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A Bacterial Consortium Interacts With Different Varieties of Maize, Promotes the Plant Growth, and Reduces the Application of Chemical Fertilizer Under Field Conditions

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The success of beneficial bacteria in improving the crop growth and yield depends on an adequate plant-bacteria interaction. In this work, the capability of *Azospirillium brasilense* Sp7, *Pseudomonas putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A to interact with six maize varieties was evaluated by both single-bacterium application and consortium application. The bacterial consortium efficiently colonized the rhizosphere of the autochthonous yellow and H48 hybrid varieties. Bacterial colonization by the consortium was higher than under single-bacterium colonization. The two maize varieties assayed under greenhouse conditions showed increased plant growth compared to the control. The effect of consortium inoculation plus 50% fertilization was compared with the 100% nitrogen fertilization under field conditions using the autochthonous yellow maize. Inoculation with the consortium plus 50% urea produced a similar grain yield compared to 100% urea fertilization. However, a biomass decrease was observed in plants inoculated with the consortium plus 50% urea compared to the other treatments. Furthermore, the safety of these bacteria was evaluated in a rat model after oral administration. Animals did not present any negative effects, after bacterial administration. In conclusion, the bacterial consortium offers a safety alternative that can reduce chemical fertilization by half while producing the same crop yield obtained with 100% fertilization. Decreased chemical fertilization could avoid contamination and reduce the cost in agricultural practices.

Keywords: consortium, bacterial safety, urea reduction, crop yield, field experiments

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INTRODUCTION

The study of the interaction of plant growth-promoting rhizobacteria (PGPR) with plants of agricultural interest has led to the development of biofertilizers or bio-inoculants (Kumar et al., 2007; Zahid et al., 2015). These rhizobacteria have the ability to increase plant growth and yield (Naveed et al., 2008). Furthermore, the rhizobacteria can protect plants from phytopathogens (Wang et al., 2012; Singh et al., 2014), increase plant tolerance to high concentrations of heavy metals (Hassan et al., 2014; Ramírez et al., 2019, 2020), and perform bioremediation (Glick, 2003; Sheng et al., 2012).

The rhizobacteria with the capability to promote the growth of plants include several species such as *Pseudomonas flourescens* and *P. aeruginosa* for maize (Adjanohoun et al., 2011); *Paenibacillus lentimorbus* and *Bacillus amyloliquefaciens* for rice (Bisht and Chauhan, 2020); *Rhizobium pisi* and *Pseudomonas monteilii* for bean (Sánchez et al., 2014); and *Bacillus subtilis* and *Pseudomonas fluorescens* for *Capsicum annumen* (Sundaramoorthy et al., 2012). Furthermore, some of those bacteria perform control of fungal diseases produced by *Fusarium solani* (Sundaramoorthy et al., 2012).

In the last two decades, the synergy of two or more than two PGPRs has been investigated after they are simultaneously inoculated in the same plant (Lally et al., 2017; Mpanga et al., 2019). The application of consortia can increase the production and growth of maize and cucumber plants compared to the inoculation of individual bacteria (Ehteshami et al., 2007; Wang et al., 2012). Co-inoculation of *Rhizobium* sp. and *Pseudomonas* sp. in *Phaseolus vulgaris* increased nodulation and growth parameters, compared to single inoculation (Sánchez et al., 2014). Chickpea inoculated with a consortium formulated with *Rhizobium* sp. and *Mesorhizobium ciceri* also increased plant production and plant growth (Shahzad et al., 2014). The study of soybeans and common beans co-inoculated with *Bradyrhizobium japonicum* and *Azospirillum brasilense* revealed an increment in the yield (Hungria et al., 2013). Regarding to microbial consortia applied to maize plants, the inoculation of *A. brasilense* and *Bacillus subtillis* improved phosphorus uptake, plant development, and corn grain yield (Pereira et al., 2020) Similarly, the consortium of *Trichoderma harzianum* OMG16 and *B. amyloliquefaciens* FZB42 increased the shoot dry matter and grain yield in maize (Mpanga et al., 2019).

In addition, the inoculation of plants with a PGPR consortium improved plant tolerance to abiotic stresses such as water deficit (Ehteshami et al., 2007; Wang et al., 2012; Shahzad et al., 2014), the tolerance to high salt concentration (Ahmad et al., 2013), and the high concentrations of heavy metals (Sheng et al., 2012; Hassan et al., 2014). Decreased application of chemical fertilization together with a good crop yield has been reported in plants inoculated with a bacterial consortium (Da Costa et al., 2013; Shahzad et al., 2013).

Traditional agricultural practices are based on the application of chemical fertilization to obtain high crop yields (Marks et al., 2013). However, the ecological costs generated by the massive application of chemical fertilizers are high, such as the contamination of agricultural soils, water, and the air with significant production of greenhouse gases (Marks et al., 2013; Coskun et al., 2017) and health damage to farmers (Hansen and Donohoe, 2003). Therefore, current agricultural practices require the adoption of new alternatives allowing high yields to be obtained with decreases in the pollutants generated by nitrogen fertilization (Hungria et al., 2013; Vacheron et al., 2013). In this context, *P. vulgaris* inoculated with selected microbial consortia showed an increment in fruit production with respect to the control, when plants were treated with 75% of chemical fertilization; similar grain yield and plant growth were obtained in treatments added with 100% of chemical fertilization (Chauhan and Bagyaraj, 2015). The co-inoculation of *A. brasilense* and *B. subtilis* in sugarcane (variety RB92579) growing in a field a 75% reduction in phosphorus fertilization increased the dry matter content, total phosphorus accumulation, and the production of stems by 38% (Rosa et al., 2020).

