COMMUNITY LEVEL PHYSIOLOGICAL PROFILING OF MICROORGANISMS IN THE RHIZOSPHERE OF TRANSGENIC PLANTS – A REVIEW

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Abstract: Since 1996, when the first genetically modified seeds were planted in field conditions, the commercial growing of genetically modified crops increased to over 134 millions of hectares in 2009 worldwide. Along with the great potential of transgenic plants for future agriculture, considerable concerns on their biosafety have been raised, including their potential impact on soil microbial communities. This review briefly summarizes the important features of soil microorganisms for plant health and ecosystem stability, the numerous methods available for microbial ecologists to study soil microbial diversity, with emphasis on the method of community level physiological profiling (CLPP) based on carbon substrate utilization patterning, and finally the use of CLPP for assessing the effects of transgenic crops on soil microbial communities.

Key words: CLPP, soil microbial community, transgenic plants, carbon sources

1. Introduction

The first transgenic plant, tobacco containing a selectable marker gene *nptII* from a bacterium conferring resistance to kanamycin, was produced at the University of Ghent, Belgium, in 1983 (ZAMBRYSKI *et al.*, 1983), only 30 years after the discovery of the structure of DNA.

Now, some 27 years later, genetically modified (or transgenic) crops became a common feature of the agricultural landscape in 25 countries of the both Americas, Europe, Asia, Africa and Australia (JAMES, 2009). The unprecedented high growth rate of adoption of transgenic crops started in the U.S.A. in 1996 with 1.7 million hectares. In 2009, the global area of genetically modified (GM) crops reached 134 million hectares (JAMES 2009), which is more than the total land area of six neighboring countries: Slovakia, Czech Republic, Poland, Hungary, Ukraine and Austria together (122 mil. ha). The first commercially grown transgenic crops contained one gene of interest or one introduced novel trait (single trait GM crops). At present, however, combination or stacking different traits or genes in one plant is rapidly gaining popularity and recent growth of GM crops hectarage over the last few years comes largely from deployment of these so called "stacked traits" GM crops (TAVERNIERS *et al.*, 2008; JAMES, 2009). Several transgenic plants containing two, three or more genes of interest are currently under evaluation by national authorities of different countries for deliberate release of transgenic plants into environment (TAVERNIERS *et al.*, 2008).

Along with the increasing potential for widespread commercial use and the benefits of transgenic crops, considerable concerns on their safety have been raised, including safety aspects relating to their potential impact on the environment (WIDMER, 2007). The rapid development of agricultural biotechnology and release of transgenic plants into environment have provided many agronomic and economic benefits, however they also raised a multitude of concerns over the potential ecological effects of transgenic plants. These concerns include unintended consequences of transgene flow to indigenous organisms through pollen transfer, plant invasiveness, the development of resistance in target pests, and direct or non-direct effects on non-target flora, fauna and microorganisms and ecosystems (SEIDLER and LEVIN, 1994; NIELSEN *et al.*, 2001; DALE *et al.*, 2002; BRUINSMA *et al.*, 2003; FILION, 2008). In addition to the relatively abundantly studied aboveground effects, underground impacts of GM crops have also been recognized as a result of recent methodological advances in soil microbial ecology (BRUINSMA *et al.*, 2003; ZHANG *et al.*, 2005). Nevertheless, the impact of GM crops on soil-associated microfauna, including soil microbial communities, still represents one of the least studied and understood areas, probably due in part to the inherent technical difficulties involved in the study of soil-borne living organisms (FILION, 2008).

Recently several excellent reviews on the effects of transgenic plants on soil microbial communities have been published (e.g. BRUINSMA *et al.*, 2003; DUNFIELD and GERMIDA, 2004; WIDMER, 2007; FILION, 2008). In this review, we focused on the methodological aspects, as well as the use of Community level physiological profiling (CLPP) based on sole carbon source utilization analysis to study the functional diversity of microbial communities in the rhizosphere of transgenic plants. We provide also a brief overview of the microbial characteristics of soils and methods used for analyses of soil microbial communities.

2. Microbial characteristics of soils

Soil is a complex and dynamic physical, chemical and biological system and a major component in agro-ecosystems (NANNIPIERI *et al.*, 2003). It is considered to be the greatest reservoir of biodiversity on the planet (CRAWFORD *et al.*, 2005). Prokaryotic organisms comprise more than half of the biodiversity on Earth and their diversity in soil has been estimated to be about three orders-of-magnitude greater than in all other environments combined (CURTIS *et al.*, 2002).

