Project title

MICROARRAY PREDICTION OF THE ABILITY OF SOIL TO SUPPRESS BLACK ROOT ROT DISEASE OF TOBACCO

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ABSTRACT

In disease-suppressive soils, plants are protected from soil-borne disease by specific root-colonizing microorganisms. Suppressiveness of Swiss soils to black root rot disease of tobacco (caused by *Thielaviopsis basicola*) has been attributed to rhizosphere pseudomonads producing the biocontrol compound 2,4-diacetylphloroglucinol, but these bacteria are also present in non-suppressive (i.e. conducive) soils. Whether non-*Pseudomonas* bacteria play also a role in suppressiveness is unknown. In this study, soils suppressive and conducive were compared by 16S rRNA gene-based taxonomic microarray (more than one thousand probes) to identify bacterial taxa that could serve as bioindicators of soil suppressiveness to black root rot. Soils were collected in Switzerland, in a similar geological region in France (Savoie) and a distant tobacco area in Hungary. Greenhouse tests, in which tobacco was inoculated with *T. basicola*, enabled verification (Switzerland) or identification (France and Hungary) of suppressive and conducive soils. Rhizosphere DNA extracts from field and greenhouse samples were analyzed using a 16S rRNA-based taxonomic microarray. The differences in rhizobacterial community composition between suppressive and conducive soils depended largely on soil origin (Switzerland vs France vs Hungary) and tobacco growth conditions (greenhouse vs field). Data suggest that farming practices and tobacco cultivars could also be significant factors, and it will be important to assess this in the future. When greenhouse tobacco was considered, several taxa e.g. *Flavobacterium*, *Pseudomonas fluorescens* complex and *Phyllobacterium* were prevalent in the rhizosphere of the suppressive soils, and others e.g. *Rhodospirillum*, *Mycobacterium* and *Bradyrhizobium* in the case of the conducive soils. When field samples were considered, *Methylobacterium* and *Azospirillum* were prevalent in the suppressive soils in addition to those already detected in greenhouse samples. In the conducive soils, several *Bacillus* spp. dominated in field. Thus, the microarray has potential for prediction of black root rot suppressiveness based on bacterial bioindicators. However, their practical application will entail a comparative approach, since there are rather quantitative than qualitative microbial differences between soils. Many of the bacterial taxa prevalent in suppressive soils are known for including strains with biocontrol or phytostimulatory effects, and therefore it could be useful to isolate such strains and test them for biocontrol of black root rot disease.

1. INTRODUCTION

1. 1. Suppressive soils

Agricultural practices applied during the 20th century, especially the excess of pesticide inputs, let to decrease of soil fertility as the consequence of lowered microbial diversity in soils. Soil microorganisms themselves may at the same time increase the availability of nutrients for plants via nitrogen fixation or phosphate solubilisation (biofertilisers; Rodriguez and Fraga, 1999; Kennedy et al., 2004). Some of them may promote plant growth directly also by phytohormone production. In addition, some soil microorgansims are antagonistic to various plant pathogens and thus protect plants from diseases (biopesticides; Raaijmakers et al., 2002). Agricultural practices that preserve natural soil biodiversity must be adopted to sustain plant production.

Each soil harbors microorganisms and possesses a low level of suppressiveness against pathogens. This phenomenon called 'general suppression' is provided by competition among microorganisms (Baker and Cook, 1974). In addition, high level of suppressiveness against a pathogen can be found in some soils. This is referred to as 'specific suppression' and it is attributed to a narrow group of soil microorganisms that are antagonistic to the pathogen (Baker and Cook, 1974). They provide high-level protection to susceptible plants. The term suppressive soil is used for the case of 'specific suppression'. The opposite is a conducive soil, i.e. a soil that permits disease development.

Soils suppressive to fungal, bacterial and nematode pathogens are found worldwide (Mazzola, 2002; Haas and Défago, 2005). The suppression is either long-termed (= natural; for example the case of *Thielaviopsis basicola* suppressive soils) or induced by monoculture (for example *Gaeumannomyces graminis* var. *tritici* suppressive soils). For both types, the evidence of biological origin of suppressiveness was provided by soil sterilisation (abolishes suppressiveness) and inoculation of a conducive soil with a small amount of suppressive soil (suppressiveness transfer). However, some abiotic factors may also contribute to soil suppressiveness (Höper et al., 1995; Rimé et al., 2003).

The key microbial components of soil suppressiveness to different pathogens correspond both to bacteria (e.g. *Pseudomonas*, *Streptomyces*, *Pasteuria*; Neeno-Eckwall et al., 1999; Weibelzahl-Fulton et al., 1996) and fungi (e.g. *Fusarium*, *Dactylella*; Lemanceau et al., 1993; Olatinwo et al., 2006). Many other microorganisms are known to be antagonistic to

plant pathogens, especially bacteria e.g. *Enterobacter*, *Burkholderia*, *Comamonas*, *Pantoea*, *Serratia*, *Stenotrophomonas* or *Bacillus* and fungi e.g. *Trichoderma* or *Glomus* (Whipps et al., 2001; Raaijmakers et al., 2002), but their role in suppressive soils remains to be shown. Some of them have been however already used successfully as commercial biopesticides (White et al., 1990).

The antagonistic interactions between pathogen and biocontrol microorganisms take place mainly in rhizosphere, which is the part of soil that is directly influenced by plant roots (typically a few millimeters around roots). There is a high flux of root exudates, which can serve as carbon sources for various microorganisms. It is colonized by highly competitive microorganisms and represents a spot of high microbial activity in soil.

Different types of interactions may take place between pathogen and suppressive microorganism. Direct antagonism includes production of antibiotics and lytic enzymes that affect pathogen cells (Elad and Kapat, 1999; Raaijmakers et al., 2002). Some biocontrol microorganisms, especially those closely related to pathogens, compete with pathogens for nutrients or infection sites at the root surface (Olivain and Alabouvette, 1999). Some other parasite the pathogens, which is often the case of nematode suppression (Siddiqui and Mahmood, 1999). Various biocontrol microorganisms act rather on plant. They may induce systemic resistance in plant, promote plant growth or abolish plant stress via phytohormone interference (van Loon et al., 1998; Glick, 2005).

These interactions have been intensively studied in the case of fluorescent *Pseudomonas*, which have been recognized as a key component in several suppressive soils, for example soils suppressive to take-all or black root rot (Weller, 2007). Fluorescent *Pseudomonas* strains often produce several antimicrobial compounds, e.g. 2,4-diacetylphloroglucinol (DAPG), phenazines, and hydrogen cyanide (Chin-A-Woeng et al., 2000; Raaijmakers et al., 2002; Haas and Défago, 2005; Weller, 2007), siderophores (for Fe sequestration; Maurhofer et al., 1994), phytohormones (Lippmann et al., 1995; Patten and Glick, 2002) and other compounds. They are highly competitive and colonize quickly the rhizosphere. They are capable to induce systemic resistance in plants (Pieterse et al., 2003). In comparison with *Pseudomonas*, only a minor attention has been paid to other biocontrol microorganisms.

1.2. Soils suppressive to black root rot of tobacco

Black root rot is caused by the soil-borne pathogen *Thielaviopsis basicola* (synanamorph: *Chalara elegans*). Besides tobacco, it causes black root rot in many cultivated plant species, for example cotton, carrots, ornamentals. It is a deuteromycete. It produces only asexual spores: endoconidia and chlamydospores, that persist in soil for several years (Adams and Papavizas, 1968). *T. basicola* is present worldwide and could be isolated from number of different plant species, even in absence of visible disease syndromes (Yarwood, 1981). The wide host range together with the fact that it does not utilize structural carbohydrates as a source of carbon, classify *T. basicola* as an obligate parasite.

T. basicola attacks tobacco either in the plant bed or in the field. Affected plants are stunted and turn yellow. In field, 'patches' of diseased plants are observed (**Fig. 1**). The affected plants have a reduced root system with brownish to black lesions (**Fig. 2**). *T. basicola* is favored by elevated soil pH and wet climate (Harrison and Shew, 2001). Crop rotation reduces the level of field infestation.

In Morens region of Switzerland (canton Fribourg), soils suppressive to black root rot disease of tobacco have been identified and studied for several years (Stutz et al., 1986, 1989; Ramette et al., 2003, 2006). Suppressive and conducive soils are present in the same area and have similar physical and chemical properties, despite a different geological origin and clay mineralogy. Disease-conducive soils are found on sandstone deposits, while disease-suppressive ones developed on morainic material brought by the Rhône glacier (Stutz et al., 1986, 1989).

Suppressiveness to black root rot has been attributed to fluorescent *Pseudomonas* (*e.g.* *Pseudomonas* strain CHA0) that are antagonistic to *T. basicola*. They produce the biocontrol compounds DAPG and HCN (Hcn+ Phl+ pseudomonads; Stutz et al., 1986). Clay mineral composition of soil was nevertheless important for suppressiveness. Suppressive soils were shown to be mainly composed of vermiculitic clays, and the conducive soils of illitic and smectitic clays (Stutz et al., 1989). It was shown that Fe^{3+} (largely liberated by vermiculite but not by illite) was essential for tobacco protection by *Pseudomonas* strain CHA0 (Keel et al., 1989). This had probably also an effect on HCN production (Voisard et al., 1989).

Further studies, based on *Pseudomonas* isolation from tobacco inoculated by *T. basicola*, showed that significant numbers of fluorescent pseudomonads were present in the tobacco rhizosphere, both in suppressive and conducive soils (Ramette et al., 2003, 2006).

These findings were however based only on the study of culturable pseudomonads isolated from tobacco plants inoculated with *T. basicola*. Therefore, cultivable fluorescent pseudomonads do not seem to be a good indicator of black root rot suppressiveness. The results of Ramette et al. (2003, 2006) and subsequent DGGE profiling of pseudomonads by Frapolli et al. (2008) raised the question whether some other bacteria could contribute to black root rot suppressiveness. There is therefore a need for a non-cultivation approach that would permit wide screening of soil bacterial communities to identify other microbial bioindicators of black root rot suppressiveness.

Fig. 1. Tobacco plants affected by *T. basicola* (red circle). Picture: R.J. Reynold, R.J. Reynolds Tobacco Company, http://www.ipmimages.org/

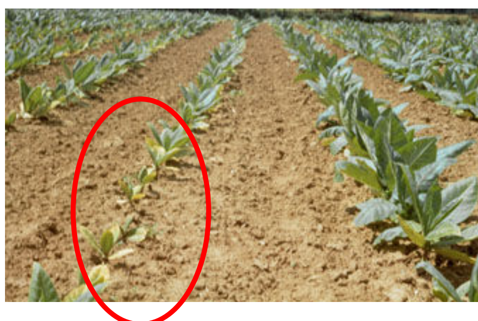


Fig. 2. Roots of tobacco affected by *T. basicola* (this study)

1.3. Microarrays as a tool for bacterial community assessment

It is estimated that only 1% of soil bacteria are cultivable. Non-cultivation approaches are therefore required to study soil bacteria communities (Schloter et al., 2000). At present, several methods are available, e.g. phospholipid ester-linked fatty acid (PLFA) profiling, 16S rRNA gene-based terminal restriction fragment polymorphism (T-RFLP) analysis, 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). All of them are fingerprinting methods suitable for sample comparisons, however, with no or only limited possibility of direct identification of bacterial

taxa. Cloning/sequencing of environmental samples provides direct information on the bacteria present, but it is still an expensive and time consuming method.