The beneficial effects on plants (plant growth and yield) inoculated with bacterial formulations (individual strains or in a consortium) could be affected by several factors, such as the bacterial genotype used for the formulation and the plant variety. These factors have been reported to play a role in sugarcane (Muñoz-Rojas and Caballero-Mellado, 2003; De Oliveira et al., 2006), corn (Mrkovački et al., 2016), tomato (Vaikuntapu et al., 2014), and bean (Sánchez et al., 2014). The analysis of several studies suggests that three key steps are required to achieve the beneficial effect of bacteria on plants: attraction of PGPR to a host plant, root colonization, and functional associative symbiosis (Drogue et al., 2012). Therefore, those steps must also be evaluated for consortium formulations (Molina-Romero et al., 2017).

Furthermore, PGPR used to formulate individual inoculants or consortia must be harmless, and they should be safe to human and animal health (Vílchez et al., 2015). The literature suggests the use of rhizobacteria classified as non-pathogenic (BSL-1), to design beneficial bacterial formulations for field application. In the case of BSL-2 microorganisms (pathogens and opportunistic pathogens), it is recommended to carry out experiment under strict containment and regulatory practices. However, applications of these microorganisms are not recommended (Keswani et al., 2019).

In our previous work (Molina-Romero et al., 2017), bacterial antagonism assays were performed among 20 strains using the double layer agar plate method. Using data on compatibility and desiccation resistance, a consortium formulated with four plant growth-promoting rhizobacteria (*A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178A) was designed. The compatibility of the selected strains for the bacterial consortium was verified under different growing conditions. All of strains were able to coexist and adapt under different environmental conditions. In addition, the strains of the consortium were experimentally evaluated for their capability to stimulate the growth of maize and their PGPR characteristics (Molina-Romero et al., 2017). *P. putida* KT2440, *Acinetobacter* sp. EMMS02, *Sphingomonas* sp. OF-178A, and the consortium displayed a high production of siderophores; *P. putida* KT2440, *Acinetobacter* sp. EMM02, and the consortium showed the highest phosphate solubilisation capacity; and *A. brasilense* Sp7

and *Acinetobacter* sp. EMM02 presented the highest production of total indole compounds (Molina-Romero et al., 2017). Those observed characteristics were in line with other studies (Gamalero et al., 2004; Gulati et al., 2009; Planchamp et al., 2014; Rojas-Tapias et al., 2014; Lin et al., 2018; Bharwad and Rajkumar, 2020).

This consortium showed a beneficial effect on autochthonous blue maize. The benefits remained after subjecting the bacterial consortium to desiccation stress prior to the plant inoculation (Molina-Romero et al., 2017). However, this consortium has not been explored in other maize varieties or under field conditions.

This research aimed to evaluate the capability of *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A (alone or in a consortium) to interact and stimulate the plant growth of 6 maize varieties, under greenhouse conditions. We selected the most promising maize variety for field application. This variety was again inoculated with the consortium and its capability to promote the plant growth was tested under field conditions, using different treatments of urea fertilization (50 and 100% of the recommended N dose). In addition, the effects of the oral administration of all PGPR used to formulate the consortium were evaluated in adult male Long Evans rats.

MATERIALS AND METHODS

Germination Rate

Fifty seeds of different maize varieties were inoculated using a bacterial mixture formulated as described previously (Molina-Romero et al., 2017). The maize varieties used were two autochthonous (yellow and red from the Huejotzingo region) and four hybrids (1463, 1069, 888, and H48), and hybrid varieties were coated with the carboxin-thiram fungicide (Abati et al., 2014; Haghanifar et al., 2018) by providers from the municipality of Huejotzingo. Inoculation was performed during 1 h by submerging seeds in the bacterial suspension containing 10^7 Colony Forming Units (CFU)/mL of *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178. After inoculation, seeds were placed on sterile filter paper moistened with sterile water deposited in a sterile petri dish and placed at 20°C for 48–72 h for germination. For the control treatment of each corn variety, seeds were submerged in distilled water only.

Adherence and Colonization

The cell suspension was prepared as described by Molina-Romero et al. (2017). Inoculation of autochthonous and hybrid seeds (coated with fungicide) was carried out by submerging them in the bacterial suspensions for 1 h. The bacterial numbers of the suspension were determined in the order of 10^6 to 10^7 CFU/mL by using the Massive Seal Drop Plate (MSDP) method (Corral-Lugo et al., 2012), selection media were used for each strain, determining the population for the individual strains and the bacterial consortium.

Fifty seeds of each variety (autochthonous or hybrid) were inoculated for 1 h with the respective bacterial suspension (single strains or the consortium). Inoculated seeds were sown in 50 mL conical tubes with 15 g of sterile vermiculite. Five milliliters of sterile MS liquid and 2 mL of water was added to each tube (Murashige and Skoog, 1962). All tubes were placed in a greenhouse with 16 h of light and a temperature of 30°C during the day and 8 h of darkness and a temperature of 25°C during the night.

Six inoculated seeds were extracted within 24 h of sowing and the bacterial number adhered to seeds was determined by the MSDP method. For this, seeds were placed in 50 mL conical tubes with 3 mL of sterile water for 1.5 h. Selective media for each bacterial strain and the bacterial consortium were used for quantification (Molina-Romero et al., 2017). Plates were incubated for 24 to 48 h at 30°C. The other inoculated seeds (with an individual bacterium or in a consortium) sown in sterile vermiculite were kept under greenhouse conditions for 21 days. Plants were watered with distilled water every 4 days. At 21 days post inoculation (dpi), six plants of each treatment were used to determine rhizosphere colonization (Rodríguez-Andrade et al., 2015). The root of each plant was placed in 50 mL conical tubes with 10 mL of sterile water for 1.5 h. To determine the bacterial population extracted from the roots, the MSDP technique was used with each selection media for each bacterium; the incubation conditions of the plates were 24 to 48 h at 30°C (Rodríguez-Andrade et al., 2015; Molina-Romero et al., 2017). The vermiculite strongly adhered to the root was removed and its dry weight determined to calculate the parameter Colony-Forming Units / gram of Vermiculite (CFU/gV) (Morales-García et al., 2011; Rodríguez-Andrade et al., 2015; Molina-Romero et al., 2017; Pazos-Rojas et al., 2019).

