The Diversity of Culture-Dependent Gram-Negative Rhizobacteria Associated with *Manihot esculenta* Crantz Plants Subjected to Water-Deficit Stress

Tatiana Zapata, Diana Marcela Galindo, Alba Rocio Corrales-Ducuara and Iván Darío Ocampo-Ibáñez *

Research Group of Microbiology, Industry and Environment, Faculty of Basic Sciences, Universidad Santiago de Cali, Cali 760035, Colombia; tatianazapata101@gmail.com (T.Z.); diana.marcela.galindo@gmail.com (D.M.G.); alba.corrales00@uscd.edu.co (A.R.C.-D.)

* Correspondence: ivan.ocampo00@usc.edu.co; Tel.: +57-518-3000

Abstract: There is a lack of studies on the root-associated bacterial microbiome of cassava plants. The identification and characterization of rhizobacteria can contribute to understanding the adaptation of the agriculturally important crop plants to abiotic stress. Rhizobacteria play a significant role in plants, as they can alleviate the drought stress by various mechanisms that enhance the plant growth under these stressor conditions. In this study, Gram-negative bacterial strains from the plant rhizosphere of *Manihot esculenta* Crantz CIAT MCOL1734 variety subjected to water deprivation were isolated, characterized according to their morphological properties, and then identified by VITEK® 2. An increase in the diversity, abundance, and species richness of Gram-negative rhizobacterial community was found in cassava plants subjected to water-deficit stress. In total, 58 rhizobacterial strains were isolated from cassava plants. The identification process found that the bacteria belonged to 12 genera: *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Buttiauxella*, *Cronobacter*, *Klebsiella*, *Ochrobactrum*, *Pluralibacter*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Sphingomonas*. Interestingly, *Pseudomonas luteola* and *Ochrobactrum anthropi* were rhizobacteria isolated exclusively from plants submitted to drought conditions. The cassava roots constitute a great reservoir of Gram-negative bacteria with a remarkable potential for biotechnological application to improve the drought tolerance of plant crops under water-deficit conditions.

Keywords: *Manihot esculenta* Crantz; plant-growth-promoting rhizobacteria; drought stress; plant water deficit

1. Introduction

Cassava (*Manihot esculenta* Crantz) is widely grown in subtropical and tropical areas worldwide, including Africa, Asia, Latin America, and the Caribbean [1,2]. In these regions, the cassava root is one of the most essential sources of calories after rice and corn and provides staple food to an estimated 800 million people [1]. In addition, the top biomass including leaves and immature stems may be used as the cassava hay for animal feeding [2,3].

The cassava crops are well adapted to several agroecological conditions; even its potential to adapt well to climate change is a factor that favors the increased production of cassava [1]. However, several factors, such as salinity and drought, may cause crop yield losses of more than 50% worldwide [4,5]. Despite these reasons, cassava can withstand relatively prolonged periods of drought during the first three months; however, after planting, the cassava crop is very sensitive to the soil water deficit [1,5–7]. At any time during this early establishment period, the water-deficit stress may affect the root system development, significantly reducing the growth of roots and shoots and, subsequently, the storage roots [1,8]. After this time, a water-deficit causes turgor loss, diminished water potential, disruption of membrane integrity along with protein denaturation, and stomatal...
closure, which may decline the rate of photosynthesis [9,10]. After planting, stakes only sprout and grow well when the soil moisture content is at least 30% of the field capacity. Once established, cassava can grow in dry areas [6,7,11,12]. Because the greatest potential for an increased yield of cassava production is in the rainfed areas and drylands of subtropical and tropical regions [1,15], the breeding of several higher-yielding varieties with improved root quality and tolerance to drought have been produced to be used in those areas [14–16]. The most common breeding objectives of these varieties include physiological mechanisms, such as delay in flowering time, reduction in transpiration rate, increased production of primary and secondary metabolites, hormone-mediated regulation of vegetative development, and an increased production of abscisic acid (ABA) that act as a mediator in the response to drought stress [17,18].

In addition to the breeding of tolerant varieties, there are natural strategies to rescue the plant growth in abiotic stressful conditions, for example, the activity of microorganisms associated with the rhizosphere [10,19,20]. Bacteria are the most abundant group in the rhizosphere, and they are referred to as plant-growth-promoting rhizobacteria (PGPR) [10,19,20]. Rhizobacteria are considered a bulwark because they colonize plant roots and possess a tremendous potential in promoting plant growth to ensure its survival under stressful conditions [10]. In particular, PGPRs have an inherent capacity to cope with abiotic stresses; for instance, they are able to boost plant tolerance to drought stress. Through a process called rhizobacterial-induced drought endurance and resilience, the PGPRs can mitigate the impact of water deprivation on plants [10]. This process includes physiological and biochemical modulations in plants, such as changes in the antioxidant defense and modifications in the production and content of phytohormones, such as indole-3-acetic acid (IAA), ABA, gibberellic acid, and cytokinin (CK) [10,21–23]. In addition, to ensure the survival of plants under drought-stressed conditions, the rhizobacteria can produce osmolytes, bacterial exopolysaccharides, heat-shock proteins, and volatile organic compounds, which may contribute to the drought tolerance of plants [10,24,25]. A large numbers of rhizobacteria strains belonging to several genera, including *Achromobacter*, *Acinetobacter*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia*, have been identified from soils and rhizospheres of diverse plant species [26,27]. Several studies have demonstrated that the inoculation of plants with rhizobacteria is a biotechnological tool that may improve the productivity of crops under abiotic stress conditions [10,23]. When these procedures were implemented particularly in crops under a drought stress environment, an enhancement in the root system was observed, which improved the plant’s ability to uptake water and enhanced the crop productivity [10,23].