Microarrays, originally developed for gene expression studies, are nowadays used also for bacterial community assessments. In comparison with the above mentioned approaches, these so-called taxonomic microarrays enable a high throughput analysis of bacterial communities and bacterial taxa identification (Loy et al., 2002; Bodrossy et al., 2003; Stralis-Pavese et al., 2004; Kelly et al., 2005; Kostic et al., 2005; Brodie et al., 2006; Sanguin et al., 2006a, 2006b). They usually target 16S rRNA genes, which are ubiquitously present in bacteria and reflect their evolutionary relationships (Woese, 1987).

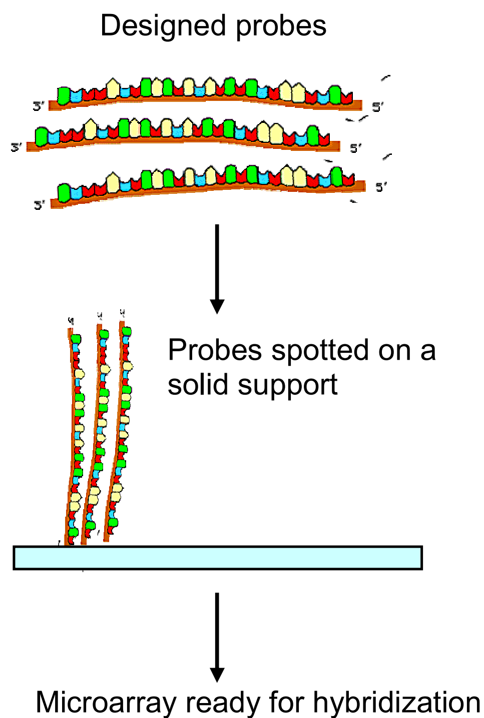
Briefly, microarrays are based on hybridization between oligonucleotide probes and their complementary DNA or RNA targets (**Fig. 3**). Hybridization is expected if there is no or little mismatch between a probe and its target. Probes are attached on solid supports, membranes, slides or gel pads (a set of probes attached on a solid support is in fact called microarray). Targets (complex samples) are labeled before hybridization, usually with some fluorescent dye. They are applied on microarray and allowed to hybridize with probes at certain temperature. The hybridization temperature determines hybridization specificity. Afterwards, microarrays are washed to remove non-attached or weakly attached targets. Thus, only specific targets strongly attached on probes (thanks to their complementarity with probes) remain on microarray. Microarrays are scanned and luminous spots are visible where fluorescently labeled targets remained attached to probes. The luminosity is proportional to target quantity. The images are numerized and the data are carefully filtered, normalized and analyzed.

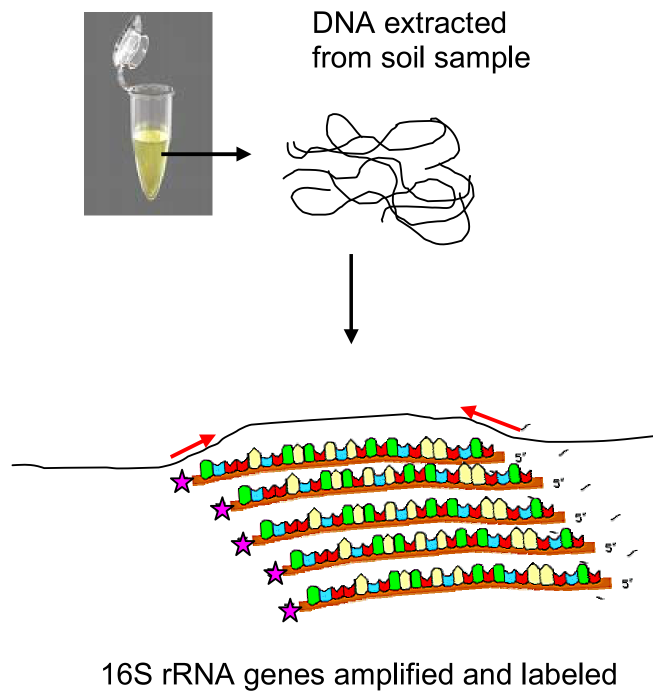
Generally, two main approaches are used for taxonomic microarray development. Brodie et al. (2006) adopted a commercial method, the Affymetrix microarray ('high-density microarray' made using photolithography). Their PhyloChip possesses about 500 thousands probes targeting 16S rRNA genes of more than 8000 bacterial taxa (genus/species level). The probes were designed automatically with an algorithm, with at least eleven probes per taxa. The PhyloChip was successfully applied in several studies, but never on soil bacteria (Brodie et al., 2006, 2007). The majority of taxonomic microarrays are 'home made' arrays. They comprise fewer probes (tens to hundreds), but each probe is defined with a lot of care. Many studies focused on prediction of probe hybridization behavior to avoid false-positive and false-negative results. Zhang et al. (2006), for example, estimated the effect of mismatch position, type of mismatch, mismatch neighborhood and the type of solid support on

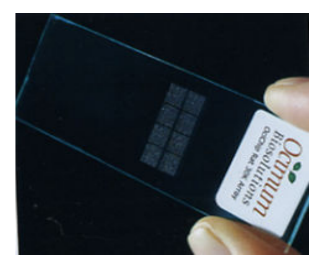
hybridization results. Liu et al. (2007) assessed the impact of target length. Some of these microarrays were used for soil community assessments (Sanguin et al., 2006a, Stralis-Pavese et al., 2004), including the case of disease suppressiveness (Lievens et al., 2007; Sanguin et al., 2008).

MICROARRAY PREPARATION

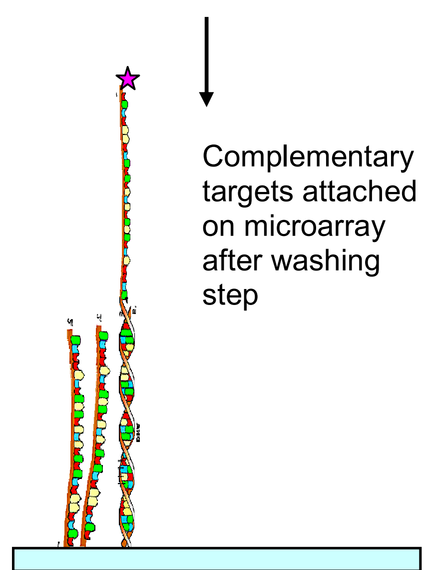
SAMPLE PREPARATION







Labeled sample hybridized on microarray



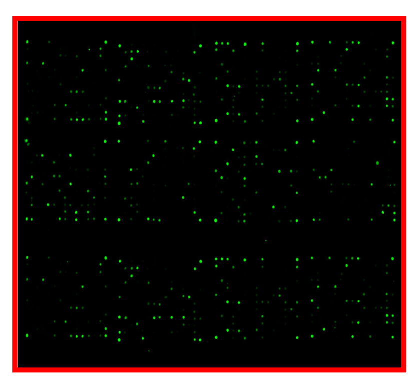


Fig. 3. Schema of hybridization

2. OBJECTIVES

2.1. Development of 16S rRNA gene-based taxonomic microarray for soil bacterial community assessment

The microarray used in this project has been based on the prototype microarray of Sanguin et al. (2006a, 2006b), developed at UMR CNRS 5557 d'Ecologie microbienne (Université Lyon 1, Villeurbanne, France) with technical support of DTAMB platform (Développement de Techniques et Analyse Moléculaire de la Biodiversité). The prototype is a 16S rRNA gene-based taxonomic microarray targeting soil *Proteobacteria*, especially α-*Proteobacteria*. It has been further extended with probes targeting different groups within the *Pseudomonas* genus and has been proved to be suitable for monitoring *Pseudomonas* in a suppressive soil, as published by Sanguin et al. (2008). The number of probes remained however quite low.

The objective was therefore to increase the number of 16S rRNA probes in order to target all important soil bacteria, with focus on antibiotic-producing Gram-positive bacteria (*Actinobacteria*, *Firmicutes*) and other plant-beneficial bacteria (mainly from α-, β-, γ-*Proteobacteria*).

2.2. Assessment of bacterial indicators of soil suppressiveness to black root rot of tobacco

In black root rot suppressive soils, *T. basicola* establishes but causes little or no damage on tobacco plants. Pathogen suppression is provided by certain soil microorganisms. In Morens, Switzerland, soils suppressive to black root rot of tobacco are well documented and can be therefore used as a reference. In the first part, the objective was to compare bacterial communities in soils from Morens known to be either suppressive or conducive to black root rot of tobacco, using a taxonomic microarray.

In the second part, the objective was to find soils suppressive to black root rot in other geographic regions than Morens, assess their suppressiveness level in a standardized inoculation experiment and their bacterial communities in a microarray experiment. Two different approaches were applied to find tobacco black root rot suppressive soils: (i)

Geological origin of soil – based on the knowledge that conducive soils are found on sandstone deposits, while suppressive ones developed on morainic material brought by glacier (Stutz et al., 1986, 1989); (ii) Experience of farmers – the fields with tobacco monoculture where black root rot causes little damage on susceptible cultivars are likely to be suppressive soils.

Finally, the objective was to identify bacterial taxa that could discriminate between *T. basicola* suppressive and conducive soils from different geographical areas including Morens. The knowledge of suppressiveness indicators may help farmers to identify fields suitable for tobacco cropping. In addition, identified bacteria can be tested as potential biopesticides for tobacco black root rot control.

3. MATERIAL AND METHODS

3.1. Soil harvesting and analysis

Soils were collected from fields from Morens region, Switzerland, in June 2006, from Nyíregyháza region, Hungary, in April 2007, and from Savoie region, France, in June 2007. In Morens region, soils to known to be suppressive (MS8), moderately suppressive (MS16), moderately conducive (MC10) and conducive (MC112; Stutz, 1985; Stutz et al., 1986; Ramette et al., 2003) to black root rot were collected. In Savoie region, two soils collected (F2 and F5) originated from sandstone bedrock and two soils (F1 and F4) originated from morainic material brought by Rhône glacier (according to a detailed geological map of the region, and confirmed by field analysis of the type of stones in soil, with kind help from Dr. Gérard Nicoud, Laboratoire *Environments, Dynamiques et Territoires de la Montagne*, Université de Savoie, Le Bourget du Lac, France). Soils F1 and F2 are located near Albens and F4 and F5 near Seyssel. At present, there is no tobacco cultivated in this region, and the soil was taken from maize fields. In Nyíregyháza region, soils were collected from fields with tobacco monoculture, two of them having problems with black root rot (H1 and H3) and two (H2 and H4) with no black root rot problems reported. Soil samples were taken from 10-30 cm depth using sterilized shovels. Root residues and stones were removed, soils were sieved (0.7 cm) where necessary. Analyses of soil physicochemical composition were done at CESAR (*Centre Scientifique Agricole Régional*), Les Soudanièrs, France (**Table 1**).

