Molecular Identification of Microorganisms Associated to the Rhizosphere of Vanilla and Their Potential Use as Biofertilizers

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Abstract

The cultivation of vanilla (*Vanilla planifolia*) is highly promissory in Colombia, where advances in crop management techniques is needed. This study describes vanilla rhizosphere microorganisms in functional groups, their molecular identification, and explores their potential use as biofertilizers. Under in vitro conditions, the most effective phosphate solubilizer was a bacterium with phylogenetic proximity to *Serratia* sp. followed species related to *Pseudomonas saponiphila* and *P. koreensis*. *Pseudomonas* sp. are among nitrogen fixing bacterium that are more abundant and fast growing. The most effective cellulolytic was a fungus phylogenetically related to *Penicillium gliseofulvum* followed by another isolate related to *Aspergillus fumigatus*. Among the five most effective proteolytic were three bacteria phylogenetically related to *Bacillus mycoides* and *B. cereus*, a bacterium related to *Serratia* sp. and another associated to *B. thuringiensis*. The most relevant phytate degrader were two isolates related to *Acremonium* sp. and *Plectosphaerella* sp. The results of this study show the high diversity of rhizosphere microorganisms in vanilla plants, some of them exhibit capacity for enhancing plant nutrients availability and, consequently, they have the potential to be used as biofertilizers.

INTRODUCTION

Vanilla plants produce vanillin, one of the most appreciated food flavoring world-wide (Spices Board, 2000). In Colombia its cultivation is highly promising because the high international price and the possibility of using it to replace illegal crops, particularly in rainy forests. However, little is known about the crop management of vanilla and the yield is commonly low (<1 kg per plant per year) likely due to inadequate plant nutrition. Higher yields are reported for this plant in Madagascar, Mexico, Costa Rica, and Brazil, (Soto-Arenas, 2006).

Availability of adequate nutrients in growth medium is one of the most critical points for production of vanilla, because it strongly affects vegetative growth, flowering and pod production of the plant (McGregor, 2005). The nutrient supply depends on the organic material composition (Sarma et al., 2010) and the biological activity of microbes (Porras-Alfaro and Bayman, 2007).

The biological management of plant nutrition of vanilla seems to be very attractive for several reasons: some authors reported that the vanilla roots are sensitive to chemical fertilizers (Fouché and Jouve, 1999); this kind of management can be compatible with a habitat requirement rich in decaying organic matter; and high acceptance of environmental-sound practices.

The objective of this study was to isolate, test, and identify microorganisms from the rhizosphere of vanilla plants. These microorganisms were studied in functional groups capable of carry out one of the following activities: nitrogen fixation (FBN), release phosphate from phytate (FIT) and rock phosphate (PSM), cellulose (CEL) and protein decomposition (PROT).

MATERIALS AND METHODS

Microbial Isolation

This study was carried out in the Laboratory of Cellular and Molecular Biology and in the greenhouse of Soil Fertility of the Universidad Nacional de Colombia at Medellin (6°14'N; 75°35'W, and 1450 m of altitude). Samples of the rhizosphere of healthy one-year-old vanilla plants and organic substrates were collected randomly from different fields of a vanilla plantation located in the town of Sopetran (Antioquia, Colombia). Samples were taken with sterilized tools with 70% ethanol and transported to the lab in plastic bags at 4°C. Then, serial dilutions (10^{-1} to 10^{-8}) were prepared with sterile distilled water. An aliquot of 50 µl was transferred into petri dishes that contained autoclaved (120°C, 0.1 MPa, 30 min) selective media to grow microorganisms in five functional groups; nitrogen fixers were isolated in the medium developed by Dobereiner and Day (1976), cellulose and protein degraders in the medium of Wood (1980), phytase activity in the medium of Tabatabai (1982) and those capable of rock phosphate dissolution with the medium developed by Osorio and Habte (2001). Petri dishes were incubated at 28°C for five days.

