Article

Use of Plant Growth Promoting Rhizobacteria in Combination with Chitosan on Maize Crop: Promising Prospects for Sustainable, Environmentally Friendly Agriculture and against Abiotic Stress

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Abstract: Faced with the problems posed by the abusive use of chemical fertilizers and pesticides, it is important to find other alternatives that can guarantee a sustainable and environmentally friendly agriculture. The objective of this study was to evaluate the tolerance of a PGPR (plant growth promoting rhizobacteria) *Pseudomonas putida* strain to different abiotic stress in in vitro conditions and the synergistic effect of this rhizobacterium in combination with chitosan extracted from crab exoskeletons on the growth of maize in greenhouse conditions. The strain of *P. putida* was put in culture at different temperatures, pH, and NaCl concentrations to determine its growth. Then, this strain in combination with chitosan extracts were tested for their ability to improve maize growth for 30 days. The results showed that the *P. putida* strain showed excellent resistance capacities to different salt concentrations, pH, and temperature variations. Moreover, an improvement in plant growth and biomass yield parameters was observed. The highest values of height, diameter, and leaf area were obtained with the plants treated with the combination of chitosan extracted from *Cardisoma armatum* and *P. putida*, with increases of 26.8%, 31%, and 55.7%, respectively, compared to the control. This study shows the possibility of using chitosan and rhizobacteria as biostimulants to improve productivity and increase maize yield in a sustainable manner.

Keywords: abiotic stress; biostimulants; chitosan; crab exoskeletons; PGPR; sustainable agriculture

1. Introduction

Agriculture plays a significant role in the economies of countries around the world. However, it is confronted with many problems leading to substantial crop losses. The constant increase in population, rapid urbanization with the decrease in agricultural land, radical change in climatic conditions, and intensive use of agrochemicals in farming practices that have caused environmental disruptions are confronting humanity with growing problems of food security and agricultural sustainability [1,2]. In addition, the excessive use of chemicals for crop fertilization may lead to the accumulation of minerals and nutrients that cannot be easily utilized for plant consumption, ultimately resulting in soil pollution and toxicity [3].

Consequently, the expansion of crop production relies on increasing soil fertility to ensure food for all under the current global food security scenario [4]. It thus becomes essential to develop different environmentally friendly methods of soil fertility improvement such as the use of natural products and/or organisms in order to reduce the effects

of harmful organisms and improve agricultural production. Among these products or organisms, we can find plant biostimulants such as chitosan (a chitin derivative extracted from the shells of crustaceans) and soil microorganisms, most especially bacteria whose overall effect promotes plant growth. These bacteria have been grouped under the name of plant growth promoting rhizobacteria (PGPR).

PGPR are bacteria that actively colonize the root rhizosphere and establish free and intimate associations with host plants [5]. They can enhance plant growth and increase plant nutrients through several mechanisms such as siderophore production, antibiotics, and phosphate solubilization [6–9]. Authors in [10] also reported that PGPR could be used as an inexpensive and environmentally friendly approach for enhancing crop growth. Furthermore, microbes have been shown to be of great help in the fight against abiotic stress, via their biological activities at the rhizosphere of plants [11]. The use of PGPR is increasing in agriculture and provides an attractive alternative to chemical fertilizers, pesticides, and food supplements [12].

Concerning chitosan, it is a derivative of chitin. Chitin is the most abundant natural polysaccharide after cellulose, and is obtained by chemical extraction of the exoskeletons of crustaceans, mollusks, and arthropods [13]. Its derivative, chitosan, is thus described as a linear, semicrystalline polysaccharide consisting of glucosamine ($C_6H_{13}NO_5$) and N-acetylglucosamine linked by β (1–4) glycosidic bonds, which differs from the chitin polymer by the presence of free amino groups on the second carbon atom of the D-glucose unit, rather than acetamide groups [14]. Chitosan is a biodegradable, antifungal, and non-toxic biopolymer with antimicrobial and plant immunity-triggering properties [15–17]. Several works refer to the stimulating effect of chitosan on plant growth and development in cucumber [18], strawberry [19], tomato, eggplant [20], radish [21], cowpea [22], and common bean [23]. Authors in [24] also reported that chitosan improves morpho-physiological parameters and attenuates the detrimental effect of abiotic stresses stimulation of stress transduction pathways. The use of these two bioproducts would be interesting to improve growth and also control pathogenic diseases. To this effect, ref. [25] in their research work, showed that the application of *Pseudomonas* sp. in combination with chitosan reduced the severity of disease caused by tomato leaf curl virus (ToLCV) by up to 90.33% in field conditions.

Based on this evidence, the use of low-input agriculture through the use of these organic sources is, therefore, becoming a necessity to achieve profitable and sustainable agriculture. It is then necessary for agricultural research to position itself in the development of processes to make chitosan-based bioproducts and beneficial microorganisms available to producers in the agricultural sector in order to improve agricultural productivity. It is in this context that this work aimed to evaluate, on the one hand, the tolerance of a PGPR *Pseudomonas putida* strain to different abiotic stresses under in vitro conditions and, on the other hand, the synergistic effect of this rhizobacterium in combination with chitosan extracted from the exoskeletons of local Benin crabs (*Callinectes amnicola* and *Cardisoma armatum*) on the growth of greenhouse maize in Benin.