3.2. Plant inoculation experiment

Growth of tobacco (*Nicotiana glutinosa* L.) and preparation of endoconidial inoculum of the fungus *T. basicola* Ferraris strain ETH D127 were performed as described in Ramette et al. (2003). Briefly, *T. basicola* endoconidia suspension (5 ml, to reach 10^3 of endoconidia cm^{-3} soil) was added to soil around the stems of 4-week-old tobacco plants on the same day of their transplantation in soils. The same volume of distilled water was added to the non-inoculated controls. The number of pots was 8 per treatment. Soil water content was adjusted to 21-35% w/w (depending on field capacity) by watering pots every 1-2 days with distilled water. Plants were cultivated at 22 °C (day, 16h) and 18 °C (night, 8h) at 70% relative humidity. Disease severity was recorded for each plant at 3 weeks after inoculation, as the percentage of root surface covered by *T. basicola* chlamydospores. Root disease level was rated visually using a height-class disease scale (Stutz et al., 1986) based on midpoints of disease level intervals.

3.3. Rhizosphere sampling and DNA extraction

In four fields in Morens (MS8, MS16, MC10 and MC112; see section 3.1), rhizosphere samples from field-grown plants (i.e. either tobacco or wheat) were sampled in June 2004 and June 2006. At the 2004 sampling, tobacco plants had 4 leaves, and in 2006 already 7 leaves. Three plants were taken in 2004, four plants in 2006. Soil tightly adherent to roots was collected in sterile tubes and kept frozen until DNA extraction. The samples from 2004 were sampled by Prof. Yvan Moënne-Loccoz and were kept frozen till DNA extraction. Rhizosphere soil from greenhouse samples was collected at 3 weeks from four inoculated plants and four non-inoculated plants. Total DNA was extracted from 250 mg of soil using PowerSoil DNA Isolation Kit (*MoBio Laboratories*, Carlsbad, CA).

3.4. PCR amplification of 16S rRNA gene

The universal eubacterial primers *T7*-pA (forward; *TAATACGACTCACTATAG*AGAGTTTGATCCTGGCTCAG) and pH (reverse; AAGGAGGTGATCCAGCCGCA) were used to amplify 16S rRNA gene from total DNA extracts (Bruce et al., 1992). Primer *T7*-pA includes at the 5' end the sequence of T7 promoter

(in italics), which enabled T7 RNA polymerase-mediated *in vitro* transcription using the purified PCR products as templates. PCR reactions were carried out in total volume of 50 µl. PCR reaction mixture contained 1× reaction buffer for *Taq* Expand High Fidelity (*Roche Applied Science*, Meylan, France), 0.25 µM of each primer, 50 µM of each dNTP, 20 ng of environmental DNA, 0.025 mg of T4 Gene 32 (*Roche Applied Science*) and 1.25 U of *Taq* Expand High Fidelity (*Roche Applied Science*). Thermal cycling was carried out with an initial denaturation step of 94 °C for 3 min, followed by 30 cycles with 45 s denaturation at 94 °C, 30 s annealing at 55 °C, 90 s elongation at 72 °C, and a final elongation step for 7 min at 72 °C. PCR products were purified with MinElute PCR purification kit (*Qiagen*, Courtaboeuf, France) according to the manufacturer's instructions. DNA concentration of purified samples was determined spectrophotometrically by measuring the optical density at 260 nm and adjusted to 50 ng μl^{-1}.

3.5. Labeling of PCR products

In vitro transcription was carried out according to Stralis-Pavese et al. (2004). Briefly, 8 µl of purified PCR product (400 ng), 4 µl T7 RNA polymerase buffer (5×, *Invitrogen*, Cergy Pontoise, France), 2 µl dithiothreitol (100 mM), 1 µl each of ATP, CTP, GTP (10 mM each), 0.5 µl 25 UTP (10 mM), 1 µl Cy3-UTP (5 mM) (*Amersham Biosciences Europe GmbH*, Saclay, France), 0.5 µl RNAsin (40 U μl^{-1}) and 1 µl T7 RNA polymerase (50 U μl^{-1}, *Invitrogen*) were added into a RNase-free 1.5 ml microcentrifuge tube and incubated at 37° C for 4 h (Spiess et al., 2003). RNA was purified immediately using the QIAGEN RNeasy kit (*Qiagen*), according to the manufacturer's instructions. Purified RNA was eluted into 50 µl RNase-free water. RNA yields and dye incorporation rates were measured by spectrophotometry. Purified RNA (50 µl containing about 2 µg RNA) was fragmented by incubating with 5.71 µl $ZnSO_4$ (100 mM) and 1.43 µl Tris.Cl (1 mM, pH 7.4) at 60 °C for 30 min. Fragmentation was stopped on ice by the addition of 1.43 µl EDTA (500 mM, pH 8.0) to the reaction. RNAsin (1 µl, 40 U μl^{-1}) was added again. Fragmented and labeled RNA targets were stored at -20 °C.

3.6. Design of probes and microarray manufacturing

Probes were designed using ARB software with its 16S rRNA database (ssu_jan04_corr_opt.arb) available at http://www.arb-home.de (Ludwig et al., 2004). The parameters of the Probe Design function were chosen according to Sanguin et al. (2006b), in particular all probes were designed to have a weighted mismatch (WMM) value below 2 with the targeted taxa and more than 2 with non-targets (The WMM value is computed based on the number, position and type of mismatch, and it is 0 in the absence of any mismatch). The probes were further tested *in silico* for the required probe/target melting temperature of 65 ±5 °C, Gibbs energy of 3' dimer formation above -5 kcal mol^{-1}, and absence of stable hairpin structures at 50 °C. The first two criteria were estimated using Oligo5 (*Molecular Biology Insights*, West Cascade, CO), by the nearest neighbor method in the case of the melting temperature, and the third criteria using the QuickFold server and (for probes forming a hairpin near 50 °C) also the DINAMelt server (both servers available at http://www.bioinfo.rpi.edu/applications/hybrid/ ; Markham and Zuker, 2005).

Probes were custom synthesized (*Invitrogen*, Cergy Pontoise, France) with a 5' NH_2-C6 group for covalent attachment onto aldehyde slides AL (*Schott Nexterion AG*, Mainz, Germany). They were resuspended in 3× SSC buffer (0.3 M sodium citrate, pH approx. 7.0, containing 3 M NaCl; *Sigma*, L'Isle d'Abeau, France) and 1.5 M betaine (*Sigma*) (Diehl et al., 2001) to a final concentration of 50 µM and spotted onto slides with a MicroGrid II spotter (*BioRobotics*, Cambridge, UK) at 50-55% relative humidity and 19 °C. After spotting, the slides were treated as described previously (Sanguin et al., 2006b). Each probe was repeated four times per slide.

3.7. Hybridization protocol

Two slides were hybridized per sample. Hybridization was carried out in a custom-tailored aluminum block used as an insert for a temperature-controlled Belly Dancer (*Stovall Life Sciences*, Greensboro, NC) set at maximum bending (Bodrossy et al., 2003). The hybridization block was preheated to 57 °C for at least 30 min to allow the temperature to stabilize. A Hybriwell sealing system (*Grace BioLabs*, Bend, OR) was applied onto the slides. Assembled slides were preheated on top of the hybridization block for at least 15 min. Hybridization mixture contained 400 ng labeled RNA, 1% SDS (*Sigma*), 1× Denhardt's reagent (*Sigma*), 6× SSC, and DEPC-treated water to reach 60 µl. Samples were preheated at 65°C for 5-10 min and applied onto assembled slides. Hybridization was conducted overnight

at 57 °C. The slides were then immersed into 2× SSC containing 0.03% SDS, and washed at room temperature by shaking successively for 5 min in fresh 2× SSC containing 0.03% SDS, 5 min in 0.2× SSC (twice) and 5 min in 0.1× SSC. Finally, the slides were dried by centrifugation.

3.8. Scanning and image analysis

The slides were scanned at 532 nm with a 10 µm resolution, using a GeneTac LS IV scanner (*GenomicSolutions*, Huntingdon, UK). Images were analyzed with the GenePix 4.01 software (*Axon*, Union City, CA). Spot quality was always visually checked, and spots of poor quality (presence of dust or saturation of the hybridization signal) were excluded from further analyses, as described previously (Sanguin et al., 2006b).

3.9. Filtration and normalization of microarray data

Data filtration was conducted with the R 2.2.0 statistical computing environment (http://www.r-project.org). Hybridization of a given spot was considered positive when 80% of the spot pixels had intensity higher than the median local background pixel intensity plus twice the standard deviation of the local background. The intensity signals (median of signal minus background) were replaced by their square root value and the intensity of each spot was then expressed as a fraction of the total intensity signal of the basic pattern it belongs to (Sanguin et al., 2006a). Finally, a given feature probe was considered as truly hybridized when (i) hybridization signals were superior to the mean signal of the negative controls and (ii) at least 3 of 4 replicate spots displayed positive hybridization.

3.10. Microarray validation by cloning sequencing

Validation of microarray data was sought by cloning sequencing for selected bacterial taxa (**Table 2**), after amplification with specific primer pairs. If possible, a probe already designed was used as a specific primer (with if necessary a modification in 3' to improve primer specificity). Otherwise, new primers were designed with ARB software (Ludwig et al., 2004). For each PCR reaction, 20 ng of purified 16S rRNA PCR product (see section 3.4) obtained with one non-inoculated plant from soil MC112 or one non-inoculated plant from

soil MS8 were used as a template. Otherwise, the PCR conditions were the same as in section 3.4. Annealing temperature for each primer pair is indicated in **Table 2**. PCR products were purified with MinElute PCR purification kit (*Qiagen*, Courtaboeuf, France) according to the manufacturer's instructions.

Purified PCR products obtained with specific primer pairs were cloned into the plasmid vector pGEM-T (*pGEMs-T Easy Vector System kit*; Promega, Charbonnieres, France) according to manufacturer's protocol. Five to six clones were sequenced on both strands (*Genome Express*, Meylan, France). Sequences were checked and edited with BioEdit version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Chimeric 16S rRNA gene sequences were identified using the chimera detection program Pintail version 0.33 (http://www.cardiff.ac.uk/biosi/research/biosoft/Pintail/pintail.html), and five putative chimeric clone were discarded. Sequence affiliation of non-chimeric sequences was performed using algorithm BlastN with default parameters at NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3.11. Statistical analysis

Analysis of variance was conducted with S-PLUS 6.1 (*Insightful Corp*, Seattle, WA). Multivariate analysis, specifically principal component analysis (PCA) and between-class analysis were done with ADE-4 (Thioulouse et al., 1997) in R environment (http://www.r-project.org).

