth in the soil profile rather than tillage management practices. Wortmann *et al.* (2008) also found that bacterial communities in the 0 to 30 cm depth, on a mass equivalent basis, were not significantly affected by tillage. It was found that richness and diversity of bacterial communities were slightly higher under zero tillage treatment, while the percentage of the majority of individual bacteria was similar under different tillage systems, except for Firmicutes and Chloroflexi, which showed different trends at $P = 0.1$ when using high throughput methods such as TRFLP and Pyrosequencing (Li *et al.*, 2010^{a, b}).

Therefore, tillage practices should be chosen according to climate, vegetation history and disease incidence. More research is needed to understand the influence of different crops and specific tillage practices on plant diseases.

The effect of Pesticides and Fumigants

Pesticides have been widely used in conventional farming systems as part of the pest control strategy. Globally, about 3×10^9 kg of pesticides is applied annually (Pan-UK, 2003). However, only 0.1% of the applied pesticides reach target organisms while the remaining amount contaminates the soil (Carriger *et al.*, 2006). Many studies have shown that pesticides adversely affect the diversity and activities of beneficial soil bacterial organisms due to their xenobiotic characteristics (Widenfalk *et al.*, 2008; Wang *et al.*, 2006; Niewiadomska and Klama 2004). There are also reports elucidating the ability of soil microorganisms to degrade pesticides and use the applied pesticides as a source of energy and nutrients to multiply. These pesticides result in increasing population sizes and activity of pesticide degrading soil microorganisms (Li *et al.*, 2012; Hussain *et al.*, 2007; Kumar and Philip, 2006; Das and Mukherjee, 2000).

However, some studies also showed no adverse side-effects of pesticides on microbial communities (Sarnaik *et al.*, 2006). Lupwayi *et al.* (2009) investigated the effect of the pesticides vinclozolin and cyhalothrin on functional soil bacterial diversity, and detected no side effects in canola rhizosphere or bulk soil. There are some cases that show microbial populations initially affected by pesticide application acclimatize after a period of time and return to normal (Filessbach and Mader, 2004; Niewiadomska, 2004).

The effect of three phenyl urea herbicides on microbial communities was examined by El Fantroussi *et al.* (1999). The number of culturable heterotrophic bacteria decreased significantly with all three herbicides. The decline of unculturable *Acidobacterium* was also observed. With an increase of bromoxynil concentrations, changes in the bacterial community were measurable. The addition of bromoxynil had a negative impact on the presence of α and γ -Proteobacteria in the soil (Baxter and Cummings, 2006). Khan *et al.* (2006) found that atrazine and isoproturon adversely affected *Bradyrhizobium* spp. It was found that mefenoxam and metalaxyl inhibited N-fixing bacteria (Monkiedje *et al.*, 2002).

However, there is some conflicting research on the effects of herbicides on soil bacterial diversity. Xia *et al.* (1995) found no changes in the bacterial community in response to the application of 2, 4-dichlorophenoxyacetic acid (2, 4-D) at the recommended application rates. Busse *et al.* (2001) showed that glyphosate caused the decline of bacterial numbers in a pine plantation. In contrast, Nicholson and Hirsch (1998) reported an increase in culturable bacterial populations in soils treated with glyphosate.

Fumigants are widely used when farming high-value cash crops to effectively control nematodes, soilborne pathogens and weeds. Ibekwe *et al.* (2001) examined the changes in soil microbial community structure in a microcosm experiment following the application of methyl bromide (MeBr), methyl isothiocyanate, 1,3-dichloropropene (1,3-D) and chloropicrin. They found that high diversity indices were maintained between the control soil and the fumigant-treated soil, except for MeBr, where MeBr has the greatest impact on soil microbial communities and 1, 3-D has the least impact.

There is only a modest amount of research on the effect of pesticides and fumigants on the diversity of microbial community. Therefore, no firm conclusions can be drawn. More studies are needed in this area.

