Frequency and biodiversity of 2,4-diacyethylphloroglucinol-producing rhizobacteria are differentially affected by the genotype of two maize inbred lines and their hybrid

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Abstract

Rhizobacteria (2808) were isolated on Pseudomonas-selective S1 medium from two maize inbred lines and from their hybrid at three plant growth stages. Positive phlD hybridization was found for 364 of them. The PhlD⁺ isolates were significantly more numerous in the rhizosphere of the hybrid than in those of parental lines. Furthermore, the frequency of PhlD⁺ was significantly higher for the hybrid at the flowering stage. An amplified rDNA restriction analysis showed that the hybrid genotype also increases the genetic diversity of PhlD⁺ populations when compared with its inbred parent lines, and this could be an effect of heterosis. Influence of the hybrid on the frequency and diversity of the bacterial PhlD⁺ population varied along the plant growth stage.

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Keywords: Plant growth-promoting rhizobacterium; 2,4-diacyethylphloroglucinol; Zea mays L.; Biodiversity; Heterosis

1. Introduction

In nature, the roots of plants interact with a large number of different microorganisms, and these interactions, together with soil and climatic conditions, are major determinants of the extent to which plants grow and spread [1]. Among soil microorganisms, several bacterial genera are known to have beneficial interactions with plants, since they suppress disease, enhance growth, fix atmospheric nitrogen, and solubilize phosphorus and other nutrients [2–6].

The ability of beneficial bacteria to suppress soilborne fungal pathogens depends, among several mechanisms, on efficient root colonization and production of antibiotic metabolites, such as pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid, and 2,4-diacyethylphloroglucinol (DAPG) [7–9]. The latter, DAPG, is considered a major factor in the biological control of a range of plant pathogens [10–12]. Bacteria able to produce DAPG play a key role in agricultural environments, and their potential for use in sustainable agriculture is promising. However, performance of root colonisation and biocontrol activity by DAPG-producing bacteria is affected by a great variety of biotic and abiotic factors [13]. For example, it is now widely acknowledged that the structure and activity of DAPG-producing bacterial populations naturally occurring in soils are modified in the rhizosphere of different plant species [7,14,15], in relation to the plant development stages [16,17] and to the root proximity [16].

To our best knowledge, the literature reports data on the diversity of DAPG-producing bacterial communities from plants of diverse species or from cultivars of the same species [14], whereas data for hybrid and respective
parent lines are not available. Taking into account that plant breeding can indirectly alter root morphology and physiology, as well as root exudation [18,19], one can hypothesise that plant breeding might also affect the structural and functional diversity of DAPG-producing bacterial communities as a result of plant genetic variation and heterosis. However, the effect of plant breeding on the diversity of DAPG-producers is not resolved enough to be understood. Thus, the aim of this research was to determine the abundance of rhizobacterial DAPG-producing populations and their genetic diversity as affected by the genotype of two maize (Zea mays L.) inbred lines and their hybrid.

2. Materials and methods

2.1. Bacterial strains

Five DAPG-producing reference strains were used. Strains 3-1 and 16-31 were isolated from maize in France [16], Q2-87 from an other monocotyledonous plant (wheat) in United States [9], CHA0 and Pf5 from dicotyledonous plants, in Switzerland and United States, respectively [3,6].

2.2. Plant material

Three maize genotypes were used: the inbred lines Lo964 and Lo1016, and their hybrid, Lo964 × Lo1016. The parents have been developed at the Experimental Institute of Cereal Crops, Bergamo, Italy, and were chosen because of their contrasting root morphology [20]. Lo964 is characterized by a very intensive root system (a dominating primary root), resulting in a deep and poorly ramified root system. Lo1016 develops a superficial and extensive root system (uniform root types). Roots of the hybrid Lo964 × Lo1016 are highly ramified, and develop both in the surface and in deeper layers.

2.3. Field experiments

The two parental inbred lines and their hybrid were sowed in spring 2002 at Cadriano (44°33’ N, 11°24’ E; Po valley, northern Italy) on a fine silty, mixed, mesic soil. All maize genotypes were tested in two trials differing in the date of sowing: the first one was sowed on 2nd May 2002, and the second one three weeks later (23rd May 2002). These two trials allowed us to mimic in the field two climatic conditions without any change in soil environmental factors (Table 1).