Table 1. Soil composition

Soil	Geological origin	Texture	Clay (%)	Silt (%)	Sand (%)	pH [water]	Organic matter (%)	$CaCO_3$ total (%)	Fe [$C_2H_4O_2$ 1:10] (mg kg^{-1})
Morens									
MS8	morainic	sandy loam	10.7	29.6	59.7	7.8	1.3	6.0	230
MC112	sandstone	sandy loam	16.1	29.9	54.0	6.8	2.2	0.0	251
MS16	morainic	sandy loam	12.2	28.9	58.9	6.6	1.6	0.0	293
MC10	sandstone	sandy loam	15.8	30.0	54.3	7.2	2.1	1.0	487
Savoie									
F1	morainic	loam	20.1	39.8	40.0	7.4	3.7	0.7	46
F2	sandstone	sandy loam	9.0	19.7	71.3	8.1	2.1	4.1	60
F4	morainic	clay loam	28.2	33.5	38.4	8.1	2.6	19.5	11
F5	sandstone	sandy loam	17.8	25.9	56.3	7.9	2.2	2.8	32
Nyíregyháza									
H1	not determined	sandy loam	15.3	26.8	57.8	6.9	1.0	0.6	122
H2	not determined	loamy sand	7.7	17.2	75.2	6.6	0.9	0.0	99
H3	not determined	sand	4.1	5.6	90.4	5.3	0.6	0.0	162
H4	not determined	loamy sand	6.9	7.0	86.1	5.5	0.7	0.0	76

Table 2. Specific primers

Target	Forward primer name	Corresponding probe	Primer sequence (5'-3')	Reverse primer name	Corresponding probe	Primer sequence (5'-3')	Annealing temperature (°C)
Azospirillum	**Azo1f**	**Azo1**	ACGATGATGACGGTAGCGTG	**AzoAr**	-	CACCGAAGTGCATGCACCCC	59
Bradyrhizobium	**Brady6Af**	**Brady6A**	TAGCAATACGTCAGCGGCAG	**BradyAr**	-	GCCACTAGTGAGTAAACCCA	55
Sphingomonadaceae	**Sphingo5Af**	**Sphingo5A**	GCTCTTTTACCAGGGATGAT	**Sphingo4r**	**Sphingo4**	CGGACAGCTAGTTATCATCG	52
Burkholderia	**Burkho4DMf**	modified **Burkho4D**	AACTGCATTTGTGACTGGC	**Burkho3r**	**Burkho3**	CCGTGGTGACCGTCCTCCTT	57
Herbaspirillum	**Herb1f**	**Herb1**	AACGGCAGCATAGGAGCTTG	**Hefri2r**	**Hefri2**	AGCCAAAACCGTTTCTTCCC	60
Nitrosospira	**Nitro1BMf**	modified **Nitro1B**	TCAGCCGGAACGAAACGGT	**NitroIr**	-	GCGGGCTTCGGTACATGTCA	62
Comamonadaceae	**Comtes2f**	**Comtes2**	TACTAGAGCGGCTGATGGCA	**Conide4r**	**Conide4**	GTTTCTAGCCCCACCTATAA	56
Mycobacterium	**MycobaBf**	-	TAGGTGGTTTGTCGCGTTGT	**Mycoba2Mr**	modified **Mycoba2**	ACGGCACGGATCCCAAGGA	55

4. RESULTS

4.1. Microarray development

In this project, about 500 probes targeting important soil bacteria from α-, β-, γ-, δ-*Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* were designed. These probes were spotted together with the probes of Sanguin et al. (2006a, 2006b, 2008 and unpublished data). The current probe set hence comprises more than 1000 probes (**Table 3**). The well-covered groups are α-, β-, γ-*Proteobacteria*, *Firmicutes* and *Actinobacteria*. Within these groups, probes at different taxonomical levels, e.g. family, genus, species were designed where possible. Other less important groups are targeted usually by one or only few probes.

The probes are 18-mer to 24-mer oligonucleotides, the majority of probes being 20-mers, with a G+C content between 45 and 70% (average 54%). The melting temperature (Tm) of probes is between 50.6 and 77.7°C, and 79% of probes have the desired Tm i.e. between 60 and 70°C (Sanguin et al., 2006b). Only probes that do not form 3’ dimers or stable secondary structures at hybridization temperature were kept in the probe set.

Table 3. Number of probes designed for different bacterial groups
(OP2 and OP11 comprise only uncultivated bacteria)

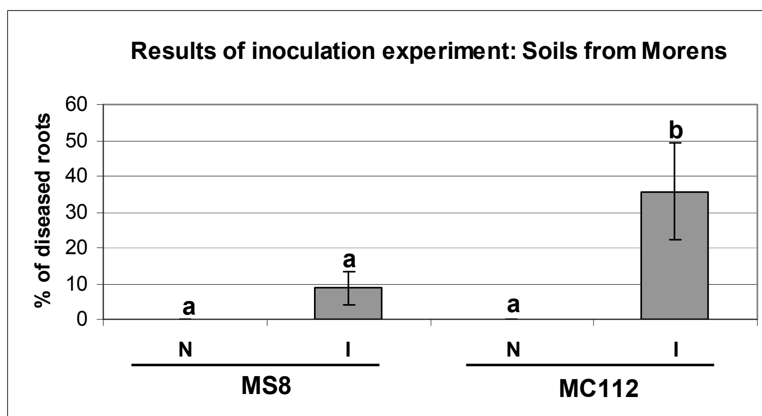
Bacterial (sub)division	**Number of probes**
Alpha-Proteobacteria	153
Beta-Proteobacteria	134
Gamma-Proteobacteria	203
Delta-Proteobacteria (*Myxobacteria*)	77
Epsilon-Proteobacteria	24
Cyanobacteria	28
Chlorobi	1
Firmicutes	209
Planctomycetes	13
Verrucomicrobia	2
Bacteroidetes	42
Fusobacteria	1
Actinobacteria	170
Nitrospira	5
Deinococcus-Thermus	1
Thermotogae	2
Thermodesulfobacteria	1
Fibrobacteres	1
Acidobacteria	9
Chloroflexi	1
Chlamydiae	1
OP11	5
OP2	1
Deferribacteres	1
Total	**1129**

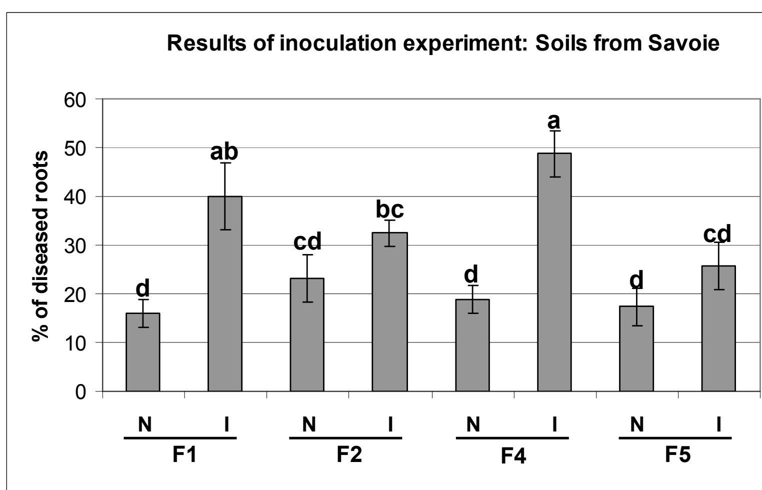
4.2. Microarray analysis of bacterial communities is soils suppressive and conducive to black root rot of tobacco

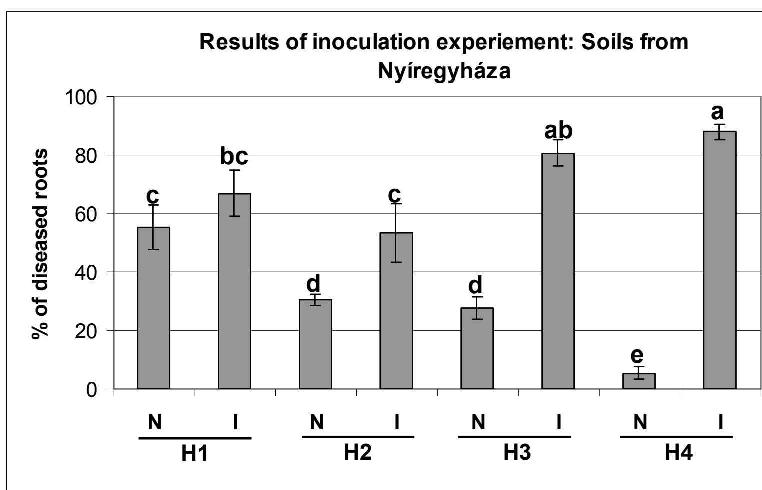
4.2.1. Indicators of black root rot suppressiveness under standardized conditions

The level of disease suppressiveness of each soil was assessed in a standardized greenhouse experiment (Ramette et al., 2003). The level of disease, expressed as the % of diseased roots, for inoculated and non-inoculated tobacco plants is shown in **Fig. 4**. Suppressive soils are characterized by (i) low level of disease even under high pressure of indigenous *T. basicola* (non-inoculated controls); (ii) limited difference between *T. basicola* inoculated samples and non-inoculated controls. On this basis, three soils were suppressive in this study: MS8 (from Morens), F2 (from Albens) and F5 (from Seyssel). Since no soil from Nyíregyháza was really disease suppressive, only non-inoculated plants from soils H1 and H4 were further analyzed as representatives of naturally infested and non-infested conducive soils, respectively.

Fig. 4. Results of greenhouse inoculation experiment. Bars represent mean % of roots with black lesions (n = 8). Standard errors are shown as error bars. Soil treatments labeled with the same letter were not significantly different (at $P < 0.05$) based on analysis of variance and Fisher post-hoc test. Treatment: N – non inoculated with *T. basicola*; I – inoculated with *T. basicola*.







