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Assessment of bacterial populations associated with banana tree roots and development of successful plant probiotics for banana crop

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ABSTRACT

Two hundred and sixty-one bacterial isolates associated with banana tree roots in organic systems were isolated from 19 farms located in four different provinces of the Dominican Republic. The isolates were analysed by means of ARDRA plus RAPD, and as a consequence 114 of them were selected for identification by means of complete 16S rRNA sequencing and phylogenetic reconstruction. The 114 isolates belonged to 20 different genera, with *Bacillus*, *Pseudomonas*, *Enterobacter* and *Stenotrophomonas* the prevailing genera. Of these, 65 isolates showed more than 99.5% similarity with a type strain, and they were assigned to 34 different species from 16 genera; 29 isolates could constitute new species and the remaining 20 isolates belonged to groups containing more than one species with identical 16S rRNA genes, and therefore they could not be assigned to any species. This result showed a higher number of bacterial taxa associated with banana tree roots than previously described. Additionally, we found seven bacterial species with significant *in vitro* plant growth promoting (PGP) activity, which had not been previously described as PGP bacteria in any crop. Field trials with isolates pre-selected based on their *in vitro* PGP activity showed that one strain of the species *Pseudomonas plecoglossicida* improved fruit yield and controlled the incidence of the disease black sigatoka caused by *Mycosphaerella fijiensis*. This activity was tentatively attributed to induced systemic resistance mechanisms. Bacterial diversity was analysed among the 261 isolates based on the Shannon index of diversity (H), calculated from the ARDRA profiles. Interestingly, the majority of the bacterial diversity was found within farms (86% of the total), being higher than the bacterial diversity between farms (14%). Moreover, the differences in the average H Index between provinces were very low. Consequently the biodiversity of the bacterial communities was little influenced by the soil characteristics. These results could work in favour of the efficient adaptation of the bacterial strains selected for use as plant probiotics in a range of soils in the region analysed.

1. Introduction

Banana is a key crop for the economy in tropical regions (De la Torre-Gutiérrez et al., 2008). Moreover, it is ranked the eighth crop worldwide for its importance as a foodstuff, and the fourth, after rice, wheat and maize when only developing countries are considered (Arias et al., 2004). Banana is a “dual crop” in many tropical countries because it is cultivated as a subsistence crop but is also one of the most important crops for export. Banana export is a technological and economic activity, different from its production as subsistence crop (Arias et al., 2004). The preferences of the international market make bananas produced in certified organic systems more competitive than conventionally produced bananas. In the Dominican Republic, organic bananas represented more than 50% of the total banana exports on average during the last five years, and over 85% of the organic bananas produced in Dominican Republic are exported (CEI-RD, 2013).

Nowadays, there is no doubt about the important role of the beneficial interactions between microbes and plants in improving agricultural production. The exploitation of the intrinsic biological potential of rhizosphere processes improves nutrient use efficiency

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(Pii et al., 2015) and plant health by different mechanisms of action (Babalola, 2010; Cummings, 2009; Lugtenberg and Kamilova, 2009; Maheshwari, 2011). In organic systems, where the use of traditional chemical products is limited and strictly regulated, the exploitation of plant–microbe interactions to improve the nutritional and health status of the plant is especially important.

The use of seeds or soil inoculants consisting of products based on soil microorganisms called bioprotectants, biofertilisers or biostimulants (Viveros et al., 2010; Prashar et al., 2014) or more general plant probiotic microorganisms (PPM) (Lugtenberg and Kamilova, 2009), is becoming more popular in agriculture. What it is expected from such inoculants is that the crops show a yield increase and a better defence against pathogens, with reduced or eliminated chemical inputs (Bhardwaj et al., 2014). These results are a consequence of a complex mix of different modes of action involving: improvement of plant nutrition through better nutrient uptake, improved nitrogen fixation, and phosphate, potassium and iron solubilisation or mineralisation; control of diseases by several mechanisms; and increase of chlorophyll content and photosynthetic activity (Mantelin and Touraine, 2004; Glick et al., 2007; Esitken et al., 2010; Singh et al., 2011; Adesemoye et al., 2009; De Souza et al., 2013; Sinha et al., 2010).

However, there are still inconsistencies in the performance of the inoculants at the field scale (Morrissey et al., 2004). To tackle this issue, research is being undertaken to learn how to prepare the rhizosphere environment for PGPR rhizosphere colonization by means of rhizosphere engineering (Ryan et al., 2009). Nevertheless, at present, the best strategy to improve the performance of the microorganisms at the field scale is to search for region-specific microbial strains, to be used as the inocula to achieve the desired effects in the crop (Deepa et al., 2010). Knowledge of the native bacterial population, its characterisation, and identification is required to understand the distribution and diversity of indigenous bacteria in the rhizosphere of specific crops in a specific region (Keating et al., 1995; Chahboune et al., 2011).