Abundant and fast-growing microbial colonies that developed surrounding halos were selected to further multiplication in nutrient-agar (for bacteria) and malt extract and PDA medium (for fungi) at 28°C for five days. Then, they were stored at 4°C for further studies.

Microbial Identification

Selected microorganisms from each functional group were subjected to nucleic acid extraction using CTAB 3X for fungi and SDS for bacteria. The integrity of the extracted DNA was determined by electrophoresis in an agarose gel (1%) with 3 µl of ethidium bromide (10 mg ml^{-1}) and visualized under an UV transiluminator (Biometra). DNA concentration was estimated by absorbance at 260 nm in a spectrophotometer Genesys 6 (Thermo Scientific).

Microbial identification was carried out by sequencing of ribosomal regions ITS and domains D1-D2 of 28S subunit for fungi and the subunit 16S for bacteria. The PCR reactions were conducted in a thermal cycler T3 (Biometra) with specific primers (Guadet et al., 1989; Kuske et al., 1997).

Amplicons were purified with the QIAquick PCR Purification Kit (Qiagen) and sequencing was done through the Big Dye Terminator Cycle Sequencing Ready Reaction System (PE Applied Biosystems) in an ABI Prism 3730xl at Macrogen (South Korea).

Sequences were edited using softwares BioEdit 6.0.6 and Chromas 1.45 in order to obtain consensus sequences. These sequences were compared by Blastn with the GenBank database. Putative sequences of identified microorganisms by Blastn were subsequently used to perform phylogenetic analysis through the Maximum Likelihood method under the Tamura-Nei substitution model (1993) with the software Mega 5 (Tamura et al., 2011). Internal topology of dendrograms was verified by a Bootstrap analysis.

In Vitro Tests for Effectiveness

A series of in vitro tests were conducted to evaluate the effectiveness of microorganisms for degrading protein and cellulose and solubilizing rock phosphate. In the case of cellulolytic activity each isolate was aseptically transferred by triplicate into petri dishes containing carboxy-methyl-cellulose as the only carbon source (Wood, 1980) and incubated during four days at room temperature in the darkness. A test for respirometry was conducted (Tate et al., 1988) and the CO_2 released was trapped into 20 ml of 0.1 *M* NaOH and calculated by titration with 0.1 *M* HCl. Inoculated petri dishes and the beaker with NaOH were placed in a 300-ml container which was hermetically closed. An uninoculated control was included for comparison.

Phosphate solubilizing microorganisms were tested by the method developed by

Osorio and Habte (2001). Each PSM isolate was aseptically transferred into 250-ml Erlenmeyers containing the medium without agar described in Table 1, in which the only phosphorus source was an insoluble rock phosphate. Erlenmeyers were incubated for five days at room temperature in orbital shaker at 150 rpm. Then, pH and soluble phosphorus concentration were determined by a pH-meter and by the blue-phosphomolybdate method (Murphy and Riley, 1962), respectively. An inoculated control was included.

In the case of protein degraders, each isolate was aseptically transferred into 250-ml Erlenmeyers containing the medium (without agar) developed by Wood (1980), in which the only nitrogen source was casein. Erlenmeyers were incubated at room temperature for five days. The effectiveness of protein degraders was measured by the release of NH_4^+, which was conducted by an ion selective electrode coupled to a potentiometer. An inoculated control was included.

In all in vitro tests, a completely randomized experimental design was used. Treatments consisted of individual inoculation of each isolate and its respective control. Significant effects of inoculations were evaluated by an ANOVA (F-test) and mean separation by a Duncan test (t-test) using a significant level of $(P)\leq 0.05$. Statistical analyses were conducted in the software STATGRAPHICS Centurion XV.

RESULTS AND DISCUSSION

The screening allowed detecting 109 microbial isolates in the five different functional groups: 25 were capable of fixing N_2, 6 exhibited capacity of producing phytase, 20 degraded inorganic phosphate, 30 degraded protein, and 28 degraded cellulose. A view of some microbial colonies growing in the media is shown in Figure 1. Most of the microbial isolates were bacteria (100) and only few fungi were found (9), which is very common in decaying organic materials and soils (Atlas and Bartha, 1997). The more adequate serial dilutions were 10^{-5} and 10^{-6}. The results showed that there is a great abundance and diversity of microbes in the five functional groups studied.