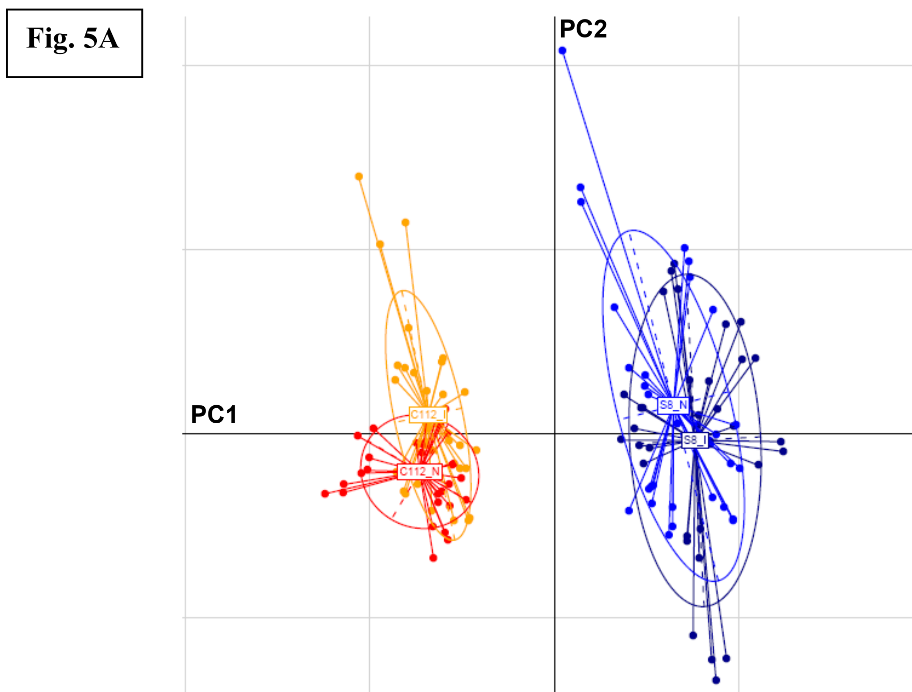
Bacterial communities present in the rhizosphere of tobacco inoculated or not with *T. basicola* were compared with microarray. About 250-300 probes were positive with each soil. **Fig. 5A** shows the results of principal component analysis (PCA) performed on microarray results obtained with a suppressive (MS8) and conducive (MC112) soil from Morens. There was a clear difference between the two soils, as they were separated by the first principal component (axis PC1). There was no difference in rhizobacterial communities between inoculated and non-inoculated tobacco plants. The probes and the corresponding bacterial taxa discriminating between the suppressive and conducive soils are marked in **Fig. 5B**. To confirm the presence of bacterial taxa indicated by the discriminant probes, some suitable probes were used as primers to amplify the corresponding bacterial groups and the PCR products were cloned and sequenced. Five to six clones from each PCR product were sequenced. The results of cloning/sequencing are shown in **Table 4**. Presence of *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Nitrosospira*/*Nitrosovibrio*, *Mycobacterium* and taxa corresponding to *Sphingomonadaceae* and *Comamonadaceae* families was confirmed in both MS8 and MC112 soils. Presence of *Herbaspirillum* was confirmed only in soil MS8, while in soil MC112, a clone of its close relative *Janthinobacterium* was found. With the exception of *Herbaspirillum* PCR product, the majority of clones were affiliated to the targeted group, indicating a good probe specificity.

When comparing the soils from Savoie (i.e. Seyssel and Albens), Nyíregyháza and Morens together, the major differences (assessed by PCA) were attributed to soil geographical origin, as shown in **Fig. 6**. To maximize the differences between suppressive soils (MS8, F2 and F5) and conducive soils (MC112, F1, F4 and H4; soil H1 was left apart), between-class analysis was performed on microarray results obtained with those soils. The suppressive and conducive soils were quite well separated along the second axis (**Fig. 6A**). The differences between defined soil groups appeared to be highly significant ($P < 0.001$) in a randomization test (not shown). The probes and the corresponding bacterial taxa discriminating between the suppressive and conducive soils are marked in **Fig. 6B**.

Fig. 5. Morens soils: Comparison of inoculated (I) and non-inoculated (N) rhizosphere samples of tobacco cultivated in soils MS8 (= S8; Suppressive) and MC112 (= C112; Conducive) under greenhouse conditions.

A. Sample separation between the first (explains 20% of variability among samples) and the second (explains 11% of variability among samples) principal components (PC), as determined by principal component analysis performed on microarray results.

B. Probes and corresponding bacterial taxa discriminating between MS8 (Suppressive) and MC112 (Conducive) soils. Taxa comprising plant-beneficial strains are indicated in yellow boxes.



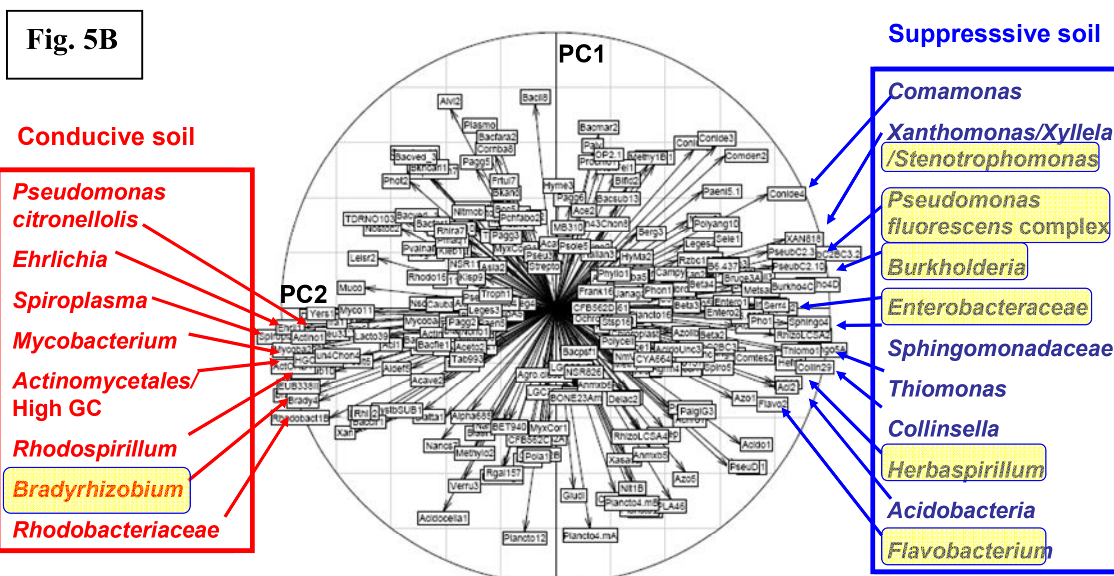


Table 4. Results of cloning/sequencing from chosen specific primers. Clones corresponding to targets are in grey, non-target clones in white.

Target (length)	Clone # and identification (The most similar cultivable strain with % of identity)	
	Conducive soil MC112	**Suppressive soil MS8**
Alphaproteobacteria		
***Azospirillum* and relatives** *(Rhodospirillacea)* (382 bp)	MC112-1: *Azospirillum* sp. B510 (97%) MC112-2: *Azospirillum* sp. AP-500 (97%) MC112-3: *Skermanella* sp. 5416T-32 MC112-4: *Hyphomicrobium* sp. W1-1B (96%) MC112-5: *Sphingomonas* sp. M30-VN10-1W (98%)	MS8-1: *Azospirillum lipoferum* B2 (99%) MS8-2: *Azospirillum lipoferum* B2 (100%) MS8-3: *Roseomonas* gsp. 6 ATCC 49961 (98%) MS8-4: *Roseomonas* gsp. 6 ATCC 49962 (100%) MS8-5: idem MS8-4
Sphingomonadaceae *(Sphingomonadales)* (380 bp)	MC112-6: *Novosphingobium* sp. K16 (99%) MC112-7: *Novosphingobium pentaromativorans* 17-34 (99%) MC112-8: *Spingobium yanoikuyae* BF-18 (99%) MC112-9: *Sphingomonas* sp. LBS8 (98%) MC112-10: *Sphingomonas* sp. Y57 (97%) MC112-11: *Kaistobacter* sp. P-55 (98%)	MS8-6: *Sphingomonas suberifaciens* IFO 15211 (99%) MS8-7: idem MS8-6 MS8-8: idem MS8-6 MS8-9: *Sphingomonas suberifaciens* IFO 15211 (99%) MS8-10: *Sphingomonas* sp. KAR7 (97%) MS8-11: *Kaistobacter* sp. P-55 (98%)
Betaproteobacteria		
Burkholderia *(Burkholderiaceae)* (843 bp)	MC112-12: *Burkholderia phenazinium* HG 8 (99%) MC112-13: *Burkholderia phenazinium* HG 8 (99%) MC112-14: *Burkholderia* sp. CCBAU 11189 (100%) MC112-15: idem MC112-8 MC112-16: *Burkholderia* sp. S2.1 (99%)	MS8-12: *Burkholderia oklahomensis* E0147 (97%) MS8-13: idem MS8-4 MS8-14: *Burkholderia oklahomensis* E0147 (97%) MS8-15: *Burkholderia glathei* LMG 14190T (97%)
Bradyrhizobium *(Bradyrhizobiaceae)* (729 bp)	MC112-17: *Bradyrhizobium* sp. KO14 (99%) MC112-18: *Bradyrhizobium* sp. KO14 (99%) MC112-19: *Bradyrhizobium* sp. P-52 (99%) MC112-20: *Bradyrhizobium canariense* MCLA23 (100%) MC112-21: *Bradyrhizobium canariense* MCLA23 (99%)	MS8-17: *Bradyrhizobium canariense* MCLA23 (99%) MS8-18: *Bradyrhizobium canariense* MCLA23 (99%)
Comamonadaceae *(Burkholderiales)* (314 bp)	MC112-22: *Variovorax paradoxus* A25 (96%) MC112-23: *Variovorax* sp. P7G10 (96%) MC112-24: *Rhodopherax* sp. Man02 (97%) MC112-25: *Hydrogenophaga carboriundus* KRH YZ (92%) MC112-26: *Xenophilus aerolata* 5516S-2 (97%)	MS8-22: *Aquabacterium* sp. P-113 (97%) MS8-23: *Ramlibacter* sp. P-8 (97%) MS8-24: *Ramlibacter henchirensis* TMB834 (97%) MS8-25: *Phenylobacterium* sp. G26 (93%)

Herbaspirillum *(Oxalobacteraceae)* (342 bp)	MC112-27: *Janthinobacterium agaricidamnosum* NCPP 39455 (98%) MC112-28: idem MC112-22 MC112-29: *Azospira oryzae* N1 (92%) MC112-30: *Lysobacter* sp. 06-03 (97%) MC112-31: *Lysobacter* sp. 06-03 (95%)	MS8-27: *Herbaspirillum frisingense* CAF265 (95%) MS8-28: *Polynucleobacter* sp. MWH-Braz-FAM3C (92%) MS8-29: *Thiobacillus denitrificans* ATCC 25259 (93%) MS8-30: *Lysobacter* sp. 06-03 (97%)
Nitrosospira and Nitrosovibrio *Nitrosomonadaceae* (280 bp)	MC112-32: *Nitrosospira* sp. ENWyke8 (100%) MC112-33: idem MC112-32 MC112-34: *Nitrosospira* sp. ENWyke8 (99%) MC112-35: *Nitrosospira* sp. Nsp41 (99%) MC112-36: *Nitrosovibrio tenuis* Nv1 (99%)	MS8-32: idem MC112-32 MS8-33: idem MC112-32 MS8-34: idem MC112-34 MS8-35: *Nitrosopira briensis* Nsp10 (99%) MS8-36: idem MC112-36
Actinomycetes		
Mycobacterium *(Mycobacteriaceae)* (280 bp)	MC112-37: *Mycobacterium* sp. CRIB-63 (100%) MC112-38: idem MC112-37 MC112-39: *Mycobacterium* sp. JLS (100%) MC112-40: *Mycobacterium brasiliensis* Rio559.03 (100%) MC112-41: *Mycobacterium* sp. MCRO 7 (100%)	MS8-37: *Mycobacterium* sp. CRIB-63 (99%) MS8-38: *Mycobacterium* sp. czh-132 (99%) MS8-39: *Mycobacterium* sp. czh-132 (100%) MS8-40: *Mycobacterium* sp. KNUC92 (99%) MS8-41: *Mycobacterium* sp. KNUC92 (99%)

Fig. 6. Comparison of inoculated (I) and non-inoculated (N) rhizosphere samples of tobacco cultivated in soils MS8 (= S8), MC112 (= C112; Morens), F1, F2 (Albens), F4, F5 (Seyssel), H1 and H2 (Nyíregyháza) under greenhouse conditions. Sample separation between the first (explains 22% of variability among samples) and the second (explains 10% of variability among samples) principal components (PC), as determined by principal component analysis performed on microarray results.