Cassava plants have a wide range of associated microorganisms that are distributed throughout the body of plants, including leaves, stems, and roots [28,29]. Previously, several studies characterized the bacterial diversity of the cassava rhizosphere and evaluated the treatment effects with PGPR on plant growth [30,31]. In this respect, a wide diversity of rhizobacteria associated with cassava has been identified including strains belonging to the genera *Bacillus*, *Micrococcus*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, and *Serratia*, which were isolated from several cassava varieties and cultivars [31,32]. When cassava stem cuttings were in vitro treated with the isolates of these genera and then planted, the production of IAA and ammonia, phosphate solubilization, and increased iron levels in the leaf were induced, suggesting that rhizobacterial isolates from cassava may have potential as plant-growth promoters [30,31,33]. Despite these studies, very few studies have studied the rhizobacteria in cassava under abiotic stress conditions, and the structure and diversity of the bacterial communities of the rhizosphere of cassava plants under these conditions are not well known [34]. Recently, several studies have shown that the microbial composition of the rhizosphere in several plant species may be affected under abiotic stress conditions, for example, increasing the relative abundance of PGPRs by root colonization under drought stress [27,35–37]. In this study, we isolated bacterial strains from the rhizosphere of cassava (*M. esculenta* Crantz) subjected to water deprivation. Once isolated, the
strains were characterized according to their morphological properties and then identified. The bacterial species here identified were reported as PGPR in previous studies.

2. Materials and Methods

2.1. Drought Stress Assays

Experiments were conducted under glasshouse conditions, such as temperature of 28–30 °C and relative humidity of 50 ± 80%, at the International Center for Tropical Agriculture (CIAT) (Palmira, Valle del Cauca, Colombia, 03°31’ N, 76°18’ W; a 1001 m.a.s.l.) between 1 August and 1 September 2018. All experiments were performed following a completely randomized design. Seven plants of CIAT MCOL1734 cassava variety [38] at vegetative stage were used to perform every assay. Five cassava plants (cp-ds-1, cp-ds-2, cp-ds-3, cp-ds-4, and cp-ds-5) were exposed to drought stress by experimentally manipulating irrigation to artificially impose water deficit for 20 days [39–41]. During this time, two cassava plants (cp-c-1 and cp-c-2) were exposed to daily irrigation and used as controls.

2.2. Strain Isolation

Taproots and adventitious roots were collected from cp-ds and cp-c plants at the beginning of assays (T0) and after 20 days of drought stress assay (T20). To isolate rhizobacteria only from the rhizoplane and endorhizosphere, the adhering soil particles were removed by washing the roots with abundant sterile distilled water. About 1 g of roots were collected and then macerated in a mortar. Next, 9 mL of Tween 80 solution (Milli-Q distilled water) was added into it to make a 10× dilution. Then, 1 mL of this mixture was used for serial 1:10 dilutions using buffered peptone water. From about 10⁻² to 10⁻⁷ dilutions were plated in triplicates on the following media: nutrient agar (NA) (Sigma-Aldrich), Ashby’s mannitol agar (AMA) (HIMEDIA), King Agar B (KAB) (Sigma-Aldrich), and eosin methylene blue (EMB) Agar (Becton Dickinson). They were then incubated at 37 °C ± 2 °C for 18–24 h [42–44]. Distinct colonies obtained from agar plates were selected and then purified by subculturing.

2.3. Strain Characterization and Identification

Distinct bacterial colonies purified by subculturing were initially examined for colony characteristics (appearance, elevation, margin, pigmentation, shape, size, and texture) [45] and cellular characteristics (cell shape, gram testing, and production of spores) via microscopy [45,46]. In addition, the identification of bacterial species was based on biochemical testing. Catalase and oxidase tests were initially performed; then, species identification was performed using the automated VITEK® 2 system, (bioMerieux, 9.02, Marcy l’Etoile, France) with the VITEK® 2 Gram-negative identification card, which is based on established biochemical methods and substrates that evaluate the use of carbon and enzymatic activities (Reference 21341, bioMerieux, Marcy l’Etoile, France).

2.4. Statistical Analysis

Species diversity indices, such as the Shannon diversity index [47] and the Pielou evenness index [48], were determined to analyze bacterial communities in each group of cassava plants; in addition, bacterial species richness and abundance were estimated. Diversity indices, richness, and abundance were calculated from standardized profiles of individual cassava samples using the number of isolates for each identified bacterial species. The Shannon diversity index was calculated as follows:

\[ H' = - \sum (p_i)(\ln p_i) \]

The Pielou evenness index was derived from the Shannon diversity index and was calculated as follows:

\[ J' = H' / H'_{\text{max}} \]
were \( H' \) is the Shannon diversity index, \( p_i \) is the relative importance value of species \( i \), \( J' \) is
the Pielou evenness index, \( H'_{\text{max}} = \ln(S) \), and \( S \) is the total number of species in \( i \)th plot.

Statistically significant differences of the bacterial diversity in cp-c and cp-ds, comparing independently T0 and T20, were analyzed and compared using Shannon diversity index through the Hutcheson t-test [49]. \( p \)-values \( \leq 0.05 \) were considered statistically significant.

All calculations were performed with the R-Project free software Version 1.1.463.

