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Full Length Research Paper

Isolation of endophytic, epiphytic and rhizosphere plant growth-promoting bacteria from cultivated rice paddy soils of the Guadalquivir river marshes

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Endophytic, epiphytic and rhizosphere bacteria were isolated from rice fields of the marshlands of the Guadalquivir River (South Spain). Paddy fields in this area are affected by two fundamental problems for rice cultivation, salinity and incidence of the fungal pathogen *Magnaporthe* sp. Four plots, in which both environmental factors with different levels of influence are combined, were selected for these studies. A total of 624 microorganisms were isolated according to their different colony morphology and pigmentation. Plant growth-promoting (PGP) activities, such as indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) degradation, chitinase activity, phosphate solubilization, and siderophores production were determined in these strains. These data allowed the selection and identification of twenty eight bacteria with the most appropriate PGP activities to use them as inoculants for rice cultivation under stressful conditions.

Keywords: rice, paddy soils, plant growth, bacteria, Guadalquivir river marshes.

INTRODUCTION

Cultivated rice (*Oryza sativa* L.) is the most important cereal crop worldwide, with a paramount importance as a staple human food source in many areas of the world and hence plays a key role in delivering food security, especially in developing countries (Britto and Kronzucker, 2004). In addition, its production, trade, and consumption in developed economies are a significant and growing feature of the world market (Choudhury and Kennedy, 2004).

The Guadalquivir river marshlands constitute one of the most important areas devoted to rice cultivation in Europe (37,000 hectares) with about 8% of the total cultivated surface. Rice paddies are highly productive (average production of 10,000 kg/ha) and constitute an essential economic resource for the surrounding population (Aguilar-Portero, 2001). However, these crops are bordering the Doñana National Park, one of the most important European nature reserves and a fundamental point in the route of migratory birds. In order to make rice cultivation more sustainable and less dependent on chemical nitrogen and phosphorus fertilizers and pesticides, the use of plant growth promoting rhizobacteria that could protect and promote rice growth would be an alternative for rice production (Alam et al., 2001; Chinnusamy et al., 2006;

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Cong et al., 2009; Mäder et al., 2012; Nguyen and Ferrero, 2006).

The objective of this study was to isolate and characterize bacteria from rice fields of the Guadalquivir river marshlands on the basis of a culturable-based approach. Two environmental conditions, considered as essential problems for rice cultivation in the studied area, were established for bacterial isolation: **i**) saline concentration of the irrigation water, and **ii**) incidence of the rice blast disease caused by the fungal pathogen *Magnaporthe* sp. In addition, two plant physiological stages were considered, the initial stage, from seed germination to the formation of the panicle, and the flowering stage.

Four plots, in which both environmental factors with different levels of influence are combined, were selected. Isolated rhizosphere, endophytic, and epiphytic bacteria were characterized in base to colony morphology and pigmentation. Plant growth promoting activities of these bacteria were analyzed. The potential use of these bacteria for plant growth promotion and protection against pathogens and salinity is discussed.

MATERIAL AND METHODS

Collection of soil and plant samples

Soil and plant samples were obtained randomly from four different cultivated rice fields located in the marshlands of the Guadalquivir River in Seville, South Spain. These areas show different salinity and rice blast incidence caused by the pathogenic fungus *Magnaporthe oryzae* Cav. The information about the soil characteristics of the different areas is shown in Table 1. Plants were studied at two physiological stages: sampling A, an initial stage (May) when the plant has up to three leaves; and sampling B, flowering stage (September). Four plants were sampled randomly from each cultivated area and tissue samples, roots and stems were examined.

Isolation of culturable rhizosphere, endophytic, and epiphytic bacteria

Freshly collected plants from the four sampling areas were carefully separated into stem and root parts. Rice roots and stems were washed in sterile NaCl 0.9% with shaking for one hour at 37 ºC to isolate rhizosphere (RA) and epiphytic bacteria (AEP). To isolate endophytic bacteria (AEN), stems were first washed with ethanol 96º for 15 minutes and then twice with sterile water. Finally, plants were shredded and mixed. Bacterial samples were serially diluted with sterile saline solution and plated in triplicates onto tryptone soy agar (TSA) (Thompson et al., 1993) supplemented with cyclohexamide (100 $\mu g \cdot ml^{-1}$) to inhibit fungal growth. Plates were incubated for 48 hours at 28 ºC and then colonies formed were counted. Those colonies with different morphology and pigmentation were isolated, purified in TSA plates, and stored at -80 ºC until use. The isolated microorganisms were named with a code that indicated the sampling area, the plant tissue (RA: rhizosphere, AEP: epyphitic, or AEN: endophytic), and the physiological plant stage (sampling A or B).

Assay for indoleacetic acid (IAA) production

Production of IAA was colorimetrically determined according to Lambrecht et al. (2000). Briefly, bacterial cultures were grown in tryptone soy broth (TSB) for 24 hours at 28 ºC and 1 ml of each culture was centrifuged at 13,000 rpm. The supernatant was transferred to a clean 10 ml tube, mixed with 4 volumes of Salkowski reagent (250 ml, 150 ml H_2SO_4 96%, 7.5 ml 0.5M $FeCl_3$ solution), and then color changes were monitored. Pure indole-3-acetic acid (Sigma, USA) was used as standard.