Sequencing results were available only for 60 isolates. The sequences obtained indicate a high level of genetic diversity in the microbial community, as shown by Blast and phylogenetic analysis (Table 1). Bacteria phylogenetically related to *P. koreensis* were the most common microorganism found in the N_2 fixer group. Fungi strains 51SEP40 and 52SEP41 are closely related to *Plectosphaerella cucumerina* and were the most active and fast growing phytate degraders. Several bacteria capable of rock phosphate dissolution were phylogenetic related to *Pseudomonas saponiphila* and *P. koreensis*. Among the protein degraders the most commonly microorganisms were bacteria closely related to *Bacillus mycoides* and *B. cereus.* Among cellulolytic microbes, *Bacillus* sp. and *Pseudomonas* sp. were the more frequently found, followed by a fungus related to *Aspergillus fumigatus*.

The in vitro test allowed detecting those microorganisms with a high potential to be used as biofertilizers. As expected, the microbial dissolution of inorganic phosphate was associated with acidity production. This mechanism consists of the production of organic acids (citric acid, oxalic acid, gluconic acid) that attack the structure of rock phosphate and release phosphate ions that became available for either microbial or plant root uptake (Osorio, 2011). The isolates 75MA194 (*Serratia* sp.) and 40MA190 (a bacterium closely related to *P. saponiphila-P. koreensis*) were outstanding in their capacity to reduce solution pH (<4.5) and consequently were significantly the most effective PSM (Fig. 2). Although other microbial isolates reduce the solution pH, they were not capable of effectively increase solution P concentration. *Serratia* sp. and *Pseudomonas* have been amply reported as effective PSM (Hameeda et al., 2006).

The in vitro test employed for testing proteolytic microbes showed that isolates 71MA226, 77SEP29, 74MA193, 75MA194, and 28SEP21 were significantly more effective to release ammonium from a protein source (Fig. 3). The three first microorganisms are phylogeneticaly related to the group formed by *Bacillus mycoides* and *B. cereus*, while the last two isolates are related to *Serratia* sp. and *B. thuringiensis*, respectively.

The fungal isolate 49CMA159 (phylogenetically related to *Penicillium gliseofulvum*) was significantly the most effective cellulose degrader (measure through a CO_2 production test) followed by the fungal isolate 108CMA163 (related to *A. fumigatus*) (Fig. 4). *Penicillium* is one of the largest fungal genera found in soil and commonly present wherever organic material is available. This genus is widely known due to its ability to produce large quantities of extracellular enzymes (Webster and Weber, 2007). Cellulose degradation is a key process in organic matter decay, because it provides simple sugar and energy easily usable for microbes (Wagner and Wolf, 1999).

Results of this research indicate that rhizosphere of vanilla plants contains a microbial community with high potential to enhance nutrient availability for commercial plantations of this crop. Further evaluations with these microorganisms are currently conducted to verify the effectiveness of the more outstanding microbial isolates under greenhouse conditions.

ACKNOWLEDGEMENTS

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Tables

Table 1. Molecular identification of microbial isolates obtained from the rhizosphere of vanilla plants.