### 3. Results

#### 3.1. Strain Isolation, Morphological Characterization, and Biochemical Identification

In total, 58 rhizobacterial strains were obtained from the rhizoplane and endorhizosphere of cassava roots, and most of them were grown on different media (Tables 1 and 2). Based on the colony morphology on NA, AMA, KAB, and EMB media, all isolates had different morphological characteristics, including round-to-irregular colonies with flat and raised elevations, smooth and irregular surfaces, and diversity of size (Tables 1 and 2). Several isolates produced pigments, with colonies that were white, off-white, milky white, purple, pink-purple, reddish, and yellowish in color, but some isolates did not produce any pigmentation (Tables 1 and 2). All isolates reacted negatively to Gram staining but reacted positively and negatively to the catalase and oxidase tests, respectively (Tables 1 and 2). VITEK® 2 identification showed that the isolates were mainly the members of genus *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Buttiauxella*, *Cronobacter*, *Klebsiella*, *Ochrobactrum*, *Pluralibacter*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Sphingomonas* (Tables 1 and 2). The probability levels of discrimination for all strains were \( \geq 95\% \) according to the VITEK® 2 system report.

| Plant ID | Strain ID | Gram Reaction | Colony Characteristics at Several Culture Media | Biochemical Analysis | Identification (VITEK® 2) |
|----------|-----------|---------------|-----------------------------------------------|----------------------|-------------------------|
|          |           |               | **Colony Characteristics at Several Culture Media** |                      |                         |
|          |           |               | NA AMA KAB EMB Catalase Test Oxidase Test |                      |                         |
| cp-c-1.1 | cp-c-1.2  | cp-c-1.3      | ![Colony images](image1) | ![Colony images](image2) | ![Colony images](image3) | + + | Pseudomonas putida |
| cp-c-1   | cp-c-1.2  | cp-c-1.3      | ![Colony images](image4) | ![Colony images](image5) | ![Colony images](image6) | + + | Pseudomonas fluorescens |
| cp-c-2   | cp-c-2.1  | cp-c-2.2      | ![Colony images](image7) | ![Colony images](image8) | ![Colony images](image9) | + + | Pseudomonas fluorescens |
|          |           |               | ![Colony images](image10) | ![Colony images](image11) | ![Colony images](image12) | + + | Pseudomonas mendocina |
| cp-ds-1.1| cp-ds-1.2 | cp-ds-1.3     | ![Colony images](image13) | ![Colony images](image14) | ![Colony images](image15) | + + | Pseudomonas putida |
|          |           |               | ![Colony images](image16) | ![Colony images](image17) | ![Colony images](image18) | + + | Pseudomonas putida |
|          |           |               | ![Colony images](image19) | ![Colony images](image20) | ![Colony images](image21) | + + | Pseudomonas putida |
|          |           |               | ![Colony images](image22) | ![Colony images](image23) | ![Colony images](image24) | + + | Pseudomonas putida |

*Note: The above table provides a summary of the biochemical analysis of the endophytic bacterial isolates of cassava roots at the beginning of drought stress assay (T0).*

The Pielou evenness index was derived from the Shannon diversity index and was calculated as follows:

\[
J' = \frac{H'}{H'_{\text{max}}} \quad \text{with} \quad H'_{\text{max}} = \ln(S), \quad S \quad \text{the total number of species in} \quad i \text{th plot.}
\]
Table 1. Cont.

| Plant ID | Strain ID | Gram Reaction | Colony Characteristics at Several Culture Media | Biochemical Analysis |
|----------|-----------|---------------|-------------------------------------------------|----------------------|
|          |           |               | NA | AMA | KAB | EMB | Catalase Test | Oxidase Test | Identification (VITEK® 2) |
| cp-ds-3  |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Achromobacter xylosoxidans |
| cp-ds-4  |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Sphingomonas paucimobilis |
| cp-ds-5  |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Sphingomonas paucimobilis |

Abbreviations: NA, nutrient agar; AMA, Ashby’s mannitol agar; KAB, King Agar B; EMB, eosin methylene blue agar.

Table 2. Cellular and colony characteristics, as well as biochemical analysis of the endophytic bacterial isolates of cassava roots after 20 days of drought stress assay (T20).

| Plant ID | Strain ID | Gram Reaction | Colony Characteristics at Several Culture Media | Biochemical Analysis |
|----------|-----------|---------------|-------------------------------------------------|----------------------|
|          |           |               | NA | AMA | KAB | EMB | Catalase Test | Oxidase Test | Identification (VITEK® 2) |
| cp-c-1   |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Rhizobium radiobacter |
| cp-c-1.4 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | – | Sphingomonas paucimobilis |
| cp-c-1.5 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | – | Sphingomonas paucimobilis |
| cp-c-1.6 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | – | Sphingomonas paucimobilis |
| cp-c-1.7 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | – | + | Cronobacter sakazakii |
| cp-c-1.8 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Rhizobium radiobacter |
| cp-c-1.9 |           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Pluralibacter gergoviae |
| cp-c-1.10|           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | – | + | Serratia marcescens |
| cp-c-1.11|           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Pseudomonas putida |
| cp-c-1.12|           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | – | + | Klebsiella pneumoniae |
| cp-c-1.13|           | –ve           | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | ![Colonies](image) | + | + | Pseudomonas stutzeri |
| Plant ID | Strain ID | Gram Reaction | Colony Characteristics at Several Culture Media | Biochemical Analysis |
|----------|-----------|---------------|------------------------------------------------|----------------------|
|          |           |               | NA | AMA | KAB | EMB | Catalase Test | Oxidase Test | Identification (VITEK® 2) |
| cp-c-2.4 | cp-c-2.5  | –ve           |     |     |     | no growth | +           | +           | Pseudomonas stutzeri |
| cp-c-2.6 | cp-c-2.7  | –ve           |     |     |     |           | +           | +           | Acinetobacter baumannii |
| cp-c-2.8 | cp-c-2.9  | –ve           |     |     |     |           | -           | +           | Rhizobium radiobacter |
| cp-c-2.10 | cp-c-2.11 | –ve           |     |     |     | no growth | -           | +           | Achromobacter xylosoxidans |
| cp-c-2.12 | cp-c-2.13 | –ve           |     |     |     |           | +           | +           | Sphingomonas paucimobilis |
| cp-c-2.14 | cp-c-2.15 | –ve           |     |     |     |           |            |             | Buttiauxella agrestis |
| cp-ds-1.4 | cp-ds-1.5 | –ve           |     |     |     |           | +           | +           | Pseudomonas putida |
| cp-ds-2.4 | cp-ds-2.5 | –ve           |     |     |     |           | +           | +           | Pseudomonas putida |
| cp-ds-2.6 | cp-ds-2.7 | –ve           |     |     |     |           | +           | +           | Pseudomonas mendocina |
| cp-ds-2.7 |            |               |     |     |     |           | +           | +           | Oxygenobacter mendocina |
| cp-ds-3.3 | cp-ds-3.4 | –ve           |     |     |     |           | +           | +           | Sphingomonas paucimobilis |
| cp-ds-3.5 | cp-ds-3.6 | –ve           |     |     |     |           | -           | +           | Rhizobium radiobacter |
| cp-ds-4.1 | cp-ds-4.2 | –ve           |     |     |     |           | +           | +           | Sphingomonas paucimobilis |
| cp-ds-5.1 | cp-ds-5.2 | –ve           |     |     |     |           | +           | +           | Pseudomonas putida |

