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Effects of plant growth promoting rhizobacteria on field grown maize

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1 SUMMARY

Previous studies have shown the role of Plant Growth Promoting Rhizobacteria (PGPR) in various improved cropping systems The effects of 15 maize field isolated PGPR on non-degraded reddish ferrous field grown maize in southern Benin were studied to (i) characterize the maize specific PGPR; and (ii) establish the efficient maize specific PGPR inoculation for an improved maize growth and productivity. The experimental device is a block of Fischer to 4 repetitions with 16 treatments (15 rhizobacteria for maize seed inoculated at concentrations of about 10^8 bacterial CFU / ml and an uninoculated control). The data for plant height and number of leaves emerged were collected on 10 plants per treatment once a week from the 8th day after sowing. This study results revealed that *Azospirillium lipoferum, Pseudomonas fluorescens* and *P. putida* are the best PGPR candidates for maize crop improvement on reddish ferrous field. An increased root biomass of 59.57% and 23.40% was recorded with *Pseudomonas fluorescens* and *P. aeruginosa*, respectively, while other members of the 15 identified PGPR showed little or no significant growth promoting effect on maize crops compared to non-PGPR colonized maize field. This work suggests that PGPR represent one of the most promising biofertilizers to promote maize crop productivity and increased biomass yield in southern Benin.

2 INTRODUCTION

Maize is one of the most important cereal crops after wheat and rice in world. In southern Benin, the yield of maize has decreased significantly for various reasons (Badu-Apraku and Yallou, 2009). One of the major causes of re-occurring yield reduction is depletion of field nutrients combined with detrimental effects of poor land fertility. Recently, plant growth promoting rhizobacteria (PGPR) have been used to enhance crop yield and improve agriculture sustainability (Nezarat and Gholami, 2009). PGPR are a group of bacteria that colonize plant roots and promote growth and yield (Kloepper *et al.*, 1980; Wu *et al.*, 2005). The mechanisms by which PGPR promote plant growth are not fully understood (Vessey,

2003). However, PGPR are known to increase root system uptake properties of rhizobacteria colonized crops (Islam *et al.*, 2009) by facilitating ion nitrate adsorption, Phosphate solubilization, and iron chelation (Islam *et al.*, 2009). Maize seeds inoculated with rhizobacteria, *Pseudomonas cepacia*, *P. fluorescens* and *Streptomyces aurantiacus* in combination with 120 kg/hectare of nitrogen increased crop yield up to 25% more than the non rhizobacterium colonized maize control (Hernandez *et al.*, 1995). The effect of rhizobacterium-nitrogen combination was found to be 60% better than that of rhizobacterium alone (Shaharoona *et al.,* 2006). They showed that Pseudomona spp (with ACC-esaminase activity) combined with optimum concentration of nitrogenous fertilizers was able to provide a substantial corn yield. PGPR are believed to increase the supply/availability of primary nutrients to the host plant (Wu *et al.*, 2005), promoting the synthesis of antibiotics, enzymes and fungicidal compounds (Bharathi *et al.*, 2004; Jeun *et al.*, 2004; Ahmad *et al.*, 2006).

In the West African state of Benin Republic, maize is mainly produced by small scale farmers with little or no financial means to buy fertilizers. Implementing the use of PGPR in farming systems represents therefore, one of the most promising alternatives to improve maize yields not only in Benin but Africa at large. Up-to-date, there is little or no information on the use of rhizobacteria in maize yield improvement under field conditions in Benin Republic. The lack of field study has hindered the ability of national scientists in Benin to efficiently use these natural bio-fertilizers. The objectives of this work were to (i) characterize the maize specific PGPR; and (ii) establish the efficient maize specific PGPR inoculation for an improved maize growth and productivity.

3 MATERIALS AND METHODS

3.1 Geographical characterization of field work: This study was carried out in experimental fields of the Center of Agricultural Research in southern Benin, situated at an altitude 105°, longitude 2° 19' East and latitude 6° 12' north. The site is characterized by a maritime subequatorial climate made of two rain and two dry seasons with 1200 mm average pluviometry, which spread over 8 months (May to November) with maximum precipitations in June and October and minimum precipitations in August. The average temperature is around 27°C. The soil is characterized by a deep reddish ferrous soil with a pH 6.2 (Aïhou, 2003). The organic matter of the soil is 1.6% with an equivalent phosphorus content of 18.5 ppm. The passive ions such as potassium, calcium and magnesium are 0.2 meq/100 g; 4.7 meq/100 g and 1.7 meq/100 g of soil respectively (Adjanohoun *et al.*, 2011).