Microorganisms are fundamentally important components of the soil habitat where they play key roles in ecosystem functioning through controlling nutrient cycling reactions essential for maintaining soil fertility, and they also contribute to the genesis and maintenance of soil structure (KIRK *et al.*, 2004). The microbial community of arable soils is a key agent in biologically mediated processes such as degradation of organic residues, transformation of organic matter, mineralization of nutrients, formation of soil aggregates, and soil nutrient balance. Soil microorganisms are also a component with multiple functions of importance to soil fertility, such as catalysis of nutrient transformations, storage of nutrients, formation and stabilization of soil structure, and control of plant pathogens (KENNEDY and PAPENDICK, 1995; PETERSEN *et al.*, 2002; GÖMÖRYOVÁ *et al.*, 2009a).

Soil microbial communities have great potential for temporal and spatial change, and thus represent a powerful tool for understanding community dynamics in both basic and applied ecological research (GARLAND, 1997). The soil microbial community structure is considered as an early and sensitive indicator of anthropogenic effects on soil ecology (NIELSEN and WINDING, 2002). Soil microorganisms are able to respond more quickly to environmental stress because, compared to higher organisms, they have a high surface-to-volume ratio, which means they are capable of a much more intense exchange of matter and energy with their environment. Therefore, changes in size, composition and activity of microbial communities can frequently be observed before detectable changes in soil physical and chemical properties occur (NIELSEN and WINDING, 2002; WIDMER and OBERHOLZER, 2003; GÖMÖRYOVÁ *et al.*, 2009a). Moreover, bacteria interact with plants during their entire life cycles and therefore can act as precise and fast indicators of environmental changes.

Plants depend on the ability of roots to communicate with microbes. The converse is also true; many bacteria and fungi are dependent on associations with plants that are often regulated by root exudates (BAIS *et al.*, 2004). Crop plants interact with soil microbial communities through a micro-zone at the root-soil interface that is under the influence of the plant root. This complex habitat composed of the fraction of soil adhering to plant roots and altered by root activity is referred to as rhizosphere. The rhizosphere environment hosts a complex microorganism network which interacts very closely with plant roots, the outcome of such interaction being seen in the strong influence plant type has on the microbial ecosystem of the soil (BRUSETTI *et al.*, 2004). It is well known, that rhizosphere exerts a selective influence on the associated microbial communities and these microorganisms in turn have a great impact on root biology, they influence plant growth, nutrition and development, and are important for long-term sustainability of agricultural soils (MANTELIN and TOURAINE, 2004). A number of studies have shown that the rhizosphere has much higher population densities of bacteria and fungi compared with bulk soil (MALONEY *et al.*, 1997; SEMENOV *et al.*, 1999).

Plants modify their rhizosphere environment through release of assimilates in the form of root exudates (UREN, 2001). The four types of main ingredients in plant root exudates are carbohydrates, amino acids, fatty acids and nucleotides, other compounds, such as organic acids, steroids, growth hormones, flavones and enzymes are also involved (ZHANG *et al.*, 2005). This complex mixture of organic compounds provides a source of reduced carbon, nitrogen, and other nutrients for soil inhabiting microorganisms (BUYER *et al.*, 2002). The composition of compounds which are released from the roots and interact with bacteria in the rhizosphere vary depending on the plant species, cultivar, and physiological status of the plants (SORENSEN, 1997; SAXENA *et al.*, 1999). Root exudates are known to mediate both positive and negative interactions in the rhizosphere. The positive interactions include enhancement of growth rates of bacteria (HARTWIG *et al.*, 1991), acting as chemoattractants to attract bacteria towards the root (DHARMATILAKE and BAUER, 1992), and acting as transcriptional signals in the communication between soil bacteria and host plants during nodule formation and biological nitrogen fixation by plants (REDMOND *et al.*, 1986). On the other side, plant-microbe interactions often involve also plant activities

to suppress growth of bacteria in the rhizosphere, e.g., by secretion of bactericidal substances (GARCIA-OLMEDO *et al.*, 1998). Bactericidal substances naturally released from the plant roots may include toxic components like benzofurans, terpenoids, butyrolactones, and other phytoalexins (BOWEN and ROVIRA, 1991).