Assay for 1-aminocyclopropane-1-carboxylate (ACC) degradation

ACC degradation was determined according to the protocol from Penrose et al. (2001). Bacteria were cultured in a minimal medium in which ACC was the only nitrogen source (1000 ml, 1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 5 mg $NaMoO_4.2H_2O$, 10 g glucose, 0.3 g ACC). As control, a medium without ACC was prepared. Those microorganisms that grew in the medium with ACC and could not grow in the absence of this compound were considered as bacteria able to degrade ACC.

Determination of the chitinase activity

Isolated microorganisms were plated in a minimal medium with chitin as unique carbon and energy source (1000 ml, 2.7 g K_2HPO_4, 0.3 g H_2PO_4, 0.7 g $MgSO_4.7H_2O$, 0.5 NaCl, 0.5 g KCl, 0.13 g yeast extract, 20 g colloidal chitin). Colloidal chitin was prepared by mixing 4 g of chitin with 36 ml of HCl. The mixture was agitated for 2 hours until chitin dissolution. After decanting, the volume was completed up to 1 l with distilled water. The solution was decanted again, the supernatant removed and the sediment was resuspended in staple water four times and three times with distilled water. After the last wash, it was vigorously mixed and passed through a 0.5 mm metallic sieve. The elution was stored at 4ºC until use. *Serratia marcescens* CECT 159 was used as a positive control (Toratani et al., 2008). Those bacteria able to degrade chitin better than the control strain were considered chitin degraders.

Phosphate solubilization assay

Isolated microorganisms were grown in National Botanical Research Institute's phosphate growth medium (NBRIP)

Table 1. Chemical and physical properties of the soils from the four sites analyzed

Origin	pH	Salinity	Soil type					organic matter %	*Magnaporthe* sp. incidence
			%Clay	%Loam	%Fine sand	%Coarse sand	%Silt		
Zone 1 (Puebla)	8.02	Low	41.8	36.3	3.2	18.7	-	2.74-3.66	High
Zone 2 (Colinas)	8.18	High	58.5	-	0.8	1.1	39.6	1.78-2.14	Medium
Zone 3 (Calonge)	8.12	Low-medium	72.1	-	0.2	1.1	26.6	3.24-4.31	Low
Zone 4 (Rincón)	8.19	Low-medium	63.9	-	0.2	0.5	35.3	3.05	Low

(Peix et al., 2001). Those microorganisms able to solubilize phosphate formed a halo around the culture. The diameter of the halo was measured after 7 days of incubation in Petri dishes at 28 ºC. *Pseudomonas fluorescens* Aur6 was used as a positive control (Lucas-García et al., 2003). Those bacteria able to solubilize phosphate better than the control strain were considered phosphate solubilizers.

Detection of siderophores

The production of siderophore-type iron-binding compounds was determined using the CAS assay (Shin et al., 2001). 10 cm diameter Petri dishes were filled with 30 ml of an appropriate solid medium to culture each strain. Once solidified, the medium was cut into halves and one of them replaced by CAS-blue agar. The halves containing the culture medium were inoculated with the isolated bacterial strains. The plates were incubated in the dark at 28 ºC for 21 days and monitored every 7 days, measuring the advance of the color-change front in the CAS-blue agar. Those bacteria that induced the formation of a halo bigger than 10 mm in 7 days were considered siderophores producers. *Pseudomonas fluorescens* wcs417r was used as a positive control. This strain induces a strong reaction in CAS medium 24 hours after inoculation (Pieterse et al., 1996; Duuff et al., 2008).

Identification of bacteria

DNA was extracted using the miniprep PureLink® Genomic DNA (Promega) following the instructions of the manufacturer, and the 16S rDNA fragment amplified using the universal primers pA and pH' (AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCCGCA, respectively) following the reaction conditions previously described (Hall et al., 1999). The specific PCR products (approximately 1,500 bp) were detected by electrophoresis, purified from the gels and sequenced in order to classify microorganisms at the Genus level. The strain numbers and Genus names are presented in Table 3.

RESULTS

Enumeration of rice rhizosphere, endophytic, and epiphytic bacteria

To estimate the number of culturable aerobic bacteria present in the different soils analyzed, plate counts were determined on TSA. The total bacterial counts on this medium differed depending on the sample: rhizosphere (RA), epiphytic (AEP) or endophytic (AEN) bacteria (Figure 1). However, the number of aerobic microorganisms was similar in the four different sampling areas studied and in both plant physiological stages: plants with three leaves (sampling A) and flowering stage (sampling B). Thus, for samples AEP-A and AEP-B, the number of cfu counts was in the range of 10^6 cfu g^{-1}, except in zone 3 (Calonge) and sampling B, in which the highest number of isolates were detected (10^7 cfu g^{-1}). For samples AEN-A and AEN-B, and for RA-A and RA-B, counts were in the range of 10^4 cfu g^{-1} and 10^5 cfu g^{-1}, respectively (Figure 1).