Fertilizer

Fertilization is an important agricultural practice used to enhance plant nutrition and achieve high yields. Fertilizers can be classified as either organic (composed of enriched organic matter; e.g.: plant or animal) or inorganic (composed of synthetic chemical or mineral). Many studies have investigated the effect of long-term fertilization on soil fertility, organic matter, physical properties and crop yield (Mallarino and Borges, 2006; Cai and Qin, 2006; Pernes-Debayer and Tessier, 2004). Since microbial populations play an important role in plant residue decomposition and nutrient cycling, it has been an area of interest to study how fertilizers affect the structure and function of these microbes. By using polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) and phospholipid fatty acid (PLFA) profiling, Clegg *et al.*, (2003) found that nitrogen fertilizer has a significant impact on the total bacterial and actinomycete community structures. It was also found that fertilizer amendment altered the abundance of bacterial

groups throughout the agriculture soil in Watkinsville, Georgia, USA by using both 16S rRNA gene clone libraries and PLFA (Jangid *et al.*, 2008). One study showed that γ -Proteobacteria were sensitive to fertilization while Acidobacteria was unaffected by fertilization (Wu *et al.*, 2011). It was found that long-term inorganic nitrogen management influenced the structure of Nitrite-oxidizing bacteria and new ecotypes of non-characterized *Nitrospira* spp. were found in fertilized soil (Freitag *et al.*, 2005). By using a combination of qPCR, T-RFLP, cloning and sequencing techniques, Chen *et al.*, (2010) found that *nirK* containing denitrifiers were more sensitive to fertilization than *nirS* containing denitrifiers in a paddy soil.

There is debate in the literature on which type of fertilizer is beneficial to the composition and function of microbial populations in the soil (Kamaa *et al.*, 2011; Shen *et al.*, 2010; Chu *et al.*, 2007; Esperschuta *et al.*, 2007). A long-term field experiment in Switzerland, using the esterlinked phospholipid fatty acid method, found that long-term organic fertilization was strongly associated with slow growing bacteria known as k-strategists (Esperschuta *et al.*, 2007). Shen *et al.*, (2010) compared soil bacterial community size and structure under different fertilization treatments including no fertilization (CK), inorganic-N fertilization (N), organic manure amendment (M) and half N-fertilizer plus half organic manure (M+N). They found that the bacterial community under N treatment was significantly different from the other treatments. The N treatment had the smallest general bacterial population and was lacking any Gamma Proteobacteria. Similarly the Kabete long-term trial in Kenya revealed that bacterial community structure and diversity was negatively affected by inorganic NP fertilizer, and that microbial communities in the soil with organic input clustered away from the soil with inorganic input (Kamaa *et al.*, 2011). Chu *et al.* (2007) found that organic manure fertilizer promoted the population of *Bacillus* spp. in the soil, compared to inorganic fertilizer. In contrast, Chen *et al.*, (2010) found that organic and inorganic fertilizer had a similar effect on the composition of soil denitrifying communities.

The interaction of fertilizers and pesticides affects microbial diversity and activity in the soil. It was reported that the structure of microbial communities was influenced by the co-treatment of inorganic fertilizer (KH_2PO_4 and NH_4HCO_3) and pesticides (Acetochlor,

Fenvalerate, Thiophanate-Methyl) (Chen *et al.*, 2006).

Analytical methods to measure dynamic changes in microbial diversity

The approaches used to characterize bacterial communities inhabiting the soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques. Biochemical-based techniques include plate counts (Sands, 1980; Savchuck, 2002), protein analysis (De Vos, 1993), fatty acid methyl ester (FAME) profiling (Stead, 1992; Vancanneyt, 1996) and comparing substrate utilization (Grimont, 1996). Molecular-based methods consist of measuring nucleic acid reassociation kinetics (Torsvik *et al.*, 1990, 1996), Guanine plus cytosine (G+C) content (Nusslein and Tiedje, 1999), DNA microarrays, DNA hybridization (Cho and Tiedje, 2001), DNA cloning and sequencing (Moore *et al.*, 1996), DNA and RNA hybridization (Amann, 1996), and Pyrosequencing (Roesch *et al.*, 2007). Other PCR-based 'fingerprinting' methods include random amplified polymorphic DNA (RAPD) analysis (Keel, 1996), restriction fragment length polymorphism (RFLP) (Mcspadden-Gardener, 2001), terminal restriction fragment length polymorphism (T-RFLP) (Liu and Marsh, 1997), rapid PCR assay (Mcspadden-Gardener, 2000), amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) (Sharifi-Tehrani, 1998) and PCR-denaturing gradient gel electrophoresis (DGGE). Every method has its advantages and disadvantages.