Previous studies revealed that qualitative and quantitative differences in root exudation could strongly affect the structure of microbial communities in the rhizosphere (OGER *et al.*, 1997; SAVKA and FARRAND, 1997; MALONEY *et al.*, 1997). The composition of microbial communities in the rhizosphere is governed mainly by the quality and quantity of carbon substrates that are released as root exudates (MALONEY *et al.*, 1997). Root exudation may create a niche that influences which microorganisms colonize the rhizosphere, thereby altering the composition and diversity of rhizomicrobial communities in a plant-specific manner (GRAYSTON *et al.*, 1998). Even small modifications, as may exist between different cultivars of the same plant species may cause altered composition of root exudates, which can eventually result in the occurrence of different microbial communities in the rhizosphere (RENGEL *et al.*, 1998).

It was shown previously that microbial population density and diversity can be influenced by introducing genes into plants for the production of novel compounds (OGER *et al.*, 1997; SAVKA and FARRAND, 1997). Genetically engineered plants might change the bacterial consortia in the rhizosphere due to the release of transgene products or an altered composition of root exudates (OGER *et al.*, 1997). The insertion of transgenes may unintentionally alter plant characteristics of the transgenic crop others then the desired trait. In turn, these changes may influence the growth and species composition of soil microorganisms associated with the roots of these plants, and therefore they may have ecological effects (DONEGAN *et al.*, 1995). An altered composition of root exudates may induce a different community of rhizosphere microorganisms and even small modifications, as may exist between different cultivars of the same plant species, can result in the occurrence of different microbial communities in the rhizospheres (RENGEL *et al.*, 1998).

Comparative studies assessing whether there are differences between microbial communities living in the rhizospheres of transgenic and non-transgenic plants represent an important first step in determining if the presence of transgenic material can catalyze changes in the environment. This knowledge of the impact of transgenic crops on soil microbial ecology is essential for understanding the long-term agronomic and environmental effects of genetically modified crops and for developing appropriate management practices for minimizing potential negative impacts (FANG *et al.*, 2007).

3. Methods of studying soil microbial communities

Over the past ten years, approaches to studying soil microbial communities changed dramatically. A number of new techniques have been developed and are now available to soil microbiologists to gain deeper insight into community-level responses to changes in soil properties or management.

Commonly applied bulk soil microbial parameters include measurement of total microbial biomass (VANCE *et al.*, 1987; LIAO and XIE, 2007; LUPWAYI *et al.*,

2007), determination of activities of enzymes involved in C-, N- and P-nutrient cycling, such as proteases, cellulases, phosphatases, dehydrogenases, arylsulfatases, and others (RASCHE *et al.*, 2006; BRADLEY *et al.*, 2007; GORLACH-LIRA and COUTINHO, 2007), basal soil respiration (VISSER *et al.*, 1994; LUPWAYI *et al.*, 2007; RAUBUCH *et al.*, 2010) and specific metabolites (RAUBUCH *et al.*, 2007). These methods can provide information on general microbial activities, but not on specific microbial groups that actually contribute to different types of soil enzymatic activities (LIU *et al.*, 2005).

The methods for the analysis of microbiological soil characteristics and assessment of the impact of environmental or anthropogenic factors, including the cultivation of genetically modified crops, on soils are generally categorized into two broad groups: (1) culture-dependent methods and (2) culture-independent methods (HILL *et al.*, 2000; WIDMER, 2007).

There are several cultivation-dependent methods which are used to retrieve microorganisms from soils. The plating method (*syn*. plate count method) offers a simple but useful tool to identify and characterize changes of specific microbial strains, or groups of species (BRUSETTI *et al.*, 2004; LIU *et al.*, 2005; FARAGOVÁ *et al.*, 2005; MULDER *et al.*, 2006). It can be used to detect effects of transgenic plants on specific soil microorganisms such as symbiotic nitrogen-fixing bacteria, degraders of recalcitrant organic matter, nitrifying bacteria, denitrifying bacteria, cellulolytic bacteria, actinomycetes, and others (BRUINSMA *et al.*, 2003; FARAGOVÁ *et al.*, 2005, FARAGOVÁ and FARAGÓ, 2010). One limitation of the method is, however, that it is affected by the cultivation bias precluding analysis of the entire microbial diversity in soils (WIDMER, 2007). Development of the metabolic fingerprinting method referred to as community-level physiological profiling (CLPP), *syn*. community-level substrate utilization (CLSU), based on parallel cultivation of soil microorgamisms on a set of different carbon sources have significantly improved the capacity and robustness of cultivation-dependent methods. This method is also a cultural technique, and as such, its use is restricted to culturable microorganisms only (PRESTON-MAFHAM *et al.*, 2002; LIU *et al.*, 2005; WIDMER, 2007). Nevertheless, CLPP is often used as an efficient method capable of revealing the effects of transgenic plants on soil microbial communities (DONEGAN *et al.*, 1995; DI GIOVANNI *et al.*, 1999; DUNFIELD and GERMIDA, 2003; GRIFFITHS *et al.*, 2000; HEUER *et al.*, 2002; TESFAYE *et al.*, 2003; BRUSETTI *et al.*, 2004; FANG *et al.*, 2005; LUPWAYI *et al.*, 2007).

