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Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain

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ABSTRACT

Biocontrol capacity of two plant growth-promoting rhizobacteria (PGPR) strains, against blast disease in rice paddy fields in Southern Spain was studied in three cropping seasons. Both strains (*Pseudomonas fluorescens* Aur 6 and *Chryseobacterium balustinum* Aur 9) had already shown biocontrol capacity against pathogens, ability to induce systemic resistance against leaf pathogens and against salt stress in different plant species. Bacterial treatments were carried out on seeds and/or on leaves. Strains were inoculated individually and in combination. Protection against natural disease incidence was evaluated, and rice production and quality measured in 2005 and 2006 trials. In 2004, natural disease incidence was low (between 0.1% and 0.35% of damaged leaf surface) due to environmental conditions; under these conditions, both strains significantly protected plants against rice blast. In 2005, disease incidence was higher than in 2004, reaching higher values of affected leaf surface in controls. In these conditions, each strain individually protected rice against rice blast, although the combination of both strains was the most effective treatment. All three treatments (Aur 6, Aur 9 and Aur 6 + Aur 9) reached 50% protection in panicles, with Aur 9 being the most effective. In 2006, the most effective treatment was the combination of both strains on leaves in three physiological stages, suggesting a biocontrol mediated protection. On the other hand, when bacteria were applied to seeds, disease incidence decreased up to 50%, suggesting induction of systemic resistance. Finally, a direct relation between protection mediated by the PGPR and the increase in rice productivity (mT/ha) and quality (weight of 1000 seeds and number of intact grains after milling) was found.

1. Introduction

About 450,000 ha of the arable land in the European Union are devoted to rice (*Oryza sativa* Linn) and almost 25% of it is in Spain. One of the most important areas devoted to this crop is the *Marismas del Guadalquivir* area in Southern Spain (38,000 ha), and accounts for 40% of the total rice production in Spain (Aguilar, 2001).

'Rice blast', caused by the fungus *Pyricularia oryzae* Cav. is the most important disease of rice worldwide, both in terms of distribution (Anonymous, 1968; Pans, 1976) and damage (Ou, 1972, 1980). The blast fungus can infect rice plants at any stage of the biological cycle. The early symptoms are whitish to greyish and brownish spots or lesions, and are followed by nodal rot and/or neck blast, which can cause necrosis and frequently breakage of the panicle (Agarwal et al., 1989). Blast epidemics are mainly dependent on climatic conditions, crop management practices, such as nitrogen inputs or water supply, and cultivar susceptibility (Suzuki, 1975; Ou, 1985; Nyvall, 1999). Its great destructive potential is such that paddy fields that register disease incidences over 30% are abandoned due to loss of potential benefits in economic terms. Controlling this disease is therefore one of the main goals of rice growers.

The severity of the damage depends on the part of the plant affected and on the cultivar. Leaf infection reduces photosynthetic area and may eventually result in plant death. Panicle infection reduces yield and therefore this involves important economic losses (Roumen, 1992). The cheapest and most effective way to control this disease is the use of resistant cultivars. However, the evolutionary potential of this pathogen has overcome plant resistance via the emergence of new fungal strains. This is especially relevant in terms of disease incidence due to the number of rice genotypes and the degree of sensitivity to new fungal races together with the influence of environmental factors (Xia et al., 1993), that strongly affect the expression of resistance (Ou, 1980).

The first reports of this disease in Spain date from 1968 (Anonymous, 1968; Benlloch, 1975). The aetiology and importance of rice blast was studied by Marín Sanchez and Jimenez-Diaz

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(1981), all over the *Marismas* area. In 1997, a highly virulent rice blast epidemy caused 15% yield losses, accounting for an important economic loss (€10 million).

Beneficial free-living soil bacteria, generally referred to as plant growth-promoting rhizobacteria (PGPR) are found in association with the roots of various plants (Glick et al., 1999). However, there are many reports of beneficial bacteria from different origins including the phyllosphere (Bashan and de-Bashan, 2002) or even endophytic strains (Di Fiore and Del Gallo, 1995). In recent years, much attention has been paid to traditional cropping practices, in an attempt to move on towards an environmentally friendly agriculture within the framework of sustainable development (Sturz et al., 2000). Irrespective of the origin of beneficial strains, PGPR may promote plant growth through several mechanisms. Indirect mechanisms involve improving nutrient availability or preventing growth of pathogenic microorganisms, while direct mechanisms, involve the plant metabolism, either altering plant's hormonal balance or inducing the plant's defensive systemic response (Ramos Solano et al., 2008a). Therefore, the application of PGPR has a great potential in agriculture since it would allow to lower agro-chemicals inputs while maintaining the biotic diversity in the plant associated bio-community, a promising approach that will alter both agricultural and horticultural practices dramatically (Glick et al., 1999).