Isolation of culturable aerobic bacteria

Aerobic culturable bacteria from rhizosphere soil samples from the four zones analyzed as well as from the different plant tissue samples, were isolated in TSA medium and identified based on colony morphology and pigmentation. A total number of 624 microorganisms were identified, 355 in sampling A, and 269 in sampling B (Table 2). In sampling A, the largest group of isolates corresponded to endophytic bacteria of zone 3 (Calonge), whereas in sampling B the

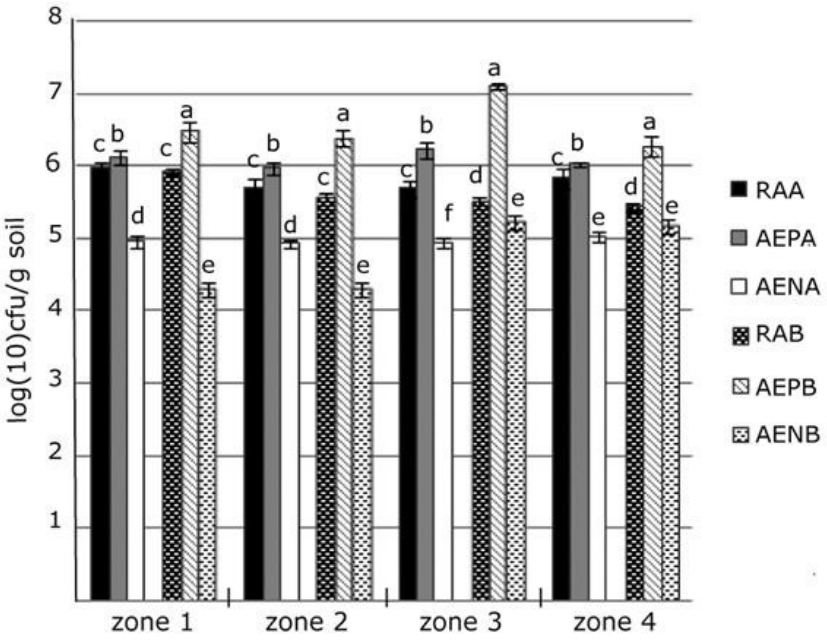

Figure 1

Table 2. Number of culturable rhizosphere, endophytic and epiphytic microorganisms

Isolates	RA-A	RA-B	EP-A	EP-B	AEN-A	AEN-B
Zone 1 (Puebla)	25	21	28	43	31	2
Zone 2 (Colinas)	34	17	20	30	24	2
Zone 3 (Calonge)	27	32	22	34	46	17
Zone 4 (Rincón)	29	29	31	30	38	15
TOTAL	115	99	101	137	139	33
	RA		**EP**		**AEN**	

largest group was composed by the epiphytic bacteria of zone 1 (Puebla). No differences in the number of culturable aerobic bacteria isolated in samplings A and B were observed. The number of isolated bacteria in zones 1 and 2 in sampling A diminished in comparison with sampling B. In contrast, in zones 3 and 4, the number of endophytic bacteria diminished in sampling B with respect to sampling A. A relation between the total number of rhizosphere, epiphytic, and endophytic bacteria isolated in samplings A and B and the four sampling areas analyzed could not be established.

Phenotypic characterization of culturable rice rhizosphere, epiphytic, and endophytic bacteria

The plant growth promoting activities of the 624 isolated microorganisms were tested. Thus, production of IAA and siderophores, ACC degradation, phosphate solubilization, and chitinase activity were determined (Figure 2).

434 of the 624 isolates produced IAA and in 93 of them, this production was higher than 3 p.p.m. Most of these bacteria were isolated from the rice rhizosphere in the early stages of plant development, mainly from zone 4 (Rincón). In all sampling areas, a decrease in the number of bacteria that produced IAA was observed in sampling B with respect to sampling A, with the exceptions of the increases of rhizosphere bacteria in zone 1 (Puebla) and endophytic bacteria in zone 3 (Calonge), both in sampling A (Figure 2a).

Zone 3 (Calonge) was the sampling area with the highest number of ACC-degrading bacteria (Figure 2b). There was an increase in the number of epiphytic bacteria able to degrade ACC in sampling A with respect to sampling B, and a decrease in the case of endophytic bacteria. In the rhizosphere isolates, the number of microorganisms that degraded ACC varied depending on the sampling area. Thus, the highest number of bacteria with this enzymatic activity was found in zones 1 and 3, with a low salt