Inherent limitations of culture-based methods have lead soil microbiologists to explore culture independent methods of microbial community analysis. These methods allow to characterize the composition and diversity of soil microbial communities based on the extraction, identification and quantification of molecules from soil that are specific to certain microorganisms or microbial groups (HILL *et al.*, 2000). Such molecules include fatty acids and nucleic acids. Phospholipid fatty acid (PLFA) analysis is based on the analysis of the presence and abundance of specific fatty acids in soil which are indicative of specific groups of microorganisms. A biochemical method fatty acid methyl ester (FAME) analysis provides information on the microbial community composition based on separation and identification of methylated fatty

acids extracted directly from soil. These methods are capable of providing a quantitative measure of the viable or potentially viable microbial biomass (LIU *et al.*, 2005). Both, PLFA and FAME are extensively used to assess the effects of transgenic plants on soil microbial communities (DUNFIELD and GERMIDA, 2003; GRIFFITHS *et al.*, 2007).

Advances in molecular biology in last years considerably improved our knowledge of morphological, physiological, biochemical and ecological features of soil microorganisms. Using the DNA based methods specific marker sequences can be analyzed in soil DNA extracts by means of polymerase chain reaction (PCR) amplification (HILL *et al.*, 2000; WIDMER, 2007). Of the various marker genes used to estimate microbial community composition and diversity, the most used are those encoding 16S ribosomal RNA (rRNA) in prokaryotic, and 18S rRNA in eukaryotic microorganisms. A number of molecular-based techniques have been developed to assess the microbial community diversity (HILL *et al.*, 2000; KIRK *et al.*, 2004; WIDMER, 2007). These include DNA guanine plus cytosine content analysis (NUSSLEIN and TIEDJE, 1999), DNA reassociation (TORSVIK *et al.*, 1990), DNA-DNA hybridization (GUO *et al.*, 1997), and different PCR-based methods such as terminal restriction fragment length polymorphism (T-RFLP) (LIU et al, 1997; RASCHE *et al.*, 2006), amplified ribosomal DNA restriction analysis (ARDRA) (LIU *et al.*, 1997; WIDMER *et al.*, 2001; LOTTMANN *et al.*, 2010), denaturing gradient gel electrophoresis (DGGE) (MUYZER *et al.*, 1993; CASTALDINI *et al.*, 2005; COSTA *et al.*, 2006), temperature gradient gel electrophoresis (TGGE) (MUYZER and SMALLA, 1998), single-strand conformation polymorphism (SSCP) (SCHWEIGER and TEBBE, 1998; MIETHLING-GRAFF *et al.*, 2010), and ribosomal intergenic spacer analysis (RISA) (FISHER and TRIPLETT, 1999; BRUSETTI *et al.*, 2004). The RFLP and ARDRA techniques distinguish sequences based on different locations of restriction enzyme recognition sites. DGGE and TGGE rely on differences in the stability of DNA duplex, while SSCP analysis detects differences in secondary structures of single stranded DNA. RISA, on the other hand, resolves length differences of the amplified marker gene sequence (WIDMER, 2007). These methods, used for DNA fingerprint analyses, enables cultivation-independent analysis of microbial community structure and diversity through the detection and characterization of microbial nucleic acid sequences within samples (LIU *et al.*, 2005), however, they do not provide quantitative analyses of microbial groups in soil. Recently, a new DNA-DNA hybridization technique, taxonomic microarray analysis, has been adopted to assess soil microbial diversity (GREENE and VOORDOUW, 2003). This technique has been shown to be able to discriminate and characterize bacterial community composition in related biological samples, offering extensive possibilities for systematic exploration of bacterial diversity in different ecosystems (SANGUIN *et al.*, 2006).