2. Materials and Methods

2.1. Material

The maize seed used was of the variety EVDT 97 STR W and came from the Centre de Recherches Agricoles du Sud (CRA-Sud) of the Institut National des Recherches Agricoles du Bénin. This variety shows good resistance to American rust, streak, helminthosporiosis, curvulariasis, and drought [26].

The chitosans used were chitin derivatives extracted from the exoskeletons of crabs (*Callinectes amnicola* and *Cardisoma armatum*) collected in Benin according to the method adapted by [27]. The extracts obtained were stored at the Laboratoire de Biologie et de Typage Moléculaire en Microbiologie (LBTMM). They are solid bioproducts containing natural polymers, biodegradable, and non-toxic for animals and plants.

The PGPR rhizobacterium named *Pseudomonas putida* was used in this study to evaluate the synergistic effect of PGPR in combination with extracted chitosans. This strain stored at -85°C in Mueller Hinton nutrient broth with addition of glycerol (10%) in the laboratory was isolated and identified by [28] and characterized by [7] for its PGPR profile.

2.2. In Vitro Evaluation of the Ability of *P. putida* to Grow under Different Environmental Stress Conditions (Temperature, pH, Salt)

2.2.1. Evaluation of the Effects of Temperature Variation on the Growth of *P. putida*

The ability of *P. putida* to grow under different temperatures (4, 16, 25, 30, 37, 40, 44, 48, and 50°C) was performed by the method adapted by [29] on Mueller Hinton (MH) liquid nutrient broth inoculated with 100 μL of the bacterial culture of the tested strain for 48 h under agitation. The growth of *P. putida* was evaluated by reading the optical density (OD) with a spectrophotometer (BioMATE 3S, Thermo scientific, Waltham, MA, USA) at the wavelength of 600 nm and the experiments were performed in three replicates. [29].

2.2.2. Evaluation of the Effects of pH Variation on the Growth of *P. putida*

The effect of pH was evaluated by the method adapted by [29] on Mueller Hinton (MH) liquid nutrient medium. The medium was adjusted to different pH values, 4, 5, 6, 7, 8, 9, 10, and 12, to evaluate the ability of the strain to grow over a wide range of pH values. The growth of the strain was evaluated by measuring the optical density of the cultures with a spectrophotometer (BioMATE 3S, Thermo scientific) at the wavelength of 600 nm and the experiments were carried out in three replicates.

2.2.3. Evaluation of the Effects of Different Salt Concentrations (NaCl) on the Growth of *P. putida*

Salt tolerance of *P. putida* was evaluated by the method adapted by [29] on Mueller Hinton (MH) liquid nutrient broth inoculated with 100 μL of one culture of each rhizobacterium and incubated at 30°C for 48 h under agitation in the presence of the following increasing concentrations of NaCl: 0, 0.2, 0.4, 0.6, 0.8, and 1 M. The optical density of each culture was read with a spectrophotometer (BioMATE 3S, Thermo scientific) at the wavelength of 600 nm and the experiments were performed in three replicates.

2.3. Evaluation of the Effects of Chitosans Produced in Benin from *C. amnicola* and *C. armatum* in Combination with *P. putida* on the Greenhouse Growth of Maize on Ferrallitic Soil

2.3.1. Extraction of Chitosans from Exoskeletons of *C. armatum* and *C. amnicola*

- The samples of land crab (*Cardisoma armatum*) and freshwater crab (*Callinectes amnicola*) used were collected from anglers and crab sellers, respectively, in the municipalities of Ajohoun and Abomey-Calavi in Benin. After the crab samples were collected, the shells were separated from the meat. These shells were washed with tap water then with distilled water before being dried. The shells were sprayed with acetone and placed in an oven at 50°C for 24 h to activate the drying process. Drying was continued in a pasteurized oven at 50°C for 10 days. After drying, all samples were ground and powdered using a Retsch mill type SM 2000/1430/Upm/Smfet [27].
- The powders of the two crab species obtained were used to obtain chitosan in four steps according to the method of [30] adapted by [27], which consists of a demineralization of the powder in acid medium, followed by a deproteinization of the powder in basic medium, then a bleaching of the powder, and finally a deacetylation of the powder by hydrothermochemical process in basic medium.

2.3.2. Refreshing of the Strain and Preparation of *P. putida* Suspensions

The *P. putida* strain previously stored at -85°C was revived by subculturing on King B agar medium at 30°C for 24 h at 30°C . After 24 h, bacterial inocula were obtained by culture in a nutrient medium liquid (Mueller Hinton broth) for 24 h at 30°C . Then, another culture was carried out from the previous 24 h at the appropriate temperatures. After the 24 h incubation, the cultures were then adjusted to a microbial concentration of about 1×10^8 CFU/mL (OD 0.45 at 610 nm) with a spectrophotometer (BioMATE 3S, Thermo scientific) according to the method described by [31].

