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Bacillus velezensis RC 218 as a biocontrol agent to reduce *Fusarium* head blight and deoxynivalenol accumulation: Genome sequencing and secondary metabolite cluster profiles

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ABSTRACT

Bacillus subtilis RC 218 was originally isolated from wheat anthers as a potential antagonist of *Fusarium graminearum*, the causal agent of *Fusarium* head blight (FHB). It was demonstrated to have antagonist activity against the plant pathogen under *in vitro* and greenhouse assays. The current study extends characterizing *B. subtilis* RC 218 with a field study and genome sequencing. The field study demonstrated that *B. subtilis* RC 218 could reduce disease severity and the associated mycotoxin (deoxynivalenol) accumulation, under field conditions. The genome sequencing allowed us to accurately determine the taxonomy of the strain using a phylogenomic approach, which places it in the *Bacillus velezensis* clade. In addition, the draft genome allowed us to use bioinformatics to mine the genome for potential metabolites. The genome mining allowed us to identify 9 active secondary metabolites conserved by all *B. velezensis* strains and one additional secondary metabolite, the lantibiotic ericin, which is unique to this strain. This study represents the first confirmed production of ericin by a *B. velezensis* strain. The genome also allowed us to do a comparative genomics with its closest relatives and compare the secondary metabolite production of the publically available *B. velezensis* genomes. The results showed that the diversity in secondary metabolites of strains in the *B. velezensis* clade is driven by strains making different antibacterials.

1. Introduction

Fusarium graminearum sensu stricto is the main causal agent of *Fusarium* head blight (FHB) in wheat in Argentina. During the last 60 years several epidemics of FHB occurred with reductions in grain yield, quality and safety due to the contamination with trichothecenes mainly deoxynivalenol (DON) (Dalcero et al., 1997; Kikot et al., 2011; Palazzini et al., 2015). This toxin, besides representing a constraint for human and animal health (Pestka, 2010), it facilitates disease development by acting as a virulence factor allowing the disease to spread during the infection process (Proctor et al., 1995; Talas et al., 2012).

Due to the toxicity of trichothecenes, the European Union has established a maximum tolerance level of 0.75 ppm (mg/kg) for DON in food commodities for human consumption (EC Regulation, 2006) and the FDA in United States has set an advisory level of DON at 1 ppm (mg/kg) in wheat products intended for human consumption (US-FDA, 2010). Strict management of FHB is required to meet these tolerance levels.

Different strategies have been used to reduce the impact of FHB including fungicides treatment, planting less susceptible cultivars, but none of them are enough to control the problem (McMullen et al., 2012; Mesterházy et al., 2011; Miedaner and Korzun, 2012). Management of the disease using biocontrol is an alternative to reduce FHB and the accumulation of DON in the frame of an integrated pest management (IPM) (Da Luz et al., 2003; Khan and Doohan, 2009; Khan et al., 2001, 2004; Schisler et al., 2002, 2004, 2006; Zhao et al., 2014). The demand of biopesticides has been increasing steadily worldwide due to increased environmental awareness and the pollution potential and health hazards from many of the chemical pesticides. Biopesticides can supplement the conventional chemical pesticides when used in IPM (Thakore, 2006).

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Members of the *Bacillus* genus are considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory of pathogenic fungi and bacteria. Among these antimicrobial compounds, cyclic lipopeptides (LPs) including surfactin, iturin and fengycin families are relevant in strains isolated as biocontrol agents (Arguelles-Arias et al., 2009; Chen et al., 2007; Dunlap et al., 2013). In addition, it has been shown that LPs induce disease resistance in plants (Jourdan et al., 2009; Kloepper et al., 2004; Yamamoto et al., 2015) and confer an advantage to the producing *Bacillus* strains in specific ecological niches (Mukherjee and Das, 2005). *Bacillus* lipopeptides are non-ribosomally synthesized via large multi-enzymes called non-ribosomal peptide synthetases (NRPSs). In addition, these *Bacillus* biocontrol strains are known to make several polyketides, such as bacillaene, difficidin and macrolactin (Chen et al., 2009). Polyketides are a large family of secondary metabolites that include many bioactive compounds with antibacterial, immunosuppressive and antitumor bioactivities. They are synthesized by type I polyketide synthases (PKSs) modularly organized assembly lines starting from acyl-CoA precursors by decarboxylative Claisen condensations. The biosynthetic pathway follows the same logic as in non-ribosomally synthesized peptides and requires at least three modular domains.

These biocontrol properties of *Bacillus* spp. have led to the isolation and characterization of many strains in this area. Unfortunately, the large and concurrent research effort has also led to confusion and inconsistencies in the taxonomic naming of these strains. However, the availability of genome sequence data and the generation of new genome sequence data of type strains has led to a definitive understanding of the taxonomy of these strains. Recent phylogenomic studies have shown that *Bacillus velezensis*, *Bacillus methylotrophicus, Bacillus amyloliquefaciens* subsp. *plantarum* and *Bacillus oryzicola* are conspecific (Dunlap et al., 2016). Under the rules of the prokaryotic naming commission, *B. velezensis* is the valid name for this species, since its description predates the other.