4. Community level physiological profiling

To understand the role of microbial communities in different environments, it is essential to have knowledge of microbial community function and functional diversity

(PRESTON-MAFHAM *et al.*, 2002). Although molecular techniques are now widely used to characterize soil bacterial communities, these techniques provide little information regarding functional properties of microbial communities, for example, on the ability of microbial communities to metabolize specific chemical compounds. To address these functional features of microbial communities, the method of community level physiological profiling (CLPP) using Biolog® microplates (Biolog, Inc., Hayward, USA) have been developed. GARLAND and MILLS (1991) were the first who introduced the use of commercially available Biolog® microtitre plate bacterial identification system based on the utilization pattern of 95 different single carbon sources, to assess functional diversity of microorganisms in environmental samples.

The Biolog® system was originally developed for rapid identification of pure bacterial cultures by sole carbon source utilization, using plastic 96 well microtiter plates containing 95 different carbon substrates in separate wells, and no substrate in one well, which is used as control. Among the 95 substrates on Biolog GN and GP plates, there are several groups of chemical compounds, such as carbohydrates, amino acids, carboxylic acids, amines, amides and polymers (Fig. 1). In addition, each well contains a colourless tetrazolium dye. Upon inoculation of plate with a pre-grown isolate, metabolism of the substrate and the production of NADH via cell respiration lead to reduction of colourless tetrazolium to violet formazan (Fig. 2). The colour development is measured colorimetrically at specific absorbance and a data set of optical density (O.D.) values is obtained (GARLAND and MILLS, 1991; PRESTON-MAFHAM *et al.*, 2002). Individual species may be identified by a specific pattern of colour change on the plate, providing an identifiable metabolic fingerprint (PRESTON-MAFHAM *et al.*, 2002). Differences in sole carbon source utilization have been used successfully to distinguish among different bacterial isolates for more than 60 years. At present, the Microbial Identification Database for Biolog Systems (Biolog, Inc.) contains more than 1950 entries for rapid identification of broad spectrum of aerobic and anaerobic bacteria, yeasts and filamentous fungi, mainly of medicinal importance.

The commercially available 96 well Biolog® microtitre plates were first used to compare metabolic activities of heterotrophic microbial communities from environmental habitats (water, soil and wheat rhizosphere) by GARLAND and MILLS (1991).

Currently several different types of Biolog plates are available to microbial ecologists, all of them containing different substrates appropriate for specific groups of microorganisms. The Biolog GN™ plates were developed for identification of Gram-negative bacteria and contain substrates appropriate for this group of microorganisms (Fig. 1A), whereas Biolog GP™ plates contain carbon sources appropriate for Gram-positive bacteria (Fig. 1B). Even though GN and GP plates were successfully used for ecological microbiology research, Biolog ECO™ plates have been developed specifically for ecological applications. Contrary to GN and GP plates, the ECO plates contain only 31 substrates (plus a control well without a substrate) in three replicates on the microtitre plate. These substrates have previously been found to have high relevance to soil bacterial communities, or are known to be included in plant root exudates (PRESTON-MAFHAM *et al.*, 2002). The tetrazolium dye used in GN, GP

and ECO plates is not metabolizable by fungi, therefore specific Biolog FF plates have been designed for identification of fungal microorganisms. The FF plates contain different carbon sources from GN, GP, and ECO plates and a modified tetrazolium dye specifically metabolizable solely by fungi. To prevent interference of growth and colour development by bacteria, an appropriate combination of antibiotics is frequently included into FF plates (BUYER *et al.*, 2001). Biolog MT plates, containing a redox dye and no substrate, are also available, which allow researchers to produce customized plates (PRESTON-MAFHAM *et al.*, 2002; STEFANOWITZ, 2006).

