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Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of *Fusarium graminearum*

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ABSTRACT

The role of endophytic communities of seeds is still poorly characterised. The purpose of this work was to survey the presence of bacterial endophytes in the seeds of a commercial wheat cultivar widely sown in Argentina and to look for plant growth promotion features and biocontrol abilities against *Fusarium graminearum* among them. Six isolates were obtained from wheat seeds following a culture-dependent protocol. Four isolates were assignated to *Paenibacillus* genus according to their 16S rRNA sequencing. The only gammaproteobacteria isolated, presumably an *Enterobactereaceae* of *Pantoea* genus, was particularly active as IAA and siderophore producer, and also solubilised phosphate and was the only one that grew on N-free medium. Several of these isolates demonstrated ability to restrain *F. graminearum* growth on dual culture and in a bioassay using barley and wheat kernels. An outstanding ability to form biofilm on an inert surface was corroborated for those *Paenibacillus* which displayed greater biocontrol of *F. graminearum*, and the inoculation with one of these isolates in combination with the *Pantoea* isolate resulted in greater chlorophyll content in barley seedlings. Our results show a significant ecological potential of some components of the wheat seed endophytic community.

1. Introduction

Wheat is a very important crop for Argentine agriculture and has become a key factor for the preservation of soil fertility because many Argentine soils are under a continuous soybean-wheat rotation scheme, and soybean is recognized as a very soil-exhausting crop (Shurtleff and Aoyagi, 2014). About 14.5 million tons of total Argentine agriculture production corresponded to wheat at the 2012/2013 campaign. Argentine was sixth among wheat exporter countries, and wheat exportations represented for our economy an income of 2.5 million dollars in that period (Barberis, 2014).

During last decades many researchers reported the presence of seed endophytes in several plant species including gramineous plants such as rice and maize, where Proteobacteria, Actinobacteria and Firmicutes were particularly dominant (Rijavec et al., 2007; Kaga et al., 2009; Ruiz et al., 2011).

Seed endophytes may come from different plant organs, being transferred to seeds *via* vascular connections or through gametes, resulting in colonisation of embryo and endosperm; and reproductive meristems may also be the source (Malfanova et al., 2013). Vertical transmition from one plant generation to the following may then occur, as suggested by several authors (Ringelberg et al., 2012; Liu et al., 2012; Gagne-Bourgue et al., 2013) and depicted recently in Truyens et al. (2015).

After seed germination, these populations are expected to increase in number and to colonise different tissues including roots, reaching the endorizosphere and probably also the exorizosphere. Mano et al. (2006) observed that although rice seed endophytes mainly colonised shoots, some strains were able to spread out into the rhizosphere and soil. Similar observations were made by Hardoim et al. (2012). López-López et al. (2010) could recover almost all bacterial genera isolated from bean seeds also from the roots of bean seedlings. Under this scenario, introduced microorganisms are expected to compete and/or to share their ecological niche with endophytic communities established in the rhizosphere, for which it is particularly necessary to gain more knowledge about seed endophytic communities in crops which are increasingly being inoculated with plant growth promoting microorganisms (PGPM), such as wheat or maize. In this sense and regarding particularly corn crop, of note are the findings of Johnston-Monje and Raizada

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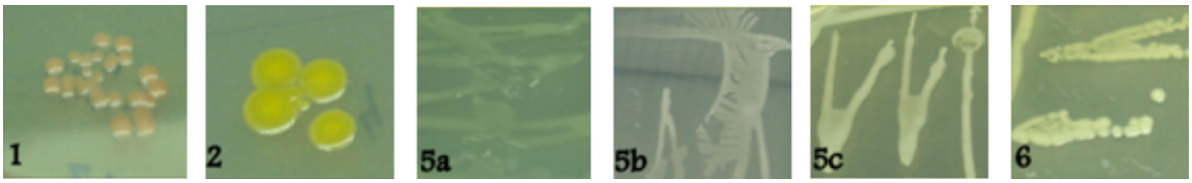

Fig. 1. Image illustrates colony morphotype of the representative endophytes isolated by a culture-dependent protocol from seeds of wheat, cultivar 75 Aniversario, according to the description in Materials and methods. Numbers identify the different isolates.

(2011), who demonstrated that in maize, a core microbiota consisting of the same bacterial species is conserved apparently from teosinte's times, in spite of evolutive and selective changes, even across the huge American continent.

The role of seed endophytes has not been unravelled yet. It has been demonstrated that some of them can increase plant growth due to the production of plant hormones or to their contribution in plant nutrients acquisition, specially nitrogen and phosphorus (Gagne-Bourgue et al., 2013; Johnston-Monje and Raizada, 2011; Xu et al., 2014). For cacti, the ecological significance of seed endophytes was demonstrated by Puente et al. (2009a, 2009b). On another hand, antifungal activity of several bacterial seed endophytes has also been recognized, involving lipopeptides like surfactin, iturin and mycobacillin (Gagne-Bourgue et al., 2013). Some strains of bacteria frequently mentioned as seed endophytes (such as *Bacillus* and *Pseudomonas*) were found to have antagonistic effects on *F. oxysporum* f.sp. lycopersici (Fol.), the causative agent of tomato wilt (Sundaramoorthy and Balabaska, 2013). Volatile antifungal compounds were also found to be involved in the biocontrol displayed by endophytic *Enterobacter* strains obtained from rice (Mukhopadhyay et al., 1996).