2.3.3. Experimental Device

The experimental design used was a randomized block of six treatments with three replicates. The treatments were defined as follows:

- T1: CTL = Uncoated seed (without chitosan and *P. putida*) (control);
- T2: Cali = Seeds coated with chitosan extracted from *C. amnicola*;
- T3: Card = Seeds coated with chitosan extracted from *C. armatum*;
- T4: Puti = Seeds inoculated with *Pseudomonas putida*;
- T5: Cali + puti = Seeds coated with the combination of *C. amnicola* + *Pseudomonas putida*;
- T6: Card + puti = Seeds coated with the combination of *C. armatum* + *Pseudomonas putida*.

2.3.4. Filling the Pots

The substrate used was an undegraded ferrallitic-type soil sampled at a depth of 0 to 20 cm using a marked shovel spade. This substrate was collected at the experimental station of the Centre de Recherches Agricoles-Sud du Bénin and dried in the sun. It was then sieved and double sterilized at 120°C for 20 minutes at an interval of 24 h [32]. Six kilograms of the substrate were weighed and poured into each pot, previously labeled. Each pot was moistened to 2/9th of the Maximum Retention Capacity (MRC) of the substrate water 24 h before sowing [33].

2.3.5. Coating of Seeds with Extracted Chitosans Based on *C. amnicola* and *C. armatum*

The coating of the seeds by the chitosans was done on the eve of sowing. Clay was used as a binder to coat the seeds. The amount of chitosan and clay used applied was 10% of the weight of the maize seeds. An amount of water equivalent to 1200 mL/kg of the product was added to obtain the mixture. The seeds were coated in each mixture, thus obtained according to the type of chitosan. They were then air-dried according to the recommendations of [34].

2.3.6. Sowing of Coated Seeds, Inoculation, and Maintenance of Pots

After placing the pots in the greenhouse, two maize seeds previously coated with each chitosan according to the defined treatments were sown after opening the pots about 5 cm deep in the center of each pot. Then, they were immediately inoculated with 10 mL of the *P. putida* bacterial suspension with a concentration of about 1×10^8 CFU/mL according to each treatment, and the pots were immediately closed again.

The pots were watered at 2/9th of their Maximum Water Retention Capacity every 48 h for 30 days. After one week, the plant was de-wedded to one plant per pot. The average temperature values inside the shelter were determined using a thermometer. To obtain the average temperature values inside the greenhouse, a thermometer was used. The average daytime temperature was 35.85°C and the average night-time temperature was 37.5°C during the test period.

2.3.7. Chemical Analysis of Soil Used in Greenhouses

The chemical analysis of part of the soil (500 g) used was carried out at the Laboratoire des Sciences du Sol, Eau et Environnement (LSSEE) of the Institut National de Recherches Agricoles du Bénin (INRAB). The analyses carried out consisted of pH measurement using an electrode pH meter in a soil/water ratio of 2/5 (g/mL), determination of organic matter [35], determination of assimilable phosphorus using the Bray-1 method [36], and of exchangeable bases (Ca, Mg, K, and Na) by the method of [37].

2.3.8. Collection of Growth and Yield Parameters

Plant growth parameters were assessed by collecting height and crown diameter data on each maize plant at the time of de-mating and every 96 h, i.e., on the 7th, 11th, 15th, 19th, 23rd, 27th, and 30th day after seeding (DAS), while data on leaf length and width were collected on the 30th DAS for leaf area determination. The height of a maize plant is the distance from the crown to the last ligule. It was measured with a tape measure. The diameter at the plant crown was measured using the calliper at the plant crown. The ligulate leaves' surface area was estimated by multiplying the length and width of the leaves by 0.75 [38]. It involved the last two ligulate leaves of each plant. This measurement was carried out only at the 30th DAS. The collected data were recorded in a register. Furthermore, the aerial parts of the plants were cut off, the roots were separated from the culture substrate, and the fresh biomasses were weighed. The dry matter (above and below ground) was determined by weighing after drying in an oven at 70 °C for 72 h.

2.3.9. Statistical Analysis of the Data

Linear mixed-effect models on longitudinal data (using nlme packages for model fitting and lsmeans packages for the calculation of fitted means) were fitted to evaluate the effects of chitosan-based treatments and its combination with *P. putida* on the growth parameters (height and crown diameter) of maize plants. These effects are graphically represented by the evolution curves per treatment and days after sowing (DAS).

As for the statistical analysis of maize plant yield performance following the different chitosan treatments, a one-factor analysis of variance was performed by yield parameters using the "car" package in the R 4.0.2 software (R Core Team, 2020). This analysis of variance was followed by a post hoc test of pairwise comparisons using Tukey post hoc test [39] was carried out to assess the statistical differences of the means using u multcomp packages. The descriptive statistics calculated required the use of the dplyr and DescTools packages. The packages ggplot2 and ggpur were used to represent the results graphically. A probability (*p*-value) < 0.05 is considered significant.