The use of PGPR as biocontrol agents is relatively low (Cook, 2000), representing about 1% of agricultural chemical sales (Lidert, 2001) while fungicides represent approximately 15% (http://www.epa.gov). Biocontrol agents can be used in situations in which there is no available chemical control, when conventional pesticides cannot be used due to residue concerns, or for certified organic production. However, the main reason for the development of biocontrol agents is the ability of pathogens to develop resistance to fungicides (Wilson, 1997). The different mechanisms of action use by the biocontrol agents in biopesticides represent a great advantage over chemicals which work on a single target. Therefore, biopesticides can be used in rotation with pesticides resulting in lowering the chances of pathogens to develop resistance. In addition, biocontrol agents can also be used in combination with reduced doses of pesticides. We do not know what challenges may arise in the future, and it seems prudent to develop a variety of options for disease control.

The aim of this work was to study the capacity of two bacterial strains, individually or in combinations, to protect rice against natural blast disease incidence under field conditions. In addition, the effect of these strains on rice production was evaluated determining production (tonnes per hectare) and quality (weight of 1000 seeds and percentage of intact grains after milling).

2. Materials and methods

2.1. Bacterial strains and plants

The two bacterial strains used were Aur 6 and Aur 9. Aur 6 was isolated from the rhizosphere of *Lupinus hispanicus* and Aur 9 from the rhizosphere of *Lupinus albus* (Gutierrez Mañero et al., 2003). Both strains were identified by FAMEs (Microbial ID, Inc. Newark, USA) and 16s DNA sequencing as *Pseudomonas fluorescens* and *Chryseobacterium balustinum*, respectively and both were deposited in the Spanish Culture Type Bank (CECT 5398 and 5399, respectively). Both strains were able to produce auxin-like compounds (1.48 and 3.7 ppm IAA-like, respectively) and Aur 6 is also able to solubilise phosphate and degrade 1-aminocyclopropane-1 carboxylic acid (ACC) (Gutierrez Mañero et al., 2003). Both strains have shown a growth promoting effect on *Lupinus* sp. (Lucas García et al., 2003), tomato and pepper (Cezón et al., 2003), pine and holm-oak tree (Lucas García et al., 2004), and have shown ability to induce systemic resistance against *Pseudomonas syringae* DC3000 in *Arabidopsis thaliana* (Ramos Solano et al., 2008b), and against salt stress (Barriuso Maicas et al., 2008). Both have also demonstrated biocontrol ability against *Xanthomonas campestris* in tomato, alone and in combination with other bacterial strains (Domenech et al., 2006).

The rice variety used was *O. sativa* var Baixet and belongs to japonica type. This variety was selected due to its high genetic susceptibility to *Pyricularia* (Galimany et al., 2006; Castejón-Muñoz et al., 2007).

2.2. Plant growth and delivery of biocontrol agents

The experiments were carried out in field conditions in *Marismas del Guadalquivir* (Seville, Spain). This area is characterised for its flat, clayey, saline soils of sedimentary origin. The 400 m^2 experimental plots were located in Utrera (230.6584.109.328 UTM) and belonged to the Federación de Arroceros de Sevilla (www.federaciondearroceros.es).

Growth conditions were set to favour natural rice blast incidence, since no inoculations with pathogens could be performed in field conditions. Plants were sown at hand at a higher dose than usual (200 kg of seeds/ha). Plots were fertilised with Blending (35:15:0) with more N than is normally used, overpassing limits for Integrated Production (>125 U.F. for japonica varieties). Herbicides were applied as usual: NOMINEE© (BISPIRIBAC-Na) in June 20th and with LONDAX© (Bensulfuron 60%), 40 days after sowing.

Bacterial inoculants were provided by AMC Chemical S.A. Bacteria were grown in 50 L fermenters on nutritive broth, reaching 10^9 cfu mL^{-1}. Both strains were compatible since they were able to grow simultaneously in the same culture media (data not shown). However, to prepare combined inocula each strain was grown independently to achieve 10^9 cfu mL^{-1} and then mixed in even proportions to achieve 10^8 cfu mL^{-1} of each. Inoculations were carried out with bacteria and its culture media, diluted with water to achieve the desired bacterial density; applications were done on seeds or on leaves, depending on the year, and are specified in each experiment. For seed inoculations, seeds were kept on a 10^8 cfu mL^{-1} bacterial solution for 4 h before sowing. Inoculation on leaves was done with bacterial suspensions at 10^8 cfu mL^{-1}, by foliar spray at 500 L ha^{-1}.

2.3. First experiment: cropping season 2004

The experimental plot was divided in 12 subplots, 10 m × 2 m (20 m^2) each. Four subplots were selected at random for each treatment (Aur 6, Aur 9 and untreated control), allowing free intervals between subplots to avoid potential cross-inoculum. Treatment with PGPR was carried out only in leaves, with a backpack fumigator to provide a constant and homogeneous dose at 500 L ha^{-1} throughout the plot.

Plots were treated with PGPR bacteria from the beginning of tillering (Lancashire et al., 1991) every 15 days on leaves, until harvest.

2.4. Second experiment: cropping season 2005

This year a random block design was made. The same experimental plot was divided in five blocks, and in each block all four treatments were carried out, in subplots of 10 m × 2 m (20 m^2). In this case, treatments were the individual bacteria Aur 6, Aur 9, the combination Aur 6 + Aur 9 and control (untreated plants). Therefore, 5 replicates of each treatment were made.

PGPR bacteria were applied to seeds and leaves as described above. A total of 7 bacterial applications were done, one on seeds