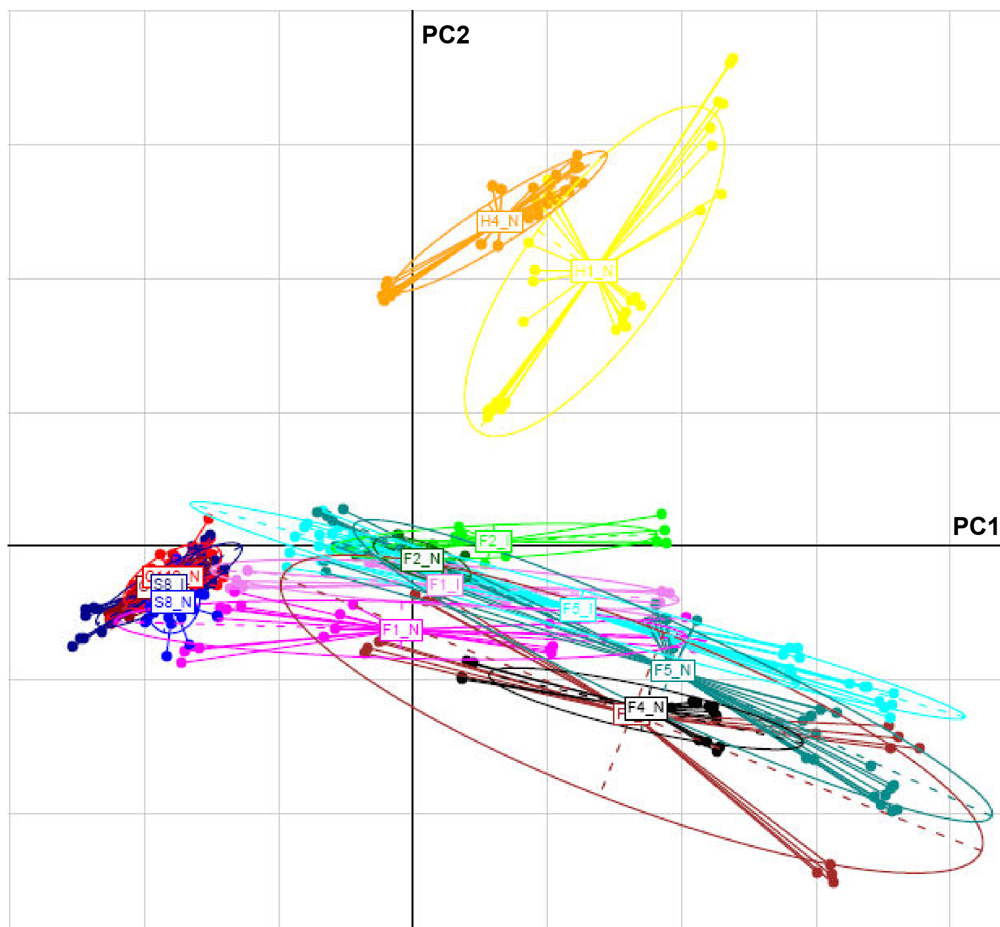


Fig. 7. Comparison of suppressive and conducive soils from different geographical regions (greenhouse samples).
A. Between-Class analysis performed on microarray results obtained with suppressive soils (MS8, F2 and F5), conducive soils (MC112, F1, F4 and H4), and soil H1.
B. Probes and corresponding bacterial taxa discriminating between suppressive and conducive soils.

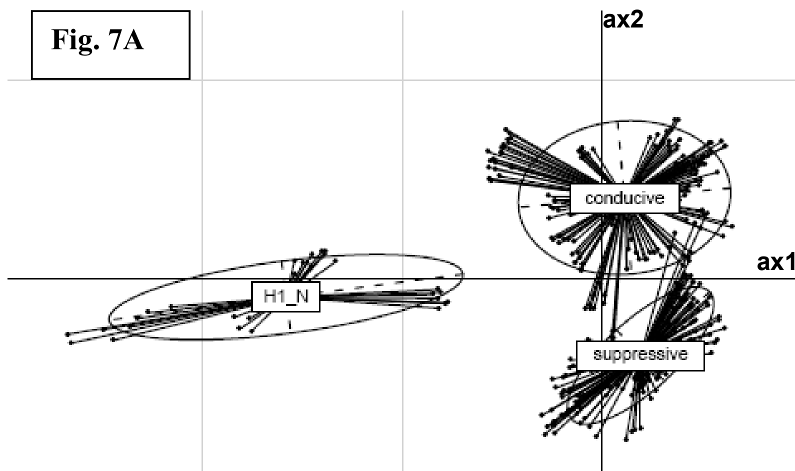


Fig. 7B

ax2

ax1

Conducive:
Ehrlichia, Spiroplasma
Rhodobacteraceae
Rhodospirillum
Rikenella
Bacillus funiculus
Pseudomonas citronellolis
Bradyrhizobium
Mycobacterium

Suppressive:
Flavobacterium
Geothrix/Holophaga
Collinsella
Bacillus simplex
Gluconacetobacter
Phyllobacterium
Acidovorax
Rhizobiaceae
Pseudomonas fluorescens

4.2.2. Indicators of black root rot suppressiveness in fields in Morens

In June 2004 and 2006, rhizosphere soil from tobacco or wheat plants actually present in four fields in Morens (MS8, MS16, MC10 and MC112) were collected and samples were analyzed with microarray in the same way as greenhouse samples. Suppressiveness of each soil, i.e. suppressive (MS8), moderately suppressive (MS16), moderately conducive (MC10) and conducive (MC112) has been determined by Frapolli (2007, PhD thesis) in 2004. Suppressiveness status of soil MS8 and MC112 was confirmed in 2006 (**Fig. 4**). The fields belong to different farmers and the information on previous crops, sampled cultivar, tillage, fertilizer inputs and chemical treatments were not available.

The soil itself had often a lower impact than other factors on plant rhizobacterial community, as determined by principal component analysis performed on field samples alone (not shown), or on both field and greenhouse samples (**Fig. 8**) from Morens. The factors of higher importance than soil type were (i) greenhouse versus field cultivation, (ii) year of sampling, and (iii) plant type (tobacco versus wheat). In addition, the between-plant variability for field samples was very large in comparison with greenhouse samples.

To maximize the differences between the soils in spite of the above mentioned differences found within soils, between-class analysis was performed on microarray results. Grouping of field samples was defined as follows: suppressive (soil MS8, tobacco sampled in 2004 and 2006, wheat sampled in 2006), moderately suppressive (soil MS16, wheat sampled in 2006), moderately conducive (MC10, tobacco sampled in 2004 and 2006, wheat sampled in 2006), and conducive (MC112, wheat sampled in 2006). The best separation of soil groups was obtained along the combination of axes 1 and 3 (P value < 0.05 – significant; **Fig. 9A**), the centers of gravity for both conducive soils and moderately suppressive/moderately conducive soils being separated from suppressive soils by the first axis. Samples from both intermediate soils were spread among those from conducive and suppressive soils, while samples from conducive and suppressive soils were not mixed (**Fig. 9A** and **B**). The probes and the corresponding bacterial taxa discriminating between field samples from suppressive and conducive soils are marked in **Fig. 9C.**

Fig. 8. Morens soils: Comparison of greenhouse and field samples by principal component analysis. Sample separation between the first (explains 17% of variability among samples) and the third (explains 8% of variability among samples) principal components (PC), as determined by principal component analysis performed on microarray results. Only non-inoculated plants from greenhouse experiment were included in the analysis. Outliers were excluded. Legend (number of plants analyzed is indicated within brackets):
S8T_greenhouse_06: tobacco plants grown in greenhouse in soil MS8 sampled in June 2006 (4)
S8T_06: tobacco plants sampled in field MS8 sampled in June 2006 (3)
S8T_04: tobacco plants sampled in field MS8 sampled in June 2004 (3)
S16W_06: wheat plants sampled in field MS16 sampled in June 2006 (4)
C10T_06: tobacco plants sampled in field MC10 sampled in June 2006 (3)
C10T_04: tobacco plants sampled in field MC10 sampled in June 2004 (3)
C10W_06: wheat plants sampled in field MC10 sampled in June 2006 (4)
C112_06: wheat plants sampled in field MC112 sampled in June 2006 (4)
C112T_greenhouse_06: tobacco plants grown in greenhouse in soil MC112 sampled in June 2006 (4)