Biochemical-based methods

Plate counts are isolate-based which characterize and use carbon sources to provide useful information to understand the growth habit, development and potential function of microorganisms from soil environments. In addition, they have the advantages of providing rapid analysis, being reproducible and being inexpensive. However, because of the need for isolation and cultivation of individual species, these methods are biased towards fast growing microorganisms and cannot detect unculturable microorganisms. Therefore, the diversity of microbes in environmental samples is underestimated.

The substrate utilization method is based on tetrazolium dye reduction as an indicator of sole-carbon source utilization to profile the physiological requirements of the microbial community. Biolog systems (Biolog GN Microplates and Ecoplates) were widely used to determine how microbial communities change in

response to various factors including soil type (Miethling *et al.*, 2000), plant species (Fang *et al.*, 2001; Siciliano *et al.*, 1998) and agricultural practices (Larkin, 2003; Lupwayi *et al.*, 1998). Although this method is able to differentiate microbial communities at the community-level using physiological profiling, it bears some fundamental problems. Primarily, it is still based on the culture-dependent method to assess microbial communities and prefers rapidly growing microbial species who can utilize the carbon substrate. Therefore this method is unlikely to provide a significantly more comprehensive understanding of microbes at the community level when compared with other media-based culture methods. Furthermore, since the Biolog system analyzes the metabolic reaction pattern, it is unable to distinguish which microbial communities are responsible for the carbon source utilization.

Fatty acid methyl ester (FAME) profiling is the other widely used bio-chemical method to assess the structure of microbial populations based on detection of the FAME, a relatively constant component of the cell membranes which can be used to differentiate major taxonomic groups within a community (Ibekwe and Kennedy, 1998). This method can differentiate the active microbial biomass and the non-living microbial biomass. Despite the advantages of this method, there are some limitations. Cellular fatty acid can be influenced by factors such as temperature and nutrition.

Molecular-based methods

It is estimated that there are nearly 10^7 microbial species in one gram of soil (Gans *et al.*, 2005). However, less than 1% of these microorganisms in soil can be cultured; the majority of them are in the 'black box'. Molecular-based methods provide an invaluable opportunity for us to expand our knowledge on soil microbial communities. These approaches have been widely used to assess the composition and function of soil microbial communities. They have also been used to monitor how different plants, soils and agricultural management practices influence soil microbial communities. Among these numerous tools available, approaches based on the amplification of target DNA and RNA sequences extracted from the soil will be addressed. The 16S rRNA genes of bacteria and archaea, and the 18S rRNA of fungi are the most commonly targeted and amplified, because they possess conserved and hypervariable regions. They are used for examining particular organisms or taxa of interest through universal or taxa-specific

primers. In the past decade, the database of 16S rRNA gene sequences has grown to 2,639,157 (Ribosomal Database Project, 2012). This enables us to differentiate more microbes and discover greater diversity in microbial communities.

Quantitative Real-Time PCR

Real-time PCR or quantitative PCR (qPCR) is used to amplify and simultaneously quantify target DNA. Unlike normal PCR, qPCR detects the PCR products at each cycle during the exponential reaction instead of at the final phase or the end point of the reaction. This allows researchers to view the accumulation of amplicons during the reaction, and therefore the amplicons can be quantified more accurately. It can be used to estimate the changes in a specific species or genus of microorganisms (using specific primers) (Dang *et al.*, 2010; Szukics *et al.*, 2010; Davis *et al.*, 2009), or shifts in microbial communities (Hollister *et al.*, 2010; Andreote *et al.*, 2009; Wakelin *et al.*, 2009). Quantitative PCR is becoming a useful tool to evaluate dynamic changes of microbial communities in complex soil environments.

Cloning Libraries and Sequencing

Using this approach, microbial populations can be identified by PCR amplification of 16S ribosomal genes, and subsequently cloned and sequenced. The microbial community composition, and the species richness and abundance can be compared to sequence information in sample libraries (Dimitriu and Grayston, 2010; Kielak *et al.*, 2008; Borneman *et al.*, 1996). Since the average size of clone libraries constructed is only 100-500, abundant microbial communities will be identified and rare species will not be detected. Therefore, in a survey of highly diverse ecosystems, this method may result in an undervaluation of species richness (Noha *et al.*, 2009).