A

A1 Water	A2 α-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl-Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Amino-Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

B

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D-Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-Phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvatic Acid Methyl Ester	F7 Succinic Acid Mono-methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

Fig. 1 Carbon sources in GN2 (A) and GP2 (B) MicroPlates (Biolog, Inc., Hayward, USA)

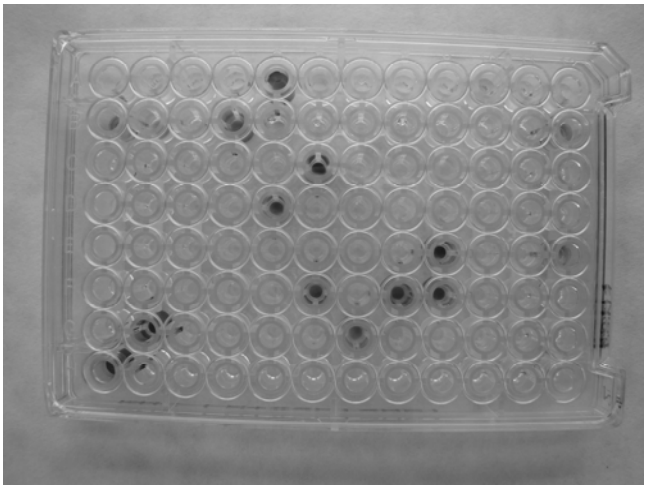

Fig. 2 Colour development in Biolog GP microplate inoculated with a microbial suspension extracted from the rhizosphere zone of transgenic alfalfa (Faragová *et al.*, not published).

The use of Biolog microtitre plates enables analyzing microbial communities on both, the individual- as well as community levels (GARLAND, 1997). At the individual-level, the analysis of microbial communities requires the isolation of microorganisms, isolation and characterization of individual isolates, and description of the community based on the relative abundances and diversities of different isolates. The community-level approach, established by GARLAND and MILLS (1991), bypasses the isolation step and the environmental samples are directly inoculated into microplates and the resulting response (colour change pattern) is used to describe differences in microbial communities (GARLAND, 1997).

Community-level Biolog analysis is generally accomplished in five consecutive steps: (1) sample processing to obtain suspension of microorganisms, (2) inoculation of aliquots of microbial suspension into wells of Biolog® microplates, (3) incubation of plates for 2-8 days in defined culture conditions, (4) regular monitoring the colour development in each well and (5) data processing and statistical analysis of results.

For analyses of pure culture isolates, the samples are prepared from freshly grown cells on a standard enriched medium (GARLAND, 1997; KONOPKA *et al.*, 1998). In contrast, analyses of environmental samples have used a number of different sample processing techniques, including direct inoculation of aquatic samples into microtitre plates (GARLAND and MILLS, 1991), shaking plant roots in a solution to release rhizosphere microorganisms (GARLAND, 1996), and extraction of microbial cells from complex soil matrices using sieving, shaking, blending and sedimentation or centrifugation of samples (HAACK *et al.*, 1999; KONOPKA *et al.*, 1998; DOBLER *et al.*, 2001). For comparisons it is important that samples are of equivalent size, in term of volume and/or weight (PRESTON-MAFHAM *et al.*, 2002).

In most cases the processed sample is diluted prior to use as inoculum. As bacterial cell density in the inoculum affects colour development in Biolog plates (GARLAND and MILLS, 1991; HAACK *et al.*, 1995; GARLAND, 1996), it is very important to

determine dilution levels that reduce additional colour development caused by excess utilizable organic matter in the sample (KONOPKA *et al.*, 1998; PRESTON-MAFHAM *et al.*, 2002). It is known, that a minimum number of about 10^8 metabolically active cells per ml in a well is required for observable colour development (HAACK *et al.*, 1995). Therefore, in dilute inocula, the kinetics of colour development will be a function of time and will follow a sigmoidal curve with an initial lag phase, followed by linear growth phase and a final plateau phase. The lag phase represents the time period during which the inoculum grows to a population density of 10^8 cells ml^{-1}, while during the linear phase the specific metabolic activity of a microorganism on a specific substrate causes accumulation of formazan and colour development. At the plateau phase the production of formazan upon the oxidation of a specific substrate by the activity of a microorganism reaches its maximum yield due to organic substrate depletion. Especially when the goal is identification of pure cultures, it is recommended to adjust all microbial suspensions to a standardized cell density prior to inoculation into Biolog® plates. Alternatively, with community-level Biolog analysis, adoption of appropriate data analysis can account for different inoculum densities (GARLAND and MILLS, 1991).

