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

Silvana Díaz Herrera[a,b,1], Cecilia Grossi[a], Myriam Zawoznik[a,*], María Daniela Groppa[a,b]

[a] *Cátedra de Química Biológica Vegetal, Departamento de Química Biológica, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina*
[b] *IQUIFIB, CONICET, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina*

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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.

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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

* Corresponding author at: Myriam S. Zawoznik, Cátedra de Química Biológica Vegetal, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Junín 956, (1113) Ciudad Autónoma de Buenos Aires, Argentina.
E-mail address: myriamz@ffyb.uba.ar (M. Zawoznik).

[1] All authors contributed equally to this work and should be considered co-first authors.

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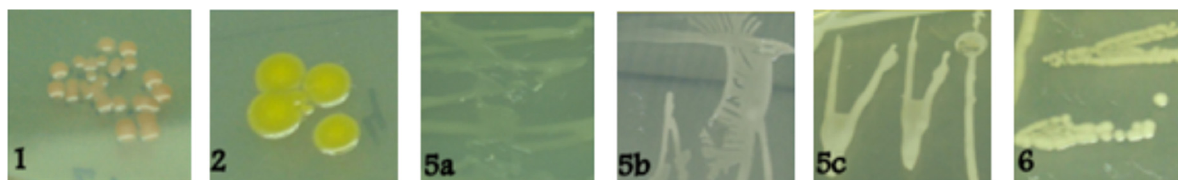

Fig. 1. Image illustrates colony morphotype of the representative endophytes isolated by a culture-dependent protocol from seeds of wheat, cultivar 75 Aniversario, according to the description in Materials and methods. Numbers identify the different isolates.

(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 Biológicos) 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

Table 1
Taxonomic features and some metabolic properties of isolated bacteria.

Isolate	Staining	Spore formation	Growth in semisolid Nfb	Phosphate solubilisation	Siderophore production*	IAA production** (μg/10^7 CFU)	Most probable affiliation based on partial 16S ARNr sequencing***	NCBI Accession Number
1	+	nd	N	N	(+)	5	*Paenibacillus* sp.	KR263165
2	–	N	Y	Y	(+++)	52	*Pantoea* sp.	KT982206
5a	+	Y	N	N	(+)	0.03	*Paenibacillus* sp.	KR263166
5b	+	Y	N	N	(+)	2.5	*Paenibacillus* sp.	KR263167
5c	+	Y	N	N	(++)	6.5	*Paenibacillus* sp.	KR263168
6	+	Y	N	N	(+)	0.002	*Bacillus sp./Fictibacillus* sp.	KR263169

nd: not determined.
N: no; Y: yes.
* Comparison based on dissolution halos diameter.
** Determined in 1 ml supernatant aliquots and expressed on 10^7 CFU basis.
*** All isolates showed 99% identity or more with deposited sequences on BLAST-NCBI database.

cultures containing only the fungus plugs at the centre served as control units.

Involvement of diffusible substances with antifungal activity released by these endophytes into the culture medium was also investigated. Four-days grown cultures in LB were centrifuged (23,400 g, 10 min) twice and filter-sterilised (Millipore–0.45 μm). The supernatants thus obtained were mixed with melted nutrient agar (45–50 °C) at a proportion of 1:4 (v/v) before filling the plates. Once solidified, a 1 cm^2 plug of *F. graminearum* obtained from an actively growing culture was placed at the centre of the plates, and further incubated for 6 days. Control plates were prepared by mixing fresh LB medium with agar nutrient (at the same proportion) before placing the fungal plug.

The production of volatile metabolites with activity against *F. graminearum* growth was also investigated. 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, the lids of these plates were replaced by the bottom of a plate containing nutrient agar inoculated with a fresh mycelial plug of *F. graminearum*. Plates were sealed together with sticky tape to minimize gas exchange and further incubated for 6 days. Controls were prepared in a similar manner but the bottom plate contained no bacteria.

In all these experiments, the radial size of the fungal colony was measured and compared to that of control units.

2.5. Biofilm formation

Biofilm formation was assessed in static conditions using the microtiter dish assay described by O'Toole (2011). In this assay, the extent of the biofilm formed by bacterial cultures on the wall and/or bottom of a microtiter dish was measured (after the removal of the liquid phase) using a 0.1% solution of crystal violet in water. To solubilise the dye, 30% acetic acid in water was used. Absorbance was read at 550 nm. Finally, data were normalised by total growth estimated by OD at 540 nm.

