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Enhanced growth of *Oriza sativa* by endophytic: *Bacillus cereus* and Paenibacillus polymxya

Abstract

The health growth of *Oryza sativa* depends on non-excess nitrogen fertilizer, one way to achieve its to inoculate its seed with endophytic plant growth promoting bacteria: *Bacillus cereus* and *Paenibacillus polymxya.* In that, sense seeds of *O. sativa* were inoculated by the variable responses as germination percent, phenology and biomass at seeding stage. Results demonstrate that germination and growing, were enhanced by the *B. cereus* and *P. polymixa*, by the invading inside of the root system protected from negative environmental factors and then by transforming seed exudates into phytohormons to induce rapid germination and healthy growth due to improve the uptake of nitrogen fertilizer to optimize it and avoid applying in excess to preserve soil productivity.

Keywords: soil, nitrogen fertilizer, endophytic beneficial bacteria, cereal

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Blanca Celeste Saucedo Martinez,[1] Maria Janet Tena Rodriguez,[1] Liliana Marquez-Benavides,1 Jose Luis Rico Cerda,[2] Juan Manuel Sánchez-Yáñez,[1]

[1]Research Institute of Chemical Biology, Environmental Microbiology Laboratory, Universidad Michoacana de San Nicolás de Hidalgo, Mexico

[2]Department of Chemical Engineer, Universidad Michoacana de San Nicolás de Hidalgo, México

Correspondence: Juan M Sánchez-Yáñez, Research Institute of Chemical Biology, Environmental Microbiology Laboratory, Universidad Michoacana de San Nicolás de Hidalgo, Mexico, Tel 00524433223500, ext. 4240, Email syanez@umich.mx

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Introduction

The world population is pressing agriculture to increase *Oryza sativa* (rice) production. Rice is one of the three leading cereals in the world and an important dietary component for more than 3.5 billion people.[1] Among the nutrients needed by the rice plants, nitrogen (N) as a nitrogen fertilizer (NIFE) is one of the most important.[2] Due to the intensive agriculture production, the use of NIFE as NH_4NO_3 as source is nowadays necessary.[3] However, the excessive utilization of NIFE in agriculture, decreases organic matter of the soil which is negative for the life of microorganisms living nutrient cycling.[4] Likewise it was reported that organic matter degradation by applying in excess NIFE affecting the soil microbiota.[5] In addition, up to 70 % of the NIFE supplied to rice fields is lost, which causes a high negative impact to the environment.[6–8] Currently bacterial inoculants are receiving special attention due to their positive effects in agriculture that can mitigate the environmental problems caused by the excessive use of NIFE. In that sense plant growth promoting bacteria (PGPB) transforming seed and root exudates into phytohormones which play an important role in NIFE availability to plants, PGPB are extensively distributed among Bacteria and Archaea domains.[9] Previous reports also indicate that the rice production can be enhanced by the use PGPB to reduce and to optimize a NIFE consumption. Das and Saha,[10] conducted a field experiment of rice seeds inoculated with *Azotobacter* strain AS8 and *Azospirillum* strain AM1, both PGPB, with 50 kg of N as NH_4NO_3 to improve NIFE uptake in the rhizosphere of rice. The authors found that both PGPB substantially increased the availability of NIFE in the rhizosphere, leading to raise crop yield of rice, due to efficiency of *Azotobacter* strain AS8 better than *Azospirillum* strain AM1. In another study, Biswas et al.[11] reported the inoculation of Pankaj rice seeds with six PGPB and found that certain strains of *Rhizobium* can enhance rice grow. The authors also concluded that these positive effects are most due, to mechanisms that involve phytohormones in seed germination and health growth root physiology at reduced dose of NIFE. García de Salamone et al.[12] also performed a rice field study by inoculation of seeds with two strains *Azospirillum brasilense*, and found that inoculation increased aerial biomass at the tillering and grain-filling stages. Although the authors found an increase in N content in rice plants by 16 and 50 kg/ha, Yanni and Dazzo,[13] assessed the inoculation of rice seeds with *Rhizobium leguminosarum*bv*trifolii.* The authors performed single of multi-strain consortia inoculation with 7 strains on 5 varieties of rice, during 5 growing seasons. An average grain-yield increase of 19.5 % was observed. In that sense, the aim of this research was to analyse the effect of endophytic *B. cereus* and *P. polymxya* in the germination adn growth of *O. sativa* at 50% of NH_4NO_3.

