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# Reprint of "An integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions"☆

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## ABSTRACT

Fusarium head blight (FHB) caused by species within the *Fusarium graminearum* complex is a devastating disease that causes extensive yield and quality losses to wheat in humid and semi-humid regions of the world. The disease can cause not only yield losses but also grain contamination with mycotoxins such as deoxynivalenol (DON). Several epidemics have occurred in Argentina, the last in 2012, with yield losses up to 70%. Control of the disease is partially achieved by chemical treatments, crop rotation, breeding for resistance and tillage practices. Biocontrol can be used as part of an integrated pest management. The objetives of this study were: 1- to evaluate the biological control effect of *Streptomyces* sp. RC 87B on FHB disease incidence, severity and DON accumulation and 2- to evaluate if *Streptomyces* sp. RC 87B reduces *F. graminearum* inoculum when applied to wheat stubble. *Streptomyces* sp. RC 87B was applied at wheat anthesis to determine effects on FHB disease parameters and over wheat stubble left after harvest to evaluate effects on the survival of *F. graminearum*. FHB incidence and severity were visually estimated and DON content was assessed by HPLC after harvest. On stubble, *F. graminearum* prevalence was monitored by using TaqMan PCR for 180 days. A significant disease severity and DON reduction were observed with the biocontrol treatments during the field trials (29–39% and 69–85%, respectively), but no reduction on disease incidence was achieved. On wheat stubble, *Streptomyces* sp. 87B reduced *F. graminearum* inoculum after 45 days (82%) and after 90 days no inoculum was detected in the first of two trials. In a second trial, a significant reduction (46%) in *F. graminearum* inoculum was observed after 90 days. The biocontrol strain *Streptomyces* sp. 87B showed high potential for contributing to the reduction of FHB on wheat at different times in the *F. graminearum* life cycle.

## 1. Introduction

Wheat (*Triticum aestivum* L.) is a critical food and feed crop around the world, and is grown on more land area worldwide than any other crop, but is not exempt from fungal diseases. Fusarium head blight (FHB), caused by species within the *F. graminearum* complex, is a devastating disease that produces extensive yield and quality losses to wheat in humid and semi-humid regions of the world. FHB is particularly destructive not only due to yield reduction but also because the occurrence of trichothecenes, among them deoxynivalenol (DON). Consumption of DON contaminated wheat based foods leads to reduced immunity, anemia, headache, and nausea in humans and food refusal, vomiting, growth retardation and reproductive disorders in animals (Karlovsky, 2011).

During the last 60 years, several epidemics of FHB of varying degrees of severity have occurred in Argentina and *F. graminearum* "*sensu stricto*" was isolated as the main pathogen associated with the disease. During 2012, a severe FHB outbreak occurred in the main wheat growing area, with estimated losses up to 70%. The extent of the damage was magnified by a considerable loss in grain trading value resulting from low grain weight, the presence of scabby grains, and DON contamination (Palazzini et al., 2015).

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Control of FHB on wheat and other small cereal crops can be done through several approaches. I was demonstrated that chemical treatments, mainly triazol-based fungicides, were effective or partially effective when applied during anthesis period, although it was observed that sometimes effectiveness was also cultivar-dependent (McMullen et al., 2012; Paul et al., 2008; Willyerd et al., 2011). Nevertheless, it has been reported that fungicide sub-lethal doses application, timing of application and spray technology may lead to diminished control of FHB and DON (Mesterházy et al., 2011; Wegulo et al., 2015). Conservative agriculture (no-, minimal, or zero tillage) is earning hectares year by year, contributing to sustainability in cropping systems (Kassam et al., 2009; Vogelgsang et al., 2011). No tillage farming left residues on the soil after harvest, *Fusarium graminearum*, as a saprophytic species, can survive on residues of crops such as maize, soybean and wheat (Palazzini et al., 2013). Such colonized residues, within and outside wheat fields, together with wind-transported spores, have been found the main sources of spores causing head blight of wheat (Dill-Macky and Jones, 2000; Edwards, 2004; Inch et al., 2005; Schmale et al., 2006; Vogelgsang et al., 2011). It was observed that FHB intensity was higher when wheat was planted after corn than when wheat followed soybean (Dill-Macky and Jones, 2000) or when no-till or minimum tillage was used in comparison with conventional tillage (Schaafsma et al., 2005). Pereyra et al. (2004) observed that wheat residues left on the soil surface provided a substrate for *F. graminearum* and that the ascospores produced on these residues after 23 months were able to induce disease. Antagonistic microorganisms applied to crop stubbles may reduce survival and multiplication of the pathogens present on the residues and prevent or delay disease epidemics (Luongo et al., 2005; Palazzini et al., 2013; A. G. Xue et al., 2014a).

Anthesis is the stage of greatest susceptibility for *F. graminearum* infection, and anthers are the common pathogen entry route into the plant (McMullen et al., 2012). Thus, antagonists with high ecological competence in this niche may prevent infection during anthesis when conditions for the pathogen and antagonists, temperature and humidity, are adequate (Khan et al., 2004). Among biocontrol agents (BCAs) used to control FHB, bacteria seem the more successful ones, being *Bacillus* spp. (Palazzini et al., 2016b; Schisler et al., 2006; Zhao et al., 2014), *Pseudomonas* spp. (Khan and Doohan, 2009; Schisler et al., 2006) and *Lysobacter enzymogenes* strain C3 (Jochum et al., 2006) the most representatives. Also yeasts belonging to the genera *Cryptococcus* have shown antagonistic activity (Schisler et al., 2014; Allen G. Xue et al., 2014a).

