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Uptake of 15N-urea and phosphates in Triticum aestivum with Pseudomonas putida and Rhizophagus irregularis endophytes of calcareous soil weeds

Blanca Valdivia Urdiales[1], Juan Luis Ignacio de la Cruz[2], Juan Manual Sánchez-Yáñez[2]

1 Universidad Autónoma Agraria "Antonio Narro"

2 Universidad Michoacana de San Nicolás de Hidalgo

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Abstract

In calcareous soil, the growth and production of *Triticum aestivu*m depends on the availability of phosphates, which in turn reduces the uptake of nitrogen in the form of urea, which causes volatilization and partial leaching of both fertilizers, contributing to the greenhouse effect, and warming global. An alternative ecological solution for *T. aestivum* is to inoculate *Pseudomonas putida* and *Rhizophagus irregularis* with endophytes that increase phosphorus uptake such as P_2O_5 and urea. The objective of this research was to analyze the uptake and distribution of ^{15}N-urea in *T. aestivum* with *P. putida* and *R. irregularis* fertilized with 50% urea and P_2O_5. In this sense, *P. putida* and *R. irregularis* isolated from roots of *Resenda luteola* and *Arista purpurea* native to the calcareous soil of northeastern Mexico. In *T. aestivum* with these endophytes, acid and alkaline phosphatase activity in root and stem, N (nitrogen) uptake from total urea (Nt) and yield (Y). The experimental data were analyzed by ANOVA/Tukey ($P<0.01$). The results showed a positive response of *T. aestivum to P. putida* and *R. irregularis* with 60 kg/ha of urea and 40 kg/ha of P_2O_5, equivalent to the 50% recommended for this region of Mexico. It was evidenced that in *T. aestivum, P. putida,* and *R. irregularis* endophytes of desert weeds improved the uptake of urea and P_2O_5 to 50% by phytohormones that optimized nitrogen with phosphatases, soil phosphate and that from the applied fertilizer. The Nt in stem and the yield of *T. aestivum* with *P. putida and R. irregularis* reached statistically different numerical values to those registered in *T. aestivum* with 120 kg/ha of urea and 80 kg/ha of P_2O_5 without *P. putida* and *R. irregularis* at 100% (relative control), consequently the performance of *T. aestivum* in calcareous soils. It is avoided by the generation of greenhouse gases, the contamination of surface water, by using the beneficial interaction of endophytes with weeds with *T. aestivum,* as well as global warming.

Blanca Valdivia-Urdiales[1], **Juan Luis Ignacio-De la Cruz**[2] and **Juan Manuel Sánchez-Yáñez**[2*]

[1] *Department of Soils, Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila, México current address, Department of Food Research, Facultad de Química, Universidad Autónoma de Coahuila V. Carranza y Gonzalo Lobo s/n 25280, Saltillo, Coahuila, Mexico.*

[2] *Environmental Microbiology Laboratory, (corresponding author: syanez@umich.mx), Chemical-Biological Research Institute, Ed-B3, Michoacán University San Nicolás de Hidalgo, Fco J Mújica s/n Col Felicitas del Rio, CP 58030, Morelia, Michoacán, Mexico*

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Introduction

In the northeast of Mexico, the soil is of calcareous origin, they have an alkaline pH with high salinity[1], which limits the absorption of nitrogenous fertilizers (urea) and PO4-3 (phosphates). In specific calcareous soil pH controls the relative availability of the two ionic chemical forms of phosphate. The $H_2PO_4^-$ ion is favored by a pH less than 7, while the divalent ion $HPO_4^=$ is above pH 7. When the pH is alkaline, the phosphate in the form of apatite (Fe2Ca3(PO4)2Ca3), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), and carbonatoapatite (Ca_3 $(PO_4)_2CaCO_3$) causes a drastic decrease in the production of *Triticum aestivum* (wheat) [2]. Besides that, it causes volatilization problems of nitrogenous fertilizers as well as the leaching of nitrogenous fertilizers and phosphates, which contributes to the generation of greenhouse gases and contamination of surface and deep waters. While evolutionarily wild plants have these interactions as prokaryotic endophytic microorganisms, such as the eukaryotic genus, that allow them to face this problem of the availability of phosphates [3][4], especially in calcareous soil, since phosphates are necessary for obtaining energy and synthesis of nucleic acids, endophytic prokaryotes with acid and alkaline phosphatase enzymes, and endotrophic mycorrhizal fungi with these enzymes and by translocation of phosphates (3-5). In general, nitrogenous fertilizers such as urea and phosphorus such as P_2O_5 (triple superphosphate) are applied to the soil in order to be effectively absorbed by the roots of *T. aestivum* [5][6]. For this reason, there are ecological solution alternatives such as weeds that grow successfully in adverse environments such as nutritional stress and tolerance to salinity, since they are associated with endophytic microorganisms that solubilize calcium phosphate complexes and less aluminum or iron phosphate compared to mycorrhizae [7][6]. Therefore, in this work, *Pseudomonas putida* was isolated and selected, an endophyte due to the synthesis of phytohormones that improves the radical uptake of minerals with nitrogen (NH_4^+) [8][9] [10][11] and a mycorrhizal fungus: *Rhizophagus irregularis* that synthesizes alkaline phosphatases for the solubilization of phosphorus [12][13], recovered from 2 weeds such as *Reseda luteola* and *Aristida purpurea* adapted to the alkalinity and salinity associated with *T. aestivum.* [14][15]. The objective of this work was to analyze the uptake and distribution of ^{15}N-urea in calcareous soil in *T. aestivum* with *P. putida and R. irregularis* at 50% urea and P_2O_5.

Materials and Methods

Isolation of *Pseudomonas putida* and *Rhizophagus irregularis* from desertic weed.