**Table 2. Cont.**

Abbreviations: NA, nutrient agar; AMA, Ashby's mannitol agar; KAB, King Agar B; EMB, eosin methylene blue agar.
3.2. Strains Diversity at the Beginning of the Drought Stress Assay (T0)

In total, 18 strains were isolated from the rhizoplane and endorhizosphere of cassava roots at T0, with 33.3% and 66.6% of bacteria isolated from roots of cp-c and cp-ds plants, respectively (Table 1). All isolates were identified at the species level using VITEK® 2. Concerning to identified isolates from the control plants, six rhizobacterial strains were the members of genus *Pseudomonas*, including four of *Pseudomonas fluorescens*, one of *Pseudomonas putida*, and one of *Pseudomonas mendocina* (Table 1). Contrariwise, 12 isolates were identified from the roots of cp-ds plants at the beginning of the assay. Members of the identified rhizobacteria in these plants were dominated by the genus *Pseudomonas* (50%), including species such as *P. putida* (*n* = 5) and *P. fluorescens* (*n* = 1). Moreover, species such as *Sphingomonas paucimobilis* (*n* = 4), *Achromobacter xylosoxidans* (*n* = 1), and *Rhizobium radiobacter* (*n* = 1) were also identified (Table 1). All the isolates produced colonies with different morphological characteristics (Table 1). According to the relative abundances, *Pseudomonas* and *Sphingomonas* were the predominant bacterial genera found in roots of cp-c and cp-ds plants at T0 (Figure 1).
3.3. Strains Diversity at the End of the Drought Stress Assay (T20)

In total, 40 rhizobacterial strains were isolated from the cassava roots of all plants after 20 days and identified (Table 2). The identified isolates produced colonies with different morphological characteristics (Table 2). In total, 21 identified strains were isolated from the cp-c plants, which underwent daily irrigation (Table 2). Bacterial species such as Acinetobacter baumannii \((n = 1)\), A. xylosoxidans \((n = 1)\), Aeromonas salmonicida \((n = 1)\), Buttiauxella agrestis \((n = 1)\), Cronobacter sakazakii \((n = 1)\), Klebsiella pneumoniae \((n = 1)\), Pluralibacter gergoviae \((n = 1)\), P. putida \((n = 1)\), P. fluorescens \((n = 2)\), Pseudomonas stutzeri \((n = 2)\), R. radiobacter \((n = 4)\), Serratia marcescens \((n = 1)\), and S. paucimobilis \((n = 4)\) were identified in the root of the control plants after 20 days with daily irrigation (Table 2). By contrast, 19 isolates were isolated from roots of non-irrigated plants and then identified (Table 2). Several rhizobacterial species, including Ochrobactrum anthropi \((n = 1)\), Pseudomonas luteola \((n = 1)\), P. mendocina \((n = 1)\), P. putida \((n = 6)\), P. stutzeri \((n = 2)\), R. radiobacter \((n = 5)\), and S. paucimobilis \((n = 4)\) were identified in cp-ds plants exposed to drought stress (Table 2). In this respect, Pseudomonas, Rhizobium, and Sphingomonas were the predominant bacterial genera found in the roots of the control cassava plants and plants exposed to drought stress (Figure 1).

3.4. Comparison of Strain Diversity for cp-c and cp-ds Plants

When populations of isolates were analyzed for the roots of cp-c and cp-ds plants, a higher diversity of rhizobacteria were found (Figure 1). First, a wider diversity of bacterial species and higher abundance were found at T20 than T0 for control plants (Table 3). The analysis of alpha diversity through Shannon diversity index revealed a marked effect of irrigation on the Gram-negative endophytic bacterial community of cassava roots after 20 days (Table 3). Daily irrigation for 20 days resulted in a significant increase in the Shannon diversity index in control plants (Table 3). In this respect, all rhizobacterial strains isolated from cp-c plants at the beginning of assay belonged to Gammaproteobacteria (Pseudomonadales) (Table 1 and Figure 1). In contrast, after 20 days with daily irrigation, the rhizobacterial community associated with the roots of these plants exhibited changes in the relative abundance of bacteria, as well as an increased diversity (Figure 1 and Table 3). In this respect, members belonging to Alphaproteobacteria (Rhizobiales and Sphingomonadales), Gammaproteobacteria (Enterobacteriales, Pseudomonadales, and Aeromonadales), and Betaproteobacteria (Burkholderiales) were notably abundant at T20 for control plants (Table 2 and Figure 1). Meanwhile, the

![Figure 1](image-url)  
**Figure 1.** The relative abundances of rhizobacterial species isolated from the cassava roots of control plants (cp-c) and plants exposed to drought stress (cp-ds), at the beginning (T0) and after 20 (T20) days of drought stress assay.
evenness index showed no significant difference when comparing the bacterial community of control plants at T0 and T20 (Table 3).