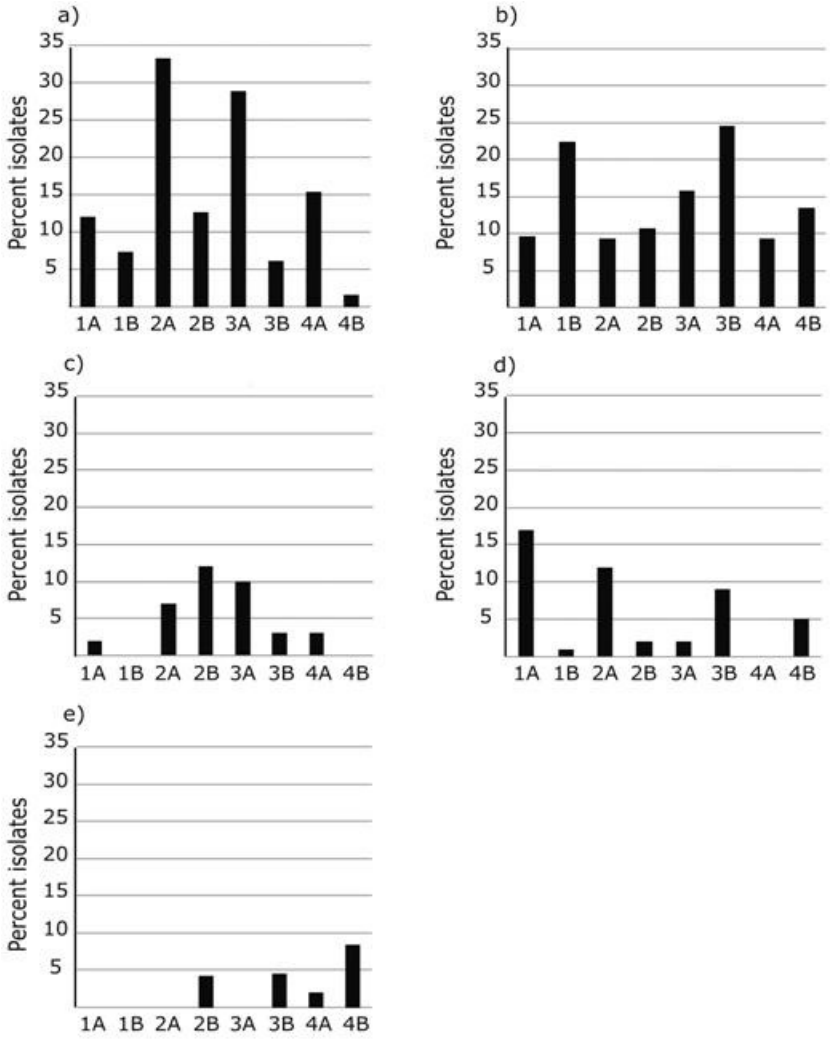

Figure 2

concentration in the irrigation water, and the lowest in zones 2 and 4, both with higher salt concentrations (Figure 2b).

Only 11 of the 624 isolated bacteria produced siderophores. 9 of these strains were isolated in sampling B and only 2 in sampling A, both in zone 4, which was the sampling area with the highest number of siderophore-producing 1 bacteria (Figure 2e). A remarkable chitinase activity was detected in only 15 of the total number of isolated bacteria. Most of these strains were isolated in zones 1 and 2, which are the areas with the highest incidence of rice blast disease caused by the fungus *Magnaporthe* sp., mainly in sampling A (Figure 2d). Finally, 12 strains showed phosphate solubilizing activity, 11 of them isolated in zone 2 (Colinas), an area with high salinity and rice blast disease incidence (Figure 2c).

Selection and identification of putative PGPR

In base to the plant growth promoting activities assayed, those microorganisms that possessed more than one of these characteristics, or a very strong positive result in any of them, were selected for further identification at the Genus level. A group of 28 microorganisms from the 624 isolated were selected. All of these bacteria were isolated mainly from zones 2, 3 and 4, and just one from zone 1 (Table 3).

These organisms were identified according to the 16S rDNA sequencing data. There was a high reiteration between the Genera found in this group (*Aeromonas*, *Pantoea*, *Enterobacter*, and *Klebsiella*). Just one organism was identified as a member the Genera *Kocuria*, *Acinetobacter*, or *Pseudomonas*.

Table 3. Selected microorganisms in base to their plant-growth promoting capacities assayed, and identification after amplification and sequencing of the 16S rDNA fragment. Each strain was named in reference to the zone in which it was isolated, the plant tissue, the plant development stage (A, early stage and B, flowering stage), and a number regarding to the position in the collection.