Some isolated bacteria were selected to confirm bacterial identity. To do it, the 16S rDNA gene was amplified. The 1.5 kb amplifications were digested with the *MspI* enzyme; subsequently, the electrophoretic separation was performed to observe the characteristic restriction pattern of the PGPR inoculated in each plant (Molina-Romero et al., 2017).

Animal Experiments

Adult male Long Evans rats (with an average weight of 250 to 270 g) were obtained from the Benemérita Universidad Autónoma de Puebla, México. The animals were housed in experimental cages; food and tap water were free to demand. Environmental conditions were maintained at a temperature of 22°C ± 2°C and 60% relative humidity, with a light-dark cycle of 12 h. The protocol in this study was carried out according to the official Mexican standard NOM-062-ZOO-1999.

The treatments established to evaluate the PGPR-rat interaction were five (bacterial consortium and the four independent strains: *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178). For the control treatment, animals were not inoculated. Four animals were inoculated for each treatment ($n = 4$ rats).

Rats were dewormed and vitaminized before inoculation. A cell suspension of each strain and consortium was formulated with a population of 1 x 10^7 CFU/mL, according to Molina-Romero et al. (2017). The administration of 1 mL of cell suspension was orally introduced with a cannula, after the sedation of the animal with phenobarbital (0.7 mL/270 g weight).

Animals of the control treatment were administered with 1 mL of physiological solution. Both inoculated and control rats were observed twice, every day, over a period of 30 days. Two independent experiments were established. The parameters evaluated in the rats were the alteration of motor activity, weight loss, diarrhea, and the presence of lethargy. In the presence of the aforementioned parameters and other symptoms that compromise the life of the animal, the slaughter would take place.

At the end of the evaluation time of the symptoms of bacterial infection (1 month), the rats were sedated and sacrificed by decapitation. The extraction of the meninges, small intestine, and a blood sample from the heart, under aseptic conditions, was performed. Organs and samples were homogenized with a physiological solution for subsequent seeding in the selection media designed for each PGPR (Molina-Romero et al., 2017). The incubation conditions of the selection media inoculated with the samples from the rats of each treatment were 48 h and 30°C. The procedures described in this study were based on the Standards for the use and care of laboratory animals of the Mexican Council of Animal Care and as indicated in NOM-062-ZOO-1999, which are also approved by the Animals and Ethics Committee of the BUAP. All effort was made to minimize the number of animals used and to ensure minimal pain and discomfort.

Determination of Growth Promotion Parameters Under Greenhouse

Fifty seeds were inoculated for each treatment and were sown in pots containing 650 g of sterile vermiculite. Irrigation for each pot was done with 500 milliliters of sterile MS nutrient solution (Murashige and Skoog, 1962) and 200 mL of sterile water. The greenhouse cultivation conditions were 16 h of light at 30°C during the day and 8 h of darkened at 22°C during the night. Seedlings were developed up to 45 dpi with regular irrigation with distilled water.

At 45 days after inoculation (dpi) plants were removed from the vermiculite and washed with water, and the excess water was dried with absorbent paper. A tape measure and Vernier were used to measure the height of the plant and the stem diameter, respectively. The fresh weight of the seedlings was determined with the analytical balance. Subsequently, the samples were dried in the oven at 75°C until they reached a constant weight. This procedure was also carried out to determine the growth parameters in the field experiment.

Field Experiment

Localization of the Field Experiment

The field experiment was carried out in the summer of 2015. The experiment was carried out in the municipality of Huejotzingo, Puebla México, located between 19° 06′ and 19° 16′ in north latitude; 98° 20′ and 98° 38′ in west longitude meridians. The climate is temperate sub-humid with rains in summer, with an average maximum temperature of 16°C and a minimum of 2°C. The average annual precipitation is 900 to 1,100 mm in the territory. Physicochemical analysis of a soil sample from the experimental place was determined as sandy clay soil, according to standard methods (Shahzad et al., 2014).

Treatments and Experimental Design

Four treatments were established in the field: (1) Bacterial consortium treatment; seeds inoculated with the consortium alone, without urea application in the field. (2) Urea treatment; seeds without bacterial inoculation with a complete dose of urea fertilization (100%) in the field. (3) 50% urea treatment plus consortium; seeds inoculated with the consortium and half dose of urea fertilization applied in the field. (4) Control treatment, non-inoculated seeds without urea application in the field.

The seeds (from the bacterial consortium and 50% urea plus consortium treatments) were inoculated as previously described in the adherence and colonization section. The seeds of the control treatment and 100% urea were immersed in sterile distilled water for 1 h to discard the effect of water. The seeds were sown manually in plots (0.8 m wide by 152 m long, with 8 replicates for each treatment) with a distance from plant to plant of 80 cm.

Nitrogen chemical fertilization was applied using the commercial urea formula (46-0-0) in only two treatments: 100% urea (69 kg of N/ha) and 50% urea (34.5 kg of N/ha) plus consortium treatments. For the irrigation in the temporary field, a water channel was used.

The design employed was randomized complete block design (RCBD) with four treatments and 8 replicates, the experimental area was divided into 973 m^2 for each treatment. The plot was separated by 1.0 m, with small furrows of approximately 1.5 m to prevent surface mixing with the bacteria and fertilizer used in each treatment. This is because of the strong rains that are common in the summer season.

Recording of Growth Parameters and Production

The corn growth period was carried out in the traditional period season from March to June 2016. Growth parameters (plant height, root dry weight, root length, and plant diameter) were evaluated using 20 plants of each treatment, through the design of random blocks. The plant growth parameters were recorded for 80 days after sowing as previously described in the methodology of the greenhouse experiment.