The field experimental layout of each trial was a randomized complete block design with three replications, adopted in order to control possible soil variation in the field. Plots were single rows 5.2 m long and spaced 0.80 m apart, and included 18 plants each. Fertilizers were applied at rates of 44 kg ha⁻¹ for P (before sowing) and 200 kg ha⁻¹ for N (half before sowing and half after thinning), while K was not applied because of its high availability in the soil. Weeds were removed mechanically. Sprinkler irrigation was made according to the crop requirements.

2.5. Selection of DAPG-producing isolates

DAPG-producing isolates were selected by colony hybridization analysis with a phI/D specific probe. For

<table>
<thead>
<tr>
<th>Plant growth stage</th>
<th>Warmest air temperature (°C)</th>
<th>Coldest air temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eg</td>
<td>A²</td>
<td>23.0</td>
</tr>
<tr>
<td>Ef</td>
<td>B²</td>
<td>29.6</td>
</tr>
<tr>
<td>Pm</td>
<td>A²</td>
<td>29.0</td>
</tr>
</tbody>
</table>

²A, maize plants sowed the 2nd May and B, maize plants sowed the 23rd May.
each sample, 52 randomly selected colonies were picked and transferred to new S1 medium plates. A total of 2808 bacterial colonies were collected in this way (52×3 plants×3 maize genotypes×3 stages of plant growth×2 dates of sowing). These 2808 bacterial colonies were transferred to nylon membranes (Roche Molecular Biochemicals) according to the manufacturer recommendation. The membranes were baked for 1 h at 80 °C and then were treated with a 2 mg ml⁻¹ proteinase K solution (0.5 ml for a membrane that was 82 mm in diameter) for 1 h at 37 °C. To remove bacterial cell debris from colony blots, the membranes were blotted between pieces of filter paper wetted with distilled water, and pressure was applied by passing a ruler over the area. The hybridization buffer used contained 50% formamide, 5×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), and 2% blocking reagent (Roche Molecular Biochemicals). Prehybridization was performed for 1 h at 42 °C, and hybridization was performed overnight at 42 °C. The membranes were washed twice for 5 min at room temperature in 2×SSC–0.1% SDS and twice for 15 min at 68 °C in 0.5×SSC–0.1% SDS.

The probe used was a 745-bp DNA fragment obtained by amplifying the DNA from DAPG-producing strain _P. fluorescens_ 3-1 [16] with primers Phl2a and Phl2b [22], which were designed on the basis of the sequence of the _phlD_ gene, one of the six clustered genes involved in DAPG biosynthesis [23]. Amplification reactions were carried out by using the protocol described below. The nucleotide sequence of the PCR product was verified by sequencing (Genome Express) before labeling the probe, which was done by using the non-radioactive digoxigenin system in accordance with the protocol recommended by the manufacturer (Roche Molecular Biochemicals). The hybridized probe was immunodetected with anti-digoxigeninalkaline phosphatase-Fab fragments and was visualized with the colorimetric substrate nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate, as described in the protocols provided by the supplier. Isolates that gave an hybridization signal with the _phlD_ gene were hereafter referred to as _PhlD⁺_ isolates. All _PhlD⁺_ isolates were preserved at −80 °C in 40% glycerol.

2.6. Amplified rDNA restriction analysis (ARDRA)

The DNA encoding the 16S rRNA of each _PhlD⁺_ isolate was amplified using the universal primers fD1 and rD1 [24], corresponding to positions 8-27 and 1524–1540 on the _Escherichia coli_ rrs gene sequence. This primer pair is capable of amplifying nearly full-length 16S ribosomal DNA from a wide variety of bacterial taxa [24]. Amplification was performed as previously described [16]; each mixture contained 2 μl of