A correlation matrix was established in order to assess the dependency between the different parameters measured. The Pearson method was used and using the package corrrplot, the coefficients were visualized.

3. Results

3.1. In Vitro Effect of *P. putida* Tolerance to Different Environmental Stress Conditions

3.1.1. Effect of Temperature Variation on the Growth of *P. putida*

The growth of *P. putida* was observed at the different temperatures tested. It was optimal at 30 °C (with an optical density equal to 1.51) but reduced at 40 °C (with an optical density equal to 1.01). At extreme temperatures (4; 16; 48 and 50 °C), *P. putida* maintained an appreciable but low growth rate (Figure 1a).

Figure 1. (a–c): Effect of temperature, pH, and NaCl on the growth of *P. putida*.

3.1.2. Effect of pH Variation on the Growth of *P. putida*

Regarding pH tolerance, *P. putida* grew in a wide range of pH from 4 to 8. At neutrality (pH = 7), *P. putida* reached its optimal growth with an OD equal to 1.61. On the other hand, inhibition of strain growth was visible from pH 9 (OD = 0.81) until the decline at pH 12 (OD = 0.12). (Figure 1b).

3.1.3. Effect of Salt (NaCl) at Different Concentrations on the Growth of *P. putida*

The growth of *P. putida* on nutrient broth was evaluated by measuring the turbidity of the culture at NaCl concentrations ranging from 0 to 1 M. A good tolerance capacity of *P. putida* to NaCl was noted with a concentration ranging from 0.2 M to 1 M. The *P. putida* strain subjected to NaCl concentration ranging from 0 to 0.4 M revealed good salt tolerance ability with OD ranging from 1.71 to 1.52. The *P. putida* strain maintained an appreciable growth rate at the concentration between 0.6 M and 0.8 M and dropped as the amount of NaCl increased (1 M) (Figure 1c).

3.2. Chemical Characteristics of the Soil

Table 1 shows the chemical characteristics of the soil used. This substrate is moderately acidic, with a very low percentage of organic matter (1.15%) and organic carbon (0.63%). The content of assimilable phosphorus (8 ppm) is very low and the sum of exchangeable bases of about 4 meq/100 g.

Table 1. Chemical characteristics of the used soil for maize grow.

Sample	pH		PA (ppm)	OC (%)	OM (%)	EB (meq/100 g)			
	Water	KCl				Ca	Mg	K	Na
Soil	5.3	4.7	8	0.63	1.15	2.63	0.61	0.13	0.22

PA: phosphorus assimilable, OC: organic carbon, OM: organic matter, EB: exchangeable bases; ppm: parts per million; meq: milliequivalents.

3.3. Effects of Chitosan-Based Treatments and Its Combination with *P. putida* on the Growth Parameters of Maize Plants

The use of chitosan-based bioproducts and PGPR is one of the alternative solutions to increase crop productivity and plant health. The results presented in Table 2 show an improvement in growth parameters at day 30 after sowing following the use of chitosans extracted from *C. amnicola* and *C. armatum* and their combination with *P. putida* on maize growth (Table 2). Moreover, the results show a significant difference between maize plant heights, crown diameters, and leaf areas according to the treatments applied (Figures 2–4).

Figure 2. Plant height development as a function of treatments and overtime. CTL = Control (Uncoated seed (without chitosan and *P. putida*); Cali = Seeds coated with chitosan extracted from *Callinectes amnicola*; Card = Seeds coated with chitosan extracted from *Cardisoma armatum*; Puti = Seeds inoculated with *Pseudomonas putida*; Cali + puti = Seeds coated with the combination of *Callinectes amnicola* + *Pseudomonas putida*; Card + puti = Seeds coated with the combination of *Cardisoma armatum* + *Pseudomonas putida*.

Figure 3. Plant diameter development as a function of treatments and over time. CTL = Control (Uncoated seed (without chitosan and *P. putida*); Cali = Seeds coated with chitosan extracted from *Callinectes amnicola*; Card = Seeds coated with chitosan extracted from *Cardisoma armatum*; Puti = Seeds inoculated with *Pseudomonas putida*; Cali + puti = Seeds coated with the combination of *Callinectes amnicola* + *Pseudomonas putida*; Card + puti = Seeds coated with the combination of *Cardisoma armatum* + *Pseudomonas putida*.

Figure 4. Effects of treatments on the foliar surface of plants. a, ab, b, bc, etc.: represent the results of the tests of comparison of means after the results of analysis of variance. CTL = Control (Uncoated seed (without chitosan and *P. putida*); Cali = Seeds coated with chitosan extracted from *Callinectes amnicola*; Card = Seeds coated with chitosan extracted from *Cardisoma armatum*; Puti = Seeds inoculated with *Pseudomonas putida*; Cali + puti = Seeds coated with the combination of *Callinectes amnicola* + *Pseudomonas putida*; Card + puti = Seeds coated with the combination of *Cardisoma armatum* + *Pseudomonas putida*.