The isolation of *P. putida* and *R. irregularis* was carried out from three weed species: *R. luteola* and *A. purpurea* associated with the *T. aestivum* crop. In the case of *P. putida*, the roots of *A. purpurea* were washed in sterile drinking water to remove the soil from the roots, which were cut into 5 cm segments and macerated. Then 0.1 g of roots were taken and 9 mL of sterile distilled water was added, dilutions were made from $1X10^{-1}$ to $1X10^{-18}$. From the $1X10^{-5}$ dilution, 0.01 mL were taken and sown on Ashby agar with the following chemical composition (g/L): mannitol, 1.5; K_2HPO_4, 0.02; $MgSO_4*7H_2O$, 0.02; NaCl, 0.02; $CaSO_4*2H_2O$, 0.01; $CaCO_3$, 0.5 and bacteriological agar, 18. The Petri dishes were incubated at 30°C/48 h, when smooth, round, cream-colored colonies appeared, with high mucilage production, similar to those of *P. putida*, they were selected and reseeded on the same agar to purify the isolates, which were then multiplied in a 250 mL flask with 50 mL of nutrient broth (Bioxon) at 30ºC/48h/150 rpm. This bacterial biomass was resuspended in sterile saline (NaCl 0.85%). For the isolation of *R. irregularis*, the roots of *R. luteola* and *A. purpurea* were washed with sterile tap water to remove the soil, then stained with the technique proposed by [16] that observed under the microscope, arbuscules and vesicles were observed inside the roots of both weeds, were cut, ground and treated with 0.1 g/l kanamycin to inhibit bacterial growth. The spores of *R. irregularis* were separated, sieved, and decanted following the technique proposed by [17].

Effectiveness test of *Pseudomonas putida* and *Rhizophagus irregularis* on *Triticum aestivum* in greenhouses conditions.

This part of the research was carried out in a greenhouse belonging to the Universidad Autónoma Agraria Antonio Narro (UAAAN), Saltillo, Coahuila, Mexico. Seeds of *T. aestivum* (var. Pavón F-76) were provided by the UAAAN, were disinfected with NaOCl (sodium hypochlorite) at 1.2%/15 min, washed five times with sterilized distilled water, and then germinated in Petri dishes with Agar-water. At emergence, were inoculation with 2.0 mL equivalent to $9.8X10^3$ CFU/mL of *P. putida* obtained by viable plate count on nutrient agar. Subsequently, the seeds were sown in the upper part of the Leonard jars, where soil and sterile sand were placed in a 1:4 ratio, while in the reservoir 500 mL of Jensen nutrient solution were added with the following composition (g/L): $CaHPO_4$, 0.1; K_2HPO_4, 0.02; $MgSO_4*7H_2O$, 0.02; NaCl, 0.02; $FeCl_3$, 0.01; pH 7; mL/L of the micronutrient solution (g/L): H_3BO_3, 0.05; $MnSO_4$, 0.05; $ZnSO_4$, 0.005; Na2MoO_4, 0.005 and $CuSO_4$, 0.002, enriched with 60 kg/ha of urea and 40 kg/ha of P_2O_5 equivalent to 50% of the recommended dose for the region. In the case of *R. irregularis*: G1 and G2, *T. aestivum* seeds were disinfected as described above, then mixed with 10% sucrose and covered with 0.1 g of *R. irregularis* inoculum. The inoculation seeds were then sown in Leonard jars and fed with Jensen's nutrient solution and 60 kg/ha of urea and 40 kg/ha of P_2O_5 equivalent to 50% recommended dose for the region of Saltillo, Coah, Mexico [18]

The response variable to determine the beneficial effect of both *P. putida* and *R. irregularis*: G1 and G2 on *T. aestivum* fertilized with urea and P_2O_5 at 50% was the total dry weight (TWD) at Feeke's stage 10.5, was compared with *T. aestivum* uninoculated fed with Jensen nutrient solution enriched with 120 kg/ha of urea and 40 kg/ha of P_2O_5 equal to 50% used as relative control (RC). Experimental data were subjected to analysis of variance (ANOVA) and Tukey's

comparison of means ($P \leq 0.01$) [19].

Sowing, inoculation, and fertilization of *Triticum aestivum* in the field.

This part of the research was carried out in a field of the UAAAN, Saltillo, Coahuila, Mexico, at coordinates 25° 25" 41' north longitude and 100° 57" 57' west longitude, with an altitude of 1742 m. The soil was classified as silty-sandy according to its physicochemical properties as shown in Table 1 [20]. The soil was then prepared in yield plots of 4.0 m^2 (2x2 m) with five rows each, spaced 30 cm apart, and a useful plot of 1.0 m^2. The seeds of *T. aestivum* variety: Pavón F-76 used in this trial were provided by UAAAN, were soaked with 10% sucrose, then mixed with 40 g of *P. putida* and 0.2 g of *R. irregularis*: G1 and G2. The seeds inoculation with *P. putida* and *R. irregularis* were sown by the broadcast method at a density of 150 kg seed/ha per treatment based on the experimental design shown in Table 2. The fertilization rates were 0, 60, and 120 kg/ha of urea and 0, 40, and 80 kg/ha of P_20_5, equivalent to 0, 50, and 100% of the recommended rates for the region, applying 0, 60 and 120 of urea and 0, 40 and 80 kg/ha of P_20_5. Both fertilizers were divided into three equal parts and applied manually in bands during the critical growth stages of *T. aestivum*: tillering, anthesis, and beginning of grain filling [21].

Table 1. Physicochemical characteristics of the soil used for planting *Triticum aestivum* inoculated with *Pseudomonas putida* and *Rhizophagus irregularis*.