Biomass and production were recorded after the maturity of the crop, 190 days after sowing, the corn was harvested and the cobs were separated, later they were weighed on a balance; the data were converted to Kg/ha, following the unit method.

Statistical Analysis

Data on plants (adherence, colonization, and growth parameters) were subjected to a one-way analysis of variance (ANOVA). Significant differences between average were obtained with Duncan multiple range test at $P \leq 0.05$, using SigmaPlot 12 (Handel Scientific Software).

RESULTS

Germination of Maize Inoculated With a Bacterial Consortium

The percentage of germination in both yellow and red autochthonous maize varieties with and without bacterial

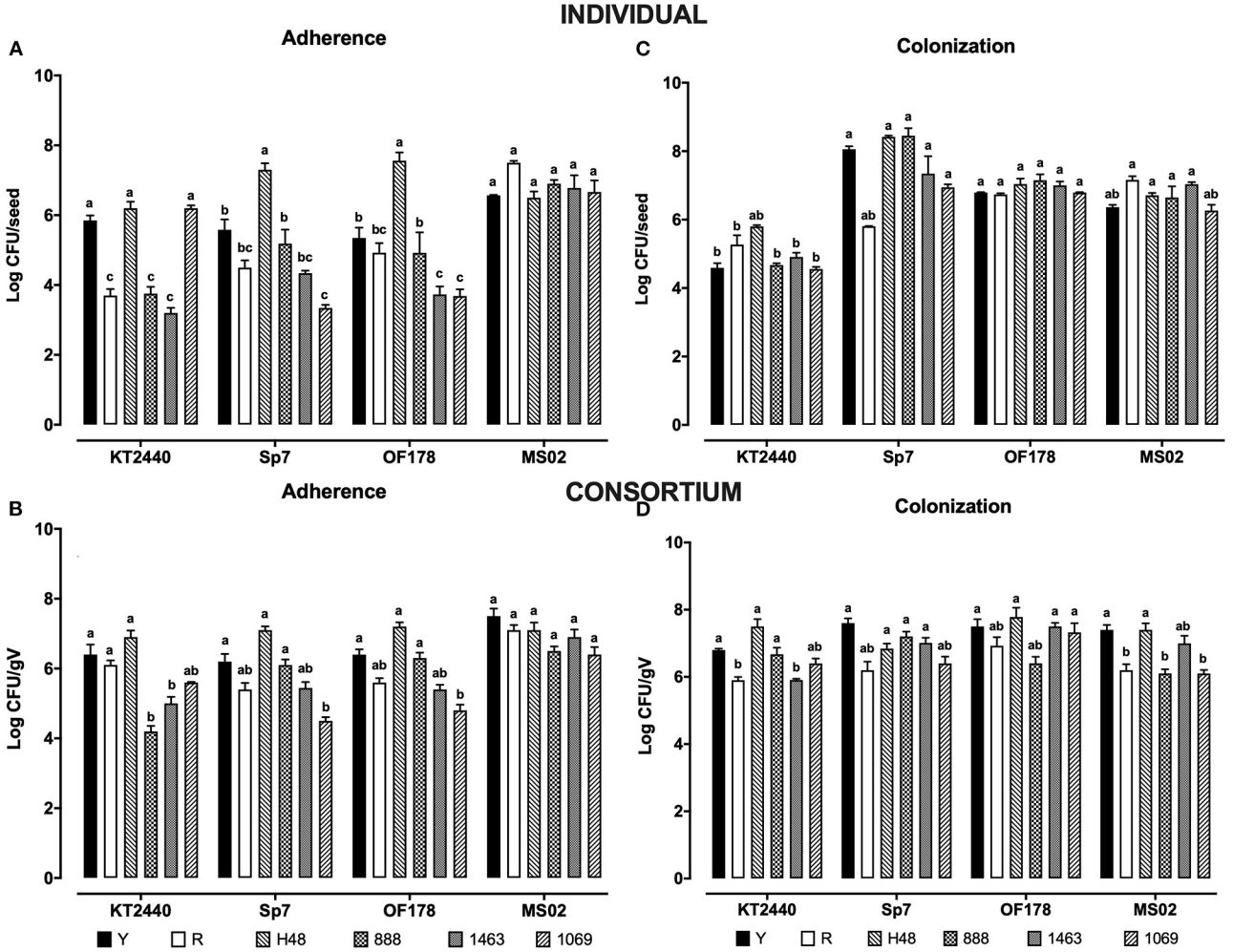

FIGURE 1 | (A) Individual adherence of PGPR to six different varieties of maize (genotype), **(B)** adherence in the consortium treatment, **(C)** individual colonization of PGPR in corn seedlings at 30 dpi in, **(D)** colonization of the consortium. Black bar: autochthonous yellow variety; white bar: autochthonous red; bar with right diagonal lines: H-48 hybrid; dotted bar: 888 hybrid; checkered bar: 1463 hybrid variety; bar with diagonal to the left: 1069 hybrid varieties. The different letters represent a statistically significant difference at $P < 0.05$ (One-way analysis of variance ANOVA).

inoculation was of 100%. All hybrid varieties showed a germination percentage of 100% when they were inoculated with the bacterial consortium. In contrast, the non-inoculated seeds of 1069 and 1463 hybrids varieties presented 96% germination. The H48 and 888 hybrids presented 98 and 100% of germination, respectively.

Adherence

The four strains (alone or in a consortium) had the ability to adhere to the six maize seed varieties under *in vitro* conditions. The individual adhesion of *Acinetobacter* sp. MS02 presented a similar trend in all varieties explored, on the order of 10^6-10^7 CFU/seed (**Figure 1A**). The adhesion of this strain increased one order of magnitude, in the autochthonous yellow and the hybrid H48 varieties, when it was inoculated in a consortium (**Figure 1B**). However, in the hybrids varieties (888, 1463, and 1069) and the red autochthonous variety, adhesion maintained the same tendency when the bacteria were inoculated individually or in a consortium.