Table 3. Abundance, species richness, Shannon diversity index, and Pielou evenness index of the endophytic bacterial isolates identified in cassava control plants (cp-c) and cassava plants exposed to drought stress (cp-ds), at the beginning (T0) and after 20 days (T20).

| Cassava Plants Treatments | Abundance | Species Richness | Shannon Diversity Index (H′) | p-Value ¹ | Evenness | p-Value ² |
|---------------------------|-----------|-----------------|------------------------------|-----------|----------|----------|
| cp-c_T0                   | 6         | 3               | 0.868                        | 0.0019    | 0.789    | 0.7070   |
| cp-c_T20                  | 21        | 13              | 2.384                        |           | 0.929    |          |
| cp-ds_T0                  | 12        | 5               | 1.350                        |           | 0.840    |          |
| cp-ds_T20                 | 19        | 7               | 1.663                        | 0.2899    | 0.855    | 0.9678   |

¹ Significance level for Shannon diversity index of rhizobacteria community of cp-c between T0 and T20 and cp-ds between T0 and T20. ² Significance level for the Pielou evenness index of rhizobacteria community of cp-c between T0 and T20 and cp-ds between T0 and T20.

On the other hand, water deprivation also influenced the Gram-negative rhizobacterial community of cassava after 20 days of drought stress. Despite cp-ds plants showing no significant change in the diversity, an increase in the Shannon diversity index of rhizobacterial endophytes of cassava plants after 20 days under water-deficit conditions was observed (Table 3). In this respect, an increase in the abundance and species richness was detected at the end of the drought stress assay (Table 3 and Figure 1). However, the proportion between *Gammaproteobacteria* (*Pseudomonadales*) and *Alphaproteobacteria* (*Rhizobiales* and *Sphingomonadales*) was conserved between T0 and T20 for the roots of cp-ds plants (Figure 1). In particular, a slight increase in the relative abundance of *Rhizobium* genus was observed at the end of the drought stress assay (Table 2 and Figure 1). Similar to control plants, the evenness index of the rhizobacteria community from cp-ds plants showed no significant difference between T0 and T20 (Table 3).

Finally, changes in the relative abundances of predominant bacterial genera and species diversity were observed in both groups of plants at T20 (Figure 1 and Table 3). In this respect, when the bacterial community associated with the roots of the control plants under full irrigation was compared to that of plants under drought conditions at the end of 20 days of treatment, a higher diversity of rhizobacteria was found in the cp-c roots (Figure 1 and Table 3). Here, *A. baumannii*, *A. salmonicida*, *B. agrestis*, *C. sakazakii*, *K. pneumoniae*, *P. gergoviae*, and *S. marcescens* were bacterial species exclusively found in control plants at T20, whereas *O. anthropi* and *P. luteola* were exclusive rhizobacterial species of plants submitted to drought conditions (Figures 1 and 2).
Figure 2. Distribution of the number of rhizobacterial isolated from cassava roots. The Venn diagram illustrates the number of bacteria isolated from cassava roots of control plants (cp-c) and plants exposed to drought stress (cp-ds), at the beginning (T0) and after 20 (T20) days of drought stress.

4. Discussion

Rhizobacteria can contribute to the adaptation of the plants to drought habitats [50]. In this study, we isolated rhizobacterial strains from the cassava M. esculenta Crantz MCOL1734, which is a variety with high tolerance capacity against drought. This study represents the first characterization of rhizobacterial community associated with cassava plants under drought stress conditions. In total, 58 Gram-negative rhizobacteria were isolated from the roots of the cassava plants exposed to daily irrigation and drought stress conditions. Previously, some studies have reported that Gram-negative rhizobacteria dominates the rhizosphere of several crop plants of agricultural importance [51,52]. According to the identification process with the VITEK® 2, we found that all the rhizobacterial strains isolated from cassava roots belonged to 12 genera: Achromobacter, Acinetobacter, Aeromonas, Buttiauxella, Cronobacter, Klebsiella, Ochrobactrum, Pluralibacter, Pseudomonas, Rhizobium, Serratia, and Sphingomonas. These genera include bacterial species previously reported as PGPRs. Several studies have showed that these taxa are dominant in the roots and tissue of plants, in most of cases as drought stress alleviators to ameliorate crop production [10,23,53]. The diversity of rhizobacteria found here was wider than that reported previously for several cassava varieties from India and China, which only included genera such as Enterobacter, Klebsiella, Pseudomonas, and Serratia [31,32].

We found that both water-deficit stress and daily irrigation changed the diversity, abundance, and richness of Gram-negative endophytic bacterial communities of cassava roots; however, changes in the evenness were not detected. In this respect, the Shannon diversity index revealed that the species diversity of bacterial isolates from roots of cp-c and cp-ds plants were higher at T20 compared to T0, although the difference was only significant in control plants. Meanwhile, the evenness index reflected the uniformity of bacterial species distribution at T0 and T20 for both cp-c and cp-ds plants, contrary to the trend found for species diversity (Table 3). Thus, the water deprivation and full irrigation had no significant effect on evenness, which suggests little effect on the relative abundances of either dominant or minor taxa. In particular, Pseudomonas, Rhizobium, and Sphingomonas were the predominant bacterial genera that we found in the roots of both cp-c
and cp-ds plants of cassava. *Pseudomonas* spp. are characteristic in agricultural soils, being one of the most dominant genus commonly reported in many plant crops [54]. Several members of *Pseudomonas* genus have been widely studied as PGPRs that contribute to plant tolerance against abiotic stress [19,51,54] by producing a set of hormones involved in the growth and development of plants [26,53,55]. In this study, we observed that species such as *P. fluorescens*, *P. putida*, *P. mendocina*, *P. stutzeri*, and *P. luteola* prevailed in all plants throughout the treatment. A previous study reported that *P. fluorescens* produce higher amounts of 1-aminocyclopropane-1-carboxylate (ACC) deaminase that can promote the plant growth under drought stress conditions [20,50], whereas *P. putida* can induce the IAA production that plays a key role in both root and shoot development in plants [56]. Interestingly, we found that *P. mendocina* and *P. stutzeri* were species shared between control plants and plants exposed to drought stress, whereas *P. luteola* was isolated exclusively from plants submitted to drought conditions for 20 days. A previous study reported the role of these PGPR–mediating drought stress tolerance in plants through several mechanisms. The inoculation of lettuce under moderate and severe drought stress with *P. mendocina* significantly enhanced the phosphatase activity in roots, and the activities of nitrate reductase (N), peroxidase and catalase, and the proline accumulation in leaves [57]. Maize plants inoculated with *Pseudomonas* spp., including *P. putida* and *P. stutzeri*, developed protection against drought stress by reducing the activity of antioxidant enzymes [58]. Meanwhile, *P. luteola* played a key role during the development of roots in *Malus domestica* due to an increased production of IAA, siderophores, and biosurfactants, as well as the solubilization of organic and inorganic phosphorus [59]. In this respect, strains of *Pseudomonas* spp. isolated from cassava roots in this study could be considered excellent candidates to promote plant growth under drought stress conditions.