Parameter	Value	Interpretation
Texture	52.5% sand, 24% silt, 23.5% clay	Silty – Sandy
pH	8.3	Medium alkaline
Electrical conductivity	0.27 S m^{-1}	saline
Organic Matter	1.92%	Medium
Total nitrogen	0.12%	Medium por
Cation exchange capacity	30.6 cmol/kg	saline
Total carbonates (CaCO3)	36.3%	Medium
Usable phosphorus	32.5 mg/kg	Low
Apparent density	1.3 g/cm^3	-

**Physicochemical characteristics in accordance with* [20].

Table 2. Experimental design to evaluate the effect of *Pseudomonas putida* and *Rhizophagus irregularis* on *Triticum aestivum* fertilized with different doses of urea and P_2O_5 in the field.

*Triticum aestivum	Pseudomonas putida	Rhizophagus irregularis		Urea (kg/ha)	P_2O_5 (kg/ha)
		G1	G2		
Irrigated with water or absolute control	-	-	-	-	-
Relative control	-	-	-	120	80
Treatment 1	+	+	-	0	0
Treatment 2	+	-	+	0	0
Treatment 3	+	+	-	60	0
Treatment 4	+	-	+	60	0
Treatment 5	+	+	-	0	40
Treatment 6	+	-	+	0	40
Treatment 7	+	+	-	60	40
Treatment 8	+	-	+	60	40

Inoculated (+); not inoculated (-).

The response variables to determine the effect of *P. putida* and *R. irregularis*: G1 and G2 on *T. aestivum* the uptake of ^{15}N-urea, the plots corresponding to the treatments were fertilized with the stable isotope of nitrogen known as ^{15}N-urea, with a 10% excess of ^{15}N atoms in seven 1.0 m^2 microplots. In addition to inoculation, the *T. aestivum* plots were fertilized with 60 kg/ha of urea and 0 and 40 kg/ha of P_2O_5. For isotopic analysis, 20 *T. aestivum* seedlings were collected from each plot, and the grain was separated and ground to a particle size of 1.0 mm. They were analyzed by mass spectrometry at the CINVESTAV unit, Irapuato, Guanajuato, Mexico, and the percentage of ^{15}N derived from urea was calculated [22]. The percentage of total nitrogen (Nt) was also quantified by the Kjeldahl method and grain yield was estimated in Ton/Ha.

Labeling of *Pseudomonas putida* on *Triticum aestivum* in the field conditions.

To evaluate the colonization of *P. putida* on *T. aestivum* roots, it was labeled by the antibiotic resistance method. For this, *P. putida* was grown in a 250 mL flask with 50 mL of nutrient broth (Bioxon) at 30°C/24 h at 150 rpm. It was then seeded on nutrient agar to determine its resistance to 12 antibiotics Gram Negative multidisc (Senofi). As well as its tolerance to 200 µg/mL of trimethoprim-sulfamethoxazole (Bactrin, Roche), to ensure its recovery from field-grown *T. aestivum* inside of roots.

Detection of acid and alkaline phosphatase in *T. aestivum* inoculated with *P. putida and R. irregularis.*

To determine the ability to solubilize phosphatase, according to [23] method was used, *P. putida* was activated in PO_4^{3-} agar with the following chemical composition (g/L): 10.0 $C_6H_{12}O_6$; 5.0 $Ca_3(PO_4)_2$; 5.0 $MgCl_2{\cdot}6H_2O$; 0.025 $MgSO_4{\cdot}7H_2O$; 0.2KCl; 0.1 $(NH_4)\ SO_4$; 0.05 bromothymol blue; 20.0 bacteriological agar pH 7, incubated at 28 ºC / 72 h a transparent halo was detected around the colony that indicated the release of PO_4^{3-} by phosphatases [6]. Quantitative analysis of

phosphatases activity in *T. aestivum* with *P. putida* and *R.irregularis* to evaluate the activity of the acid and alkaline phosphatases of *P. putida* and *R. irregularis* in *T. aestivum* by release of PO_4^{3-} from p-nitrophenyl phosphate with the method of [23], the following was prepared: a 0.9%-0.1% detergent saline solution as absolute control; the roots and the stem of *T.aestivum* inoculated and uninoculated with *P. putida* and *R. irregularis* were taken at flowering 60 days after planting, then were disinfected with 1% chlorine for 3 min, rinsed 5 times with sterile water, then with 70% alcohol for 3 min, were rinsed 7 times with sterile water, were transferred to a sterile mortar, were macerated with 10 mL of sterile 0.9%-1% detergent saline solution, 5mL was taken, 45mL of sterile distilled water was added with 20mL of buffer adjusted to pH 5.5 for the determination of acid phosphatase and pH 9.0 for the alkaline, the mixture was homogenized at 800 rpm/30 seconds, then 3mL of the suspension was used to 1mL of p-nitrophenyl phosphate (0.025 M) were left for 3h/37°C, centrifuged at 2000rpm/10min at 0.5mL of the 4.5mL of NaOH (0.5M) was added to the supernatant, the p-nitrophenol released was measured in a spectrophotometer at 410nm, which indirectly detected the release of PO_4^{3-} from p-nitrophenyl phosphate by acid phosphatases /alkaline of *P. putida* and *R. irregularis* in *T. aestivum*.

The experimental data were analyzed by the statistical test ANOVA/ Tukey HSD P<0.01 with Statgraphics Centurion XVI.I [19].