3.2 Rhizobacterial inoculum and maize seed treatment: The PGPR used in this work (Table 1) have been characterized in our previous report (Adjanohoun *et al.*, 2011). PGPR inoculum was prepared in LB growth media and incubated for 24 hours at 28-30°C for Pseudomonas sp, 37°C for Bacillus sp, 30°C for Streptomyces and Azospirillum sp. The inoculum was used at 10^8 UFC/ml for maize seed treatment.

For seed treatment in the field, maize variety EVDT 97 STR C1 (Badu-Apraku and Yallou, 2009), was used. Field plots of 4 m x 3.2 m (12.8 m^2) were considered. The experiment was arranged in a randomized design with four replicates and 16 batches of inoculated maize seeds were used. Two maize seeds were put together with the inoculums in about 5 cm deep well. 10 individual plants were considered per rhizobacterial colony. Four seeding lanes of 4 m long were made per field plot. The inter-row spacing within a plot was kept at 0.80 m and distance between hills within a row at 0.40 m resulting to a density of 31,250 plants/hectare.

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Table 1: Plant Growth Promoting Rhizobacteria identified from maize grown field soils in southern Benin.

Identified Plant Growth Promoting Rhizobacteria (PGPR)
Pseudomonas putida
Pseudomonas aeruginosa
Pseudomonas fluorescens
Streptomyces hygroscopicus
Streptomyces rimosus
Streptomyces fasciculatus
Azospirillium lipoferum
Bacillus coagulans
Bacillus thurengensis
Bacillus pumilus
Bacillus polymixa
Bacillus licheniformis
Bacillus lentus
Bacillus circulans
Bacillus fimosus

3.4 Data collection and statistical analysis: In order to evaluate the effects of PGPR on crop growth and subsequent yield, we measured the height and number of maize leaves (10 seedlings per inoculation) every week from 8 days post seeding until harvest. In addition, the root and shoot biomass sections of the plants were measured at harvest time (at 87 days post seeding). The height of maize plants was measured from the surface to the roof of the top sheet, which emerged more than 50%. (Describe the method that was used to measure the plant height.)

The data were subjected to a statistical analysis of variance where the inoculum treatment was considered as a fixed factor and the replications as a random factor. The least square means of every parameter were extracted from each 16 rhizobacterial treatment. These values were then subjected to a numerical classification (algorithm of Ward 1963) in order to generate homogenous rhizobacterial groups according to their similar morphological growth effects on colonized maize plants. The numerical classification is preferred to the traditional Newman and Keuls test of separation of means due to the high number of "Treatments" used in this study. When the number of data to be analyzed per factor is up to 16, the tests of separation of means using test of Student-Newman-Keuls become inaccurate (Dagnelie, 1998). Numerical classification and means of dendrometric parameters were used instead to establish and characterize the evolutionary relationship of homogenous rhizobacterial groups and to construct a dendrogram representation of identified rhizobacteria.

4 RESULTS

4.1 Effect of rhizobacteria on maize growth parameter: Base on functional similarity effects, PGPR were clustered according to the numerical classification depending on height recorded mean value for 10 consecutive weeks as depicted in the dendrogram (Figure 1). The dendrogram profile revealed three main groups (G1, G2 and G3) of rhizobacteria represented by 76.3% of similarity effect within the group. PGPR that has less than 76.3% similarity with a characterized PGPR group is considered to belong to another group. In t our study, G1 of PGPR was represented by *Azospirillium lipoferum*, *Pseudomonas fluorescens* and *Pseudomonas putida*. Group 2 PGPR was composed of *Pseudomonas aeruginosa, Bacillus coagulans, Bacillus thurengensis, Bacillus pumilus, Bacillus polymixa, Bacillus licheniformis, Bacillus lentus, Bacillus circulans and Bacillus fimosusaeruginosa*. Group 3 PGPR was composed of *Streptomyces hygroscopicus*, *Streptomyces fasciculatus* and *Streptomyces rimosus* (Figure 1).