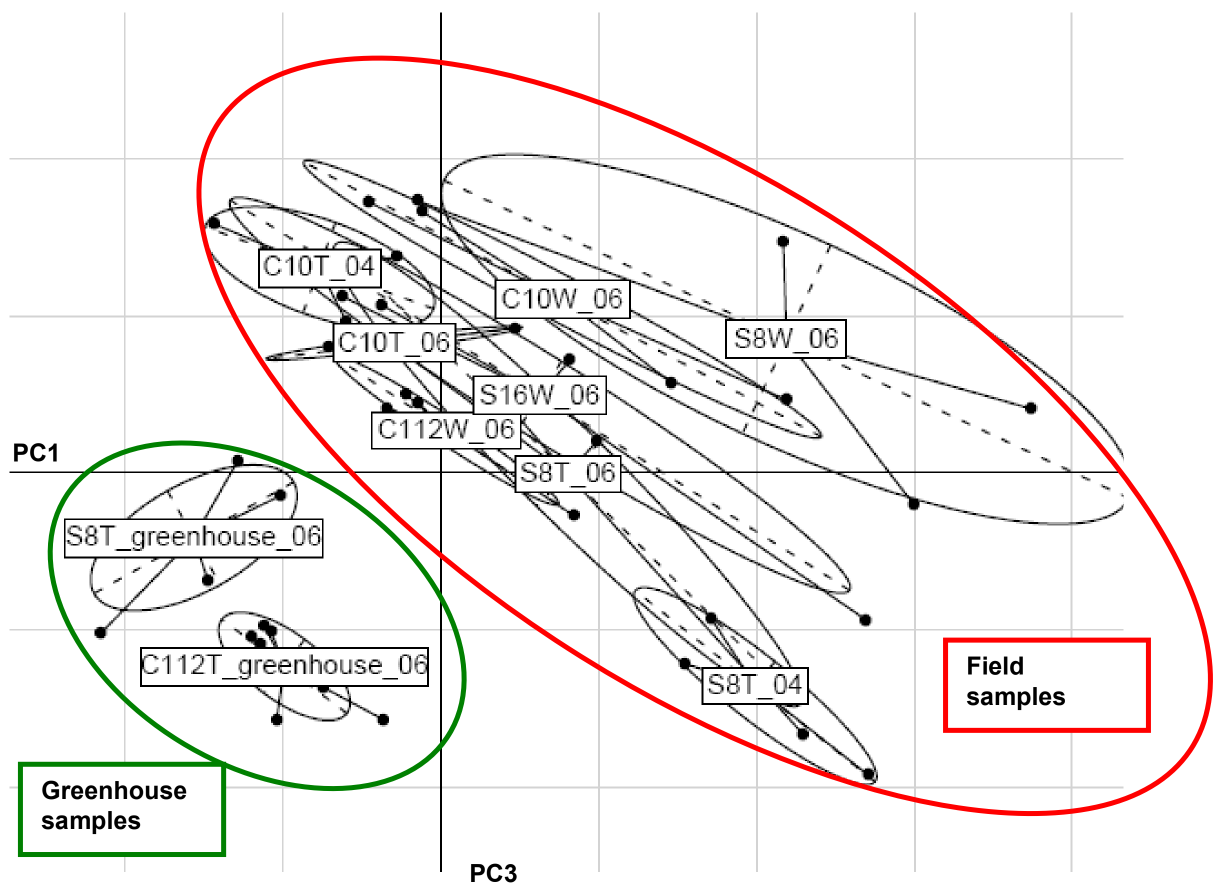
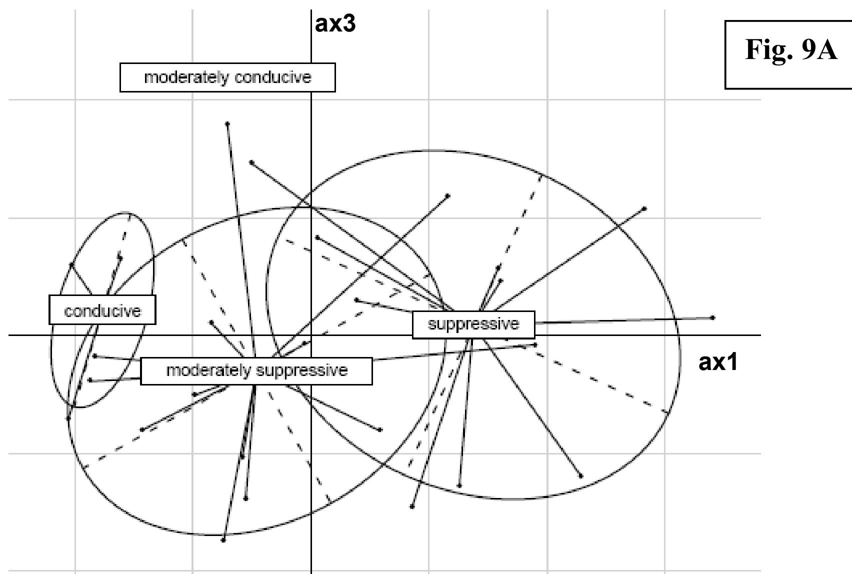
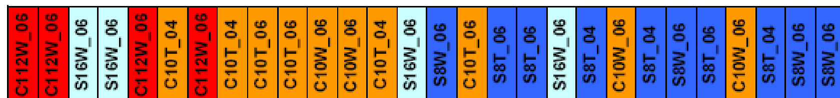
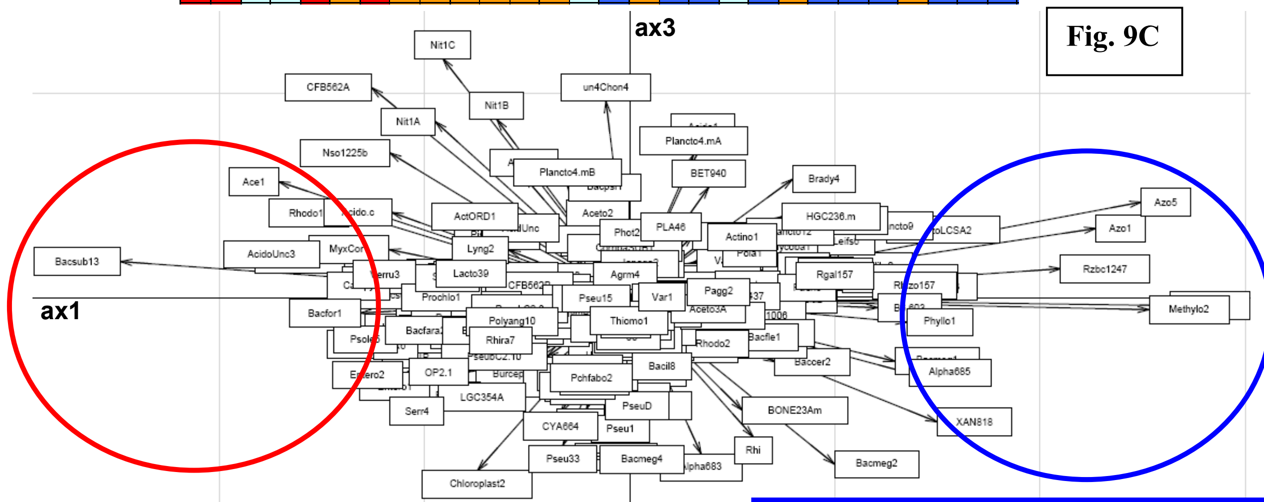


Fig. 9. Morens soils: Comparison of field samples.
A. Between-Class analysis performed on microarray results obtained with suppressive (MS8), moderately suppressive (MS16), moderately conducive (MC10) and conducive (MC112) soil.
B. Position of samples along the first axis. For legend, see **Fig. 8**.
C. Probes and corresponding bacterial taxa discriminating between suppressive and conducive soils.







Conducive fields:

Bacilllus subtilis/licheniformis
Bacillus funiculus
uncultured *Acidobacteria*
Rhodothermus
Campylobacter

Suppressive fields:

Methylobacterium
Geothrix/Holophaga
Azospirillum
Rhizobiaceae
Xanthomonas/Xylella/Stenotrophomonas
Ochrobactrum
Phyllobacterium

5. DISCUSSION

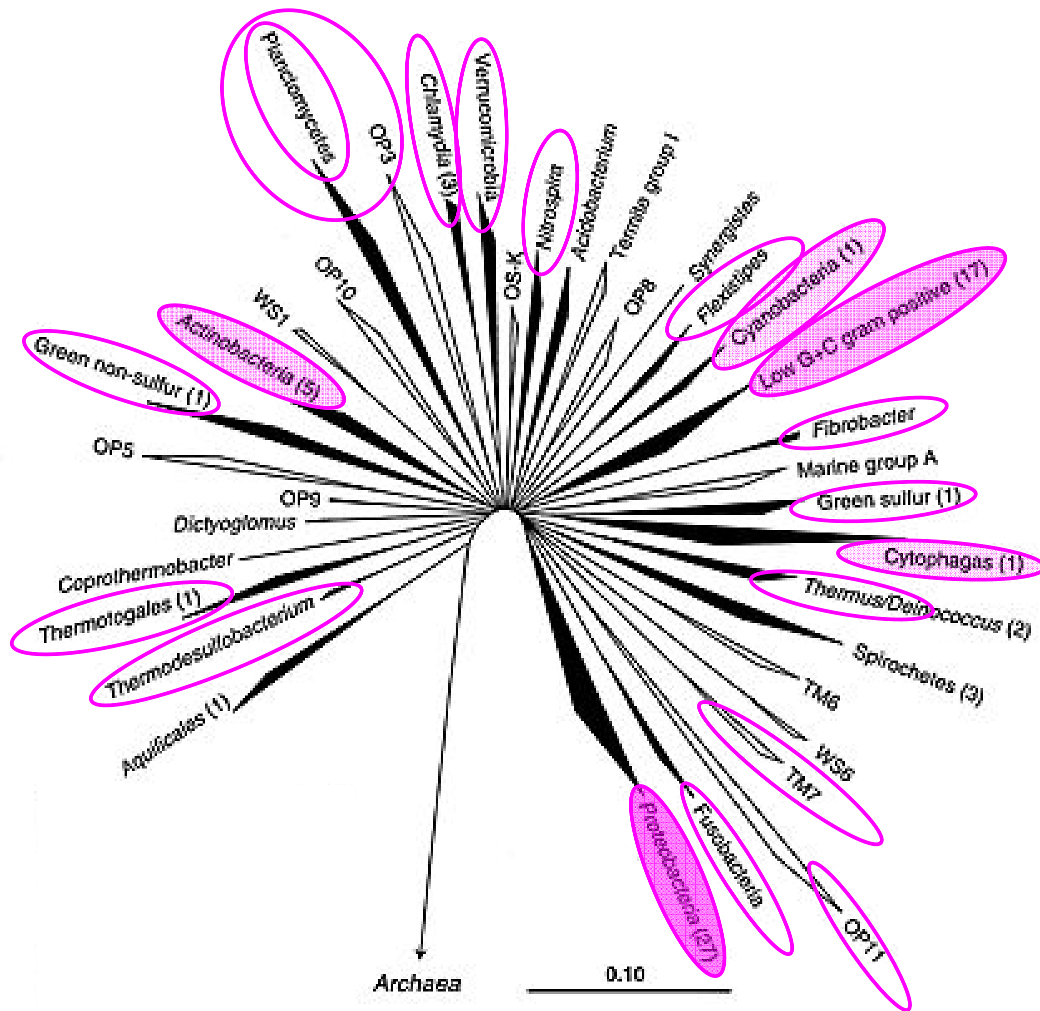
5.1. Microarray development

The number of probes already designed by Sanguin et al. (2006a, 2006b, 2008) for a prototype taxonomic microarray was insufficient for soil bacterial community comparisons, and it was also too focused on α-*Proteobacteria* and *Pseudomonas* (within the γ-*Proteobacteria*). Therefore, the number of probes has been doubled and other important soil bacteria have been covered in this project. We have particularly focused on β- and γ-*Proteobacteria* that comprise many plant-beneficial bacteria with both phytoprotection and biofertilizer properties. Further, we have focused on antibiotic-producing *Firmicutes*, *Actinomycetes* and *Myxobacteria* (δ-*Proteobacteria*), because they comprise potential antagonists of plant pathogens. The probes for antibiotic producers were designed in collaboration with M. Marečková and J. Kopecký (Dept. of Plant Pathology, Crop Research Institute, Praha-Ruzyně, Czech Republic) who have experience of antibiotic production by bacteria. All probes were designed according to Sanguin et al. (2006b), so the probes have the same hybridization properties and may be used together in one experiment.

