EDITORIAL

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Plant-associated microorganisms: a view from the scope of microbiology

Microorganisms interact with plants because plants offer a wide diversity of habitats including the phyllosphere (aerial plant part), the rhizosphere (zone of influence of the root system), and the endosphere (internal transport system) [3, 5]. Interactions of epiphytes, rhizophytes or endophytes may be detrimental or beneficial for either the microorganism or the plant and may be classified as neutralism, commensalism, synergism, mutualism, amensalism, competition or parasitism.

Plant-associated microorganisms play essential roles in agricultural and food safety, and contribute to the environmental equilibrium. Their study has been classically based on cultivation-dependent methods, which often recover only 0.01–10% of direct counts. However, studies based on molecular analysis have estimated more than 4,000 species per gram of soil. Most of these microorganisms are probably non-cultivatable, such as the plant symbiotic vesicular-arbuscular mycorrhiza (VAM) endomycorrhizae common in many angiosperms and gymnosperms, or are transiently in a viable but non-cultivatable state [1]. Microbial composition and population levels in the rhizosphere and phyllosphere are determined mainly by carbon sources released as root exudates and often change with cultivars of the same plant species. The degree of change in microbial composition of the phyllosphere or rhizosphere is associated even with the type of genetic modification in some transgenic plants.

Cultivation-dependent methods based on PCR, RFLP, fatty acid profiles (FAME), nutritional (Biolog) and many others have been used largely to characterize specific groups of plant-associated bacteria and fungi. However, conventional cultivation-dependent methods are biased towards the selective enrichment of fast-growing microorganisms adapted to high substrate concentrations, which can represent a minor fraction of the microbial community. Cultivation-independent PCR-based fingerprinting techniques to study small-subunit (SSU) rRNA genes (rDNA) in the prokaryote microbial fraction have been developed to study diversity, structural composition and dynamics of microbial communities associated with plants. Amplicons are separated by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), or submitted to terminal restriction fragment length polymorphism (T-RFLP) analysis. For example, using T-RFLP in a study of corn-associated bacteria, signals related to *Cytophaga*/*Bacteroides*/*Flavobacterium* phylum, *Holophaga*/*Acidobacterium* phylum, α-proteobacteria, β-proteobacteria and γ-proteobacteria were detected, whereas cultivation-based methods detected mainly γ-proteobacteria [4].

Crop production losses due to biotic agents (pests, diseases and weeds) are controlled mainly by spraying crops with a vast amount of synthetic chemical pesticides, which can severely affect planet health due to their non-target effects. New methods of crop protection have been developed based on historical observations in agriculture and forestry of the benefits obtained from naturally occurring microbial communities. Beneficial interactions may result from competition, antagonism and hyperparasitism against plant pathogens, insects and weeds. In most cases, the practice of biological control implicates the introduction of massive amounts of microbial pesticides into the environment. Therefore, specific analysis methods are necessary to study the traceability, residue analysis and environmental impact of a pesticide. However, the search for specific genotypic markers in microbial pesticides is difficult and time-consuming because it is accomplished by DNA fingerprinting methods to identify strain-specific DNA fragments that can be sequenced and characterized (SCAR), and used to develop real-time PCR (quantitative PCR) analysis. The use of reporter gene systems in plant-associated microorganisms has also provided

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powerful tools for evaluating in situ gene expression, colonization, mechanism of action, and monitoring of release. The most successful methods are based on the chromogenic gusA (a β-glucuronidase from *Escherichia coli*), luminescent *lux/luc* (luciferases from *Vibrio fischeri* or firefly lantern) or fluorescent *gfp* (green fluorescent proteins from jellyfish).