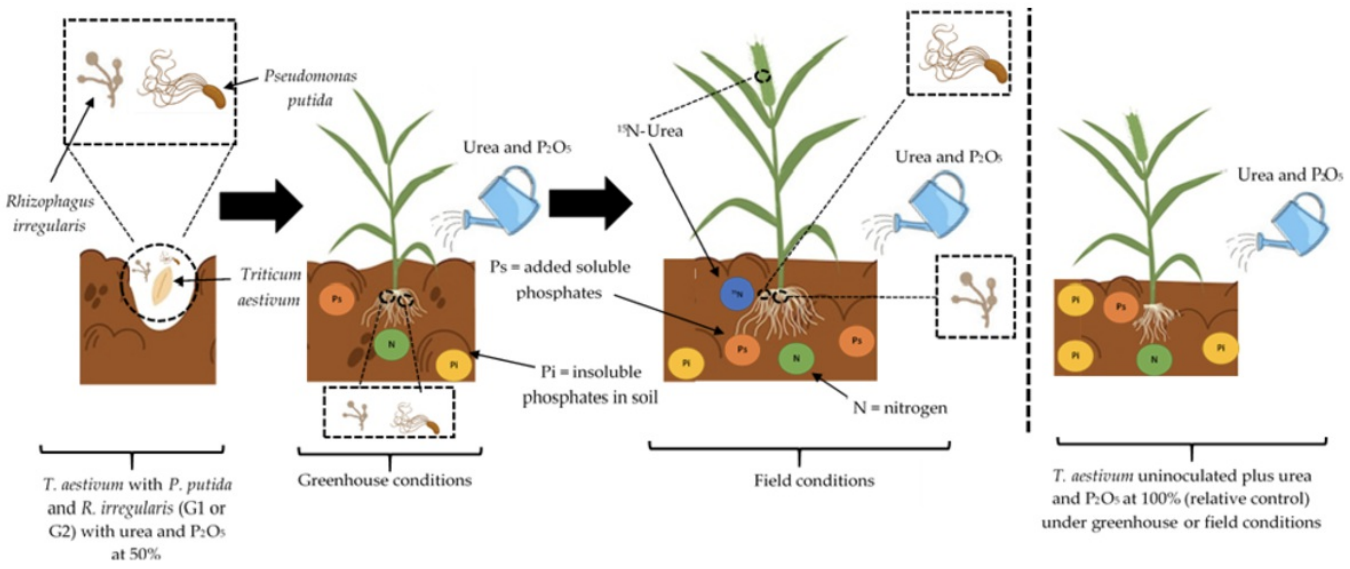

Figure 1. Shows the inoculation of *Pseudomonas putida* and *Rhizophagus irregularis* in *Triticum aestivum* to optimize root uptake 60 Kg/Ha of ^{15}N urea and 40 kg/ha of P_2O_5 in greenhouse and/or field.

Results

Figure 1 summarizes the inoculation of *T. aestivum* with *P. putida* and *R. irregularis* with 50% urea and phosphates in comparison with the application of the 100% dose of urea and phosphates in *T. aestivum* uninoculated with *P. putida* or *R. irregularis* used as relative control (RC)

In the effectiveness test of *P. putida* and *R. irregularis* coded as G1 and G2 in *T. aestivum* fertilized with 60 kg/ha of urea

and 40 kg/ha of P_2O_5 equivalent to 50% of the recommended dose, induced an increase in total dry weight (TDW), similar to that registered in *T. aestivum* uninoculated and fertilized with 120 kg/ha of urea and 80 kg/ha of P_2O_5 Kg/ha equivalent to 100% referred to as relative control (data not shown).

Nt content in the grain of *T. aestivum* inoculated with *P. putida* and *R. irregularis*

Figure 2 shows the amount of Nt in the grain of*T. aestivum* with *P. putida* and *R. irregularis* (G1) with 60 kg/ha of urea and 40 kg/ha of P_2O_5 equivalent to 50%, there is registered 3.31%, a numerical value statistically different compared to 2.58% of Nt in grain of *T. aestivum* uninoculated, fertilized with 120 kg/ha of urea and 80 kg/ha of P_2O_5 equivalent to 100% or relative control (RC). As well as with 2.59% Nt in *T. aestivum* grain with *P. putida* and *R. irregularis* (G2) fertilized with urea and P_2O_5 at 50%. In the grain of *T. aestivum* with *P. putida* and *R. irregularis* at 50% urea and phosphate doses, the nitrogen concentration was equal to or higher than the nitrogen concentration in *T. aestivum* with the 100% urea dose and phosphates recommended for that agricultural zone of northeastern Mexico.