By contrast, orders such as *Rhizobiales* and *Sphingomonadales* include several PGPR that elicit plant drought tolerance [23]. In this respect, we detected the isolates of *R. radiobacter* and *S. paucimobilis* in the roots of cassava plants after 20 days of drought stress. *Rhizobium* spp. can produce CK that increase the development of principal and adventitious roots [60,61]. In addition, when plants of *Phaseolus vulgaris* were inoculated with *Rhizobium* spp. overexpressing the trehalose-6-phosphate synthase gene, an increase in the drought tolerance was observed [62]. Regarding *Sphingomonas* genus, some species have the capacity to grow around the root zone and promote the plant growth by producing primary metabolites [63]. *Sphingomonas* spp. synthesize siderophores that favor and promote the absorption of minerals and other ions by plants [64]. The inoculation of *Dendrobiurn officinale* with *S. paucimobilis* promoted the growth of seedlings through a combination of phytohormones and nitrogen fixation [63]. Moreover, *Arabidopsis thaliana* plants under drought stress increased the growth rate when they were inoculated with strains of *Shigromonas* sp. [65]. Therefore, the rhizobacterial strains of *R. radiobacter* and *S. paucimobilis* identified in this study have a great potential as PGPR strains that could contribute to plant tolerance against drought stress conditions.

Furthermore, we also identified other PGPRs genera such as *Acinetobacter*, *Achromobacter*, *Aeromonas*, *Klebsiella*, *Serratia*, and *Ochrobactrum*, but they were less abundant in the cassava roots. According to previous reports, several species of these rhizobacterial genera can induce the increase in plant growth and resistance to abiotic stresses through various mechanisms [20]. In this respect, *Acinetobacter* spp. have played an essential role in the alleviation of drought stress in the plants of *Vigna radiata* and *Vitis vinifera* through the production of IAA [66]. Likewise, strains of *Serratia* sp. induced drought tolerance in cucumber plants and promoted growth through IAA, ACC deaminase activity, and production of CK, siderophore production, and hydrogen cyanide [67]. *Achromobacter* spp. alleviated the adverse effects caused for abiotic stresses, including drought and oxidative stress, in tomato and peppers plants [68]. Thus, the inoculation of plants with *Achromobacter* sp. increased the biomass and significantly contributed to the reduction of ethylene levels in the stressed plants [68]. Finally, *Ochrobactrum* spp. strains grow in environments where water availability is a limiting factor [69]. This bacterial genus is involved in the release of
organic acids and the efflux of hydrogen peroxide, which is a way of drought tolerance in plants [50]. Interestingly, we found that O. anthropi is exclusively present in cassava roots under water-deficit conditions, further suggesting that this rhizobacterial species is contributing to enhancing the tolerance to drought stress in cassava plants.

5. Conclusions

This study’s results showed that the Gram-negative rhizobacteria community was associated with M. esculenta Crantz MCOL1734 using culture-dependent approaches. A wider diversity of the root-associated bacterial microbiome was found in these plants. Our results demonstrate that the water-deficit stress clearly influences the species diversity of rhizobacterial community associated with cassava. In this respect, the water deprivation leads to changes in the diversity, relative abundances, and species richness of Gram-negative endophytic bacterial communities associated with cassava roots; however, no significant effect on evenness index of rhizobacterial species was found after drought stress assay. Moreover, a great diversity of bacterial species reported as PGPR species in previous studies were isolated from the roots of cassava plants under drought stress conditions and then identified. The rhizobacterial strains could improve the resistance and tolerance of cassava plants to drought throughout water stress while maintaining their viability. The cassava roots constitute a great reservoir of potential Gram-negative rhizobacteria with remarkable biotechnological applications. These strains can be used for including plant inoculation or development of bio-inoculants to improve the drought tolerance of plant crops under water-deficit conditions.

Author Contributions: Conceptualization, A.R.C.-D. and I.D.O.-I.; methodology, T.Z. and D.M.G.; formal analysis, T.Z., D.M.G., A.R.C.-D. and I.D.O.-I.; investigation, T.Z., D.M.G., A.R.C.-D. and I.D.O.-I.; resources, A.R.C.-D. and I.D.O.-I.; data curation, T.Z. and I.D.O.-I.; writing—original draft preparation, T.Z. and I.D.O.-I.; writing—review and editing, I.D.O.-I.; visualization, T.Z. and I.D.O.-I.; supervision, A.R.C.-D. and I.D.O.-I.; project administration, A.R.C.-D.; funding acquisition, A.R.C.-D.

All authors have read and agreed to the published version of the manuscript.