Table 2. Mean value ($\pm$ standard error) of the effect of chitosans produced in combination with *P. putida* on maize plant growth parameters.

Observed Data	Treatments						p-Value
	CTL	Cali	Card	Puti	Cali + puti	Card + puti	
Height (cm)	14.2 ^a $\pm$ 0.12	15.0 ^{ab} $\pm$ 0.03	16.6 ^{bc} $\pm$ 0.23	16.2 ^{bc} $\pm$ 0.58	17.0 ^c $\pm$ 0.51	18 $\pm$ 0.71	0.0001 ***
Diameter (cm)	0.58 ^a $\pm$ 0.03	0.63 ^{ab} $\pm$ 0.02	0.71 ^{bc} $\pm$ 0.04	0.69 ^{bc} $\pm$ 0.03	0.71 ^c $\pm$ 0.01	0.76 $\pm$ 0.03	0.0006 ***
Leaf Area (cm ²)	55.7 ^a $\pm$ 2.09	66.1 ^{ab} $\pm$ 2.57	66.7 ^{ab} $\pm$ 3.58	73.9 ^{bc} $\pm$ 2.32	81.4 ^{cd} $\pm$ 3.10	86.7 $\pm$ 2.44	2.8 $\times$ 10 ^{−6} ***

***: indicates a very significant probability at the 5% threshold. a, ab, b, bc, etc.: represent the structuring of the averages after the post. CTL = Control (Uncoated seed (without chitosan and *P. putida*); Cali = Seeds coated with chitosan extracted from *Callinectes amnicola*; Card = Seeds coated with chitosan extracted from *Cardisoma armatum*; Puti = Seeds inoculated with *Pseudomonas putida*; Cali + puti = Seeds coated with the combination of *Callinectes amnicola* + *Pseudomonas putida*; Card + puti = Seeds coated with the combination of *Cardisoma armatum* + *Pseudomonas putida*.

3.3.1. Height

Figure 2 shows the evolutionary trend of maize plants as a function of treatments over time. Good plant width development from emergence until 30 DAS. The results of the linear mixed-effects model showed that the development in plant diameter depends on the treatments but also varies with time (p -value < 0.001). The height of all the plants evolved as a function of time but from 11 DAS to 30 DAS, there was a clear difference in the evolution of the height of treated and untreated plants. Indeed, from the 7th to the 10th, the plants' height knew a similar evolution, but from the 11th to the 30th of DAS, we noted a clear difference in the development of the plants according to each treatment. Thus, the plants treated with the combination of chitosan extracted from *C. armatum* and *P. putida* were clearly more important than the other treatments. The average heights measured on the 30th day after sowing varied between 14.2 ± 0.12 cm and 18 ± 0.71 cm (Table 2). The lowest values were recorded by control plants while the highest values were recorded by plants treated with the combination of chitosan extracted from *C. armatum* and *P. putida*. The treatment based on chitosan extracted from *C. armatum* alone resulted in a 17% increase in plant height compared to the control. In comparison, its combination with *P. putida* resulted in a 26.8% improvement in plant performance compared to the control. The same observations were made with the treatment under chitosan extracted from *C. amnicola* slightly induced an increase of 6%. Furthermore, when combined with *P. putida*, the increase was 20% compared to the control (Table 2).

3.3.2. Diameter

Figure 3 shows the width development of the plants from emergence until 30 DAS. The results of the linear mixed-effects model show that the development in plant diameter depended on the treatments but also varied as a function of time (p -value < 0.001).

In fact, between the 7th and 20th day, plants treated with the combination of chitosan extracted from *C. amnicola* and *P. putida* had the best development in diameter, followed by plants treated with the combination of chitosan extracted from *C. armatum* and *P. putida* and plants treated with *P. putida* only which had the same development (Figure 3). After the 20th day, the development of the plants treated with chitosan extracted from *C. armatum* and *P. putida* was significantly higher than the other treatments. The evolution of the plants under the combination of chitosan extracted from *C. amnicola* and *P. putida* and chitosan extracted from *C. armatum* and *P. putida* was similar (Figure 3). The mean diameters measured on the 30th day after sowing varied between 0.58 ± 0.03 cm and 0.76 ± 0.03 cm (Table 1). The control plants were those with the low development in diameter. At the same time, those treated with the combination of chitosan extracted from *C. armatum* and *P. putida* induced the high development in diameter. In fact, the treatment based on chitosan extracted from *C. armatum* alone ensured an increase of 24% compared to the control. In comparison, its combination with *P. putida* induced an improvement in plant performance of 31% compared to the control plants. The combination of chitosan extracted from *C. amnicola* and *P. putida* induced an increase of 23% compared to the control. On the

other hand, plants treated only with *P. putida* resulted in a 20% increase compared to the control plants (Table 2).