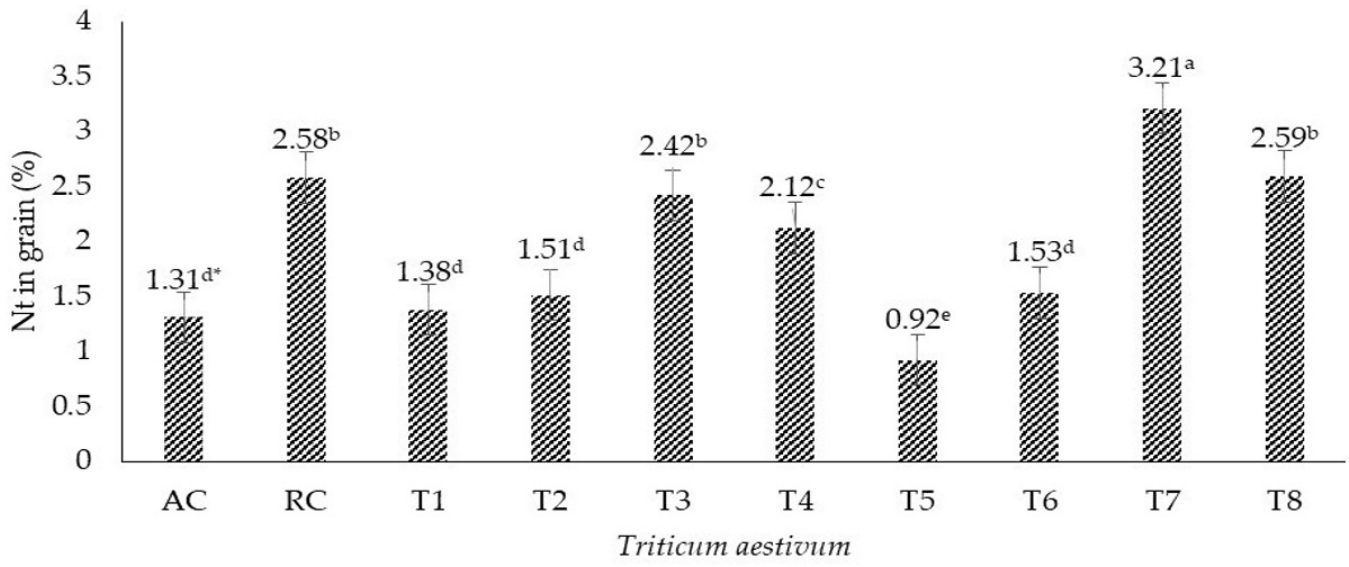

Figure 2. Effect of *Pseudomonas putida* and *Rhizophagus irregularis* on total N in *Triticum aestivum* grain with different levels of urea and P_2O_5 in Kg/Ha.

Absolute control (AC): T. aestivum uninoculated irrigated with water; Relative control (RC): T. aestivum uninoculated plus urea and P2O5 at 100% (120 and 80 kg/ha respectively); Treatment (T) 1: T. aestivum with P. putida and R. irregularis (G1) with urea and P2O5 at 0%; T2: T. aestivum with P. putida and R. irregularis (G2) with urea and P2O5 at 0%; T3: T. aestivum with P. putida and G1 with urea at 50% and P2O5 at 0%; T4: T. aestivum with P. putida and G2 with urea at 50% and P2O5 at 0%; T5: T. aestivum with P. putida and G1 with urea at 0% and P2O5 at 50%; T6: T. aestivum with P. putida and G2 with 0% urea and 50% P2O5; T7: T. aestivum with P. putida and G1 with urea and P2O5 at 50%; T8: T. aestivum with P. putida and G2 with urea and P2O5 at 50%.

*numerical values with the same letters between bars do not differ statistically (ANOVA-Tukey, $P \leq 0.01$).

Effect of *Pseudomonas putida* and *Rhizophagus irregularis* on the yield of *Triticum aestivum* at different doses of urea and phosphates.

Figure 3 shows the yield of *T. aestivum* with *P. putida* and *R. irregularis* (G1) fertilized with 60 kg/ha of urea and 40 kg/ha of P_2O_5 equivalent to 50%, which reached 3.39 To/Ha, a numerical value statistically similar compared to achieved in uninoculated *T. aestivum* fertilized with 120 kg/ha of urea and 80 kg/ha of P_2O_5 equal to 100% used as RC.

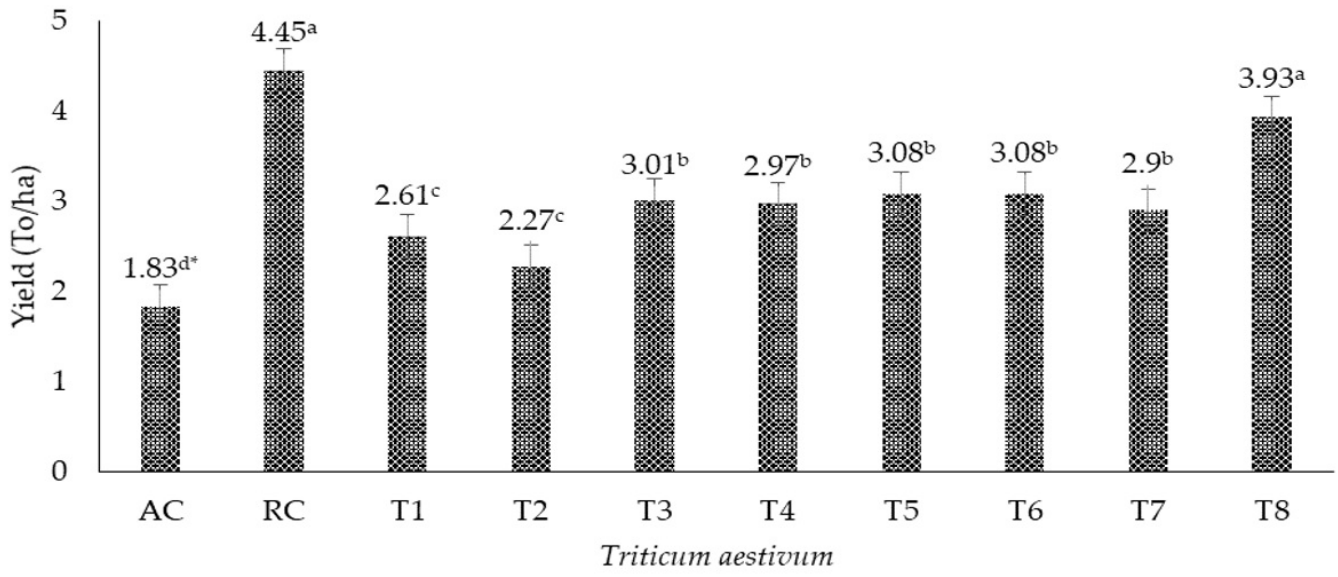

Figure 3. Effect of *Pseudomonas putida* and *Rhizophagus irregularis* on yield of *Triticum aestivum* at different levels of urea and P_2O_5 (Kg/Ha).