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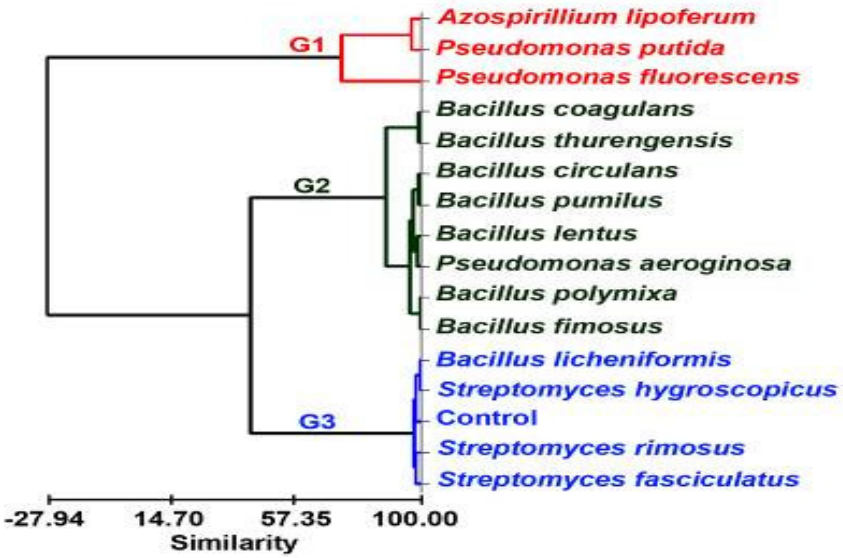

Figure 1: PGPR dendrogram representation depending on numeric classification of plant height adjusted averages

Not surprisingly, the Bacillus were clustered together in G2 and the Streptomyces were here clustered together with non inoculated plants used as negative control in G 3, indicating that G3 rhizobacteria showed reduced or no significant effect on maize growth development compared to the control. The rest of the 15 rhizobacteria (Azospirillium sp. and the Pseudomonas sp.) were clustered in G 1 (Figure 1). The G 1 PGPR (Azospirillium sp. and the Pseudomonas sp.) has the best promoting effect ($p < 0.001$) on the plant height followed by the G 2 PGPR, and the least was G3 PGPR (Figure 2). The effect of G 2 and G 3 on plant height was similar to each other until 63 days after inoculation. Above 63 days post inoculation, the effect of G 2 PGPR was significantly ($p < 0.001$) better than that of the G 3 PGPR (Figure 2). These results suggest that *Azospirillium lipoferum*, *Pseudomonas fluorescens* and *Pseudomonas putida* could be considered as the most prominent PGPR to improve maize crop development as revealed by higher average plant height data (224.27 cm) / at 77 days post inoculation. This data proved to be 8.25% higher than the height values recorded with *Pseudomonas aeruginosa, Bacillus coagulans, Bacillus thurengensis, Bacillus pumilus, Bacillus polymixa, Bacillus licheniformis, Bacillus lentus, Bacillus circulans* and *Bacillus fimosus* colonized maize crop.

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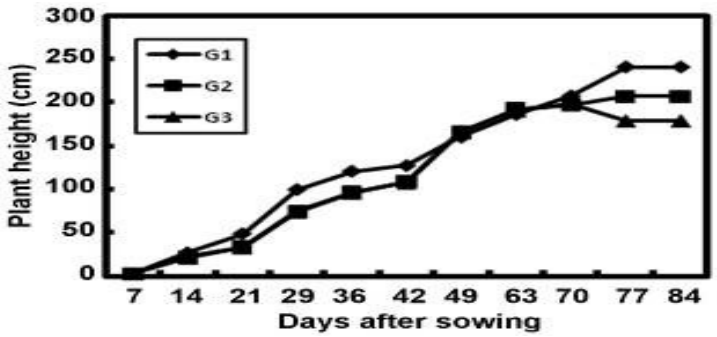

Figure 2: PGPR group dependent improvement of plant heights. G1 = Group 1 PGPR, G2 = Group 2 PGPR, G3 = Group 3 PGPR