lysed cell suspension in 25 μl of reaction buffer containing 1.5 mM MgCl₂, 150 ng of each primer, each deoxynucleoside triphosphate at a concentration of 250 mM, and 0.5 U of Taq DNA polymerase. The reaction mixtures were incubated in a thermocycler at 95 °C for 1.5 min and then subjected to 35 cycles consisting of 95 °C for 30 s, the annealing temperature for 30 s, and 72 °C for 4 min. The annealing temperature was 60 °C for the first five cycles, 55 °C for the next five cycles, and 50 °C for the last 25 cycles. Finally, the mixtures were incubated at 72 °C for 10 min and then at 60 °C for 10 min. Five microliter of each amplification mixture was analyzed by agarose (1.2% w/v) gel electrophoresis in Tris-borate-EDTA (TBE) buffer. Restriction analysis was performed with 5 μl of amplified product and 15 μl of restriction buffer containing 3 U of either _AluI_, _HinfI_, _MspI_ or _RsaI_ (Roche Molecular Biochemicals). After a 3-h digestion at the appropriate temperature, the enzyme was inactivated by heating the preparations at 70 °C for 15 min, and the restriction fragments were separated by gel electrophoresis (2% agarose) in TBE buffer. A 1-kb DNA ladder (Invitrogen) was used as molecular size marker. For each isolate, PCR amplification and restriction analysis were performed at least three times.

2.7. Statistical analysis

The frequency of _PhlD⁺_ isolates was subjected to analysis of variance (ANOVA) according to a split-plot design with three replications, repeated in time (sowing dates). The genotypes were considered as whole units and the plant growth stages as subunits. Moreover, the two degrees of freedom corresponding to the variation among genotypes were further partitioned in “Lo964 vs. Lo1016” and “inbreds vs. hybrid”, with one degree of freedom each. Data were subjected to angular transformation before ANOVA and non-transformed means are reported in the figures.

The analysis of molecular variance (AMOVA) procedure was used to estimate the variance components for ARDRA patterns by partitioning the variations among maize genotypes, plant growth stages and/or climatic conditions. The AMOVA technique is a method for analyzing molecular variance that produces estimates of variance components reflecting the correlation of haplotypic diversity at different levels of a hierarchical subdivision. The significance of the variance components is tested by a permutational approach [25]. The vectors for the presence of ARDRA markers (1 for the presence of each band on a gel; 0 for the absence of each band on a gel) for each strain were used to compute the genetic distance for each pair of strains. The parameter used was the Euclidean metric measurement (E) of Excoffer et al. [25], as defined by Huff et al. [26] as follows: $E = \sqrt{(1 - 2n_{xy}/2n)}$, where $2n_{xy}$ is the number...
of markers shared by two strains and \( n \) is the total number of polymorphic sites. All analyses were performed by the Arlequin program [27], which is used in several scientific fields (microbiology, medicine, population genetics) and is available at the following URL: http://anthropologie.unige.ch/arlequin/.

3. Results

3.1. Isolation of PhlD\(^{+}\) bacterial strains

Bacteria isolated on solid S1 medium were counted seven days after plating for each of the three maize genotypes examined. Climatic conditions, plant growth stages and plant genotype had no effect on the densities of the total cultivable bacterial populations, since they ranged independently from \(4 \times 10^7\) to \(2 \times 10^8\) cfu g\(^{-1}\) of dry roots.

Positive \( phlD \) hybridisation was found for 364 isolates of the total of 2808 colonies randomly tested, corresponding to the frequency of 13.0\%. Climate, mimicked by the two different date of sowing, had no significant influence on the frequency of the PhlD\(^{+}\) isolates, since 175 of them were isolated from the rhizospheres of plants sowed on the first date (2nd May), and the other 189 from plants of the second sowing date (May 23), corresponding to frequencies of 12.5\% and 13.5\%, respectively. On the contrary, ANOVA (not shown) showed a highly significant variation among genotypes due to the contrast “inbreds’ mean vs. hybrid”, while the contrast “Lo1016 vs. Lo964” was non-significant. In particular, three times more PhlD\(^{+}\) isolates (\( P = 0.0001 \)) were obtained from the rhizosphere of the hybrid genotype (214 PhlD\(^{+}\) isolates, corresponding to 22.9\%) than from the rhizosphere of the inbred lines (74 and 76 PhlD\(^{+}\) colonies were obtained from Lo964 and Lo1016, corresponding to 7.9\% and 8.1\%, respectively). Plant age also had a significant influence on the number of PhlD\(^{+}\) isolates. The frequency of PhlD\(^{+}\) isolates increased from the germination stage (60 isolates, corresponding to 16.5\% of the total PhlD\(^{+}\) isolates) to the flowering stage (242 PhlD\(^{+}\) isolates, corresponding to 66.5\%), and decreased with the physiological maturation (62 PhlD\(^{+}\) isolates, corresponding to 17.0\%). This phenomenon was observed for both sowing dates and was particularly evident for the hybrid (Fig. 1(a) and (b)). ANOVA (not shown) revealed that also the interactions were significant, even though the magnitudes of corresponding variances were much lower than those of main factors.