2.6. Antibiotic resistance

Increasing concentrations (25, 50, and 100 $\mu g\,ml^{-1}$) of ampicillin, kanamycin, chloramphenicol and tetracycline (SIGMA Aldrich, USA) were tested in solid LB to characterise the antibiotic resistance pattern of the seed endophytes obtained. Four replicated Petri dishes were prepared for each antibiotic concentration and for controls.

2.7. Kernel bioassay

Surface disinfected (as described earlier) seeds of wheat cv. 75 Aniversario and barley cv. Josefina INTA were immersed for 2 h at room temperature in a diluted cell suspension of isolates 5a, 5b, 5c or 2. These suspensions were obtained by centrifuging (23,400 g 15 min) overnight-grown cultures in LB and resuspending the pellets in sterile saline solution (0.85% NaCl), to reach a cell density of about 1×10^5 CFU ml^{-1}. Seeds were then placed (half and half at the same plate) on plates containing nutrient agar previously inoculated with 100 μl of a spore suspension (2×10^5 spores ml^{-1}) of *F. graminearum*. Controls were prepared by immersing seeds in distilled sterile saline solution for two hours. Plates were incubated at room temperature; fungal advance on the seeds was checked 15 days later.

2.8. Inoculation assays

Seeds of barley cv. Josefina INTA were sown on vermiculite. Seedlings were inoculated with the endophytes according to the following treatments: (1) isolate 5c inoculated at sowing (5c); (2) isolate 2 inoculated at sowing (P); (3) isolate 5c + 2, both inocu-

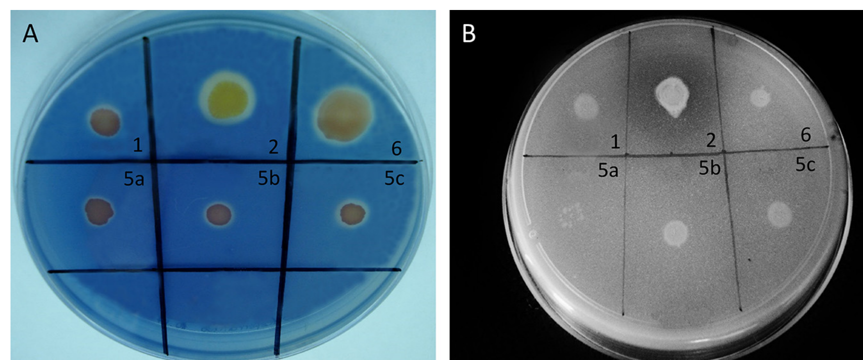

Fig. 2. Siderophore production (A) and phosphate solubilisation (B) assays. Images are representative of four replicated experimental units, obtained from three independent experiments.

Table 2
Antibiotic resistance pattern of isolated bacteria.

Isolates	Ampicillin (ppm)			Kanamycin (ppm)			Chloramphenicol (ppm)			Tetracycline (ppm)		
	25	50	100	25	50	100	25	50	100	25	50	100
1	+	+	+	–	–	–	–	–	–	–	–	–
2	+	–	–	–	–	–	+	–	–	–	–	–
5a	+	+	–	+	+	+	–	–	–	–	–	–
5b	+	+	–	+	+	+	–	–	–	–	–	–
5c	+	+	–	+	+	+	–	–	–	–	–	–
6	+	+	+	–	–	–	–	–	–	–	–	–

lated at sowing (5c + Pi); (4) isolate 5c + 2; the second one inoculated 5 days after sowing (5c + P5); (5) uninoculated (C). Inoculation was performed by adding on the crown of each emerging plantlet 10 μl of the corresponding cell suspension in sterile saline. Bacterial concentrations were in the order of 10^8 CFU ml^{-1}. Control plants received sterile saline instead of bacterial inoculum. Plants developed for 20 days in a growth chamber at 20 °C under a photoperiod of 10/14 (light/dark); light intensity was 5,000 lx. During this period, plants were watered with Hoagland solution diluted at half every other day. Three pots with five plants each were prepared, pots were distributed in the chamber following a completely randomized block design. Fresh and dry biomass and chlorophyll content were evaluated at day 20.

2.9. In vitro *compatibility of endophytes 2 and 5c*

Isolate 2 (100 μl; overnight culture) was spread on the surface of Petri dishes with nutrient agar, then isolate 5c was drop-inoculated (10 μl; overnight culture) at four equidistant points of the plate; and vice versa. Plates were incubated at 25 °C for 3 days and bacterial development was assessed. Four replicated plates were prepared.