PCR-based fingerprinting methods

The strength of fingerprinting techniques allow for large amounts of samples to be analysed and compared. These techniques are facile tools which assess the composition and structure of microbial communities in certain assemblages, or assess microbial changes between samples or treatments.

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two widely used tools based on similar principles. The theory of DGGE and TGGE is that two similarly sized DNA fragments differing in a single base can be

separated from each other by passing through acrylamide gel having either a chemical gradient (DGGE) or temperature gradient (TGGE) (Myers *et al.*, 1985). Subsequently, DNA fragments form multiple bands that are used to calculate richness, diversity and similarity index of microbial communities of samples. The differences in the banding patterns can be used to differentiate the genotype of specific microbial taxa (Smalla *et al.*, 2007; Freitag *et al.*, 2005; Bergsma-Vlami *et al.*, 2005). Furthermore, specific DGGE bands can be excised from gels, re-amplified, sequenced or transferred to membranes, and hybridized with specific primers to obtain information about the specific taxonomic groups within the community (Chong *et al.*, 2009). With the use of internal standards during DNA extraction and PCR-DGGE, this method can even be used to estimate relative abundance and diversity (Petersen and Dahllö, 2005). This method has the advantages of being reproducible, rapid and reliable. Furthermore, a large number of samples can be analyzed simultaneously. Meanwhile, the limitations of DGGE include PCR bias; only dominant species being detected and co-migration which means one band can represent more than one species (Jennifer *et al.*, 2004).

Random amplified polymorphic DNA (RAPD) is another fingerprinting tool for studying microbial diversity. Instead of amplifying the 16S ribosomal gene, it works by amplifying random genomic sequences. This method requires no prior genetic information. RAPD randomly amplifies segments of DNA using short primers (8-12 nucleotides). Normally, several primers are used and the one which yields the best discrimination of microbial communities is chosen (Denman *et al.*, 2005). The drawbacks of RAPD techniques are that they are very laborious, not reproducible and the results are difficult to interpret.

Ribosomal intergenic spacer analysis (RISA) is the method in which the rRNA gene operons of the intergenic spacer region (so-called ISR) between 16S rRNA and 23S rRNA are amplified by using primers containing conserved regions of the 16S and 23S genes. The strength of RISA lies in its ability to generate significant length heterogeneity in the ISR (range between 150-1500 bp) (Ranjard *et al.*, 2001; Borneman and Triplett, 1997) and to discriminate between closely related strains (Denman *et al.*, 2005). It has been used to construct bacterial community structures associated with different perturbed soils including metal pollution (Ranjard *et al.*, 2000), antibiotic treatments (Robledo *et al.*,

1998) and different vegetation covers (Borneman and Triplett, 1997). Restriction fragment length polymorphism (RFLP), a technique involving the amplification of total bacterial DNA and digestion of resulting PCR products, is a rapid screening method that presents the ribosomal diversity of microbial populations (Denman *et al.*, 2005).

Terminal restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA) are two methods which have been developed to improve the detection and resolution of fragment analysis by utilizing fluorescently labelled oligonucleotide primers for PCR amplification and automated systems for separation and detection of PCR fragments. Compared to methods that use standard gel electrophoresis detection, automated electrophoresis systems offer high throughput and rapid analysis of microbial community structure. In addition, the high sensitivity and precision of fluorescence detection increase the number of peaks detected and allows a more accurate comparison of community profiles based on the band intensity.

T-RFLP primarily amplifies small subunit (16S or 18S) rRNA genes from the entire microbial community using PCR with fluorescent dye labelled primers. The resultant PCR products are then digested with restriction enzymes which have 4 or 6 base-pair recognition sites. Only terminal restriction fragments (T-RFs) tagged with fluorescently labelled dyes can be automatically checked, and their relative abundance and size are measured by a DNA sequencer or a specialized T-RFLP machine. Since the different lengths and patterns of T-RFs reflect the difference in sequence of 16S or 18S rRNA, the structure and composition of microbial communities can be determined. One advantage of T-RFLP is that it is the only method "offering phylogenetic information directly without further sequencing of the fragments compared to other fragment analysis methods" (Kent and Triplett, 2002). Because it is very cost-effective and relatively simple, T-RFLP has been widely used to assess changes in the structure and composition of various microbial communities including bacteria, archaea and fungi residing in different environments (Yan *et al.*, 2009; Pérez-Piqueres *et al.*, 2006; Wu *et al.*, 2006; Nabla *et al.*, 2005; Kennedy *et al.*, 2005; Leybo *et al.*, 2006; Johnson *et al.*, 2004; Sessitsch *et al.*, 2001; Lukow *et al.*, 2000; Liu and Marsh, 1997). Additionally, T-RFLP has been used to analyze functional genes including methane oxidation