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4.2 Effect of PGPR on emerging leaves: The effect of PGPR on maize crop improvement was further assessed by systematically counting the number of emerging leaves from the seedling emergence until the harvest. Numerical classification that applied to the least square means of the number of emerging leaves was used to classify the PGPRs. PGPRs belonging to the same group where classified by 71.4% of functional similarity within each other. The 15 PGPR were clustered into three main groups as represented by the dendrogram (Figure 3). The G1 is represented by *Azospirillum lipoferum*, *Bacillus thurengensis*, *Streptomyces fasciculatus*, *Bacillus fimosus*, *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus polymixa*, *Bacillus lentus* and *Bacillus pumilus*. The G2 was composed of *Bacillus coagulans, Pseudomonas fluorescens, Streptomyces hygroscopicus* and *Streptomyces rimosus*, while, G3was composed of *Pseudomonas aeruginosa* and *Pseudomonas putida*.

The overall effect of each group of PGPR on the emerging maize leaves was not statistically significant.

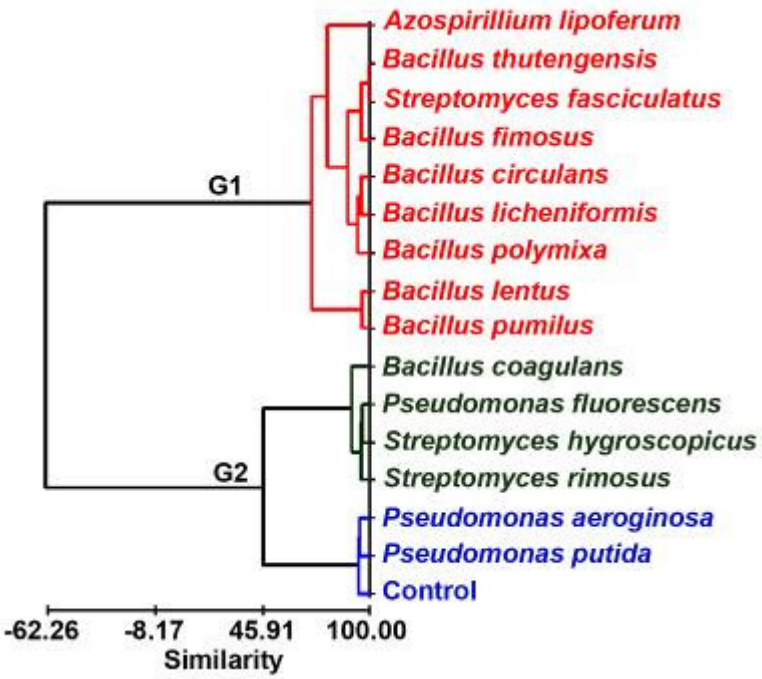

Figure 3: PGPR dendrogram representation depending on numeric classification of plant leaf number adjusted averages.

4.3 Effect of PGPR on shoot and root fresh biomass yield: The numerical classification applied to the recorded weight profile of shoot and root was used to establish different PGPR functional groups (Table 3). The PGPRs displaying 83.4% ($p < 0.001$) similar effect with each other belong to the same functional group. Accordingly, two PGPR with less than 83.4% ($p < 0.001$) of functional similarity with each other belong to two different groups. Our analysis revealed six different PGPR groups. Group 1 was composed of *Pseudomonas fluorescens* with an average shoot weight value of 127.5 g per rhizobacterial colonized plant compared to *Pseudomonas aeruginosa*, which belongs to G2 with a shoot weight value of 90 g per rhizobacterial colonized plant, while Pseudomonas putida belongs to G3 (Table 3).

Group 4 PGPR was composed of *Bacillus lentus* with an average shoot and root weight values ranging between 315 g and 327.5 g per colonized plant. *Bacillus licheniformis* and *Bacillus fimosus* belong to G 5 PGPR with an average shoot weight value of