A. brasilense Sp7 and *Sphingomonas* sp. OF178 had a similar adhesion capability in most varieties explored. They adhered at high numbers (10^5-10^7 CFU/seed) to the yellow autochthonous variety, and to the hybrid varieties H48 and 888. However, they adhered at low numbers (10^3-10^4 CFU /seed) to the red autochthonous variety and the hybrid varieties 1069 and 1463 (**Figure 1A**).

The adhesion of *P. putida* KT2440 to the autochthonous red variety, and the 888 and 1463 hybrids was lower, with bacterial numbers on the order of 10^3 CFU/seed. However, KT2440 showed a high adhesion in the H48, 1069, and yellow autochthonous varieties (10^5-10^6 CFU/seed).

Interestingly, *A. brasilense* Sp7, *P. putida* KT2440, and *Sphingomonas* sp. OF178 inoculated in a consortium, showed an increased adhesion (one or two orders of magnitude) (**Figure 1B**)

compared to their individual adhesion in hybrid varieties 888, 1463, and 1069, and the autochthonous yellow and red varieties (**Figure 1B**). For the H48 hybrid, adhesion of the bacterial strains remained similar under individual or consortium inoculation (10^7 CFU /seed).

Rhizosphere Colonization

Sphingomonas sp. OF178 and *Acinetobacter* sp. MS02 showed a similar rhizosphere colonization in the six corn varieties, 10^6-10^7 CFU/gV and 10^5 to 10^6 CFU/gV, respectively (**Figure 1C**).

In the yellow autochthonous variety, and the H48 and 888 hybrids, *A brasilense* Sp7 presented a higher individual colonization (10^8 CFU/gV) (**Figure 1C**) than was observed in the red autochthonous variety and 1463 and 1069 hybrids (10^6 CFU/gV).

The low individual colonization of *P. putida* KT2440 (10^4 to 10^5 CFU/gV) was observed in the autochthonous and hybrid varieties (**Figure 1C**).

The colonization of *A. brasilense* Sp7, *Sphingomonas* sp. OF178, and *Acinetobacter* sp. MS02 strains in the consortium showed a rhizosphere colonization ranging from 10^6 to 10^7 CFU/gV, for all tested varieties (**Figure 1D**). In several cases, bacterial colonization in the consortium was increased. The rhizosphere colonization of *P. putida* KT2440 increased in the consortium inoculation treatments, in most of maize varieties evaluated, with the exception of the autochthonous red variety, in which the colonization remained similar to individual rhizosphere colonization (**Figure 1D**).

Interaction Tests of PGPR With the Rat Animal Model

The effect of PGPR inoculation on the rat model was evaluated. The rats inoculated with *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178A, or the bacterial consortium did not show any changes in their motor activity, and there was not weight loss or lethargy observed from the time of inoculation until 30 days after inoculation (**Table 1**). Therefore, rats inoculated with the bacterial consortium or individual treatments did not exhibit any alterations, indicating that there was no damage to their health. Interestingly, bacteria were undetectable, in blood, meninges, and small intestine samples from rats treated with the bacterial consortium or individual strains (**Table 1**).

Effect of Consortium Inoculation on Growth Promotion in the H48 Hybrid and Yellow Autochthonous Varieties Under Greenhouse Conditions

The bacterial consortium showed an adequate capacity to adhere to and colonize the seeds of the H48 hybrid and yellow autochthonous varieties. The adherence was 10^6 to 10^7 CFU/seed, and colonization rate was 10^6 to 10^7 CFU/gV in the two varieties. Likewise, when the seeds of these two varieties were inoculated with the four PGPR strains individually, they presented similar adherence and colonization.

Based on these results, the H48 hybrid and autochthonous yellow varieties were inoculated with the bacterial consortium to evaluate whether the consortium promotes plant growth under greenhouse conditions.

The dry weight of the aerial parts of the H48 hybrid plants inoculated with the bacterial consortium was 7.23 g ($\pm$0.81), and it was 5.62 g ($\pm$0.58) for control plants. The difference was significant statistically ($p \leq 0.05$) (**Figure 2E**). The dry weight of the aerial part of the autochthonous yellow variety inoculated with the consortium (5.43 g $\pm$ 0.38) was significantly higher than that of the control plants 4.08 g ($\pm$0.55) (**Figure 2A**).

The root dry weight of both varieties inoculated with the consortium was significantly higher (5.33 g for H48 and 3.04 g for indigenous yellow) than that of the control plants (3.82 and 1.87 g, respectively) (**Figures 2B,F**).

Likewise, the H48 hybrid and indigenous yellow varieties inoculated with the consortium surpassed the control treatment in the parameters of height and diameter, with a significant difference (**Figures 2C,D,G,H**).

Autochthonous Yellow Variety Under Field Conditions

The native yellow maize was the variety showing the most stimulation after the inoculation of the bacterial consortium, under greenhouse conditions. Also, this variety is of great interest to farmers in the region Huejotzingo region of Puebla, because they prefer to cultivate indigenous corn varieties. Therefore, we decided to evaluate the effect of bacterial inoculation on the autochthonous yellow variety under field conditions.

The results of the field experiment showed that treatments with bacterial inoculation increased the plant height, dry root weight, and root length of the yellow variety compared to the control plants.

Plants subjected to the 50% urea plus consortium treatment and the plants fertilized with 100% urea presented the higher values of plant height, root dry weight, root length, and plant diameter. In general, parameters of growth between these two treatments were not significantly different (**Table 2A**).