Absolute control **(AC)**: *T. aestivum* uninoculated irrigated with water; Relative control **(RC)**: *T. aestivum* uninoculated plus urea and P_2O_5 at 100%; 120 and 80 kg/ha respectively; Treatment **(T) 1**: *T. aestivum* with *P. putida* and *R. irregularis* (G1) with urea and P_2O_5 at 0%; **T2**: *T. aestivum* with *P. putida* and *R. irregularis* (G2) with urea and P_2O_5 at 0%; **T3**: *T. aestivum* with *P. putida* and G1 with urea at 50% and P_2O_5 at 0%; **T4**: *T. aestivum* with *P. putida* and G2 with urea at 50% and P_2O_5 at 0%; **T5**: *T. aestivum* with *P. putida* and G1 with urea at 0% and P_2O_5 at 50%; **T6**: *T. aestivum* with *P. putida* and G2 with 0% urea and 50% P_2O_5; **T7**: *T. aestivum* with *P. putida* and G1 with urea and P_2O_5 at 50%; **T8**: *T. aestivum* with *P. putida* and G2 with urea and P_2O_5 at 50%.

*numerical values with the same letters do not differ statistically (ANOVA-Tukey, $P \leq 0.01$).

Radical uptake of ^{15}N-urea by *Triticum aestivum* with *Pseudomonas putida* and *Rhizophagus irregularis.*

Figure 4 shows the uptake of urea labeled with the stable isotope ^{15}N in the stem and grain of *T. aestivum* with *P. putida* plus G1 with 60 kg/ha of urea and 40 kg/ha of P_2O_5 equivalent to 50%, that registered up to 68% and 45% of the uptake of fertilizer derived nitrogen (NdF). This result was statistically different with 44% and 38 of NdF in stem and grain of uninoculated *T. aestivum* with 120 kg/ha of urea and 80 kg/ha of P_2O_5 equivalent to 100% or RC.

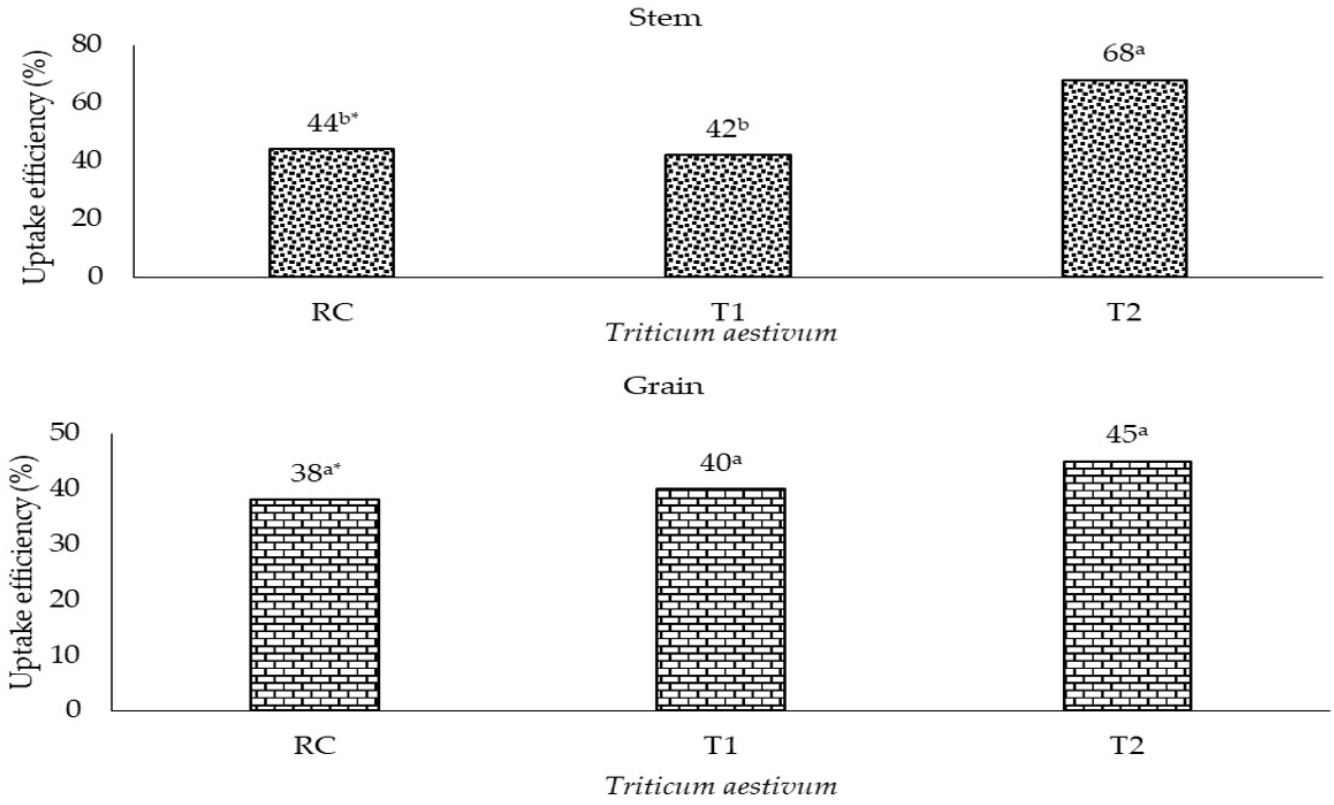

Figure 4. Effect of *Pseudomonas putida* and *Rhizophagus irregularis* on ^{15}N-urea uptake in stem and grain of *Triticum aestivum* fertilized with 60 kg/ha of urea and 40 kg/ha of P_2O_5. Relative control **(RC)**: *T. aestivum* uninoculated plus urea and P_2O_5 at 100% (120 and 80 kg/ha respectively); Treatment **(T) 1**: *T. aestivum* with *P. putida* and *R. irregularis* (G1) with urea and P_2O_5 at 50%; **T2**: *T. aestivum* with *P. putida* and *R. irregularis* (G2) with urea and P_2O_5 at 50%.