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310 g per plant, while G 6 PGPR was composed of *Bacillus pumilus* and gives an average shoot weight value of 7.5 g per colonized plant. The non-colonized control plants were similar to the effect of G 3 PGPRs. Under this classification, *Bacillus circulans* belongs simultaneously to G 3 and G 4 PGPR. Similarly, *Azospirillum lipoferum, Streptomyces fasciculatus*, *Bacillus thurengensis*, *Streptomyces rimosus* and *Bacillus polymixa* belong simultaneously to G 4 and G 5 PGPRs, while *Bacillus coagulans* and *Streptomyces hygroscopicus* belong simultaneously to G5 and G 6 PGPRs. The two main PGPR groups with 89.2% of functional similarity within members of the same group were clearly identified based on their effect on shoot and root biomass promotion. Group 1 PGPR was, therefore, composed of the highest (11) members of PGPRs such as *Pseudomonas putida, P. aeruginosa, Streptomyces rimosus, S. hygroscopicus, Bacillus fimosus, B. polymixa, B. lentus, B. licheniformis, B. pumilus, B. circulans and B. thurengensis* with an average root fresh biomass weight ranging between 79 and 86 g per plant (Table 3).

G 2 PGPR was composed of a single member of rhizobacterium; *Pseudomonas fluorescens. Bacillus coagulans*, *Azospirillum lipoferum* and *Streptomyces fasciculatus* were members of PGPR that clustered under both g G 1 and G 2 with an average root fresh biomass weight of 73.5 g, 73.75 g and 75.75 g per plant, respectively, (Table 3).

Table 2: Analysis of variance on repeated measures on treatment and period dependent effect of PGPR on maize plant heights..

Source	DF	SSE	MSE	F	Prob.
Period (P)	9	3117344	346372	6075.1	<.0001
Rhizobacteria (Rh)	15	40460.5	2697.4	33.9	<.0001
P x Rh	135	59016.5	437.2	7.7	<.0001
Replication (R)	3	1278.4	426.1	5.4	0.003
P x R	27	4875.6	180.6	3.2	<.0001

DF: degree of freedom; SSE: Sum of square error; MSE: Mean square error; F: value of Fisher- Snedecor statistic; Prob.: Probability level.

Table 3: Biomass production of PGPR colonized maize plants

Treatment	Shoot biomass fresh Weight	Root biomass fresh Weight
Control	352.5 c	60.25 b
P. fluorescens	562.5 a	65.25 b
B. coagulans	302.5 ef	73.5 ab
A. lipoferum	325 de	73.75 ab
S. fasciculatus	315 de	75.75 ab
S. hygroscopicus	297.5 ef	79 a
B. thurengensis	325 de	79.75 a
B. circulans	342.5 cd	80 a
B. pumilus	302.5 f	81 a
P. aeruginosa	435 b	81.25 a
B. licheniformis	310 e	81.5 a
S. rimosus	317.5 de	81.75 a
B. polymixa	317.5 de	82 a
B. lentus	327.5 d	82.5 a
B. fimosus	310 e	83.25 a
P. putida	345 c	86 a

The means with different letters are significantly different with probability level of 1%.

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5 DISCUSSION

Understanding the biological mechanisms of PGPRs in improved crop production is a long-standing goal in agricultural sustainability. PGPRs have been known to promote growth when added to seeds, roots or tubers in a wide range of crop species (Kloepper *et al.*, 1980), increasing both growth and yield (Wu *et al.*, 2005). A key missing element in optimizing the use of PGPRs is the lack of available characterization of crop species-specific PGPRs that will be appropriate for species dependent improvement. In this study, we provide evidence for the use of *Azospirillium lipoferum*, *Pseudomonas fluorescens* and *Pseudomonas putida* as the best maize crop specific PGPRs to improve field maize crop development. We found that *Streptomyces hygroscopicus*, *Streptomyces fasciculatus* and *Streptomyces rimosus* do not have any special promoting effect on maize plants as revealed by plant height data in comparison with non-colonized control plants. However, these PGPR were also isolated and characterized from maize crop field, indicating that not all PGPRs isolated from a crop specific soil will display a positive crop promoting factor on corresponding crop species. The mechanisms by which PGPRs promote plant growth are not fully understood (Wessey, 2003). Members of PGPRs *(Azospirilliumlipoferum, Pseudomonas fluorescens and Pseudomonas putida*) isolated from maize grown field were found to have significant (p <0,001) growth promoting effect on maize crops, while the other members (*Pseudomonas aeruginosa, Bacillus coagulans, Bacillus thurengensis, Bacillus pumilus, Bacillus polymixa, Bacillus licheniformis, Bacillus lentus, Bacillus circulans and Bacillus fimosus*) showed little or no positive effect on maize crops compared to non colonized plants.