3.2. ARDRA analysis

The 16S rDNA of 348 out of 364 PhlD\(^{+}\) isolates were successfully amplified (data not shown). The remaining isolates were not investigated further, since they did not survive the cryoconservation in glycerol. The results of amplified 16S rDNA restriction analysis (ARDRA) with the four enzymes \( AluI, HinI, MspI, \) and \( RsaI \) were subjected to cluster analysis. The different patterns obtained with each of the four endonucleases were readily
distinguishable one from the other and highly reproducible from one experiment to the other. ARDRA revealed a considerable level of genetic diversity between the isolates, since 45 clusters were found (designated ARDRA-1 to ARDRA-45). Patterns obtained for the isolates were all different from the patterns obtained from the 16S rDNA of the five reference strains used. The restriction pattern of the main ARDRA clusters and of the reference strains are presented in Table 2. One cluster (ARDRA-1) was predominant and found in 238 (68.4%) isolates. Three other clusters, (ARDRA-2, ARDRA-3 and ARDRA-4), included 35 (10%), 21 (6%) and 13 (3.7%) isolates, respectively. Cluster ARDRA-1 was present in maize rhizosphere of plants from both sowing dates (the two climatic conditions), through the subsequent stages of plant growth and for the three plant genotypes (Table 3). All the three other main clusters (ARDRA-2, -3 and -4) were found for plants representing both climatic conditions, through the subsequent stages of plant growth and for the three plant genotypes (Table 3). All the three other main clusters (ARDRA-2, -3 and -4) were found for plants representing both climatic conditions. However clusters 3 and 4 were absent in early growth stage. As for their distribution among genotypes, clusters 2, 3 and 4 were found for only one of the two inbred lines, but were always present in the rhizosphere of the hybrid (Table 3). The other 41 clusters (ARDRA 5–45) were represented by only one isolate.

### 3.3. AMOVA analysis

Each ARDRA cluster (1–45) was compared with the other clusters, and an Euclidean distance matrix (E) was calculated (data not shown). Date of sowing (climatic conditions), maize genotypes and stages of plant growth were considered three different grouping criteria, to evaluate independently their respective effect on the biodiversity of PhlD⁻ isolates.

The AMOVA data revealed that the genetic variability between the PhlD⁺ isolates was poorly related to the date of sowing, independently on the maize genotype considered (not more than 1.81%) (Table 4). However, the genetic variability between the isolates of the two climatic conditions was not significant only in case of the hybrid.

To investigate the effect of maize genotype on the diversity of PhlD⁺ isolates, we analyzed all the maize genotypes together, as well as all the possible combinations of two maize genotypes. When considering all the three maize genotypes together, AMOVA data revealed that a part of the biodiversity of PhlD⁺ isolates could be attributed to the maize genotype. Interestingly, the data obtained (Table 4) clearly showed that the factor “maize genotype” varies its influence on the gen-