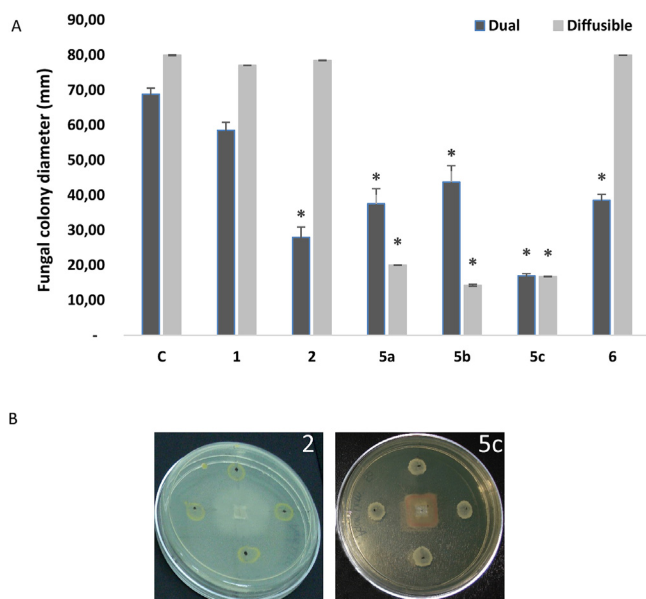

Fig. 3. Antagonism against *Fusarium graminearum*. (A) Comparative effects of seed endophytes (dual assay) and of their supernatants (diffusible substances) on the radial size of *Fusarium graminearum*. Four replicated experimental units obtained from three independent experiments were prepared. Bars represent standard error of the mean (SEM). Asterisks indicate significant differences ($P < 0.05$). Three independent experiments were carried out. B) Illustrative images showing *F. graminearum* growth in dual assays with isolates 2 (left) and 5c (right).

2.10. Statistical analysis

Data shown in Figs. 3 and 5 are mean values of three independent experiments. Standard errors of the means (SEM) are presented. Differences among treatments were analysed by one-way ANOVA followed by Tukey's multiple range test using InStat^{TM} software (Graph Pad Software, San Diego, CA, USA), at different significance levels ($^*P < 0.05$, $^{**}P < 0.01$).

3. Results and discussion

Much effort has been made to increase crop yields without taking into account the role of endophytic communities of seeds, still poorly characterised. In this research, a narrow range of endophytic microorganisms were obtained from wheat seeds following a culture-dependent protocol. Four distinct isolates (originally designated 1, 2, 5 and 6) were considered representative of the bacterial cultivable community of these seeds. Isolate 1, 2 and 6 formed small round colonies and isolate 5 developed an extensive, and massive, and irregular growth. As the appearance of this material was not uniform, particularly under longer incubation periods (≥ 6 days), three distinct parts of this massive culture were subcultured and maintained separately, handled as distinct isolates. This rendered a total of 6 isolates designated 1, 2, 5a, 5b, 5c and 6 (Fig. 1). Low numbers of bacterial genera recovered from seeds were generally related to the challenges imposed by the specific habitat they are derived from and to the limitations of culture-dependent techniques (Truyens et al., 2015).

Taxonomic features and some metabolic properties of the isolated bacteria are summarised in Table 1. A clear predominance of sporulated bacilli was found. Most of them were assigned to *Paenibacillus* genus according to their 16S rRNA sequencing (isolates 1, 5a, 5b and 5c), while isolates 2 and 6 were assigned to genus *Pantoea* and *Fictibacillus/Bacillus,* respectively. All nucleotide sequences have been submitted to GenBank Database and received the accession numbers shown on Table 1.

Most seed endophytic bacteria isolated from plants belong to the phylum γ-Proteobacteria; Actinobacteria, Firmicutes and Bacteroidetes were also found inside plant seeds in a lesser extent. *Bacillus* and *Pseudomonas* were the most frequently genera found in plant seeds, *Paenibacillus, Micrococcus, Staphylococcus, Pantoea* and *Acinetobacter* were also reported (Truyens et al., 2015 and references therein). In a previous work, eight isolates assigned to *Pseudomonas, Azospirillum*, and *Bacillus* genera were retrieved from barley seeds under selective pressure for nitrogen-fixing microorganisms (Zawoznik et al., 2014).