3.3.3. Leaf Area

In terms of leaf area, combinations between chitosans and *P. putida* gave the best development (Figure 4). In addition, the plants treated with the combination of chitosan extracted from *C. armatum* and *P. putida* provided the best leaf development at the end of the greenhouse trials (30th day after sowing), and the average value of the leaf area of these plants was $86.7 \pm 2.44 \text{ cm}^2$, an increase of more than 55% of the leaf area of the plants compared to the control plants. The plants treated with chitosan extracted from *C. amnicola* in combination with *P. putida* were the second most important with an average of $81.4 \pm 3.10 \text{ cm}^2$ and ensured an increase of about 46%. Treatments based solely on chitosan or *P. putida* gave intermediate results, however, clearly superior to the control treatment. However, it should be noted that the plants treated with *P. putida* showed a greater development than those treated with chitosan. Thus, treatments with *P. putida*, chitosan extracted from *C. armatum*, and chitosan extracted from *C. amnicola* induced, respectively, an increase in leaf area of 32%, 19%, and 18% (Table 2).

3.3.4. Effects of Chitosan-Based Treatments and Its Combination with *P. putida* on Maize Plant Yield Parameters

Table 3 shows the average biomass obtained from maize plants per treatment. The results indicate that the effect of the different treatments on the performance of the maize plants was significant regardless of the type of biomass measured.

Table 3. Mean value ($\pm$ standard error) of the effect of chitosans produced in combination with *P. putida* on the biomass yield parameters of maize plants.

Observed Data	Treatments						p-Value
	CTL	Cali	Card	Puti	Cali + puti	Card + puti	
FAB (g)	$8.48^a \pm 0.6$	$9.99^a \pm 0.6$	$13^b \pm 0.76$	$14.3^{bc} \pm 0.56$	$13.3^b \pm 0.42$	$16.7^c \pm 0.47$	$1.1 \times 10^{-7} ***$
DAB (g)	$1.03^a \pm 0.04$	$1.21^a \pm 0.07$	$1.83^b \pm 0.03$	$1.94^b \pm 0.07$	$1.82^b \pm 0.07$	$2.33^c \pm 0.15$	$7.5 \times 10^{-9} ***$
FUB (g)	$3.75^a \pm 0.38$	$5.28^{ab} \pm 0.56$	$7.72^c \pm 0.30$	$7.85^c \pm 0.18$	$6.52^{bc} \pm 0.22$	$7.92^c \pm 0.27$	$2.0 \times 10^{-7} ***$
DUB (g)	$0.66^a \pm 0.09$	$1.04^{ab} \pm 0.05$	$1.56^{bc} \pm 0.12$	$1.74^c \pm 0.10$	$1.54^{bc} \pm 0.14$	$1.84^c \pm 0.2$	$1.6 \times 10^{-5} ***$

***: indicates a very significant probability at the 5% threshold. ^{a, ab, b, bc, etc.}: represent the structuring of the averages after the post hoc Tukey test. CTL = Control (Uncoated seed (without chitosan and *P. putida*); Cali = Seeds coated with chitosan extracted from *Callinectes amnicola*; Card = Seeds coated with chitosan extracted from *Cardisoma armatum*; Puti = Seeds inoculated with *Pseudomonas putida*; Cali + puti = Seeds coated with the combination of *Callinectes amnicola* + *Pseudomonas putida*; Card + puti = Seeds coated with the combination of *Cardisoma armatum* + *Pseudomonas putida*; FAB: fresh aerial biomass; DAB: dry aerial biomass; FUB: fresh underground biomass; DUB: dry underground biomass.

In fact, an increase of between 18% and 97% in the fresh aerial biomass of the treated plants was observed compared to the control plants. The average fresh aerial biomass (FAB) observed was respectively 16.7 g, 14.3 g, 13.3 g, 13 g, and 9.99 g for plants treated with chitosan extracted from *C. armatum* and *P. putida*, plants treated with *P. putida*, plants subjected to chitosan extracted from *C. amnicola* and *P. putida*, plants subjected to chitosan extracted from *C. armatum*, and chitosan extracted from *C. amnicola*.

Statistically, the combination of chitosan extracted from *C. armatum* and *P. putida* gave very different results from the other treatments (Table 3). Chitosan extracted from *C. armatum* and the combination chitosan extracted from *C. amnicola* and *P. putida* gave similar results while chitosan extracted from *C. amnicola* gave similar results to the control. The same trends observed in the fresh aerial biomass (FAB) were observed in the dry above-ground biomass (Table 3).

The effect of the treatments on the fresh underground biomass (FUB) was greater compared to the results for the fresh above-ground biomass. An increase of between 40% and 110% compared to the control was induced depending on the treatment. Thus, the fresh underground biomass (FUB) was 7.92 g for the combination of chitosan extracted from *C. armatum* and *P. putida*; 7.85 g for *P. putida*; 7.72 g for chitosan extracted from *C. armatum*;

6.52 g for combination of chitosan extracted from *C. amnicola* and *P. putida*; and 5.28 g for chitosan extracted from *C. amnicola*. The effects induced by these treatments were significant with four groups. Indeed, the treatments *C. armatum* and *P. putida*, *P. putida*, and chitosan extracted from *C. armatum* formed the first group with similar effects then came groups 2 and 3 constituted, respectively, by the combined treatment of chitosan extracted from *C. amnicola* and *P. putida* and chitosan extracted from *C. amnicola* which give intermediate results relative to the last group constituted by the control (Table 3).