### Table 2

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>ARDRA cluster</th>
<th>Reference strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aal</em></td>
<td>1 380, 280, 210, 180</td>
<td>3-1 410, 240, 210, 200, 170</td>
</tr>
<tr>
<td><em>Hinf</em>I</td>
<td>2 410, 240, 210, 200, 170</td>
<td>Q2-87 410, 240, 210, 200, 170</td>
</tr>
<tr>
<td><em>Msp</em>I</td>
<td>3 410, 240, 210, 200, 170</td>
<td>CHA0 550, 390, 210, 100</td>
</tr>
<tr>
<td><em>Rsa</em>I</td>
<td>4 410, 240, 210, 200, 170</td>
<td>Pf5 550, 390, 210, 100</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>ARDRA cluster</th>
<th>Sowing date</th>
<th>Plant growth stage</th>
<th>Maize genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A  115</td>
<td>Eg  34</td>
<td>Lo964</td>
</tr>
<tr>
<td></td>
<td>B  123</td>
<td>Ef  172</td>
<td>Lo1016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pm  32</td>
<td>Lo964 × Lo1016</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a A, 2nd May; B, 23rd May.
b Eg, end of germination; Ef, end of flowering; Pm, physiological maturation.
c Lo964 and Lo1016, parental inbred lines; Lo964 × Lo1016, hybrid.
The genetic diversity of the bacterial PhiD⁺ community according to the plant growth stage from which the bacteria were isolated. In fact, when AMOVA analysis was done on bacteria isolated at the end of maize germinating stage, most of the genetic variability observed between PhiD⁺ isolates was due to differences between maize genotypes (70.57%, \( P = 0.03 \)). An important part of the biodiversity of the PhiD⁺ community isolated at the end of the growing season (phase of maturation) was also due to the maize genotypes (15.82% , \( P = 0.04 \)), while the effect of the maize genotypes on the biodiversity of PhiD⁺ isolates was the lowest when plants were at the flowering stage (1.03%, \( P = 0.002 \)). Considering all of the possible combinations of two maize genotypes, AMOVA data clearly revealed a higher effect of the plant genotypes on the biodiversity of PhiD⁺ isolates when confronting PhiD⁺ isolates of the hybrid vs. those of one inbred line (Lo964 as well as Lo1016), than when confronting together the two inbreds. It is worth noting that this phenomenon was observed independently from the plant growth stage analyzed.

Similar analyses carried out on plant growth stages showed that this factor contributed part of the genetic variability among PhiD⁺ isolates (4.27%, \( P = 0.01 \)). When considering maize genotypes individually, the plant growth stage effect on the biodiversity of the PhiD⁺ isolates seems to have the same tendency (up to 54.55% for the inbreed line Lo1016), even if those variance components are not significant.

4. Discussion

The difference among parental plant lines and their progeny regarding their effect on the rhizosphere microflora has often been analyzed for rhizobial and phytopathogenic populations. Published results indicate that host-controlled nodulation or resistance is inherited through generations [28,29], thus demonstrating a genetic basis. Less numerous are the results about the effect of the plant genotype on beneficial rhizobacterial populations and, to our knowledge, no data exist on the inheritance of the maize genotype effect on the rhizo-