Experimental

Isolation and culture of *B. cereus* and *P. polymxya*

Leonard jars, were prepared with soil sieved using a mesh No. 20, then soil was exposed to the sunrays during 48 h.The soil was then sterilized and 1 kg of treated soil was placed in the upper vessel, whereas the lower vessel of 900 ml, was completed filled with water or with a mineral solution (MS) containing NH_4NO_3. The chemical composition of the MS was the following, in g/L: KH_2PO_4, 3.0; K_2HPO_4, 3.5; $MgSO_4$, 1.5; $CaCl_2$, 0.1; $FeSO_4$, 0.5; and 1.0 ml/L of a trace metal solution (H_3BO_3 2.86; $ZnSO_4.7H_2O$ 0.22; $MnCl_2.7H_2O$ 1.81; K_2MnO_4, 0.09, as N source, this MS contained either 30 or 15 g/L of NH_4NO_3.The endophytic strains of *B. cereus* and *P. polymxya* were isolated from the root system of *Zea mays var mexicana* (teocintle well known maize ancestor). Since both were forming spores to active them, each of them was suspended in 3 ml of a saline solution at 0.85% of NaCl and 0.01 % of commercial detergent, at 72 °C for 10 min. *B. cereus* and *P. polymxya* were cultivated in agar plates containing (g/L): meat extract, 3.0; gelatine peptone, 5.0; agar-agar, 15; at pH of 7, plate was incubated at 32 °C/ 24 h.

Antibiotic marks in endophytic *B. cereus* and *P. polymyxa* to detect colonization of *O. sativa*

The colonization of endophytic *B. cereus* and *P. polymyxa*, inside of the tissues of *O. sativa,*was evaluated starting with the method proposed by Kirby-Bauer[14] by using the maximum antibiotic concentration of 102 µg mL^{-1} of ampicillin and 23.2 µg mL^{-1} of tetracycline for *B. cereus* in nutrient agar. Contrarily, 5.6 µg mL^{-1} and 15.0 µg mL^{-1} of the same antibiotics were utilized in nutrient agar for *P. polymyxa* and thiabendazole in both cases, to avoid the growing of fungi. To assure that mutation was no occurred during antibiotic treatment and to proof the atmospheric N_2-fixation capacity of *P. polymyxa,* was also cultivated in a Burk medium containing, in g/L: glucose, 10.0; KH_2PO_4, 2.0; $MgSO_4$, 3.0; and 1 ml of trace metal solution in g/l: H_3BO_3, 2.86; $ZnSO_4.7H_2O$, 0.22; $MnCl_2.7H_2O$, 1.81; $KMnO_4$, 0.09; bromothymol blue, 10; agar, 18.0, the pH was adjusted to 7.5.

Inoculation of rice seeds with endophytic *B. cereus* and P. polymyxa

The rice seeds were disinfected with a sodium hypochlorite solution (1 % v/v) during 5 min. The seeds were fivefold rinsed with sterilized water, then in ethanol solution at 75% during 5 min and rinsed sixfold with sterilized water (Table 1). Then 1 mL of each in saline solution at 85% containing the harvested bacteria grew in agar, were separately inoculated on 20 rice seeds previously disinfected. For co-inoculation of *B. cereus* and *P. polymyxa,* 0.5 mL of each solution were well-mixed and then inoculated on 20 rice grains. The sowing and growing of the seeds occurred in Leonard jars, fed with MS and NH_4NO_3 was applied. for instance, seeds inoculated with *B. cereus* or *P. polymyxa* or both, and fed with MS and NH_4NO_3 were performed and labelled as a *B. cereus* or *B. cereusand P. polymxya*, respectively. In addition, an absolute control (AC) of non-inoculated seeds sowing and irrigated with water; whereas non-inoculated seeds fed with a MS containing NH_4NO_3 (30 and 15 g/L) were referred as a relative control, RC-1 and RC-2, respectively. Table 1 shows the experimental conditions used in each assay.