Since the introduction of genetically modified (GM) crops in 1996, their impact on associated microbiota, and horizontal transfer of antibiotic selectable markers have both been a cause of concern for large-scale commercial introduction. World areas of GM crops have increased at a sustained rate, with the principal crops being soybean, corn, cotton and canola. Most commercial transgenic plants contain selectable markers under bacterial promoter control such as antibiotic-resistance genes, the most common being the *bla* gene, which codes for a TEM β-lactamase (ampicillin resistance), and the *npt*II gene, which codes for a neopentenyltransferase (kanamycin resistance). The acquisition of genes coding for antibiotic resistance from transgenic plants by plant-associated bacteria, and their subsequent transfer from these bacteria to human or animal bacterial pathogens, has been a major concern in the large-scale use of transgenic crops. The process would require a sequence of coordinated and complex events, and the occurrence of such a transfer under natural conditions would be rare, but there is no reason to assume that the phenomenon would not take place, even at a reduced probability, in nature. Gene transfer from transgenic plants to bacteria has been observed only under laboratory conditions, when certain barriers were absent, in complex model systems consisting of two bacterial species and transgenic tobacco with high transgene copy number and a high degree of sequence homology [2]. However, very few field studies have addressed this issue. The high proportion of ampicillin-resistant bacteria occurring naturally in agricultural fields fertilized often with manure, may put into perspective the potential impact of the antibiotic selectable marker acquisition by corn-associated microbiota.

The structural and functional analysis of microbial genomes and the proteins encoded by genes of important plant-associated microbes will provide insights into several aspects of these molecular interactions and will be crucial for the development of more efficient control measures, and accurate diagnostic and monitoring methods. More than 10,000 species of fungi, bacteria and viruses are estimated to cause plant diseases, several of which are of major economic importance. The automation of sequencing technologies and advancements in bioinformatics provide tools leading to better knowledge not only of the genome of plant pathogens or microorganisms beneficial to plants but also of ways of incorporating genes from microbes into plants as microbial-derived resistance. The genomes of 733 prokaryotic and eukaryotic organisms have been completely sequenced or are ongoing (146

organisms have been completely sequenced and 587 are ongoing), and those of 944 viruses have been completed. However, of the completed genomes, only 9 are plant-associated bacteria and 44 plant viruses. Of the ongoing 587 sequences, only 32 (14 prokaryotes and 18 eukaryotes) pertain to bacteria or fungi associated with plants. Of the 1,677 genomes sequenced or ongoing, only 85 correspond to plant-associated organisms. Most plant-associated microorganisms whose genomes have already been sequenced are bacteria, including *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *campestris*, *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *citri* and *Xylella fastidiosa* (several strains), *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, and *Mesorhizobium loti*. Ongoing genome analyses include bacteria such as *Clavibacter michiganensis* subsp. *sepedonicus*, *Azotobacter*, *Bacillus thuringiensis*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, and fungal pathogens including *Aspergillus* spp., *Botryotinia fuckeliana*, *Fusarium* spp., *Mycospherella graminicola*, *Ustilago maydis*, and *Phytophthora* spp. (http://ergo.integratedgenomics.com/GOLD/).

The easy commercial exchange of agricultural products (fresh products and plant material) has caused the highest spread of plant pathogens and diseases ever observed. Quarantine systems to prevent the introduction of new plant pathogens in protected areas, and eradication measures to avoid epidemic spread are the first protection barriers. However, many pathogens remain latent in the plant material and in very low numbers, and methods with high sensitivity, specificity and reliability are required. New methods of plant-pathogen analysis have evolved parallel to the advancement of methods of analysis in human and veterinary microbiology. Automation and electronic data management are essential for the increase in productivity and efficiency of routine analysis for the detection of plant pathogenic bacteria and viruses. The specificity of detection by serological techniques has improved greatly with the use of specific monoclonal and recombinant antibodies, enrichment-ELISA protocols, and on-site-testing using tissue print-ELISA and lateral flow devices. Molecular techniques based on hybridization or amplification and especially PCR have been refined by co-operational PCR, multiplex PCR, and multiplex-nested RT-PCR. In many cases, the need for quantification and field compatibility of the technique has led to new devices for portable rapid cycling real-time PCR [6]. However, new methods with a high potential for determining many pathogens simultaneously including in-situ fluorescence hybridization and DNA microarrays are under development.

These are only a few examples of the fact that microbiology, through its different projections and specifically via the approaches of molecular microbiology and microbial ecology, plays an essential role in providing answers to the main questions related to plant health.

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