(Mohanty *et al.*, 2006; Horz *et al.*, 2000, 2001) and nitrogen fixation (Rosch *et al.*, 2006; Tan *et al.*, 2003). However, since multiple copies of rRNA and other functional genes exist in microbial species, and since the copy number varies among species, T-RFLP results are both difficult to adjust for and can result in bias. In addition, incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn *et al.*, 2000).

Pyrosequencing

Pyrosequencing, a relatively new high-throughput sequencing technique, is becoming a powerful approach in 16S gene-based microbial diversity surveys. Pyrosequencing technology is based on the detection of released inorganic pyrophosphates during DNA synthesis. The general principle of a pyrosequencing reaction is that a pyrophosphate (PPi) is released and subsequently converted to ATP, by ATP sulfurylase, when nucleotides are incorporated into a nucleic acid chain by DNA polymerase. The ATP provides the energy to luciferase to oxidize luciferin and generate light, which can be detected by a photodiode, photomultiplier tube or a charge-coupled device camera (CCD). By choosing proper primers and robust community comparison methods, and by reassembling 100-400 nucleotides of rRNA sequences produced by pyrosequencing, it is possible to taxonomically cluster different microbial communities at the genus level with high accuracy (Liu *et al.*, 2007; Roesch *et al.*, 2007). The advantages of this approach are: (1) rare members of the microbial community can be detected even with inadequate sampling since a large number of 16S rRNA gene sequences are produced (*e.g.*: Roche 454 GSXLF pyrosequencing machine can produce 400Mb per run) (Noha *et al.*, 2009); (2) mixed DNA samples can be sequenced without constructing clone libraries. Current bioinformatic tools are outstripped by the abundance of data generated by next generation sequencing technologies representing a bottleneck for pyrosequencing and leading to problems using and interpreting the data (Hugenholtz and Tyson, 2008). Currently, this technique is used to assess highly diverse microbial communities inhabiting the soil (Li *et al.*, 2012; Kim *et al.*, 2008; Teixeira *et al.*, 2010; Roesch *et al.*, 2007).

Metagenome

“Soil is the most bio-diverse environment on the earth: it is estimated to contain approximately 1,000 Gbp of microbial genome sequences per gram of soil! Compared with the Human Genome Project (in which 3 Gbp were

sequenced)” (Vogel *et al.*, 2009). Soil sequencing is in contention to be the next major global metagenomics initiative since the soil metagenome would lead to new environmental breakthroughs and economic opportunities. Recently high-throughput sequencing methods have provided powerful tools to interpret the soil metagenome. Large-scale metagenomic sequencing efforts will provide sufficient data to understand soil microbial community diversity and function under different perturbations. This sequencing project would provide insight into the ecology of microorganisms that are beneficial to, or threaten crop production. The launched soil metagenomics project, TerraGenome International Sequencing Consortium, will contribute significantly to the goal of sustainable agriculture.

CONCLUSIONS

The studies discussed in this review examine the dynamic changes of bacterial and fungal communities under different cropping systems and elucidate some of the mechanisms of complex soil microbes. However, they are still far from understanding the whole picture. New tools in molecular and genomics research enable us to further investigate microbial communities in different soils. Genetic research in the field of plant microbe interaction enhances our understanding of the mechanisms involved in plant resistance to pathogens and the interactions between plants and beneficial microbes. These results can be applied to crop breeding to reduce the use of chemical pesticides while increasing crop yields. Soil type is another important driver of microbial community structure by favouring different microbes. There is a difference of opinion on whether plant species or soil type is the major factor in selecting soil microbes. It is possible that they exert different and complementary roles in selecting microbes. Soil hosts microbial communities, while plants influence the microbes dwelling in the soil. In addition, cropping systems also play an important role in influencing the changes in microbial structure. Crop rotation, tillage, pesticides and fertilizers all have an effect on the microbial communities.

An improved understanding of how plant species, soil types and cropping systems influence the fluctuation of microbes is necessary in developing truly sustainable agriculture.

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