. In general, the maximum plant height was obtained with seed inoculated with *Azospirillium lipoferum*, *Pseudomonas fluorescens* and *Pseudomonas putida*. These, therefore, can be considered as the maize specific PGPRs to improve field maize crop productivity. Similar results have been reported on maize field trial with Azospirillium sp. treatment (Bashan and Holguin, 2004; Cakmaki *et al.*, 2006). Plant inoculation with Azospirillum sp. was found to induce significant changes of growth parameters, including plant height (Bashan and Holguin, 2004), confirming the usefulness and specificity of Azospirillum sp. as maize crop specific PGPR. Further, our study is supported by recent report of Kang *et al.*(2010), demonstrating that Bacillus spp, Pseudomonas spp and *Azospirillum lipoferum* have a significantly positive effect on maize and wheat improving the crop growth.. Furthermore, Nezarat and Gholami (2009) showed that *Pseudomonas putida* R-168, *P. fluorescens* R-93, *P. fluorescens* DSM 50090, *P. putida* DSM291, *Azospirillum lipoferum* 1691 DSM and *A. brasilense* 1690 DSM significantly improved maize seed germination and plant growth. These PGPRs have been reported to improve nitrogen uptake by the colonized plants compared to non-PGPR colonized plants (Shaharoona *et al.*, 2006). *Azospirillum lipoferum* DSM 1691, *Azospirillum brasilense* DSM 1690, *Azotobacter chroococcum* DSM2286 have been proved to significantly increase plant height and seed dry biomass (Biari *et al.*, 2008). This improvement seemed to have been induced by improved nutrient uptake such as N, P, K, Fe, Zn, Mn and Cu by PGPR colonized crops (Shaharoona *et al.*, 2006; Biari *et al.*, 2008).The effect of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* on shoot fresh biomass was found to be 59.57% and 23.40% respectively.

These results are in agreement with previous study (Hernandez *et al.*, 1995), showing that *Pseudomonas cepacia*, *P. fluorescens* and *Streptomyces aurantiacus* are the most effective PGPRs on crops grown on reddish ferrous soil. However, our results indicate that *Pseudomonas putida* and *Bacillus circulans* have no significant effect (p> 0.5) on shoot fresh biomass weight compared to non-PGPR colonized control plants. These results indicate that *Pseudomonas putida* and *Bacillus circulans* might have no special role on shoot biomass in reddish ferrous maize fields. Interestingly, we observed a negative effect (p <0,001) of *Bacillus fimosus*, *B. lentus*, *B. polymi*, *B. licheniformis*, *B. pumilus*, *B. thurengensis*, *B. coagulans*, *Streptomyces rimosus*, *S. hygroscopicus*, *S. fasciculatus* and *Azospirillium lipoferum* on maize shoot fresh biomass weight. In addition, *Pseudomonas fluorescens, Bacillus coagulans, Azospirillumlipoferum* and *Streptomyces fasciculatus* showed no specific effect on root fresh weight biomas scompared to non colonized plants, while *Pseudomonas putida, P. aeruginosa, Streptomyces rimosus, S. hygroscopicus, Bacillus fimosus, B. polymixa, B. lentus, B. licheniformis, B. pumilus, B. circulans and B. thurengensis* were shown to have highly significant

effects (p.<0.001) on root fresh weight biomass. It is evident that PGPRs induced improved seed germination and plant development (Ahmad *et al.*, 2006) PGPR effect is generally beneficial to a wide range of crop production including maize (Esters *et al.*, 2004). This positive effect of PGPRs could reasonably be attributed to induction of different vegetative hormones displaying a well programmed pleitropic effects (Contesto *et al.*, 2008).

This study has demonstrated the effect of *Azospirilliumlipoferum, Pseudomonas fluorescens, P.putida, P. aeruginosa, Bacillus coagulans, B. thurengensis, B.pumilus, B. polymixa, B. licheniformis, B. lentus, B.circulans* and *B. fimosusas* efficient PGPRs to improve maize crop development.

.In this regard, our results have identified and characterized *Azospirillium lipoferum*, *Pseudomonas fluorescens* and *P. putida* as maize specific PGPR that can be used as efficient alternative biofertilizers for maize production.

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