### Table 4

<table>
<thead>
<tr>
<th>Variance component</th>
<th>Percentage of variance between sampling</th>
<th>Percentage of variance within sampling</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Climatic condition</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All maize genotypes A vs. B</td>
<td>1.30</td>
<td>98.70</td>
<td>0.009</td>
</tr>
<tr>
<td>Lo1016 A vs. B</td>
<td>1.21</td>
<td>98.79</td>
<td>0.012</td>
</tr>
<tr>
<td>Lo964 A vs. B</td>
<td>1.81</td>
<td>98.19</td>
<td>0.008</td>
</tr>
<tr>
<td>Lo964 × Lo1016 A vs. B</td>
<td><strong>1.34</strong></td>
<td><strong>98.66</strong></td>
<td><strong>0.22</strong></td>
</tr>
<tr>
<td><strong>Maize genotype</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All stages [Lo964] vs. [Lo1016] vs. [Lo964 × Lo1016]</td>
<td>3.43</td>
<td>96.57</td>
<td>0.009</td>
</tr>
<tr>
<td>[Lo964] vs. [Lo1016]</td>
<td><strong>1.91</strong></td>
<td><strong>98.09</strong></td>
<td><strong>0.19</strong></td>
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<tr>
<td>[Lo964] vs. [Lo964 × Lo1016]</td>
<td>4.00</td>
<td>96.00</td>
<td>0.01</td>
</tr>
<tr>
<td>[Lo1016] vs. [Lo964 × Lo1016]</td>
<td>8.53</td>
<td>91.47</td>
<td>0.02</td>
</tr>
<tr>
<td>Eg [Lo964] vs. [Lo1016] vs. [Lo964 × Lo1016]</td>
<td>70.57</td>
<td>29.43</td>
<td>0.03</td>
</tr>
<tr>
<td>[Lo964] vs. [Lo1016]</td>
<td>52.22</td>
<td>47.78</td>
<td>0.05</td>
</tr>
<tr>
<td>[Lo964] vs. [Lo964 × Lo1016]</td>
<td>71.17</td>
<td>28.83</td>
<td>0.04</td>
</tr>
<tr>
<td>[Lo1016] vs. [Lo964 × Lo1016]</td>
<td>72.22</td>
<td>27.78</td>
<td>0.03</td>
</tr>
<tr>
<td>Ef [Lo964] vs. [Lo1016] vs. [Lo964 × Lo1016]</td>
<td>1.03</td>
<td>98.97</td>
<td>0.002</td>
</tr>
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<td>[Lo964] vs. [Lo1016]</td>
<td>0.95</td>
<td>99.05</td>
<td>0.002</td>
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<tr>
<td>[Lo964] vs. [Lo964 × Lo1016]</td>
<td>2.25</td>
<td>97.75</td>
<td>0.005</td>
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<tr>
<td>[Lo1016] vs. [Lo964 × Lo1016]</td>
<td>5.58</td>
<td>94.42</td>
<td>0.01</td>
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<tr>
<td>Pm [Lo964] vs. [Lo1016] vs. [Lo964 × Lo1016]</td>
<td>15.82</td>
<td>84.18</td>
<td>0.04</td>
</tr>
<tr>
<td>[Lo964] vs. [Lo1016]</td>
<td>1.15</td>
<td>98.85</td>
<td>0.003</td>
</tr>
<tr>
<td>[Lo964] vs. [Lo964 × Lo1016]</td>
<td>16.76</td>
<td>83.24</td>
<td>0.04</td>
</tr>
<tr>
<td>[Lo1016] vs. [Lo964 × Lo1016]</td>
<td>52.00</td>
<td>48.00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Values in boldface type are not significant.**

<sup>a</sup> Probability of a more extreme variance distribution.

<sup>b</sup> A, sowing date the 2nd May; B, sowing date the 23rd May.

<sup>c</sup> Lo964 and Lo1016, parental inbred lines; Lo964 × Lo1016, hybrid.

<sup>d</sup> Eg, end of germination; Ef, end of flowering; Pm, physiological maturation.
bacterial DAPG-producing populations. Our experiments in the field showed that (i) the DAPG-producing bacteria were more abundant in the hybrid rhizosphere than in those of the two parental lines ($P = 0.0001$); (ii) the DAPG-producing populations were very diverse, and (iii) such genetic diversity was significantly higher for the hybrid than for the two parental lines. In addition, the influence of hybrid roots on the frequency of the bacterial DAPG-producing population varied along the plant growth stage, while climatic conditions did not show any significant influence.

Concerning the frequency of the DAPG-producing bacterial populations, we observed similar maize-genotype independent temporal shifts: DAPG-producers were few in the first stage of plant growth, dramatically increased at the flowering stage, and then decreased at the maturation phase. This result is consistent with published data for maize [16,30], as well as for other plant species [31–33], which showed similar temporal changes in Plant Growth Promoting Rhizobacteria (PGPR) populations. The present findings of a high frequency of DAPG-producers at the end of the flowering stage could be explained by assuming that there was a positive selection of these microorganisms due to a particular composition of the root exudates, which are known to evolve with plant development [1]. However, it is important to note that the shifts of the DAPG-producer frequency, here observed to be dependent on plant growth stages, were larger for the hybrid genotype than for the parents. Hence, we can speculate that the variation in root exudate was more pronounced for the hybrid than for the two parental lines. Unfortunately, literature does not yet account for such difference, since most of the studies were only done on a single hybrid [16], or in comparison with other hybrids (cultivars) [14,34], but not with parental lines.