			Plant-growth promoting activities				
				AIA			
Strain	**Isolation zone and plant's stage**	**16S rRNA**	**ACC**	**AIA (p.p.m.)**	**Chitinase**	**Phosphate solubilization**	**Siderophores**
2RA-A 15	Zone 2, rhizosphere, early stage	*Klebsiella* sp.		+ (22.066)			
2RA-B	Zone 2, rhizosphere, early stage	*Aeromonas* sp.		+ (8.504)	+		
2RA-A 27B	Zone 2, rhizosphere, early stage	*Klebsiella* sp.	+	+ (6.824)			
2AEP-A 3	Zone 2, epiphytic, early stage	*Erwinia* sp. / *Pantoea* sp.	+	-		++	
2AEP-A 6	Zone 2, epiphytic, early stage	*Klebsiella* sp.		+ (6.214)		++	
2AEP-A 19	Zone 2, epiphytic, early stage	*Enterobacter* sp.	+	+ (6.366)			
2AEN-A 16	Zone 2, endophytic (stem), early stage	*Pantoea* sp. / *Erwinia* sp. / *Margalefa* sp.		-		++	
3RA-A 21X	Zone 3, rhizosphere, early stage	*Enterobacter* sp.	+	+ (3.720)		++	
3RA-A 21Z	Zone 3, rhizosphere, early stage	*Aeromonas* sp.	+		+	+	
3AEP-A 11	Zone 3, epiphytic, early stage	*Aeromonas* sp.			+	+/-	
3AEN-A 5A	Zone 3, endophytic (stem), early stage	*Enterobacter* sp.	+	+ (2.318)		++	
3AEN-A 7	Zone 3, endophytic (stem), early stage	*Enterobacter* sp.	+	+ (2.049)			
4RA-A 28	Zone 4, rhizosphere, early stage	*Aeromonas* sp.	+	+ (14.630)	+		
4AEN-A 1B	Zone 4, endophytic (stem), early stage	*Pantoea* sp.	+	+ (8.030)			
1AEP-B 21B	Zone 1, epiphytic, flowering stage	*Acinetobacter* sp.		+ (43.840)			
2RA-B 6	Zone 2, rhizosphere, flowering stage	*Enterobacter* sp.				++	
2RA-B 23	Zone 2, rhizosphere, flowering stage	*Enterobacter* sp.	+			++	
2AEP-B 11	Zone 2, epiphytic, flowering stage	*Enterobacter* sp.	+			++	
2AEP-B 15	Zone 2, epiphytic, flowering stage	*Pantoea* sp.		+ (14.190)		++	+
2AEP-B 4	Zone 2, epiphytic, flowering stage	*Pantoea* sp.		+ (8.350)		++	+
2AEP-B 26A	Zone 2, epiphytic, flowering stage	*Pantoea* sp.	+	+ (6.610)		++	
3RA-B 4B	Zone 3, rhizosphere, flowering stage	*Pseudomonas* sp.		-		++	
3AEP-B 17	Zone 3, epiphytic, flowering stage	*Pantoea* sp.		+ (8.480)			+

Table 3. Continue

3AEP-B 28B	Zone 3, epiphytic, flowering stage	*Pantoea* sp.		+ (15.610)		++	+
3AEP-B 9B	Zone 3, epiphytic, flowering stage	*Pantoea* sp.		+ (13.830)		++	+
3AEP-B 20B	Zone 3, epiphytic, flowering stage	*Enterobacter* sp.	+	+ (7.840)			
4AEP-B 12	Zone 4, epiphytic, flowering stage	*Pantoea* sp.		+ (9.460)			+
3AEN-B 2	Zone 3, endophytic (stem), flowering stage	*Kocuria* sp.		+ (50.290)			

DISCUSSION

In this work, a complete screening of bacteria that interact with rice plants and could potentially improve its growth has been carried out. Soil and plant samples were collected from four cropping areas with different salinity and rice blast disease incidence. In addition, two different plant physiological stages were selected.

Enumeration studies showed that the highest bacterial numbers from the different samples corresponded to epiphytic bacteria, then rhizosphere, and finally, endophytic bacteria. The number of rhizosphere bacteria did not change in the samples obtained from the two plant physiological stages analyzed in zones 1 and 2. However, a significant decrease in the number of microorganisms isolated was observed in zones 3 and 4 (Figure 1). A significant increase in the number of epiphytic bacteria and a clear reduction of endophytic bacteria were observed in the areas analyzed (Figure 1). The number of culturable bacteria isolated from the zones without biotic and abiotic stresses were higher than those obtained in the areas where rice plants were stressed (Figure 1). Therefore, the environmental conditions could be a factor affecting bacterial populations. However, results shown in this work indicate that the plant could also influence the number of microorganisms that form bacterial communities. Thus, the number of isolates was different in the rice rhizosphere, the surface or the interior of the plant shoot, and changed depending on the physiological stage of the plant (Figure 1). In sampling A, roots were not completely developed yet but showed an intense nutrient absorption. Therefore, there is an active interchange of substances between the plant and the rhizosphere that could attract and increase bacterial populations as they use root exudates as carbon and energy sources (Newman, 1985; Whipps and Lynch, 1986). In sampling B, the plant was in reproductive stage. Higher total leaf surface would suggest larger bacterial communities. Thus, in this physiological stage, a high number of epiphytic bacteria were observed. However, remarkable differences in the number of endophytic bacteria were observed in zones 1 and 2, as in sampling A there were 10-fold more bacteria than in sampling B (Figure 1).

Data shown in Figure 1 would indicate quantitative differences in the rhizosphere and plant-associated bacterial populations but not changes in biodiversity. The distribution in the number of aerobic bacteria isolated in base to their morphology and pigmentation was different in each zone, probably due to the soil characteristics and biotic and abiotic stresses. The stage of plant development is a factor that clearly affects the qualitative and quantitative composition of root exudates (Lynch, 1990). Our results showed that rhizosphere and epiphytic bacteria were more abundant during flowering (sampling B) than in the initial stages of the plant development (sampling A). Despites roots are more active in sampling A, biodiversity seemed to be lower (Table 2). These results could indicate a specialization of the colonizers that would allow more proliferation but less diversity and adaptation. It is worth noting that zone 2, which is the area with stronger biotic and abiotic stresses, showed the highest diversity of rhizosphere microorganisms in sampling A. These results correlate with the fact that the production of organic acids by plant roots is stimulated by nutritional deficiencies, ionic toxicity, and pathogen attacks (Mimmo et al., 2008).