For dry subsurface biomass (Table 3), the results were also broadly similar to the results obtained for fresh subsurface biomass. However, while for the fresh subsurface biomass, the chitosan treatment extracted from *C. armatum* was in the first group, for the dry subsurface biomass, these effects were similar to those of group 2 (chitosan extracted from *C. amnicola* and *P. putida*).

3.3.5. Correlation of the Various Parameters

The measured linear dependence between the growth parameters (height, diameter, and leaf area) and yield (fresh and dry aerial biomass, fresh and dry underground biomass) of the plants is presented in Figure 5. All correlations were positive and important as shown by the size of the circles and the intensity of the colors ($r_{corr} > 0.70$). A linear relationship between growth and yield parameters can be deduced from this, the most important of which is the relationship between plant height and diameter ($r_{corr} = 0.99$) then the relationship between fresh aerial biomass (FAB) and diameter/height ($r_{corr} = 0.95$). The relationship between leaf area and fresh underground biomass (FUB) is the least important ($r_{corr} = 0.72$).

Figure 5. Correlation coefficients of the different parameters. FAB: fresh aerial biomass; FUB: fresh underground biomass.

4. Discussion

The deteriorating environmental effects arising from the misuse of pesticides and chemical fertilizers in agriculture has resulted in the pursuit of eco-friendly means of producing agricultural produce without compromising the safety of the environment [40]. These ecological means include the use of PGPR such as *P. putida* and chitosan, a bioproduct derived from chitin extracted from crustaceans. The knowledge on the tolerant effect of

P. putida to abiotic stress under in vitro conditions as well as the synergistic effect of this strain in combination with chitosan on maize cultivation under greenhouse conditions was the subject of this study.

4.1. Effect of Temperature, pH, and Salinity on the Growth of *P. putida* Strain

A number of abiotic factors, including temperature, salinity, drought, pesticide and fertilizer application, soil pH, and heavy metal contamination, also impede crop productivity [41]. Among these factors, temperature, salinity, and acidity are among the main causes of abiotic stress in plants, causing adverse consequences on the development and quality of harvested products.

The growth of the *P. putida* strain was observed at the different temperatures tested (Figure 1a). It reached its optimum growth at 30 °C but reduced from 40 °C. At extreme temperatures (4, 16, 48, and 50 °C), this strain maintained an appreciable but low growth rate. These results are similar to those of [29] with the *Pantoea agglomerans* lma2 strain that was positive at the different temperatures tested. Indeed, temperature is one of the most important factors that governs the physiology and growth of microorganisms [42].

For the growth of the strain under different pH variations, it was noted that *P. putida* grew from pH 4 to pH 8 (Figure 1b). The ability of the strain to grow over a wide range of pH is indicative of its survival ability. This is in agreement with the work of [43] on *Pantoea agglomerans* CPA-2, able to grow at pH 4.5 to pH 8.6. The same is true for the work of [29], which showed that *Pantoea agglomerans* lma2 grew in a wide range of pH from pH 4 to pH 8. The use of PGPR will go a long way in supporting the plant to develop both intrinsic and extrinsic ability to tolerate stressful conditions and sustain yield [11].

Concerning salinity, *P. putida* was found to be salt tolerant at different concentrations (0.2 M to 1 M) (Figure 1c). It retained a strong growth capacity in the presence of NaCl at a concentration of 0.2 to 0.4 M, but from a NaCl concentration equivalent to 0.8 M, the growth of the strains slowed down. Salinity is considered among the abiotic stresses most impacting agriculture for its ability to interfere with crop development and quality. For this reason, practices and innovations that could contain the deleterious effects of such stress are of pivotal importance for maintaining acceptable crop yields [44]. In the last few years, research has shown that the use of salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) in saline agriculture can be harnessed for enhancing productivity and improving soil fertility as well [45]. Authors in [46] also reported that under stress conditions, PGPR can improve the injurious impacts and enhance the yield production under salt conditions.

4.2. Effects of Combination Chitosan and *P. putida* on Growth Parameters

This study shows the positive effect of the combination of chitosan and PGPR on the growth of maize plants. Indeed, inoculation of seeds with chitosan extracted from *C. amnicola* and *C. armatum* and their combination with *P. putida* on day 30 after sowing induced an improvement in growth parameters, which vary from one treatment to another (Table 2). In addition, the results (Figures 3–5) show a significant difference between maize plant heights, crown diameters, and leaf areas depending on the treatments applied.