Concerning the biodiversity of DAPG-producing bacteria, we could evidence, by ARDRA and AMOVA analysis, that the isolates from hybrid rhizosphere show more genetic diversity than those from both inbred lines at all growing stages. This was clearly observed when comparing variance between hybrid and parental lines of young plants, as well as of mature plants. The few literature reports on the diversity of PGPR populations colonizing different maize genotypes were done on different cultivars [30,35], none of them on hybrid and respective parent lines. Furthermore, they were generally done at only one stage of the plant growth. However, these reports are generally in accord with our findings on the hybrid genotype, when maize plants are considered at the same growth stage, and thus support them. For example, da Mota et al. [35] showed that the Paenibacillus polymyxa strains isolated from the rhizosphere of 90 days old maize plants (corresponding to the maturation phase) were genetically significantly diverse according to the cultivars. On the other hand, Dalmastri et al. [30] observed, on 60 days old maize plants (corresponding to the flowering stage), a very small cultivar effect on the genetic diversity of the root-associated Burkholderia cepacia populations.

The observation that genetic diversity and numerical differences among DAPG-producers were always greater between the hybrid and each of the parental lines than between the two parental lines, lets us suppose that the hybrid could have more chances than its parents of selecting strains with higher biocontrol potential, thus enhancing both its resistance to pathogenic fungi and its yield. The superiority of the hybrids in comparison with their parents, defined as heterosis by Shull [36], has been well described and exploited in case of maize [37,38]. Heterozygous maize hybrids display high vigour and also stability of yield across very different environmental conditions [38]. This is in accordance with the observation made in the present research that the genetic variability between the PhlD$^+$ isolates from the two climatic conditions was not significant in case of the hybrid.

The average superiority of a modern maize hybrid, with respect to its parental mean, ranges from 150% to over 300% for yield, and for the genotypes investigated in this study is about 200% (data not published). The biological basis of heterosis is still largely unknown, and the two main hypothesis that have been proposed as the genetic basis of heterosis are the dominance hypothesis and the overdominance hypothesis [38]. The first hypothesis suggests that favorable dominant alleles, complementary provided by each parental inbred line, mask deleterious recessive alleles in the heterozygote, while overdominance suggests that the heterozygote is inherently superior to the homozygotes. The interpretation of the most recent results is that probably both of them are correct [39]. However, studies on genome expression found that an heterotic hybrid do express more genes than the better parent [40], thus resulting in production of a higher variety of proteins [41]. As for our results, the most obvious explanation of the differences in DAPG-producers root colonization between the hybrid and its parental lines would be the existence of differences in the quantity and/or quality of root exudates. In fact, the two inbred lines have very contrasting root morphology [20], and different cold tolerance [42], and both these characteristics are known to be related to the composition of root exudates [43–46]. In this case, the hybrid could have inherited both the different, complementary, alleles from each parent and may have became able to select different microbial populations [33]. This interpretation is in agreement with the result, reported in the present experiment, that the level of genetic diversity among isolates was higher in the rhizosphere of the hybrid than in those of the inbred lines.

In the light of these observations, our results support the hypothesis that maize genotype influence on root
colonization by DAPG-producers is a quantitatively inherited trait. In the literature, it was demonstrated that different recombinant tomato inbred lines differentially support the growth of a biological control agent in the rhizosphere, after seed inoculation [47]. As colonization is an important feature of successful biocontrol [47,48], a maize breeding strategy directed toward hybrids that support large populations of DAPG-producers would be an interesting approach to the development of sustainable low input production systems. In fact, plant breeding programs are generally designed to improve crop agronomic characteristics such as yield, fertilizer use efficiency, and disease resistance. However, these programs do not generally take into the right account the interactions between plants and beneficial soil microflora. Our results gave evidence that it would be possible to increase the agronomic potential of maize while conserving soil and system sustainability, by a breeding program directed toward genotypes able to support large populations of DAPG-producers. In this context, we are extending field investigations on the frequency and diversity of DAPG-producers, as well as of rhizobacteria with other PGPR functions, on several couples of parents and hybrid of maize, and other crop plants. Furthermore, it will be interesting to investigate the genetic basis of plant ability to positively interact with disease-suppressive microflora.

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