The height, root length, and dry weight of the plants inoculated with the consortium compared to the controls showed significantly higher values: 204.62 and 184.63 cm of plant height, 36.28 and 33.38 cm root length; and 7.33 and 6.11 g of dry weight, respectively (**Table 2A**). However, the diameter of the plants inoculated with the bacterial consortium was similar to the values recorded for the control plants, without any statistically significant differences (**Table 2A**).

The plants treated with 100% urea and the control plants showed the highest biomass production under field conditions. In contrast, the treatments resulting in the lowest biomass production included the plants inoculated with the bacterial consortium alone and plants treated with 50% urea plus the consortium (4.74 and 4.59 t/ha, respectively) (**Table 2B**).

The 50% urea plus consortium treatment resulted in the highest grain yield, followed by the 100% urea treatment (5353.50 and 4914.50 kg/ha, respectively). In contrast, the bacterial consortium treatment resulted in the third highest in grain

TABLE 1 | Effects of PGPR on the rat animal model.

Treatment	Motor activity	Lethargy	Weight loss of the animal	Animal sacrifice	PGPR isolation, after oral inoculation		
					Blood	Meninges	Small intestine
Control	N	A	N	A	NI	NI	NI
A. brasilense Sp7	N	A	N	A	NI	NI	NI
P. putida KT2440	N	A	N	A	NI	NI	NI
Acinetobacter sp. EMM02	N	A	N	A	NI	NI	NI
Sphingomonas sp. 0F178A	N	A	N	A	NI	NI	NI
Consortium	N	A	N	A	NI	NI	NI

N, normal; A, absent; NI, not isolated. The control treatment was not PGPR-inoculated. Four animals were inoculated for each treatment, individual and consortium (n = 4). Data were obtained from two independent experiments.

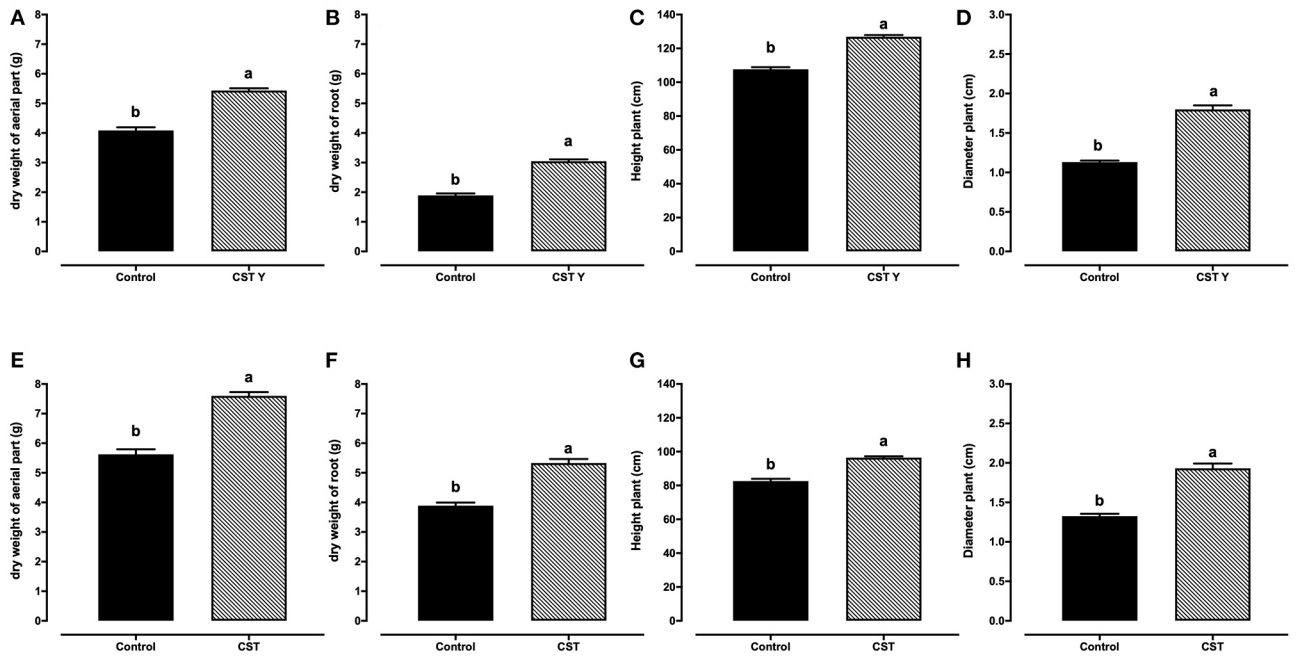

FIGURE 2 | Promotion of the growth in maize plants variety of the H-48 and autochthonous yellow varieties inoculated with the consortium (CST) and control treatment (C) under greenhouse conditions, in 30 dpi seedlings. Autochthonous yellow maize, **(A)** dry weight of aerial parts, **(B)** dry weight of roots, **(C)** height, and **(D)** diameter. H48 hybrid, **(E)** dry weight of aerial parts **(F)** dry weight of roots, **(G)** height and **(H)** diameter. The different letters represent a statistically significant difference at $P < 0.05$ (*t-test, Student*).

production; and the lowest production was presented by the control (4549.3 and 3916.0 kg/ha, respectively) (**Table 2B**).

An increase of 1437.5 kg/ha was obtained with the 50% urea plus consortium treatment compared to the control (**Table 2B**).

DISCUSSION

Bacterial establishment in the rhizosphere is a crucial step to obtain the beneficial effect of PGPR on the host plant. Therefore, an adequate adhesion and colonization must occur to obtain the desired effects (Drogue et al., 2012; Shahzad et al., 2013). Individual and consortium bacterial adhesion to maize for *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A was variable among the maize seed varieties explored in this work, suggesting differences in bacterium-seed affinity. It is important to highlight that the adhesion was improved when bacterial strains were applied in the consortium to all corn varieties used and was in agreement with our previous observations in the blue autochthonous variety (Molina-Romero et al., 2017).