*numerical values with the same letters between bars do not differ statistically (ANOVA-Tukey, $P \leq 0.01$).

Table 3. Activity of acid and alkaline phosphatases of *T. aestivum* at the flowering stage at 60 days after sowing with 50% urea and P_2O_5 fertilizer with and without *P. putida* plus *R. irregularis* isolates G1 and G2.

Triticum aestivum	Activity	P-nitrophenol released(µg/mL)
Saline solution (absolute control)	Acid	-
	Alkaline	-
Uninoculated stem	Acid	0.13^{f**}
	Alkaline	0.45^{f}
Uninoculated root	Acid	0.10^{f}
	Alkaline	1.49^{e}
P. putida and *R. irregularis* G1 on stem	Acid	102.52^{d}
	Alkaline	170.53^{b}
P. putida and *R. irregularis* G1 on root	Acid	170.36^{b}
	Alkaline	220.29^{a}
P. putida and *R. irregularis* G2 on stem	Acid	110.15^{d}
	Alkaline	145.20^{c}
P. putida and *R. irregularis* G2 on root	Acid	135.01^{c}
	Alkaline	250.10^{a}

*Number repetitions (n) = 4, **values with the same letters do not differ statistically (ANOVA-Tukey, P ≤ 0.01).

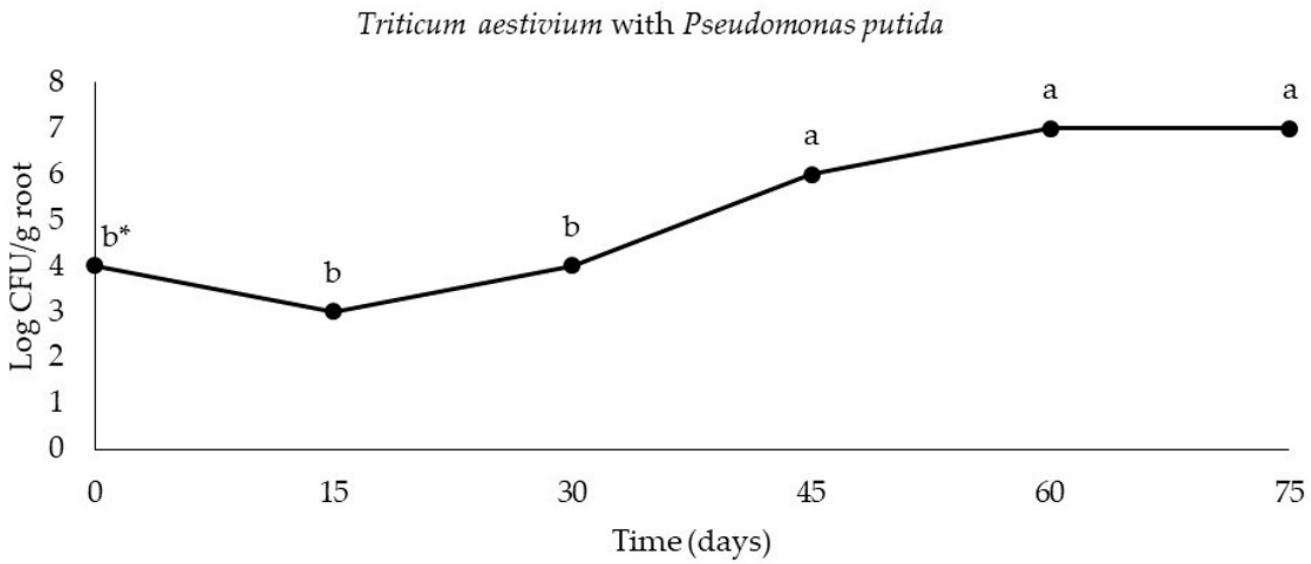

Figure 5. Colonization of the interior of the roots of *T. aestivum* by *Pseudomonas putida*.

*Numerical values with the same letters do not differ statistically (Tukey, P ≤ 0.01).

Discussion

According to the literature, populations of native microorganisms in the root system are stimulated by crops like*T. aestivum* [24][25]. An underexplored strategy is the isolation of endophytic microorganisms from wild plants, specific sites

where this problem is critical [14]. Since endophytic microorganisms have an advantage compared to rhizobacteria isolated from domestic plants, their ability to interact intimately with the diversity of wild or domestic plant species including their adaptability to adverse environments: stress caused by lack of soluble PO_4^{-3}as well as other basic minerals for plant nutrition [26][27]. Therefore, wild plants growing in soils such as calcareous benefit from these endophytic microorganisms, which are able to enhance the uptake of basic nutrients: inorganic nitrogen, PO_4^{-3}, and other microelements to deal with water stress and salinity. In Saltillo, Coah, Mexico. Temperatures between 8 and 10°C in the morning, between 29 and 35°C at midday, and in the afternoon are frequent, with higher temperatures the nitrogen and phosphorus cycle can be altered [28]. Wild plants and weeds are harmful to conventional agriculture, but persistent and successful growth in nature despite efforts for its control and/or elimination is an option to isolate endophytic plant growth-promoting microorganisms such as *P. putida* isolated from *A. purpurea* and *R. irregularis*: G1 and G2 from *R. luteola* both have the ability to positively interact with both wild and domestic plants given the evolutionary versatility of its genome reason why are import tool to enhance in *T. aestivum* the uptake of urea and P_2O_5 in calcareous soil [1][2][5][7].