Most studies of soil microbial community composition and diversity employing Biolog® plates use fixed incubation temperatures between 15 and 28°C (PRESTON-MAFHAM, 2002). Another important variable in Biolog analysis is incubation time, as both the colour intensity in individual wells, and the number of positive wells will increase with incubation time. Too short incubation times (48 h and less) may cause absence of colour development in some wells (especially with those of slow growing microorganisms), while longer incubations (>48 h) will result in more positive responses and reaching the colour saturation levels in some wells (KONOPKA *et al.*, 1998; PRESTON-MAFHAM *et al.*, 2002). To reduce the effects of differential rates of colour development due to inoculum density and inoculation time, appropriate transformations of original raw data are recommended (GARLAND and MILLS, 1991; PRESTON-MAFHAM *et al.*, 2002).

In principle, the Biolog assay is done by colorimetrically measuring tetrazolium dye reduction that is coupled to substrate oxidation. Any microtitre plate reader equipped with an appropriate filter (590 nm) can be used to quantify the colour development in individual wells of plates. The degree to which each of the 95 substrates is oxidized is determined after a fixed incubation period. A positive response is identified as an absorbance or optical density value greater than that occurring in the blank well (HAACK *et al.* 1995). The number and types of utilized substrates, as well as developed colour intensities, constitute a data set from which the functional diversity of microbial communities involved may be assessed. Colour development in each well reflects species activity and density, as well as the ability of the bacterial community to respond to particular substrates (ZAK *et al.*, 1994). The data obtained by quantifying the colour development in individual wells are usually expressed as individual well optical densities or average well colour development (AWCD) (GARLAND and MILLS, 1991; PRESTON-MAFHAM *et al.*, 2002). The AWCD value for each microplate is obtained by calculating the raw difference between the optical density in each well and that in the control well and then summing

all these values and dividing by 95. On the other hand, SIGLER (2004) uses two parameters to describe the microbial community structure: the average metabolic response (AMR), and community metabolic diversity (CMD). AMR describes the average respiration of the C-sources by the microbial community and provides a single metric by which communities can be compared. The AMR is calculated as the average of the mean difference between the O.D. of the C-source-containing wells and the control well. The second parameter, CMD, represents the number of substrates utilized by the microbial community and is analogous to community functional richness. CMD is calculated by summing the number of positive responses (violet-coloured wells) observed following incubation (SIGLER, 2004).

The CLPP generates a rich data set of 95 values for each sample (if GN or GP plates are used) or 31 values in triplicate (if ECO plates are evaluated). Data are corrected against the control well (without a carbon substrate) or the initial reading at time zero, before subjecting to multivariate statistical analysis. Regardless of the chose of absorbance measurement, i.e. net O.D. of individual substrates, AWCD, or parameters received from kinetic analyses, the use of multidimensional statistical analyses is necessary (KONOPKA *et al.*, 1998; STEFANOWICZ, 2006). The most commonly used method is principal component analysis (PCA) of the quantitative colour data, which reduces the number of variables to a few significant principal components. The use of cluster analysis based on the presence or absence of colour in wells (ZAK *et al.*, 1994) is also possible. In kinetic studies, one may process the data by calculating the area under curve for each well O.D. for the entire period of incubation, or by estimation of kinetic parameters by fitting the curve of O.D. versus time to a density dependent logistic growth equation (GUCKERT *et al.*, 1996; LINDSTROM et al, 1998; INSAM and GOBERNA, 2004).

Analysis of microbial community functional diversity using sole carbon source utilization profiles is, despite some limitations (GARLAND, 1997; KONOPKA *et al.*, 1998; PRESTON-MAFHAM *et al.*, 2002), a rapid, reproducible and useful tool to discriminate among bacterial communities from diverse environmental samples. Its strength lies in the low manpower it requires, which enables intensive sampling across temporal and spatial scales, and the reliance of the method on metabolic traits that could lead to functionally relevant characterization of changes in microbial communities (GARLAND, 1997). Although CLPP is a culture based assay, it has been found that non culturable cells also respond to this assay, and therefore this assay seems not to be as biased as the classical culture-based techniques (PRESTON-MAFHAM *et al.*, 2002).