Recovering of endophytic *B. cereus* and *P. polymyxa* from *O. sativa* tissues

Four seeds of *O. sativa* were sowing for the determination of endophytic *B. cereus* and *P. polymyxa* were utilized in each treatment. Two seeds inoculated with *B. cereus* were rinsed with sterilized water and afterwards 1 g of tissue from leaves, stem and root were obtained and separately suspended in 5 mL o saline solution to strike on nutrient agarwith the maximum ampicillin and tetracycline concentrations. In the case of the tissues of *O. sativa* inoculated with *P. polymyxa,* a similar procedure was followed, except that the saline solution contains the required maximum ampicillin and tetracycline concentrations determined for *P. polymyxa*. However, for co-inoculated with *endophytic B. cereus* and *P. polymyxa*, the tissue suspension in 5 mL of a solution which contains 2.5 mL of each bacteria. From each solution of tissue, 2.5 mL were incubated at 32°C for 24 h. The remaining 2.5 mL were pasteurized at 72°C for 10 min, cooled in an ice bath during 5 min and finally incubated at 32 °C for 24 h. The tissue from the leaves, stem and roots of the remaining from each treatment were extracted, separately disinfected with sodium hypochlorite solution (1% v/v) and then rinsedwith sterilized water. 1g of disinfected tissue was ground in a mortar with 9 mL of saline solution at 0.85%from this solution, 1 mL was added into 4 ml of the corresponding nutrient agar with antibiotics, or in a mixture of both bacteria on nutrient agar as in the case of co-inoculation. From this suspension, 2.5 mL were directly incubated at 32°C for 24 h and the remaining was pasteurized prior to incubation, at similar experimental conditions. The CFU was determined for each tissue incubated culture. The response parameters for measured the effect of *B. cereus* and *P. polymxya* on *O. sativa* were the germination percentage, at seedling and flowering stage by the phenology: plant height, radical length, biomass: fresh, dry, areal and radical weights were determined. The experimental data were evaluated using ANOVA/Turkey software ($P<0.05$).

Results

Table 1 summarised the experimental design followed with *O. sativa* inoculated with endophytic *B. cereus* and *P. polymyxa.*

Table 2 showed the effects of inoculation on the phenology at seedlings stage. This table indicates that percentage of germination, evaluated 10 days after inoculation was better in rice seeds with endophytic *B. cereus*. Moreover, *O. sativa* at dose of 30 g/L ofNH_4NO_3 instead of 15 g/L (relative control-1 and relative control-2 respectably) enhanced the phenology and biomass weight of *O. sativa*. However, comparing with *O. sativa* as a relative control -2 with those results obtained, which were grown with half dose of NH_4NO_3, the improving achieved by *B. cereus* is remarkable. The inoculation with *B. cereus* and co-inoculation with *B. cereus* and *P. polymyxa* showed the best results, improving plant high, radial length and biomass weight. The phenology parameters using *P. polymyxa* as inoculant, were also enhanced compared to those observed with non-inoculation *O. sativa* used as a relative control.

The density of endophytic *B. cereus* and *P. polymyxa* in rice seedlings, after inoculation was performed and the results are showed in Table 3. The location of both genus in the seedling was evaluated, this demonstrates that inoculation with endophytic *B. cereus* and *P. polymyxa* indeed, enhanced the growing of rice seeds. Independently of disinfection, and pasteurization of *O. sativa*, the absence of *B. cereus* and *P. polymyxa* in leaves is clearly observed in this table. This indicates the positive effect of endophytic *B. cereus* and/or *P. polymxya* on the growth of *O. sativa* with these bacteria, is centred on stem and roots due that both bacteria colonized these specific tissues of the plant because the organic compound produced by photosynthesis which does not exist in leaves tissue. Comparing the results presented in Table 3 related to stem and roots, the most remarkable effect is observed in roots of the non-disinfected, non-pasteurized rice, which showed a density of both of them around 251 and $270x10^6$ CFU/g, when the seeds were inoculated just with *B. cereus* alone as well as only with *P. polymxya* and with both bacteria, respectively. It is worth therefore that disinfection and pasteurization of the *O. sativa* seeds, showed negative effects. The information related to the inoculation of *B. cereus* and *P. polymyxa* is scarce, especially when the beneficial effect is due to an invasion of the root system and the stem to take advantage of metabolites of photosynthesis in phytohormons that increased the capacity of rice for optimization of NIFE and consequently now there is an better option for healthy plant growth for *O. sativa* production.[15,16]