Differences in bacterial genera found in the seeds of different plant species could be related to the type of plant exudates that attract specific microorganisms; also to the ability of microorganisms to colonise plant tissues, to survive inside the plant and to be transmitted to the seeds (Truyens et al., 2015 and references therein).

In this work, the only gammaproteobacterium isolated, presumably an *Enterobactereaceae* of *Pantoea* genus designated as isolate

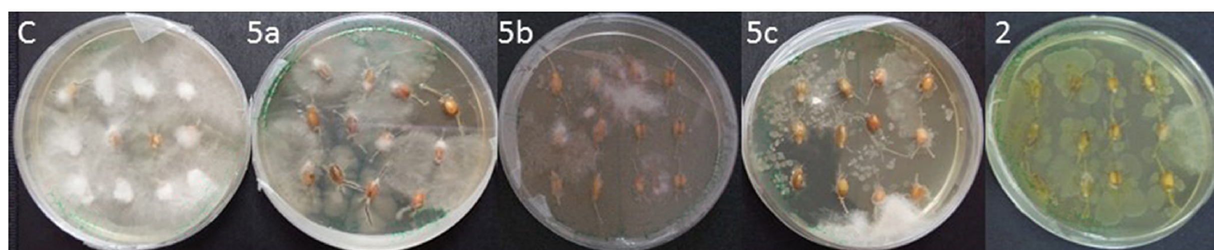

Fig. 4. Bioassays including wheat and barley kernels. Seeds of barley and wheat were pretreated with diluted cell suspensions of isolates 5a, 5b, 5c and 2, and then exposed to *F. graminearum*, as described in Materials and methods section. Left of each plate: barley kernels; right: wheat kernels.

2, was particularly active as IAA (Table 1) and siderophore producer, and also solubilised phosphate (Fig. 2A and B). Only this microorganism could grow on N-free semisolid medium.

Table 2 shows the antibiotic resistance pattern of the isolates obtained. It may be noted that isolate 1 resisted up to 100 ppm of ampicillin, but was sensitive to the other antibiotics. Isolates 5a, 5b and 5c showed moderate resistance to ampicillin (up to 50 ppm), but high resistance to kanamycin (up to 100 ppm). All the isolates were sensitive to tetracycline, while only isolate 2 resisted a low level (25 ppm) of chloramphenicol.

Isolate assigned to *Pantoea* genus (2) and three of the four isolates assigned to *Paenibacillus* genus (5a, 5b and 5c) restrained *F. graminearum* growth on dual culture, as may be observed on Fig. 3. Among these isolates, only those assigned to *Paenibacillus* seemed to have released to the culture medium antifungal substances capable to restrain *F. graminearum* growth by themselves (Fig. 3). No effect attributable to volatile substances could be proved (data not shown).

We considered of interest to validate our *in vitro* results regarding biocontrol potential of *Paenibacillus* and *Pantoea* isolates in a setting in which the kernel, as a mainstay of any beneficial-pathogenic interaction during plant life cycle, could be included. Only a few papers investigated bacterial isolates as biocontrol agents on kernels (Shi et al., 2014; Abd El Daim et al., 2015). To address this issue, we designed a simple bioassay suitable to expand our analysis by including at the same experimental units another graminaceous species usually attacked by *F. graminearum*: barley.

As depicted in Fig. 4, the growth of this phytopathogen on the kernels was markedly restrained when wheat and barley seeds were previously immersed in diluted cell suspensions of isolates 5b, 5c and 2, while isolate 5a had a minimum effect on fungal advance.

Isolates 5c and 5b displayed an outstanding ability to form biofilm on an inert surface, of about 7-fold as compared to the well-recognized PGPR *Azospirillum brasilense* strain Az39, which was used here as reference strain (Fig. 5A and B). The production of biofilm is generally regarded as a mechanism of plant protection against pathogenic bacteria, since once established on plant roots, these microbial biofilms may act as physical and chemical barriers. Likewise, these biological matrexes can also limit the release of root exudates avoiding pathogen growth (Haggag, 2010). Haggag and Timmusk (2008) also highlighted the importance of biofilm formation by *Paenibacillus polymyxa* strains in controlling crown root rot disease, associated to *Aspergillus niger*.