In order to determinate the potential plant growth promoting characteristics of the isolated bacteria, a preliminary study of the production of the hormone indol-3-acetic acid (IAA) was carried out. IAA is an auxin that promotes the elongation of the plant roots, allowing a better water and nutrients absorption (Malamy, 2005). About 69% of the 624 isolated bacteria showed IAA production (Figure 2a). As previously described (Khalid et al., 2004), IAA production was more common among rhizosphere microorganisms. By contrast, this activity was scarce in endophytic bacteria, despites some endophytic bacteria can produce high 1 amounts of this phytohormone (more than 160 mg l^{-1}) in several plant development stages, which could provide a great benefit for growth promotion and fitness of their host, as described by Wang et al. (2013). In fact, just a few IAA producers were isolated as endophytic bacteria among the microorganisms

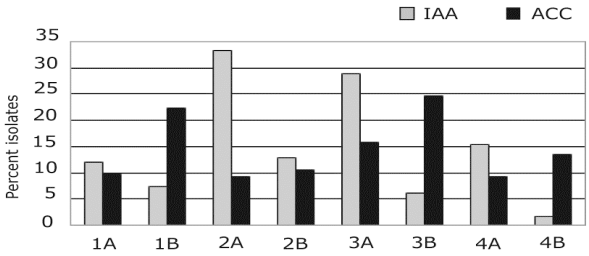

Figure 3

isolated in this study. However, the best IAA producer was an endophytic microorganism, AMG596, which was selected as an isolate with potential PGP activities interesting for agricultural use (Table 3). The concentration of the IAA produced by selected microorganisms ranged between 2-20 p.p.m. with the exceptions of the epiphytic bacterium AMG409 and the endophyte AMG596 that produced 43.84 and 50.29 p.p.m., respectively. Curiously, those samples with a higher proportion of bacteria able to produce IAA in sampling A, suffered a drastic reduction in sampling B. This fact could be due to a regulation of IAA production by plants to avoid an excess of this hormone that could have deleterious effects on plants at high concentrations (Lambrecht et al., 2000; Long et al., 2008).

Under biotic and abiotic stress, plants produce high quantities of ethylene. This hormone regulates many aspects of plant growth through multiple pathways (Tsai et al., 1996). However, high concentrations of ethylene inhibit plant roots development (Saleem et al., 2007). Therefore, the reduction of the concentration of ethylene induced by salinity would diminish the negative impact caused by an excess of this hormone. Thus, degradation of 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene, by the isolated bacteria was studied. Results showed a higher proportion of ACC-degrading microorganisms in developed plants (Figure 2b, sampling B) that would correlate with higher concentrations of ACC in the rhizosphere due to a massive production of ethylene by plants as a consequence of the stress caused by salinity.

In spite production of IAA 1 stimulates root elongation, this auxin is also involved in biochemical processes related to ACC and therefore with the ethylene cycle (Saleem et al., 2007). IAA induces the expression of the ACC synthase, which converts S-adenosylmethionine (SAM) in ACC. Glick et al. (1998) proposed a model in which a significant quantity of ACC could be exuded by plant roots and then used by organisms that posses the ACC deaminase activity. Thus, the equilibrium between the internal and external levels of ACC is maintained by exudation of this molecule to the rhizosphere. Therefore, microorganisms that degrade ACC also stimulate the production and exudation of this plant compound, providing the bacterial community with an important nitrogen source and promoting the elongation of the plant roots. When comparing our results regarding the diversity of microorganisms that produce IAA and degrade ACC (Figure 3), equilibrium between these two bacterial populations was observed, and they were complementary spread (Figure 2, panels A and B). These results are in agreement with the fact that synthesis of IAA and ACC degradation could be considered complementary in the ethylene pathway: IAA induces the synthesis of ethylene in seeds and shoots and increases the expression of the enzyme that synthesizes ACC, which is a precursor of ethylene. Thus, there is a positive feedback in the ethylene cycle (Peck and Kende, 1998). In addition, at high ethylene concentrations, IAA transporters are inhibited, reducing the effects of this plant hormone (Morgan and Durham, 1973). As suggested by Long et al. (2008), IAA shows a beneficial effect on plants growth only if the concentration of this auxin is low. At high concentrations, IAA inhibits root elongation. The relationship between bacterial ACC deaminase and IAA could therefore regulate the mode of action of this plant hormone.

Other PGP activities studied in this work are related to the antagonism against pathogens. Thus, production of siderophores, molecules able to chelate Fe^{3+}, reduces its availability for other microorganisms (Príncipe et al., 2007). Enzymes that degrade chitin, the main component of fungal cell walls, could also play an important role in protection against pathogens. There was no correlation between the biodiversity of microorganisms that produce siderophores and those that degrade chitin, as previously described by other authors (Ahmad et al., 2008), suggesting that these mechanisms work independently. Nevertheless, our results showed that most of the microorganisms that degrade chitin were localized in those areas with higher incidence of rice blast disease - zones 1 and 2 - (Figure 2d), and mainly in sampling A, when plants are more sensitive to fungal pathogens. However, bacteria able to produce siderophores were mainly found in zone 4, an area with a low incidence of the fungal disease and medium saline concentration (Figure 2e). In the Guadalquivir marshes, iron is not a limiting element, and therefore the potential mechanism used by these bacteria

to protect against pathogens is not probably the reduction of the availability of Fe^{3+}.