Funding: The Dirección General de Investigaciones of Universidad Santiago de Cali under grant number DGI: 511-621117-C7 funded this research.

Acknowledgments: The authors thank Luis Augusto Becerra who is the leader of Cassava Program at International Center for Tropical Agriculture (CIAT) for supporting the execution of this study, German Patiño Romero for technical assistance, and Sandra Rivera Sanchez for her technical support at the laboratory. This research has been funded by Dirección General de Investigaciones of Universidad Santiago de Cali under call No. 01-2021.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Howeler, R.; Lutaladio, N.; Thomas, G. Save and Grow: Cassava, a Guide to Sustainable Production Intensification; Food and Agriculture Organization of the United Nations (FAO), Ed.; FAO: Rome, Italy, 2013.
2. Wanapat, M.; Kang, S. Cassava chip (Manihot esculenta Crantz) as an energy source for ruminant feeding. Anim. Nutr. 2015, 1, 266–270. [CrossRef]
3. Santos, V.L.F.; Ferreira, M.A.; Siqueira, M.C.B.; Melo, T.T.B.; Silva, J.L.; Andrade, I.B.; Soares, A.A.; Costa, C.T.F. Rumen parameters of sheep fed cassava peel as a replacement for corn. Small Rumin. Res. 2015, 133, 88–92. [CrossRef]
4. Jauhar, P.P. Modern biotechnology as an integral supplement to conventional plant breeding: The prospects and challenges. Crop Sci. 2006, 46, 1841–1859. [CrossRef]
5. Tofíño, A.; Ceballos, H.; Romero, H.M. Posibilidades de expansión del cultivo de yuca (Manihot esculenta Crantz) en el Caribe ecológico colombiano a partir de investigación multidisciplinaria. Actual. Biológicas 2008, 30, 15–27.
6. El-Sharkawy, M.A. Drought-tolerant Cassava for Africa, Asia, and Latin America. Bioscience 1993, 43, 441–451. [CrossRef]
7. El-Sharkawy, M.A. Stress-tolerant cassava: The role of integrative ecophysiology-breeding research in crop improvement. Open J. Soil Sci. 2012, 2, 162–186. [CrossRef]
8. Pardales, J.R., Jr.; Esquibel, C.B. Effect of drought during the establishment period on the root system development of cassava. Jpn. J. Crop Sci. 1996, 65, 93–97. [CrossRef]
9. Hoekstra, F.A.; Golovina, E.A.; Buitink, J. Mechanisms of plant desiccation tolerance. Trends Plant Sci. 2001, 6, 431–438. [CrossRef]
10. Kaushal, M.; Wani, S.P. Plant-growth-promoting rhizobacteria: Drought stress alleviators to ameliorate crop production in drylands. *Ann. Microbiol.* **2016**, *66*, 35–42. [CrossRef]

11. Agili, S.M.; Pardales, J.R., Jr. Influence of moisture and allelopathic regimes in the soil on the development of cassava and mycorrhizal infection of its roots during establishment period. *Philipp. J. Crop Sci.* **1997**, *22*, 99–105.

12. Hershey, C.H.; Alvarez, E.; Aye, T.M.; Becerra, L.A.; Bellotti, A.C.; Ceballos, H.; Fahney, K.; Howeler, R.H.; Lefroy, R.D.B.; Osipina, B.; et al. Eco-efficient interventions to support cassava’s multiple roles in improving the lives of smallholders. In *Ecoefficient: From Vision to Reality*; CIAT: Cali, Colombia, 2012.

13. IWMI. *Water for Food, Water for Life. A Comprehensive Assessment of Water Management in Agriculture*; Molden, D., Ed.; IMWI: Colombo, Sri Lanka; Earthscan: London, UK, 2007.

14. Kawano, K. Cassava. In *Hybridization of Crop Plants*; Fehr, W.R., Hadley, H.H., Eds.; ASA & CSSA: Madison, WI, USA, 1980; pp. 225–233.

15. Kawano, K. Thirty years of cassava breeding for productivity—Biological and social factors for success. *Crop Sci.* **2003**, *43*, 1325–1335. [CrossRef]

16. Ceballos, H.; Hershey, C.H.; Becerra-López-Lavalle, L.A. New Approaches to Cassava Breeding. In *Plant Breeding Reviews, Volume 36*; Janick, J., Ed.; Wiley-Blackwell: Hoboken, NJ, USA, 2012; pp. 427–504.

17. Farooq, M.; Wahid, A.; Kobayashi, N.; Fujita, D.; Basra, S.M.A. Plant drought stress: Effects, mechanisms and management. *Agron. Sustain. Dev.* **2009**, *29*, 185–212. [CrossRef]

18. Turyagyenda, L.F.; Kizito, E.B.; Ferguson, M.; Baguma, Y.; Agaba, M.; Harvey, J.J.W.; Osiru, D.S.O. Physiological and molecular characterization of drought responses and identification of candidate tolerance genes in cassava. *Aob Plants* **2013**, *5*, 1–17. [CrossRef]

19. Mutumba, F.A.; Zagal, E.; Gerding, M.; Castillo-Rosales, D.; Paulino, L.; Schoebitz, M. Plant growth promoting rhizobacteria for improved water stress tolerance in wheat genotypes. *J. Soil Sci. Plant Nutr.* **2018**, *18*, 1080–1096. [CrossRef]

20. Etesami, H.; Maheshwari, D.K. Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: Action mechanisms and future prospects. *Ecotoxicol. Environ. Saf.* **2018**, *156*, 225–246. [CrossRef]

21. Etesami, H.; Allikhani, H.A.; Mirseyed Hosseini, H. Indole-3-Acetic Acid and 1-Aminocyclopropane-1-Carboxylate Deaminase: Bacterial Traits Required in Rhizosphere, Rhizoplane and/or Endophytic Competence by Beneficial Bacteria. In *Bacterial Metabolites in Sustainable Agroecosystem. Sustainable Development and Biodiversity*; Maheshwari, D., Ed.; Springer: Cham, Switzerland, 2015.