Fusarium graminearum is the causative agent of wheat head blight, a worldwide fungal plant pathogen impacting severely on cereals production and quality, as this microorganism is a source of mycotoxins, which affect human and animal health. The purpose of this work was to survey the presence of bacterial endophytes in the seeds of a commercial wheat cultivar widely sown in Argentina called 75 Aniversario, to identify the most abundant genera and to screen these isolates for some features which are involved in direct or indirect mechanisms of plant growth promotion. Their abilities to inhibit the growth of the plant pathogen *F. graminearum* and to promote barley growth were also investigated.

2. Materials and methods

2.1. Isolation and identification of bacterial endophytes

Seeds of wheat (*Triticum aestivum* L.) cultivar 75 Aniversario (seed supplier: Buck S.A.) were surface disinfected using ethanol 70% (30 s), followed by 1% active Cl_2 (2.5 min) and again, ethanol 70% (30 s), with three rinses in sterilised distilled water. One ml of the last rinse water was added to 10 ml of liquid LB culture medium and incubated for 48 h to check complete external disinfection. Ten intact disinfected seeds were placed on Petri dishes containing LB and incubated at 24 °C. Five replicates were prepared. After 7 days of incubation at 28 °C, some representative colonies appearing on the majority of the plates were selected and phenotypically characterised; following repeated subcultures several isolates were obtained. Standard identification protocols based on morphology, Gram staining, spore formation and certain biochemical properties were followed to characterise these isolates. 16S rDNA partial sequencing was performed to get identification at genus level; comparisons with deposited sequences in BLAST database were made for this purpose. Total DNA was extracted from randomly chosen colonies of each selected isolate using the AxyPrep bacterial genomic DNA Miniprep Kit (Axygen Biosciences). A fragment of around 1500 bp of the 16S rRNA gene was amplified using the universal primers 27F and 1492R. PCR products were purified with the DNA-Clean Up (PB-L Productos Bio-Lógicos) and sequenced at the Genomic Unit of the Biotechnology Institute of CNIA-INTA (http://www.inta.gov.ar/biotec) using a capillary automatic sequencer model ABI3130XL (Applied Biosystems, USA). The Naïve Bayesian Classifier utility (Wang et al., 2007) from the RDP Release 10 (http://rdp.cme.msu.edu) was used to assign the obtained sequences into the new bacterial taxonomy at genus level with 95% of confidence.

2.2. Indolacetic acid and siderophore production

In vitro IAA biosynthetic ability of the isolates obtained was estimated by Salkowski colorimetric technique in 1 ml-supernatant aliquots of 5-days old cultures grown on LB amended with L-tryptophan (100 $\mu g\,ml^{-1}$), as described by Glickmann and Dessaux (1995); cultures were run in triplicate and three supernatant aliquots of each bacterial culture were processed. Final bacterial counts were calculated by the drop plate technique, as described by Herigstad et al. (2001); IAA production was expressed on 10^7 CFU basis.

Siderophore production was investigated using the O-CAS assay (Pérez-Miranda et al., 2007), a fast and universal method of siderophore detection in which an overlay of the CAS medium of Schwyn and Neilands (1987) without nutrients is applied on agar plates containing cultivated microorganisms to reveal siderophore production. Halos surrounding colonies demonstrate siderophore production.

2.3. Phosphate solubilisation in solid media

Basal Sperber medium supplemented with 2.5 $g\,l^{-1}$ of $Ca_3(PO_4)_2$ (TCP) was used to test the ability of the isolates to solubilise inorganic phosphate, as described by Alikhani et al. (2006). The pH of the medium was adjusted to 7.2. The surface of the solidified medium was divided into equal parts at the centre of which a 5-μl drop of each bacterial culture (OD = 1.00) was applied. Inoculated plates were incubated in dark at 25 °C; at day 10 plates were observed in order to establish the formation of clear zones (halo) surrounding colonies capable of solubilise TCP.

2.4. In vitro *antagonism against* F. graminearum

These experiments were based on those described by Abdulkareem et al. (2014) with some modifications.

The possibility of antagonistic effects of the isolates obtained against *F. graminearum* was assessed on dual cultures on nutrient agar. Each bacterial strain (overnight cultures; OD = 1.00) was drop-inoculated (10 μl) at four equidistant points of the plate and incubated at 25 °C for 3 days. Then, a 1 cm^2 plug of *F. graminearum* obtained from an actively growing culture was placed at the centre of the plates, and plates were further incubated for 6 days. Control