In summary, the microarray covers nearly all known bacterial divisions at least with one probe (**Fig. 10**). Groups comprising important soil bacteria are covered by tens to hundreds of probes at different taxonomic levels. Most probes target bacterial genera (e.g. *Bacillus*) or groups of species (e.g. *Bacillus subtilis* and *Bacillus licheniformis*). It is sometimes difficult to distinguish two bacterial species by a 16S rRNA probe, so rather probes for groups of closely-related species were designed in that case. In addition, some probes target higher taxonomic levels like family (e.g. *Sphingomonadaceae*). These high taxonomic level probes are particularly important for detection of unknown bacterial species, for which species-specific probes are missing.

Taxonomic probes are usually validated by hybridization with pure strain DNA (Loy et al., 2002; Bodrossy et al., 2003; Günther et al., 2006). By this approach, the % of false-positive and false-negative probes is determined. Within the current probe set, several subsets of probes have been validated by this approach by Sanguin et al. (2006a, 2006b, 2008 and unpublished). In this project, the actinomycete probe subset has been validated carefully (Kyselková et al., accepted for publication; see Appendix). The rest is being validated at this moment.

Fig. 10. Coverage of bacterial divisions with microarray probes. Circle around division name indicates that it is covered by a least one probe. Intensity of circle coloration corresponds to number of probes designed for that division.



5.2. Indicators of black root rot suppressiveness

To find bacterial taxa that correlate with black root rot suppressiveness, rhizobacterial communities in suppressive and conducive soils were compared with microrarray. First, the level of suppressiveness had to be assessed for each soil in a standardized inoculation experiment. It is important to repeat the experiment even with the reference soils because the level of suppressiveness may vary between seasons (Ramette et al., 2003). Second, rhizobacterial communities from suppressive and conducive soils were analyzed by microarray. Finally, multivariate analysis of microarray data was performed to find bacterial taxa that correlated with suppressive vs. conducive soils.

The multivariate analyses were either principal component analysis (PCA) or between-class analysis. PCA transforms huge number of variables (in our case, about 300 probes per analysis) in a smaller number of virtual variables (so called principal components) which correlate with the real variables (probes). The principal components are defined so that the first principal component explains the majority of variance between samples, the second principal component explains most of the variance that remains unexplained by the first component etc. The principal components are thus uncorrelated and orthogonal. Therefore, each principal component may be shown as an axis and the positions of samples or probes may be projected between the axes. Euclidean distance between samples indicates their similarity. Cosinus of angle between probes or probes and axes is the correlation (it means: small angle, strong correlation), the length of probe arrow indicates the variance of the probe. Generally, only two or three principal components are used to create two (or three) dimensional images with sample/probe projections. The images are usually very clear and intuitive. The inconvenience of this method is however the lost of some variability (as less strong principal components are omitted). In comparison with PCA, the between-class analysis maximalizes the differences between predefined groups – it means that the analysis includes a hypothesis given a priori (while with PCA, we find some groups of samples and these groups may be a posteriori attributed to some hypothesis). Similarly to PCA, samples or probes may be projected according to the axes. In addition, a randomization test may be performed to assess the significance of the differences found between predefined groups.

The results of greenhouse inoculation experiment confirmed the suppressivity status of the two soils from Morens, i.e. MC112 conducive and MS8 suppressive. When compared with microarray, there was no difference between inoculated and non-inoculated tobacco

samples (**Fig. 5A**). This was surprising, because the damaged roots release more exudates than the healthy roots. The lack of difference was not due either to a problem of detection limit, as the detection threshold of the microarray approach is good (as little as 0.03% of a target may be detected) (Sanguin et al., 2006a). Differences could perhaps be found after longer cultivation. There was however a clear difference in rhizobacterial communities between the two soils. The suppressive soil was dominated by *Herbaspirillum*, *Burkholderia*, *Comamonas*, *Thiomonas* (β-*Proteobacteria*), *Enterobacteriaceae*, *Xanthomonas*/*Xylella*/*Stenotrophomonas*, *Pseudomonas fluorescens* (γ-*Proteobacteria*), *Sphingomonadaceae*, *Azospirillum* (α-*Proteobacteria*), *Flavobacterium* (*Capnocytophaga*/*Bacteroidetes*), *Geothrix*/*Holophaga* (*Acidobacteria*) and others (**Fig. 5B**). In conducive soil, *Actinobacteria*, especially *Mycobacterium*, α-*Proteobacteria* (*Bradyrhizobium*, *Rhodospirillum*, *Rhodobacteriaceae*) and *Pseudomonas citronellolis* (γ-*Proteobacteria*) were prevalent. Besides *Pseudomonas fluorescens*, many others prevalent in suppressive soil are known to include biocontrol bacteria (**Fig. 5B** in yellow). For example, certain *Burkholderia* strains produces various antibiotics, some of the latter being the same as those produced by *Pseudomonas* (Cartwright et al., 1995), and they contribute to antagonism towards *Rhizoctonia solani*; *Stenotrophomonas* strains producing chitinases are antagonistic to *Magnaporthe* (Kobayashi et al., 1995). Interestingly, the only plant-beneficial bacteria that strongly correlated with the conducive soil, *Bradyrhizobium*, was shown to be little compatible with *Pseudomonas* in the rhizospere (Siddiqui and Shaukat, 2002).

To prove the presence of bacterial taxa indicated by probes, we used some suitable probes as PCR primers and we cloned and sequenced the PCR products (**Table 4**). We found that *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Nitrosospira*/*Nitrosovibrio*, *Mycobacterium* and some *Sphingomonadaceae* and *Comamonadaceae* were present in those soils. Presence of *Herbaspirillum* was confirmed only in soil MS8. In a majority of cases, the sequences obtained corresponded to probe targets, which confirms probe specificity. The sequence results however do not indicate the quantity of present targets, for this purpose quantitative PCR should be performed. In conclusion, the differences between conducive and suppressive soils from Morens were not restricted to fluorescent pseudomonads, but many other taxa of plant-beneficial bacteria correlated with suppressive soils. These findings were encouraging but they were based only on comparison of two soils, so generalization was needed.

To find some bacterial taxa that would correlate with black root rot suppressive soils in general, more soils had to be analyzed. We therefore sampled more soils from different

geographical regions that could be suppressive and conducive to black root rot. The prediction was based either on geological origin of soil (the case of soils from Savoie region in France) or on farmer experience (the case of soils from Nyíregyháza, Hungary). For soils from Savoie, soils F1 and F4 were expected to be suppressive as they were of morainic origin while F2 and F5 were expected to be conducive as they were on sandstone bedrock. Surprisingly, the result was opposite (**Fig. 4**). In spite of the findings of Stutz et al. (1986 and 1989), the geological origin of soil was not a good indicator of soil suppressiveness at a larger scale. Unfortunately, no soil from Hungary was suppressive despite the fact that the farmers had never problem with black root rot of tobacco in fields H2 and H4. Tobacco in both soils turned diseased when inoculated with *T. basicola* under standardized conditions. So the field conditions did not permit the pathogen to cause serious damage, but the soils were not really suppressive. Disease level of inoculated tobacco in soil H1 did not differ significantly from non-inoculated control. However, the level of disease in control samples was already very high and this soil could not be considered as suppressive. Therefore, only non-inoculated tobacco samples from soils H1 and H4 were analyzed as representatives of naturally infested and not infested (but conducive) soils. PCA performed on microarray results obtained with all greenhouse tobacco samples revealed that the maximum variability between samples was explained by soil geographical origin (as the soils from the same area grouped together), soils from Morens and Savoie being closer to each other (**Fig. 6**). Between-class analysis further revealed the differences between suppressive and conducive soils despite their geographical origin (**Fig. 7**). In this case, *Pseudomonas fluorescens*, *Flavobacterium*, *Geothrix*/*Holophaga*, *Collinsela* (*Actinobacteria*), *Phyllobacterium* (α-*Proteobacteria*), *Rhizobiaceae* (α-*Proteobacteria*), *Gluconacetobacter* (α-*Proteobacteria*), *Variovorax*/*Acidovorax* (β-*Proteobacteria*) and *Bacillus simplex* (*Firmicutes*) were prevalent in suppressive soils. All of them with the exception of *Bacillus simplex* were correlated with the reference soil MS8 (**Fig. 5B**) and many of them are plant-beneficial bacteria. For example, *Gluconacetobacter* and *Phyllobacterium* are non-leguminous nitrogen fixers (Bally and Elmerich, 2007), *Variovorax* cleaves pathogen signalization molecules (Molina et al., 2003), certain *Acidovorax* strains are biocotrol agents as well (Fessehaie et al., 2005). Similarly, taxa correlating with reference conducive soil MC112 were prevalent in all conducive soils (**Fig. 5B** and **Fig. 7B**), with the exception of *Bacillus funiculus*. The bacterial taxa prevalent in different suppressive soils can be considered as indicators of black root rot suppressiveness.

The indicators were however assessed under standardized conditions and it was not therefore clear whether the same bacteria were prevalent is suppressive vs. conducive soil under field conditions. Rhizosphere soil was therefore sampled from plants actually present in four fields belonging to different farmers in Morens in Spring 2004 and 2006. The fields represented suppressive (reference MS8) and conducive (reference MC112) as well as one moderately suppressive soil and one moderately conducive soil (as assessed by M. Frapoli, 2007, PhD thesis). Despite all the variability due to climate, sampling season, plant species/variety and state of development, tillage and fertilizer application, common features for suppressive vs. conducive soils were found (**Fig. 9**). The taxa prevalent in suppressive soils in field are first of all *Methylobacterium*, *Azospirillum* and *Rhizobiaceae* (all of them α-*Proteobacteria*), *Methylobacterium* and *Rhizobiaceae* already correlated with different suppressive soils under greenhouse conditions. *Methylobacterium* may provide disease control by inducing resistance in plants (Indiragandhi et al., 2007). In addition, other bacterial taxa that already correlated with suppressive soils under greenhouse conditions were prevalent in suppressive soils from fields, e.g. *Phyllobacterium, Xanthomonas*/*Xylella*/*Stenotrophomonas* or *Geothrix*/*Holophaga*. In conducive soils, *Bacillus subtilis*/*licheniformis* dominated. Only some bacterial taxa, e.g. *Bacillus funiculus* and *Actinobacteria* correlated with conducive soils both from field and greenhouse experiment. In conclusion, it is possible to distinguish between suppressive and conducive soils based on various field samples, the indicators however differ from those defined under standardized conditions.

CONCLUSIONS AND PERSPECTIVES

The presented microarray has potential for prediction of black root rot suppressiveness based on defined indicators. However, these microbial bioindicators seem more prevalent in disease suppressive soils versus conducive soils, rather than just being present in the former and absent from the latter. Therefore, their practical application will entail a comparative approach, since we demonstrated that several other factors besides suppressivity status can also influence prevalence of these microbial bioindicators (e.g. geographic origin of soil, etc.). Many of the bacterial taxa prevalent in suppressive soils are known for including strains with biocontrol or direct plant-beneficial effects, but have not been studied extensively. Therefore,

such potential biocontrol strains could be isolated from black root rot suppressive strains and tested for their ability to control black root rot.

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