22. Etesami, H.; Beattie, G.A. Plant-Microbe Interactions in Adaptation of Agricultural Crops to Abiotic Stress Conditions. In *Probiotics and Plant Health*; Kumar, V., Kumar, M., Sharma, S., Prasad, R., Eds.; Springer: Singapore, 2017.

23. Vurukonda, S.S.K.P.; Vardharajula, S.; Shrivastava, M.; SkZ, A. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* **2016**, *184*, 13–24. [CrossRef]

24. Berjak, P. Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Sci. Res.* **2012**, *22*, 1–15. [CrossRef]

25. Ryu, C.M.; Farag, M.A.; Hu, C.H.; Reddy, M.S.; Kloeper, J.W.; Paré, P.W. Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol.* **2004**, *134*, 1017–1026. [CrossRef] [PubMed]

26. Kim, W.L.; Cho, W.K.; Kim, S.N.; Chu, H.; Ryu, K.Y.; Yun, J.C.; Park, C.S. Genetic diversity of cultivable plant growth-promoting rhizobacteria in Korea. *J. Microbiol. Biotechnol.* **2011**, *21*, 777–790. [CrossRef]

27. Hartman, K.; Tringe, S.G. Interactions between plants and soil shaping the root microbiome under abiotic stress. *Biochem. J.* **2019**, *476*, 2705–2724. [CrossRef] [PubMed]

28. Zhang, L.; Zhang, J.; Wei, Y.; Hu, W.; Liu, G.; Zeng, H.; Shi, H. Microbiome-wide association studies reveal correlations between the structure and metabolism of the rhizosphere microbiome and disease resistance in cassava. *Plant Biotechnol. J.* **2021**, *19*, 689–701. [CrossRef] [PubMed]

29. Frediansyah, A. The Microbiome of Cassava (Manihot esculanta). In *Cassava—Biology, Production, and Application*; Frediansyah, A., Ed.; Headquarters IntechOpen Limited: London, UK, 2021; ISBN 978-1-83968-909-3.

30. Kawano, K. Thirty years of cassava breeding for productivity—Biological and social factors for success. *Crop Sci.* **2003**, *43*, 1325–1335. [CrossRef]

31. Suja, S.P.; Hegde, V.; Makeshkumar, T.; Anjanadevi, I.P. Screening of rhizobacteria associated with cassava for plant growth promotion and biocontrol potential. *J. Root Crop.* **2014**, *40*, 66–73.

32. Li, H.; Yan, C.; Tang, Y.; Ma, X.; Chen, Y.; Chen, S.; Lin, M.; Liu, Z. Endophytic bacterial and fungal microbiota in different cultivars of cassava (*Manihot esculenta* Crantz). *J. Microbiol.* **2020**, *58*, 614–623. [CrossRef]

33. Freitas, M.A.; Medeiros, F.H.V.; Carvalho, S.P.; Guilherme, L.R.G.; Teixeira, W.D.; Zhang, H.; Paré, P.W. Augmenting iron accumulation in cassava by the beneficial soil bacterium Bacillus subtilis (GB03). *Front. Plant Sci.* **2015**, *6*, 596. [CrossRef]

34. Zeng, H.; Xu, H.; Liu, G.; Wei, Y.; Zhang, J.; Shi, H. Physiological and metagenomic strategies uncover the rhizosphere bacterial microbiome succession underlying three common environmental stresses in cassava. *J. Hazard. Mater.* **2021**, *411*. [CrossRef]

35. Barnard, R.L.; Osborne, C.A.; Firestone, M.K. Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *ISME J.* **2013**, *7*, 2229–2241. [CrossRef] [PubMed]
64. Takeuchi, M.; Sakane, T.; Yanagi, M.; Yamasato, K.; Hamana, K.; Yokota, A. Taxonomic study of bacteria isolated from plants: Proposal of Sphingomonas rosa sp. nov., Sphingomonas pruni sp. nov., Sphingomonas asaccharolytica sp. nov., and Sphingomonas mali sp. nov. Int. J. Syst. Bacteriol. 1995, 45, 334–341. [CrossRef] [PubMed]

65. Luo, Y.; Wang, F.; Huang, Y.; Zhou, M.; Gao, J.; Yan, T.; Sheng, H.; An, L. Sphingomonas sp. Cra20 increases plant growth rate and alters rhizosphere microbial community structure of Arabidopsis thaliana under drought stress. Front. Microbiol. 2019, 10, 1221. [CrossRef] [PubMed]

66. Rolli, E.; Marasco, R.; Vigani, G.; Ettoumi, B.; Mapelli, F.; Deangelis, M.L.; Gandolfi, C.; Casati, E.; Previtali, F.; Gerbino, R.; et al. Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environ. Microbiol. 2015, 17, 316–331. [CrossRef]

67. Wang, C.J.; Yang, W.; Wang, C.; Gu, C.; Niu, D.D.; Liu, H.X.; Wang, Y.P.; Guo, J.H. Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting rhizobacterium strains. PLoS ONE 2012, 7, e52565. [CrossRef] [PubMed]

68. Ojuederie, O.B.; Olanrewaju, O.S.; Babalola, O.O. Plant growth promoting rhizobacterial mitigation of drought stress in crop plants: Implications for sustainable agriculture. Agronomy 2019, 9, 712. [CrossRef]

69. Mishra, S.K.; Khan, M.H.; Misra, S.; Dixit, V.K.; Khare, P.; Srivastava, S.; Chauhan, P.S. Characterisation of Pseudomonas spp. and Ochrobactrum sp. isolated from volcanic soil. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 2017, 110, 253–270. [CrossRef]