Therefore, the present work analyses the culturable bacterial communities associated with the roots of banana trees in the Dominican Republic, with the objective of designing plant probiotics based on microorganisms for such crops. As each plant species selects the bacteria associated with them by means of several mechanisms, it is important to know the regional bacterial strains associated with a given crop in order to design successful inoculants. Consequently the specific objectives were: i) to identify the cultivable bacterial populations associated with banana tree roots; ii) to test the effect of strains with *in vitro* PGP traits in field conditions; iii) to investigate the biodiversity of the bacteria associated with the banana tree roots and their determinant factors; iv) to work out practical considerations for the design of successful plant probiotics based on microorganisms for banana crops.

2. Materials and methods

2.1. Isolation of bacteria

The isolation of bacteria associated with the roots of banana trees (*Musa* AAA cv. 'Dwarf Cavendish') was carried out in 19 farms under organic management, from the four provinces that are the main producers of banana in the Dominican Republic (Fig. 1). The farmers planted the crop from plants produced *in vitro*. The sampling was made in the third, fourth or fifth ratoon depending on the farm, at the phenological stage of bunch emergence (E). A brief summary of the soil characteristics is Table 1. The complete soil analyses are in Table S1. For the isolation of endophytic bacteria, the roots were washed with tap water to eliminate adhered particles of soil, following sterilization of the outer part of the roots with 70% ethanol for 1 min and 7% NaClO for 5 min, and three successive washings with sterile distilled water. Five grams of the surface-sterilised roots were macerated with 45 ml of sterile saline solution and dilution series were prepared with the saline solution. Aliquots of 100 μl from 10^{-1} to 10^{-3} dilutions were plated onto petri dishes with TSA medium (SIGMA Cat. No. 22091) supplemented with 1 mg l^{-1} cycloheximide to prevent fungal growth. For rhizospheric bacteria extraction, roots were brushed with a No. 4 brush, and 1 g of the soil obtained was placed in 9 ml of sterile saline solution and dilution series were prepared as indicated above, and plated onto petri dishes with the same medium. In both cases, individual colonies were tested for purity in other petri dishes with TSA medium, and used for the next steps.

2.2. Amplified 16S rRNA restriction analysis (amplified ribosomal DNA restriction analysis, ARDRA)

DNA bacterial extraction was carried out as indicated by Álvarez-Martínez et al. (2009). Amplification of the 16S rRNA gene was performed from bacterial genomic DNA by PCR. The primers 1522R (5′-AAGGAGGTGATCCANCCRCA-3′) and 8F (5′AGAGTTTGATCCTGGCTCAG-3′) were used to amplify almost the full length of the 16S rRNA gene. The primers were purchased from ISOGEN, and the kit used for the PCR reaction from Promega Corporation (USA) ("Go Taq Flexi DNA Polymerase"). Each PCR mixture (50 μl) contained a reaction mix of 1X buffer, 200 μM of each dNTP, 0.12 μM of each primer, 2 mM $MgCl_2$, 0.85 U of the polymerase and approximately 100 ng template DNA. PCR amplification was performed under the following conditions: 5 min at 95 °C for initial denaturation; 35 cycles of 1 min at 94 °C for denaturation; 30 s at 45 °C for annealing; 1 min 30 s at 72 °C for extension; and 7 min at 72 °C for a final extension. Five microlitres of the PCR product was used for electrophoresis in a 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 g/ml ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as a size marker. One single band corresponding to the 16S rRNA gene was typically visualised.

After PCR amplification of the 16S rRNA, 6 μl of each product was digested separately by the restriction enzymes (RE) BanI, EcoRI, HinfI and StuI (Thermo Fisher Scientific, USA) and Sau3AI (New England Biolabs Inc. USA) following the manufacturer's instructions, and subjected to electrophoresis on a 2% (w/v) agarose gel prepared in the same way as indicated above. The electrophoresis conditions, the staining procedure and the photography process were also the same as above.

The band pattern obtained from the ARDRA, was coded in a presence/absence matrix in order to calculate the Shannon index of diversity (H index) (Shannon and Weaver, 1949). A principal component analysis (PCA) was carried out for the 18 soil physico-chemical characters analysed (Table S1) plus the H index, for the 11 farms (cases) with a complete soil analysis (the rest of the farms had to be discarded because several parameters of the soil analysis were missing). The data matrix was log10 transformed for standardisation, and then the variance-covariance matrix was obtained and the PC (principal components) extracted, and finally rotated using the varimax method. A biplot was drawn for the two first PC for the 11 farms and the 19 characters analysed. All the analyses were carried out by means of the software SPSS Statistics (IBM Corp. Released, 2010).

Additionally, the band pattern model for each isolate and each RE was recorded.