In previous studies we demonstrated the ability of *Bacillus* sp. RC 218 to reduce disease severity and DON accumulation *in vitro*, under greenhouse and field trials (Palazzini et al., 2007, 2009, 2013, 2016; Palazzini, 2010). The aims of this study were: (i) to evaluate under field trial the selected potential of biocontrol agent *Bacillus* sp. RC 218 to reduce FHB and deoxynivalenol accumulation, (ii) to sequence the genome of the selected agent *Bacillus* sp RC 218, (iii) to make the phylogenomic analysis of the strain, (iv) to determine the potential for secondary metabolite production.

2. Material and methods

2.1. Biocontrol strains, biomass production and formulation

Bacillus subtilis RC 218 used in this study was originally isolated from wheat anthers as potential biocontrol agents against *F. graminearum* in Argentina (Palazzini et al., 2007, 2009). Biomass of *B. subtilis* RC 218 was produced in liquid basic medium (sucrose 10 g/l, yeast extract 5 g/l) modified with NaCl (a_W 0.97) (Costa et al., 2001) with an incubation of 48 h at 28 °C in a rotatory shaker (150 rpm). Biomass was produced in a 50 l fermentor by Bio-ferm GmbH (Tulln, Austria) in order to obtain bacterial spores. These bacterial spores were freeze dried and tested in the field trial.

2.2. Pathogen inoculum production

Two strains of *F. graminearum*, RC276 and KRC7, were used in the field trials. These strains were isolated from head blight infected ears from commercial fields located in Pergamino, Buenos Aires, Argentina. Toxigenic profiles were determined in a previous study (Palazzini et al., 2007). *F. graminearum* conidia were produced in Mung bean broth (Rosewich Gale et al., 2002). After 7–10 days of incubation at 25 °C and 200 rpm on a rotatory shaker, cultures were centrifuged (7000 rpm; 5 min), re-suspended in sterile distilled water plus Tween 80 (0.05%) and filtered through sterile gauze to obtain a conidia suspension. Conidia concentration was determined using a haemocytometer and was adjusted to 5×10^5 conidia/ml (1:1 mixture of RC276 and KRC7 strains).

2.3. Field trial conditions

The field trial was conducted in Marcos Juarez, Córdoba province, Argentina, during the 2014 harvest seasons. The wheat cultivar BioInta 1005 (susceptible *to F. graminearum*) was sown at the end of July. The experimental plots consisted of 3 rows (2 m/row, 0.2 m between rows; 250 heads per plot) with three replicates per treatment. The experiments were done in a random block design with 1 m separation between plots.

The application of the biocontrol agents was done at the anthesis stage with the inoculation of *B. subtilis* RC 218 and *F. graminearum* strain mixture. The anthesis stage was considered at the period where 50% of the heads in the plots were at flowering stage (Feekes stage 10.5.2–10.5.3; Wiese, 1987). Before applications of the BCA, wheat heads were always misted with water for 2 min in order to increase the humidity in the heads. The formulated biocontrol agent were resuspended in sterile distilled water + Tween 80 (0.05%) and allowed to stabilize for 30 min before application. The viability of the biocontrol agents was evaluated by plate counting. Control negative plots were treated with sterile distilled water + Tween 80 (0.05%).

Bacterial and *F. graminearum* suspensions were applied using a commercial sprayer consisting of 5 linear sprinklers and a CO_2 pressure source. The sprayer was adjusted to 30 mbar and flow to 15 ml per second. Application was done at a rate of 15 ml per linear meter for all treatments. Wheat plots were misted with water for 5 min every 30 min from 8:00 am to 18:00 pm for six days after inoculation. Water sprinklers (fine misting) were located between the plots and also surrounding them.

2.4. FHB disease evaluation

FHB disease incidence and severity were evaluated 21 days after inoculation with *F. graminearum*. FHB incidence was determined by counting infected heads and divided from the total spikes of the plot (treatment replicate); disease severity was evaluated by observing symptomatic spikelets (decoloured, browny) and visually compared with a 0–100% scale proposed by Stack and McMullen (1995) (Supplemental Fig. S1).

2.5. Deoxynivalenol accumulation in wheat heads

At harvest, wheat heads were collected to determine DON concentration in the entire heads. Toxin extraction was done using Mycosep 225 (Romer Labs) according to the manufacturer conditions. Briefly, 25 g of grains were milled and extracted with 100 ml acetonitrile:water (84:16 v/v) and shaken for 30 min. The suspension was then filtered through Whatman N°1 and 5 ml were transferred to the column and cleaned by push-through. After cleaning the filtered, 2 ml were taken and evaporated to dryness by N_2. The sample was then redissolved in 400 μl of mobile phase (methanol:water, 12:88) for HPLC quantification. Quantification was relative to external standards of DON (Biopure, Romer; 0.5, 1, 2 and 4 μg/ml). DON concentration was determined by liquid chromatography using the methodology described by Palazzini et al. (2007).