It has been reported that members of *Bacillus* and *Paenibacillus* have yielded several potent antimicrobial lipopeptides including iturins, surfactins, fengycins, fusaricidins, polymyxins and others (Cochrane and Vederas, 2014). A *Paenibacillus ehimensis* strain produced extracellular organic compounds that inhibited *F. oxysporum* f. sp. lycopersici conidial germination in *in vitro* assays (Naing et al., 2015), and *P. polymyxa* BRF-1 showed *in vitro* antifungal activity on the pathogenic fungus *Phialophora gregata*, the causative agent of the brown stem rot in soybean (Zhou et al., 2008). Very recently, Nguyen et al. (2015) reported for the first time the antagonistic activity of butyl 2,3-dihydroxybenzoate (B2,3DB) isolated from *Paenibacillus elgii* HOA73 against *Fusarium oxysporum* f. sp. lycopersici.

Regarding biocontrol abilities among *Paenibacillus* recovered as endophytes from wheat seeds, our results placed isolate 1 apart from isolates 5. Genotypic relationships in line with these findings were already established through a phylogenetic analysis (Grossi et al., 2015).

Pantoea genus includes several species that are generally associated with plants, either as epiphytes or as pathogens, and some species can cause disease in humans (Deletoile et al., 2009). At present 23 species are included in the genus with valid names (http://www.bacterio.net/-allnamesmr.html). Strains belonging to the most recently proposed species have been isolated from eucalyptus leaves showing symptoms of bacterial blight and die-back in Uganda, Uruguay and Argentina, and from maize suffering from brown stalk rot in South Africa (Brady et al., 2014). Kang et al. (2007)

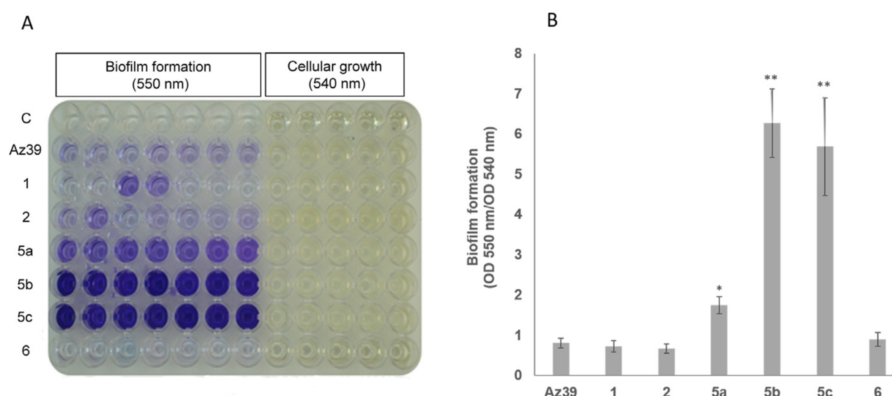

Fig. 5. Biofilm formation assay by crystal violet staining (A). Absorbances at 550 nm were normalised by total bacterial growth estimated by OD at 540 nm (B). Bars represent standard error of the mean (SEM). Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$). Three independent experiments were carried out.

demonstrated that a *Pantoea* strain colonised pepper stems in high numbers; this bacterial endophyte lacked of plant pathogenicity and stimulated specially root growth. On another hand, it also elicited induced systemic resistance against the leaf spot disease causative agent, *Xanthomonas axonopodis* pv. vesicatoria. *Pantoea vagans* strain A-9 (formerly *Pantoea agglomerans*) is commercially registered for biological control of fire blight, another bacterial disease of pear and apple trees caused by *Erwinia amylovora*. Biological control of fungal phytopathogens was also documented for several *Pantoea* isolates: a *P. agglomerans* strain isolated from a rhizospheric Mexican soil showed antifungal activity against the corn phytopathogenic fungi *Stenocarpella maydis* (Petatán-Sagahón et al., 2011), while a phosphate-solubilizing *Pantoea* strain isolated from Indian soils was reported to possess multiple plant growth attributes (IAA production, siderophore production, HCN production) and to antagonise with the phytopathogenic fungi *Penicillium chrysogenum*, *Aspergillus niger* and *Geotrichum candidum in vitro* (Dastager et al., 2009). Endophytic *Pantoea* isolated from different cotton cultivars were reported to show antagonistic potential against some strains of *Verticillium dahliae* and *F. oxysporum* f.sp. *vasinfectum* (Li et al., 2010). Of note is that several *Fusarium* genes related to fungal defense and/or virulence and cell division (among others) were induced or repressed by *P. agglomerans* (Pandolfi et al., 2010).