Regarding growth parameters, such as height, crown diameter, and leaf area of the plants, the highest values were obtained with plants treated with the combination of chitosan extracted from *C. armatum* and *P. putida*, which induced increases of 26.8%, 31%, and 55.7%, respectively, with respect to the control. This rate of improvement in height is higher than the 17% obtained by [47] following the inoculation of maize under greenhouse conditions with chitosan extracted from lobsters combined with strains of *Azospirillum lipoferum*–*P. fluorescens*–*P. putida*. Authors in [48] observed a significant increase in crop yield of 30–50% compared to plants not inoculated with the PGPR strain *Azospirillum brasilense* (HM053). Various research works have focused mainly on the evaluation of rhizobacteria for their ability to induce improvements in growth and yield parameters in cereals under controlled conditions [12,47,49]. Ref. [50] mentioned that the use of microbial

inoculants is a reliable alternative to the use of chemical inputs because these microbial inoculants can act as biofertilizers, bioherbicides, biopesticides, and biocontrol agents. As for the work of [51], it revealed that the application of chitosan increased the percentage of black pepper height and diameter compared to control. Indeed, chitosan has an excellent film-forming property, facilitates the formation of a semi-permeable film on the seed surface that can maintain seed moisture and absorb soil moisture, and thus can promote seed germination [52]. The work of [27] also showed the positive effects of chitosan extracted from the exoskeletons of *C. amnicola* and *C. armatum* on in vitro maize seed germination. Several authors have reported the beneficial effects of chitosan application on legume development [52,53]. Authors in [16] reported in their study that the integrated application of PGPR with chitosan significantly reduced symptoms caused by papaya ringspot virus-W (PRSV-W) and tomato chlorotic spot virus (TCSV). The improved effect of chitosan and PGPR has already been reported for other plants and has significantly reduced the severity of tomato leaf curl virus (ToLCV) disease [25] and cucumber mosaic virus [54]. Authors in [20] also reported in their study that foliar application of oligochitosans at different concentrations played a positive role on the growth of tomato and eggplant plants. Chitosan is efficient in agriculture by inducing a signal to synthesize plant hormones such as gibberellins and improve growth and development through a signaling pathway related to auxin biosynthesis [55]. The application of chitosan and PGPR as individual treatments or in combination was effective in improving plant growth and suppressing the severity of disease caused by the Squash Mosaic Virus (SqMV) on cucumber plants [56]. Authors in [57] also showed in their research that the application of chitosan could promote plant germination and growth and reduce harmful salinity.

4.3. Effects of Chitosan and *P. putida* on Biomass Yield Parameters

Table 3 shows the average biomass obtained from maize plants per treatment. The results indicate that the effect of the different treatments is very significant on the performance of the maize plants regardless of the type of biomass measured. In fact, an increase of between 18% and 97% in the fresh aerial biomass of the treated plants was observed compared to the control plants, whereas an increase of 40.8% to 111.2% was observed for the underground biomass. This improvement could be explained by the fact that PGPRs readily colonize the root rhizosphere and establish both free life and intimate associations with host plants [5], which could promote plant growth. The stimulating effect of chitosan, on the other hand, can be attributed to increased availability and absorption of water and essential nutrients by adjusting the osmotic pressure of cells, and reduced accumulation of harmful free radicals by increasing antioxidant and enzyme activities [58]. This effect can also be attributed to an increase in the key enzymatic activities of nitrogen metabolism (nitrate reductase, glutamine synthetase, and protease) and to improved nitrogen (N) transport in functional leaves, as well as to an increase in photosynthesis, which promoted plant growth and development [59]. Ref. [60] reported also that PGPRs can help plants absorb nutrients, control the proliferation of plant pathogens, and can have a positive impact on the natural biodiversity of the soil, thus contributing to plant growth and development. These bacteria also serve as plant growth regulators by producing the phytohormones indole-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), cytokinins, and gibberellins (GA) [61,62].

Furthermore, ref. [23] reported that foliar-applied chitosan, in particular 200 mg/L, increased common bean plant growth, yield, and its quality, as well as physiological constituents in plant shoot under stressed or non-stressed conditions as compared to chitosan untreated plants of common bean. The application of chitosan to rice grains resulted in a significant increase in yield. This increase was due to the induction of phytoalexin accumulation in plant tissues [63]. Given the importance of chitosan and PGPRs in agriculture, their use could therefore reduce dependence on chemical fertilizers and pesticides in the future.

5. Conclusions

Agricultural practices that reduce or eliminate chemical fertilizers and pesticides through the use of natural products are of paramount importance today for a sustainable and environmentally friendly agriculture. To this end, the application of bioproducts such as PGPR and chitosan in agriculture is one of the promising alternatives to combat abiotic stress in crops and to ensure food and health security of the population. This study revealed through in vitro tests that the strain of PGPR under study, *P. putida*, showed potentialities of tolerance to abiotic stress such as temperature, pH, and salinity. This study also showed that the application of each of the chitosans extracted from local crabs (*C. amnicola* and *C. armatum*) alone or in combination with the rhizobacterium *P. putida* improved the growth and biomass yield parameters of the maize plants. Plants treated with the combination of chitosan extracted from *C. armatum* with *P. putida* induced the best results. This study therefore proves the possibility of using rhizobacteria and chitosan as biostimulants in agricultural production for sustainable and environmentally friendly agriculture in the face of climate change. However, real-world field experiments are needed to better establish the best treatment option for improving maize grain yields.

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