Code	Highest score BLAST hit	Phylogenetic affinity
20SEP14[a], 21SEP15[a], 33MA221[a], 46MA223[a]	*Pseudomonas* sp.	*Pseudomonas koreensis*
15MA247[d]	*Pseudomonas stutzeri*	NE
84SEP33[e]	*Pseudomonas syringae*	NE
100SEP37[a]	*Pseudomonas* sp.	*Pseudomonas putida*
64MA224[a], 36MA222[b], 25SEP18[c], 26SEP19[c], 27SEP20[c], 39SEP23[c], 69SEP26[c], 40MA190[c], 31SEP22[d], 48SEP24[d], 58SEP25[e], 68MA225[d]	*Pseudomonas* sp.	*P. saponiphila-P. koreensis*
104SEP38[a], 89SEP34[e]	*Chromobacterium* sp.	*Chromobacterium* sp.
96MA199[d]	*Acidovorax* sp.	*Acidovorax oryzae*
90SEP35[c], 76SEP28[d], 99SEP36[d]	*Acinetobacter* sp.	*Acinetobacter* sp.
80MA227[e], 105MA228[a]	*Citrobacter* sp.	*Citrobacter freundii*
78SEP30[c], 72MA192[d]	*Klebsiella* sp.	*Klebisella* sp.-*K. pneumoniae*
70SEP27[d], 75MA194[d], 175MA201[d], 275MA231[d], 81MA195[e]	*Serratia* sp.	*Serratia* sp.
22SEP16[b] 23SEP17[b], 97MA200[d]	*Enterobacter* sp.	*Enterobacter asburiae-E. cloacae*
37MA189[d]	*Flavobacterium* sp.	*Flavobacterium* sp.
59BMA249[e]	*Curtobacterium* sp.	*Curtobacterium* sp.
155MA230[e]	*Streptomyces* sp.	NE
55MA248[e]	*Rhodococcus* sp.	*Rhodococcus* sp.
79SEP31[c], 87MA197[e]	*Bacillus megaterium*	*Bacillus megaterium*
28SEP21[d], 82BMA196[e], 82SEP32[e]	*Bacillus cereus*	*Bacillus thuringiensis*
77SEP29[d], 43MA191[d]74MA193[d], 95MA198[d], 71MA226[d]	*Bacillus cereus*	*Bacillus mycoides-B cereus*
51SEP40[b], 52SEP41[b]	*Plectosphaerella* sp.	*Plectosphaerella cucumerina*
35MA158[b]	*Acremonium* sp.	*Plectosphaerella* sp.
49CMA159[e], 53SEP54[d]	*Penicillium citrium*	*Penicillium griseofulvum*
50MA160[e]	*Bionectria* sp.	*Bionectria ochroleuca*
108CMA163[e], 108VMA164[e]	*Aspergillus fumigatus*	*Aspergillus fumigatus*

a: FBN, b: FIT, c: PSM, d: PROT, e: CEL, NE: not evaluated.

Figures

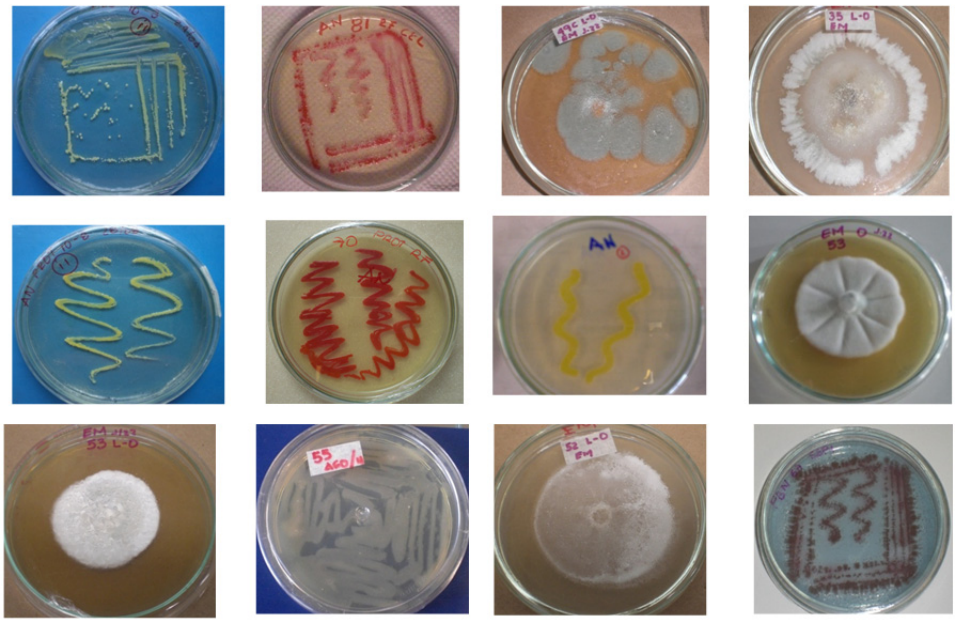

Fig. 1. View of petri dishes containing selective media and some selected rhizosphere microorganisms.