5. Assessing the effect of transgenic crops on soil microbial communities using CLPP

The CLPP using Biolog® plates has been used to date for characterizing bacterial communities from a range of environments, including comparisons of rhizosphere bacterial communities of transgenic vs. non-transgenic plants. As the method can provide important insights into ecosystem function and stability, it was used to study the metabolic responses of microbial communities from rhizosphere and bulk soils

(SÖDERBERG *et al.*, 2004), from the rhizosphere of different plant species (GRAYSTON *et al.*, 1998; MIETHLING *et al.*, 2003) or from different parts of the rhizosphere along the root (BAUDOIN *et al.*, 2001). The Biolog method is also frequently applied for estimating the influences of different cropping systems (LARKIN and HONEYCUTT, 2006), agronomic procedures such as tillage (GRIFFITHS *et al.*, 2007), soil management (GOMEZ and CORREA, 2008), or factors involved in ecosystem changes (GÖMÖRYOVÁ *et al.*, 2009b) on microbial community physiological profiles. Currently one of the most frequent applications of Biolog® system is for assessing the impacts of different stressing factors, such as heavy metals (DOBLER *et al.*, 2001; LIAO and XIE, 2007), herbicides (MIJANGOS *et al.*, 2009), explosive elements (ANDERSON *et al.*, 2010) and high salinity and low pH (PANKHURST *et al.*, 2001).

DONEGAN *et al.* (1995) first used the Biolog GN plates to assess changes in soil microorganisms associated with transgenic cotton plants expressing the *Bacillus thuringiensis* endotoxin (Bt). From that time, the CLPP method using Biolog® GN, GP or ECO plates proved to be a quick, reproducible and useful method to discriminate among microbial communities associated with the roots of transgenic vs. non-transgenic plants (DI GIOVANNI *et al.*, 1999; SCHMALENBERGER and TEBBE, 2002; TESFAYE *et al.*, 2003; BRUSSETTI *et al.*, 2005; FANG *et al.*, 2005; MULDER *et al.*, 2006; WATRUD *et al.*, 2006; LUPWAYI *et al.*, 2007; TRAVIS *et al.*, 2007 and many others).

Some authors (BRUSSETTI *et al.*, 2005; FANG *et al.*, 2005; GRIFFITHS *et al.*, 2007) could not find any statistically significant differences in functional diversities of rhizosphere microorganisms between transgenic and non-transgenic plants using Biolog® microplates. In these studies, whether in greenhouse or field conditions, soil texture, crop type and pest management regime, rather than cultivation of transgenic plants affected the rhizosphere microbial communities. Other authors reported very few changes in functional diversity of rhizosphere soil bacteria associated with growing GM crops (LUPWAYI *et al.*, 2007), or significant shifts in the carbon utilization patterns of culturable microbial communities in the rhizospheres of transgenic plants, or soils amended with residues from transgenic plants (DI GIOVANNI *et al.*, 1999; TESFAYE *et al.*, 2003; FANG *et al.*, 2007; TRAVIS *et al.*, 2007). At least some of these differences in carbon source utilization patterns may be explained by the possible effects of site of insertion or simply somaclonal variation on the availability of endogenous nutrients in the soil mix on microbial populations (WATRUD *et al.*, 2006). FANG *et al.* (2007) hypothesized, that amendment of soils with Bt corn residues may affect selected activities of soil bacteria carried by specific enzymes. Altered composition of root exudates of transgenic plants may also induce a different community of rhizosphere microorganisms.

In general, however, if shifts in bacterial communities due to cultivation of transgenic plants were observed, these were similar to those observed when non-transgenic cultivars are compared. In comparison with other factors, the impact of the genetic modification of the plant on soil microbial communities was usually minor and transient, or comparable to variations due to plant genotype, plant physiological and developmental stage, soil type, agricultural practice used and pathogen exposure.

Nevertheless, despite the research performed up to now, it is not yet clear whether GM plants or their products exert any ecological effect on the microbiota and microbial processes in soil. To elucidate this question, more studies on the impact of transgenic plants on soil microbial communities is needed, preferably employing the polyphasic approach using different methods of soil microbial communities analysis, including plate count, biochemical and molecular methods.

6. Conclusions

Community level physiological profiling is a quick and easy method of investigating the functional diversity of microbial communities from a range of environmental habitats. The method, which employs Biolog® microplates containing 95 different carbon sources, are gaining increasing importance not only in general soil ecology, but also is increasingly used as a method which is able to provide functional information on the microbial community structure and diversity in the rhizosphere of transgenic plants. The simplicity, speed, and low cost of the method compared with other approaches are very attractive to microbial ecologists and other microbiologists. However, the researcher should keep in mind, that to fully exploit its potential the technique requires very careful data acquisition, appropriate statistical analysis and cautious interpretation of results.

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