In Figure 2, it is supported that the combination of*P. putida* and *R. irregularis* was sufficient to improve the efficiency of root uptake of urea into the soil by a hormonal effect that increased the root uptake surface [9][15]. While in grain of *T. aestivum* uninoculated, fertilized with urea and P_2O_5 at 100%, a relatively low concentration of % Nt was determined, indicating that the root system was unable to uptake urea from the soil, phosphates in alkaline soils react to generate low solubility phosphorus compounds, which remain for a long time in less soluble forms, little available to plant roots [6][3][12][13].

In this sense, the alkaline phosphatases of*P. putida*, as well as the various strategies of *R. irregularis*, allow the translocation of insoluble phosphates, and the optimization of reduced soluble phosphates as was evident in treatments 3, 7, and 8 since the activity of alkaline phosphatases increases the capacity of *T. aestivum* roots to uptake not only the insoluble phosphate from the soil, but also the phosphate that is applied as fertilizer, while *R. irregularis* inside the root system of *T aestivum* does it through a synergistic action with *P. putida* and simultaneously by the mechanism of translocation of insoluble phosphates from the soil, it also optimizes the uptake of phosphorous fertilizer and urea at 50% of the recommended value, to improve the health of *T.aestivum*. In addition to avoiding the release of greenhouse gases, as well as contamination of surface and underground water [7][3][14][16][23].

In Figure 3, based on these results, it is estimated that one way to solve urea uptake in alkaline soil is not necessary to increase the dose of urea and P_2O_5, as observed in this work, but due to inoculation of the seed with endophytic*P. putida* that increase the root system to improve urea uptake and with *R irregularis* that solubilize P_2O_5 due phosphatases both microorganisms are working synergistically inside the root system of *T. aestivum* [11][4]. The positive effect on the yield of *T. aestivum* with *P. putida* and *R. irregularis* indicates that both contribute to a higher uptake of urea and P_2O_5 by alkaline phosphatases inside the root system of *T. aestivum* to maintain an acceptable yield in comparison to*T. aestivum* used as an RC [10][15]. The effect of *P. putida* and *R. irregularis* has been reported in nature, due to ecological and/or symbiotic interactions: root-microorganism, as in the case of calcareous soils that are deficient in these essential elements for agricultural crops like *T. aestivum*. Based on the above, there is evidence that*P. putida* a genus and species of

phosphate-solubilizing endophytic bacteria interact with *R. irregularis* a vesicular-arbuscular mycorrhiza or VAM that induces a synergistic relationship with the root of plants for a better use of poorly soluble P [25][28][29]. The results obtained in this work demonstrated that it is possible that the phosphate solubilized by the phosphatase of P. putida is effectively absorbed by the roots of *T. aestivum* through a biological or mycorrhizal bridge that captures the insoluble phosphate and translocates it from the soil including the root and includes the N of urea, [21][22][29]. This was demonstrated with phosphorus 32 (^{32}P), with phosphate solubilizing, that associated with *R. irregularis* increased the effective uptake of nitrogenous and phosphorous fertilizers reduced to 50% [23][24][25].

In Figure 4, the increase in the uptake of NdF labeled as ^{15}N-urea in *T. aestivum* with *P. putida* indicated a positive activity of alkaline phosphatases and phytohormones inside of root system [11][26][27] that induced a more abundant root system that increased urea uptake [27][29]. These results support that an adequate selection of *P. putida* and *R. irregularis* endophytic maximizes the radical uptake of nitrogen fertilizer as urea by *T. aestivum*. Simultaneously phosphatase activity of *R. irregularis*, inside of the root system P_2O_5 solubilization occurred to solubilize inorganic no soluble phosphates [6][12]. These data supported the need to correctly select a combination of endophytic bacteria and endomycorrhizal fungi working in a synergistic action on urea and P_2O_5 uptake [4][8][16][30].

In the root system of *T. aestivum* inoculated with *P. putida*, a bacterial density between $4.0X10^4$ and $7.0X10^7$ CFU/g root was registered, reported as sufficient to exert a positive effect on root uptake of urea by *T. aestivum* [23][31]. It is also reported that *P. putida* is competitive with the native microbial population of calcareous soil, due to its adaptation to this alkaline environment. In this sense, *P. putida* was isolated from *A. purpurea*, which similarly to *T. aestivum* is a gramine, and this could lead to the adaptability and population development of *P. putida* [32][33]. No statistical difference was established in the population density of *P. putida* in the root system of *T. aestivum* at different levels of urea or P_2O_5 at the 10.5 growth stage of Feeke's *T. aestivum*, supporting that the *P. putida* population dominated the endophytic root zone of this plant to cause a positive effect on its healthy growth [2.23]

Conclusions

The results obtained in *T. aestivum* with *P. putida* and *R. irregularis*: G1 and G2 23isolated endophytic from the root system of *A. pupurea* and *R. luteola* associated with the crop of this gramine, demonstrating a positive effect on the uptake of nitrogen as urea applied as ^{15}N-urea, in comparison to uninoculated *T. aestivum*, *P. putida* and *R. irregularis* are biological tool to increase urea and phosphates under alkaline conditions due to phytohormones and phosphatases, in consequences yield of *T. aestivum* was able to uptake insoluble phosphates from soil as well as 50% of phosphates and urea applied as fertilizers.

Author Contributions

Experimentation BVU.; conceptualization, JMSY.; methodology BVU.; software, BVU, JIDC; validation, JIDC results, BVU, JIDC and JMSY investigation BVU and JMSY resources, JMSY writing—original draft preparation, BVU, JIDC, and JMSY

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Conflicts of Interest

The authors declare no conflicts of interest.

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