Fig. 2. Phosphate concentration in the medium inoculated with different microorganisms isolated from the rhizosphere of vanilla.

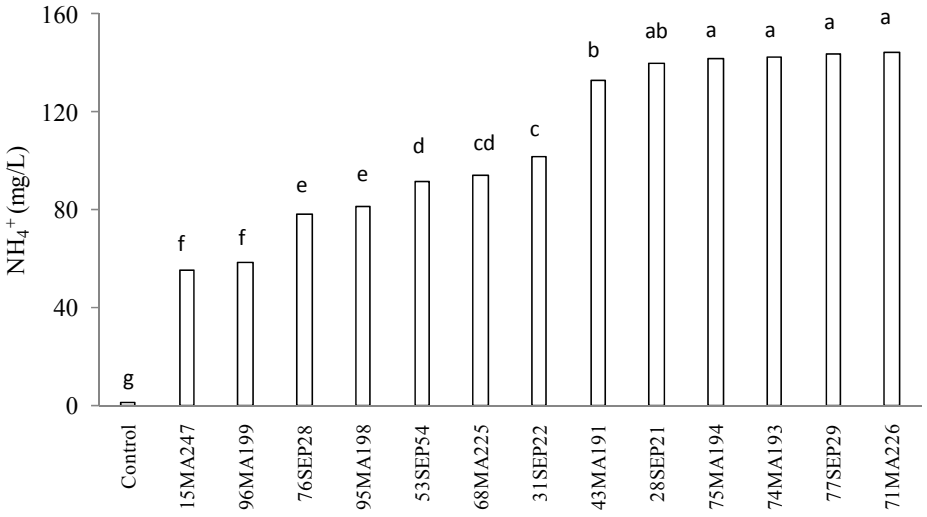

Fig. 3. Ammonium concentration in the medium inoculated with different microorganisms isolated from the rhizosphere of vanilla.

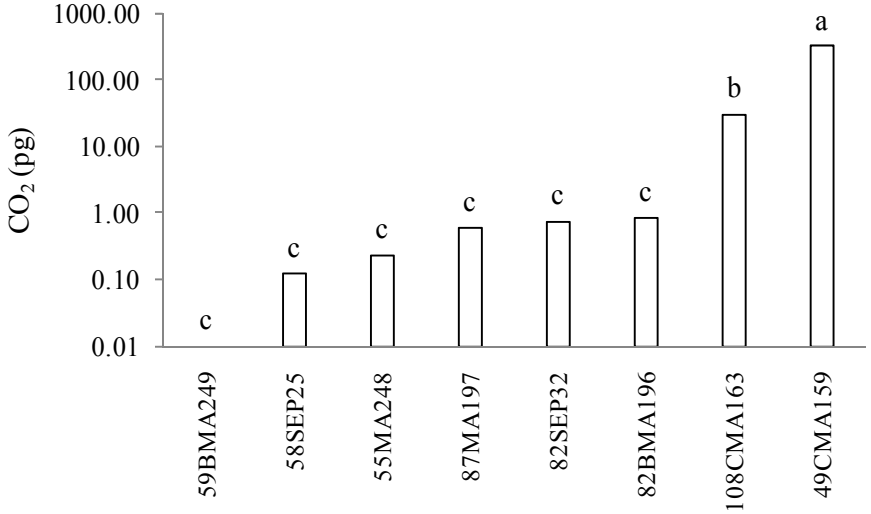

Fig. 4. Concentration of CO_2 as a result of inoculation of cellulose-containing-medium with different microorganisms isolated from the rhizosphere of vanilla.