A correlation between colonization capacity and the ability to stimulate plants has been reported (Moradtalab et al., 2020). For example, a colonization on the order of 10^7 CFU/g root dry weight stimulates the plant biomass of the forage maize (Piromyou et al., 2011). In our work, the colonization following individual and bacterial consortium inoculation in the 888, 1069, 1463, and red-autochthonous varieties was less efficient compared to that in the H48 and autochthonous yellow

TABLE 2 | Effect of the consortium inoculation on the growth of autochthonous yellow maize under field conditions using different levels of urea fertilization.

(A)

Treatment	Plant Height (cm)	Dry root weight (g)	Root Length (cm)	Plant diameter (cm)
Control	184.63 ± 6.64 c	6.11 ± 0.51 c	33.38 ± 2.35 c	8.55 ± 0.64 b
Consortium	204.62 ± 13.17 b	7.33 ± 0.74 b	36.28 ± 2.19 b	9.55 ± 0.73 ab
Urea 100%	221.50 ± 17.07 a	7.23 ± 0.60 b	40.28 ± 2.39 a	10.32 ± 0.89 a
Urea 50% + consortium	228.84 ± 11.24 a	8.30 ± 0.69 a	42.52 ± 2.94 a	10.44 ± 0.69 a

(B)

Treatment	Total biomass t/ha	Grain production t/ha
Control	5.10 ± 2.4 a	3.91 ± 0.26 c
Consortium	4.74 ± 1.6 ab	4.54 ± 0.24 b
Urea 100%	5.15 ± 3.9 a	4.91 ± 0.25 ab
Urea 50% + consortium	4.59 ± 1.9 b	5.35 ± 0.15 a

(A) *Treatments: consortium (consortium alone), 100% urea (fertilization with full dose of urea), urea 50% + consortium (inoculation of the consortium plus fertilization with half the dose of urea). The autochthonous yellow maize variety was used for field experiments, and evaluation was performed at 80 days after sowing. Data represent the means and SD of 20 replicates, analyzed with one-way ANOVA followed by a Tukey-test ($p \leq 0.05$).* ***(B)*** *total biomass per hectare at the time of harvest (190 days after planting); Total grain production per hectare. Data represent the means and SD of eight replicates. Different letters in each column indicate significant differences. The biomass and grain production data were analyzed with the Duncan multiple range test at $p \leq 0.05$.*

varieties. However, the inoculation of the bacterial consortium improved the bacterial colonization capacity compared to the individual treatments. However, a correlation between adequate colonization and the growth promotion of the plants of the H48 hybrid and autochthonous yellow varieties was observed.

Bacterial colonization depends on the plant variety. The colonization of a consortium formulated with *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia tropica* was found to differ in two varieties of sugar cane (SP70-1143 and SP 813250) (De Oliveira et al., 2006). The colonization capability of other consortia (*Pseudomonas striata* and *Piriformospora indica*) is also plant-dependent in maize varieties or mung beans varieties (Singh et al., 2009). In the present study, we also observed the influence of the maize variety on the bacterial colonization.

Few studies have evaluated the microbiological safety of PGPR before field application. Bacterial strains used to improve plant growth must be safe for human health and the environment (Kampers et al., 2019; Visnovsky et al., 2020). An example in which bacterial innocuity was explored is provided by the work reported by Chávez-Ramírez et al. (2020) in *Paenibacillus polimyxa* (NMA1017), whose safety was tested on tobacco leaves and the *Galleria mellonella* larvae, reinforcing its safe application as a biocontrol agent (Chávez-Ramírez et al., 2020). In the present work, we used a rat model to explore whether the bacterial strains provoke adverse effects on the animals after the oral administration of 1 mL containing 10^7 CFU/mL. The absence of signs of disease and the lack of bacteria in the blood, meninges, and small intestines of the animals inoculated with the bacterial consortium or individual strains, suggested that the bacterial strains were innocuous to the rats and they could be safe for human health when applied to the crops.

It is important to verify the safety of PGPR before they are used as biofertilizers, especially if they are closely related to pathogenic bacterial strains. For example, *Bacillus* sp. (RZ2MS9) and *Burkholderia ambifaria* (RZ2MS16) present a potential risk due to their taxonomic proximity to pathogenic groups (Batista et al., 2018; Ferreira et al., 2019), especially when a formulation includes bacteria considered to be opportunistic pathogens, such as *P. aeruginosa* (Lavakush et al., 2014). By conducting this verification, we can ensure that the application in the field does not represent a health risk to people who are in contact with the formulation when it is applied to plants.

Regarding the plant growth promotion under greenhouse conditions, the autochthonous yellow variety and the H48 hybrid inoculated with the bacterial consortium showed significant increases in height, diameter, the dry weight of the aerial part and the roots compared to the control. The benefit to growth parameters was probably due to the ability of the bacteria of the consortium to produce indoles, solubilise phosphates, and produce siderophores (Molina-Romero et al., 2017). These beneficial effects agree with the previous observations made in blue maize inoculated with this same consortium (Molina-Romero et al., 2017). Similarly, an increase in root dry weight was recorded in jolly hybrid maize, when it was inoculated with a *Bacillus subtilis* FZB24 and *B. subtilis* GB03 consortium (Myresiotis et al., 2015). In contrast, a consortium of *Azospirillum lipoferum, P. fluorescens, P. putida,* and chitosan did not increase the height, diameter, or number of leaves per plant but significantly increased the dry weight of the roots and the aerial parts of the maize under greenhouse conditions (Agbodjato et al., 2016). The co-inoculation of maize with *A. brasilense* and *B. subtillis* has also shown greater benefits than individual inoculation (Pereira et al., 2020).