The capacity to solubilize phosphate by the bacterial isolates was also tested because phosphate is a limiting element in crops nutrition. Results shown in this work showed that there were many isolates that possessed this capacity. This is in agreement with previous works in which these phosphate solubilizers have been described as dominant groups in various soils (Yang et al., 2012). Thus, these bacteria were isolated from all the screening areas during the flowering stage, in which availability of nutrients is essential for plants.

Once isolated and phenotypically studied, those bacterial strains with more than one PGP activity were selected for further experiments and characterized in base to 16S rDNA sequence. As shown in Table 3, there were only a few genera represented in this group of selected microorganisms. These bacteria are well represented in soils from paddy fields (Verma et al., 2001, Thakuria et al. 2004, Park et al., 2005; Bal et al., 2013), and have shown their capacities to improve plant growth, especially those belonging to the genus *Pantoea* and *Enterobacter*, which are predominant among the selected microorganisms and have been described as common rice colonizers (Verma et al., 2004). Several authors have pointed their capacity to promote plants growth through phosphate solubilization (Yang et al., 2012), production of IAA and ACC-deaminase (Bhattacharjee et al., 2012), production of siderophores or antifugal activity (Laslo et al., 2012). Most of these bacteria were isolated from zone 2, the area with highest rice blast disease incidence and salinity. This fact could suggest a plant capacity to select those microorganisms with beneficial enzymatic activities through plant root exudates. Plants are able to modify soil microbial communities and stressful conditions stimulate root exudation (Lynch, 1990; Bednarek et al., 2010). Therefore, the diversity of bacteria able to face these environmental conditions is usually higher in soils that have suffered a stress period.

Plants are involved in the selection of indigenous microbial populations in the rhizosphere and each species is thought to select specific populations that could better contribute to their fitness, creating a very selective environment and limited diversity (Berg and Smalla, 2009). In this context, the selection of plant-growth promoting microorganisms adapted to specific environmental conditions would be more successful in the rhizosphere of the selected plant.

Currently, the most representative microbial group isolated as result of these experiments (*Pantoea* sp.) is being phylogenetically identified using finest techniques as LMSA in order to get a better knowledge of them that allows their efficient use for sustainable rice cultivation, especially in areas like this with a high ecological value.

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REFERENCES

Aguilar-Portero M (2001). Cultivo del arroz en el sur de España. Sevilla, Caja de ahorros Cajasol.

Ahmad F, Ahmad I, Khan MS (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163: 173-181.

Alam SM, Cui ZJ, Yamagishi T, Ishii R (2001). Grain yield and related physiological characteristics of rice plants (*Oryza sativa* L.) inoculated with free-living rhizobacteria. Plant Prod. Sci. 4: 125-130.

Bal HB, Das S, Dangar TK, Adhya TK (2013). ACC deaminase and IAA producing growth promoting bacteria from the rhizosphere soil of tropical rice plants. J. Basic Microbiol. 0: 1-13.

Bednarek P, Kwon C, Schulze-Lefert P (2010). Not a peripheral issue: secretion in plant microbe interactions. Curr. Opin. Plant Biol. 13: 378-387.

Berg G, Smalla K (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol. Ecol. 68: 1-13.

Bhattacharjee RB, Jourand P, Chaintreuil C, Dreyfus B, Singh A, Mukhopadhyay SN (2012). Indole acetic acid and ACC deaminase-producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth, and in the process undergo colonization and chemotaxis. Biol. Fertil. Soils 48: 173-182.

Britto DT, Kronzucker J (2004). Bioengineering nitrogen acquisition in rice: can novel initiatives in rice genomics and physiology contribute to global food security?. Bioessays 26: 683-692.

Chinnusamy M, Kaushik BD, Prasanna R (2006). Growth, nutritional, and yield parameters of wetland rice as influenced by microbial consortia under controlled conditions. J. Plant Nutr. 29: 857-871.

Choudhury ATMA, Kennedy IR (2004). Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biol. Fert. Soils 39: 219-227.

Cong PT, Dung TD, Hien TM, Hien NT, Choudhury ATMA, Kecskés ML, Kennedy IR (2009). Inoculant plant growth-promoting microorganisms enhance utilisation of urea-N and grain yield of paddy rice in southern Vietnam. Eur. J. Soil Biol. 45: 52-61.

Duuff BJ, Erkelens A, Bakker PAHM, Schippers B (2008). Influence of pH on suppression of *Fusarium* wilt of Carnation *Pseudomonas fluorescens* WCS417r. J. Phytopathol. 143: 217-222.

Glick BR, Penrose DM, Li J (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190: 63-68.

Hall V, O'Neill GL, Magee JT, Duerden 1 BI (1999). Development of amplified 16S ribosomal DNA restriction analysis for identification of *Actinomyces* species and comparison with Pyrolysis-Mass Spectrometry and conventional biochemical tests. J. Clin. Microbiol. 37: 2255-2261.

Khalid A, Arshad M, Zahir ZA (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96: 473-480.