Isolate 2, which showed high identity to *P. agglomerans* at NCBI database, displayed several plant growth promotion features *in vitro* (Table 1). Several reports documented growth promotion caused by *P. agglomerans* inoculation in tomato, cucumber, maize, chickpea and rice (Dursun et al., 2010; Taurian et al., 2010; Mishra et al., 2011; Fei et al., 2011). Both *Paenibacillus* and *Pantoea* are known to produce auxins and auxins-like compounds, also cytokinins and gibberellins. In conjunction with nitrogen fixation and phosphate solubilisation, these features were related to plant growth promotion (Chauhan et al., 2015).

Isolate 2 restrained *F. graminearum* growth both in dual culture (Fig. 3) and in a tripartite bioassay (Fig. 4). This isolate also increased root growth and mitigated some adverse effects of saline stress in barley seedlings in a previous pot experiment in which barley plants were grown for 10 days (unpublished data). Now we conducted a new pot experiment of greater duration (20 days) and included the combination of two isolates: 5c and 2. Under our experimental conditions, inoculated treatments did not show significant changes in plant biomass; however, an increased chlorophyll content – normally considered an index of improved plant nitrogen status –, was found under coinoculation with isolate 5c and isolate 2 (Fig. 6A). In an *in vitro* compatibility assay, these isolates showed no any adverse interaction between them (Fig. 6B).

Several bacteria are emerging as novel PGPR, with a wide range of positive effects on plant production, including the biocontrol of different fungal and bacterial plant diseases, and *Paenibacillus* and *Pantoea* members – found in this study as wheat seed endophytes – repeatedly appear among these novel PGPR in current literature (Chauhan et al., 2015 and references therein). These microorganisms stimulated plant growth and increased plant yields by acting alone or in combination with other well-known rhizospheric microbes; however as far as we know, there are no reports in which the combination of these two microorganisms had been tested.

4. Conclusions

Our results show a significant ecological potential of some components of the wheat seed endophytic community. Seed endophytic microorganisms probably play important roles during plant development; in fact, they often possess attractive characteristics that could turn into biotechnological applications, including biological control. New inoculants based on spermospheric communities may not only improve cereal yields, they may also contribute to reduce the incidence of fusariosis, and combinations of strains are usually tested. However, more research is needed to shed light on the determinants which define seed colonisation. For instance, it is necessary to understand if seed endophytes are actively selected by their host plants to their own benefits or if seeds are just a biological vehicle to survive and eventually colonise new habitats. Our understanding of microbe interactions at seed stage and their biological relevance may just be starting.

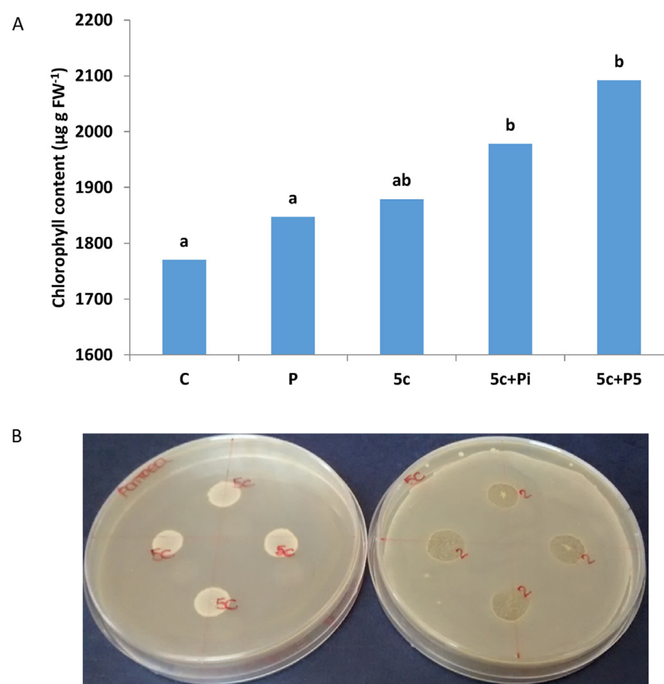

Fig. 6. Inoculation assays. (A) Chlorophyll content of barley seedlings grown in a growth chamber as described in Materials and methods section during 20 days. Treatments: 5c: isolate 5c inoculated at sowing, P: isolate 2 inoculated at sowing, 5c + Pi: isolate 5c + 2, both inoculated at sowing, 5c + P5: isolate 5c + 2; the second one inoculated 5 days after sowing, C: uninoculated. Different letters indicate significant differences. (B) Illustrative images showing the results of *in vitro* compatibility assay. Left: Isolate 2 inoculated at the surface of the plate and isolate 5c drop-inoculated; right: vice versa.

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