In the present fieldwork, the inoculation of the bacterial consortium in the autochthonous yellow variety produced a beneficial effect on the plants, increasing the height, dry root weight, and root length of the plant and yield. However, the total biomass and the diameter of the plants inoculated with

the consortium were similar to the control. These growth-promotion results showed a trend similar to the trend reported in a field study indicating that the maize inoculation with a consortium with *Azotobacter chrococcum* and *A. liporefum* resulted in increments in shoot and seed dry weight, plant height, and yield compared to the individual inoculation of PGPR and the control (Biari et al., 2008).

In this study, nitrogen fertilization at 100% and the treatment with the consortium plus 50% urea resulted in the greatest increments in height, diameter, dry root weight, and grain production in comparison to non-inoculated plants. These results showed that the consortium stimulates the growth of autochthonous maize when a half dose of mineral nitrogen used in traditional Mexican agricultural practices is added, which generated results similar to the application of a complete nitrogen dose.

Similar trends have been reported in other studies. For example, the rice inoculation with a consortium (combined *Pseudomonas* culture, *A. chroococcum,* and *A. brasilense*) plus the application of 50% mineral phosphorus resulted in results similar to the complete dose of phosphorus plus the consortium (Lavakush et al., 2014). Additionally, the hybrid maize (33M15) has been inoculated with *Pseudomonas thivervalensis* (STF3) (Shahzad et al., 2013), the hybrid corn (786) has been inoculated with *P. fluorescens* biotype G (N3) (Naveed et al., 2008), and safflower has been inoculated with *Azospirillum* sp. and *Azotobacter* sp. (Nosheen et al., 2016). In sunflower, the highest grain production, oil, and protein content were observed in association with the consortium (*Azotobacter* sp. and *Azospirillum* sp.) plus 50% nitrogen fertilization (Naseri and Mirzaei, 2010).

Most of the studies in which plants were inoculated with PGPR or consortia have reported an increase in yield and biomass (Ehteshami et al., 2007; Kumar et al., 2007; Shahzad et al., 2013). In this work a higher grain yield was observed following the application of the consortium plus 50% urea while the biomass production was lower compared to the control, consortium inoculation alone, or 100% urea treatment. Most likely, during the interaction with bacteria, nutrients in the plant are directed to grain formation instead of other plant structures (Okuno et al., 2014), which should be clarified in the future. The results observed in maize inoculated with the consortium and treated a half of the dose of nitrogen fertilization contrasts with the direct correlation established between nutrition and higher grain production, particularly when abundant mineral nitrogen is available (Rozier et al., 2017; Bisht and Chauhan, 2020).

Plants interact with efficient indole-producing and phosphate solubilizing bacteria under low-nutrient conditions. However, in a moderate nutrient scheme, plants selectively associate with bacteria with a higher capacity for phosphate solubilization (Da Costa et al., 2013; Pii et al., 2019; Bisht and Chauhan, 2020). As observed in this work, under treatment with 50% urea plus the consortium, we registered a higher promotion of plant growth and grain production due to the beneficial effect of the consortium. This effect could be due to a high phosphate solubilization capability and indole production among the strains of the consortium (Molina-Romero et al., 2017). However, it is necessary to do more work addressing this topic, perhaps using mutants defective in these mechanisms to verify their roles in the interaction.

Under conditions of abundant nutrients, this selective interaction with bacteria is abolished (Da Costa et al., 2013; Di Salvo et al., 2018), showing that fertilization affects the PGPR-plant interaction. More work is required to define the role of bacterial mixtures on plant inoculation under field conditions. However, the eco-friendly technologies for increasing crop yields are promising (Morales-García et al., 2019).

The application of consortia in agricultural practice is offered as an alternative to implementing sustainable agriculture practices (García De Salamone et al., 2010), due to the reduction of chemical fertilization without compromising the grain yield (Tilman et al., 2002). Only 50% of nitrogen fertilizer applied into the field is absorbed by plants; the remaining half is lost to the environment in the form of ammonia, nitrate, and nitrous oxide, contributing to the pollution of soil, water, and air and to climate change (Coskun et al., 2017; Schröder et al., 2018). Increasing the application of mineral nitrogen fertilizer in rice, corn, wheat, and barley can increase methane and nitrous oxide emissions from agriculture, which directly impacts global warming, due to the characteristics of methane and nitrous oxide as greenhouse gases (Bodelier et al., 2000; Good and Beatty, 2011).

The bacterial consortium studied in this work presents the characteristics of biofertilizer: inducing efficient interaction with the plant, and ecological nature, and contributions to lowering the costs of maize production due to the reduced of mineral nitrogen requirement. Consequently, a decrease in diminution of pollution caused by nitrogen fertilization occurs. Furthermore, the consortium is safe to handle when applied under field conditions.

CONCLUSION

This research presents the potential of a second-generation consortium formulated with *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A. The bacterial strains of the consortium are compatible, resistant to desiccation, and efficient for field applications. Since the bacteria of this consortium interact efficiently with the autochthonous yellow maize variety, they trigger a beneficial effect on the grain yield. In addition, this bacterial consortium offers an alternative allowing the efficient use of half the recommended amount of nitrogen fertilizer. The use of the consortium allows a 50% reduction in mineral nitrogen application and generates important benefits for agricultural practices such as lower costs to the producer and a significant decrease in environmental pollution. Based on the results of the animal model, we suggest that the bacterial consortium is a safe formulation for use and manipulation under field conditions and does not cause health problems to animals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The experiments with animals were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The animal study was reviewed and approved by Committee of Benemerita Universidad Autonoma de Puebla.

AUTHOR CONTRIBUTIONS

BV, YM-G, and JM-R: data curation. DM-R and YM-G: funding acquisition. BV, DM-R, and JM-R: research. SJ-S, CO-G, and DM-R: methodology. SJ-S and CO-G: resources. BV, AB, and DM-R: software. JM-R: supervisión. AB, YM-G, and DM-R: writing—original draft. DM-R, AB, and JM-R: writing—review and editing. All authors have read and agree to the published version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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