The rice growing enhancement resulted after inoculation,was in agreement with other studies that reportedthe inoculation of rice seeds with other genus of plant growth promoting bacteria different than *B. cereus and P. polymyxa* depends its capacity to transform organic metabolites from steam and root metabolism into phytohormons for enhancing uptake radical absorption of NIFE reduced at 50% dose which avoiding soil losing productively and surface and underground pollution by the runoff of NIFE applying in excess.[11–13]

Citation: Martinez BCS, Rodriguez MJT, Marquez-Benavides L, et al. Enhanced growth of *Oriza sativa* by endophytic: *Bacillus cereus* and Paenibacillus polymxya. *Horticult Int J.* 2020;4(5):208–211. DOI: 10.15406/hij.2020.04.00184

Table 1 Experimental design of the effect of endophytic *Bacillus cereus* and *Paenibacillus polymyxa*on *Oriza sativa*

Treatments	O. sativa	B. cereus	P. polymyxa	NH4NO3 in mineral solution		Water
				30g/L	15 g/L	
Absolute control	+	-	-	´-	-	+
Relative control-1	+	-	-	+	-	-
Relative control-2	+	-	-	-	+	-
Bacillus cereus	+	+	-	-	+	-
Paenibacillus polymyxa	+	-	+	-	+	-
B. cereus+P.polymyxa	+	+	+	-	+	-

Table 2 Effect of endophytic *Bacillus cereus* and *Paenibacillus polymyxa* in germination and seedlings stage of *Oriza sativa* with mineral solution and NH_4NO_3 at 50% dose

Treatment* Oriza sativa	Germination, % (10 days after inoculation)	Phenology (cm)		Biomas weight (g)			
		Plant height	Radical length,	Fresh aerial	Fresh radical	Dry aerial	Dry radical
Absolute control (water)	77f**	27.91b	13.75a	0.17b	0.12d	0.02b	0.01c
Relative control-1 (NIFE 100%)	81e	25.42b	12.17a	0.23a	0.23b	0.03a	0.05a
Relative control-1 (NIFE 50%)	91b	22.92c	9.08b	0.17b	0.14c	0.02b	0.02b
B. cereus NIFE 50%	93a	32.15a	14.33a	0.24a	0.28a	0.03a	0.02b
P. polymyxa NIFE 50%	88c	28.41b	9.91b	0.22a	0.17b	0.03a	0.02b
B.cereus/P.polymxya NIFE 50%	88c	27.68b	12.25a	0.23a	0.19b	0.03a	0.05a

Table 3 Distribution and density of vegetative and sporulation cells of endophytic *Bacillus cereus* y *Paenibacillus polymyxa* in the tissue of *Oriza sativa*

Treatment of O. sativa			CFU x106/g tissue ofO. sativa			
			B. cereus	P. polymyxa	B. cereus+P.polymyxa	
					B. cereus	P. polymyxa
Non-disinfected	Non-Pasteurization	Leaves	0	0	0	
		Stem	40	31	22	33
		Roots	251	135	180	90
	Pasteurization	Leaves	0	0	0	
		Stem	43	36	18	25
		Roots	62	34	27	39
Disinfected	Non-Pasteurization	Leaves	0	0	0	
		Stem	25	23	19	24
		Roots	28	17	0	58
	Pasteurization	Leaves	0	0	0	
		Stem	35	29	0	
		Roots	33	44	48	22

Conclusion

The inoculation and co-inoculation of *O. sativa* with endophytic *B. cereus* and *P. polymyxa* is here in reported. The results indicated an improvement on the rice growth, dueits capacity of these endophytic bacteria mainly located in the stem and roots. Interestingly, the enhancement in the growing of *O. sativa* showed that 50% of NH_4NO_3 could be optimized by the activity of these endophytic PGPB with beneficial effect for the environment since hyper-fertilization could be avoided.

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Conflicts of interest

Authors declare no conflict of interest exists.

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