Lambrecht M, Okon Y, Vande Broek A, Vanderleyden J (2000). Indole-3-acetic: a reciprocal signaling molecule in bacteria-plant interactions. Trends Microbiol. 8: 298-300.

Laslo E, György E, Gyöngyvér M, Tamás E, Ábrahám B, Lányi S (2012). Screening of plant growth promoting rhizobacteria as potential microbial inoculants. Crop Prot. 40: 43-48.

Long HH, Schmidt DD, Baldwin IT (2008). Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS ONE 3: e2702.

Lucas-García JA, Schloter M, Durkaya T, Hartmann A, Gutiérrez-Mañero FJ (2003). Colonization of pepper roots by a plant growth promoting *Pseudomonas fluorescens* strain. Biol. Fertil. Soils 37: 381-385.

Lynch JM (1990). The rhizosphere. Chichester, John Wiley and sons.

Mäder P, Kaiser F, Adholeya 1 A, Singh R, Uppal, HS (2011). Inoculation of root microorganisms for sustainable wheat-rice and wheat-black gram rotations in India. Soil Biol. Biochem. 43: 609-619.

Malamy JE (2005). Intrinsic and environmental response pathway that regulate root system architecture. Plant Cell Environ. 28: 67-77.

Mimmo T, Ghizzi M, Marzadori C, Gessa CE (2008). Organic acid extraction from rhizosphere soil: effect of field-moist dried and frozen samples. Plant Soil 312: 175-184.

Morgan PW, Durham JI (1973). Leaf age and ethylene-induced abscission. Plant Physiol. 52: 667-670.

Newman EI (1985). The rhizosphere: carbon sources and microbial population. In: Pitter AH (ed.) Ecological interactions in soil, plants, microbes and animals. Oxford (UK), Oxford Blackwell Scientific, pp. 107-121.

Nguyen VN, Ferrero A (2006). Meeting the challenges of global rice production. Paddy Water Environ. 4: 1-9.

Park M, Kim C, Yang J, Lee H, Shin W, Kim S, Sa T (2005). Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. Microbiol. Res. 160: 127-133.

Peck SC, Kende H (1998). A gene encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase produces two transcripts: elucidation of a conserved response. Plant J. 14: 573-581.

Peix A, Rivas-Boyero A, Mateos PF, Rodríguez-Barrueco C, Martínez-Molina E, Velázquez E (2001). Growth promotion of chickpea and barley by phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. Soil Biol. Biochem. 33: 103-110.

Penrose DM, Moffatt BA, Glick BR (2001). Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. Can. J. Microbiol. 47: 77-80.

Pieterse CMJ, van Wees SCM, Hoffland E, van Pelt JA, van Loon LC (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell 8: 1225-1237.

Príncipe A, Álvarez F, Castro MG, Zachi L, Fisher SE, Mori GB, Jofré E (2007). Biocontrol and PGPR features in native strains isolated from saline soils of Argentina. Curr. Microbiol. 55: 314-322.

Saleem M, Arshad M, Hussain S, Bhatti AS (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J. Ind. Microbiol. Biot. 34: 635-648.

Shin SH, Lim Y, Lee SE, Yang NW, Rhee JH (2001). CAS agar diffusion assay for the measurement of siderophores in biological fluids. J. Microbiol. Meth. 44: 89-95.

Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro RC, Khan MR (2004). Characterization and screening of bacteria from rhizosphere of rice grown in acidic soils of Assam. Curr. Sci. 86: 978-985.

Thompson P, Bailey MJ, Ellis RJ, Purdy KJ (1993). Subgrouping of bacterial populations by cellular fatty acid composition. FEMS Microbiol. Lett. 102:75-84.

Toratani T, Shoji T, Ikehara T, Suzuki K, Watanabe T (2008). The importance of chitobiose and N-acetylglucosamine (GlcNAc) uptake in N, N'-diacetylchitobiose [$(GlcNAc)_{2]}$ utilization by *Serratia marcescens* 2170. Microbiology 154:1326-1332.

Tsai FY, Hung KT, Kao CH (1996). An increase in ethylene sensitivity is associated with jasmonate-promoted senescence of detached rice leaves. Plant Growth Regul. 15: 197-200.

Verma SC, Ladha JK,Tripathi AK (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J. Microbiol. 91: 127-141.

Verma SC, Singh A, Chowdhury SP, Tripathi AK (2004). Endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp. using green fluorescent protein reporter. Biotechnol. Lett. 26: 425-429.

Wang S, Wang W, Jin Z, Du B, Ding Y, Ni T, Jiao F (2013). Screening and diversity of plant growth promoting endophytic bacteria from peanut. African J. Microbiol. Res. 7: 875-884.

Whipps JM, Lynch JM (1986). The influence of the rhizosphere on crop productivity. Adv. Microbiol. Ecol. 6: 187-224.

Yang PX, Ma L, Chen MH, Xi JQ, He F, Duan CQ , Mo MH, Fang DH, Duan YQ, Yang FX (2012). Phosphate solubilizing ability and phylogenetic diversity of bacteria from P rich soils around Dianchi Lake Drainage area of China. Pedosphere 22: 707-716.