Screening for biocontrol antagonists to reduce both FHB and DON accumulation has been widely and successfully evaluated. The reduction of *Fusarium* species inoculum in crops debris through microbial interactions was also documented and reviewed (Gilbert and Haber, 2013). Nevertheless, the utilization of the same strain to control *Fusarium graminearum* both on crop residues and on spikes is limited. A similar study was done by using *Clonostachys rosea* ACM941 (Xue et al., 2014a, 2014b). These studies showed a biocontrol activity against FHB and DON accumulation in wheat and a reduction of perithecial production on corn, soybean and wheat residues. Regarding bacteria, a *Pseudomonas fluorescens* LY1-8 was effective in reducing both FHB and *Fusarium* crown root at greenhouse and field level (Wang et al., 2015). Actinobacteria have been used as biocontrol against *Fusarium* wilt in interaction with fungicides on pigeon pea (Singh and Chhatpar, 2011) or in watermelon (Faheem et al., 2015); and also against *Fusarium graminearum* under *in vitro* conditions (Baharlouei et al., 2011) or under field trials in wheat (Jung et al., 2013).

In a previous study, we reported the efficacy of *Streptomyces* sp. RC 87B as a biocontrol agent against FHB under greenhouse conditions (Palazzini et al., 2007). In the present study the biocontrol activity of *Streptomyces* sp. RC 87B was evaluated on: 1) the reduction of FHB and DON accumulation on wheat under field trials and, 2) the efficacy of the strain in controlling *F. graminearum* inoculum levels on wheat stubble left after harvest during a two year trial.

## 2. Materials and methods

### 2.1. Biocontrol strain and culture conditions

The strain evaluated was *Streptomyces* sp. RC 87B, originally isolated from wheat anthers, showed effective activity against *F. graminearum* under *in vitro* and greenhouse conditions, reducing both FHB severity and DON accumulation (Palazzini et al., 2007). Biomass of *Streptomyces* sp. RC 87B was produced in 250 ml Erlenmeyer flasks in 100 ml of liquid basic medium (sucrose 10 g/l, yeast extract 5 g/l) (Costa et al., 2001), incubated at 28 °C for 48 h in a rotatory shaker (150 rpm). Biomass was obtained by centrifugation (10,000 rpm, 10 min) and resuspended in sterile distilled water plus Tween 80 (0.05%). The viability of the biocontrol agent was evaluated by plate counting on agarized basic medium (Costa et al., 2001) and adjusted to $1 \times 10^7$ colony forming units per ml (cfu/ml).

### 2.2. Field trials and F. graminearum inoculum production

Field trials were carried out in Marcos Juarez, Córdoba province, Argentina, during the 2013/14 and 2014/15 harvest seasons. The bread wheat (*Triticum aestivum* L.) cultivar BioInta 1005 (susceptible to *F. graminearum*) was sown at the end of June during both trials. Biocontrol assays at wheat anthesis stage started at the wheat flowering period (end of September) till harvest (mid December); period in which the biocontrol at wheat stubble assays started (mid December to mid June) (Fig. 1).

A mixture of *Fusarium graminearum* strains RC276 and KRC7 was used as inoculum at anthesis stage. Toxigenic profiles of the strains were determined in previous studies (Palazzini et al., 2016a, 2007). *Fusarium graminearum* conidia were produced in Mung bean broth (Gale et al., 2002) and after 7 days of incubation at 25 °C and 200 rpm on a rotatory shaker, cultures were centrifuged (7000 rpm; 5 min), resuspended in sterile distilled water plus Tween 80 (0.05%) and filtered through sterile gauze to obtain a conidia suspension. Conidia concentration was adjusted to $5 \times 10^5$ conidia/ml (1:1 strain mixture) by using a haemocytometer.

### 2.3. Biocontrol of Fusarium head blight at anthesis stage

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 uninoculated plots serving as buffer plots.

Treatments were applied at the anthesis stage period, when 50% of

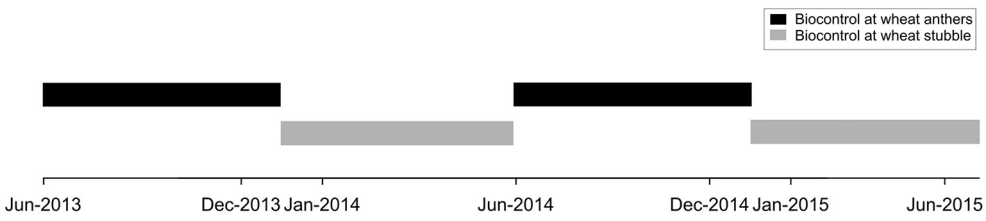


**Fig. 1.** Schematic diagram showing development of field trials.

the heads in the plots were at flowering stage (Feekes stage 10.5.2–10.5.3) (Wiese, 1987). Before treatment applications, wheat heads were always misted with water for 2 min in order to increase the humidity in the heads. *Streptomyces* sp. RC 87B (first) and *F. graminearum* strain's mixture (immediately after) were applied using a commercial sprayer at a rate of 15 ml per linear meter. Treatments were done by triplicate. Positive control plots were inoculated only with *F. graminearum* strain's mixture and negative control plots were inoculated with sterile distilled water plus Tween 80 (0.05%). Humidity in the field was achieved by using water sprinklers (fine misting), and where turned on for 5 min every 30 min from 8 am to 6 pm for six days after inoculations.

FHB disease incidence and severity were evaluated 21 days after inoculations. 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 (Stack and McMullen, 1995).

At harvest, wheat heads were collected to determine DON concentration in grains. Toxin extraction was done by mixing 15 g of milled grains and acetonitrile:water (84:16, 100 ml), shaken for 30 min, filtered by Whatman No. 1 and 5 ml of the filtrated was passed through a clean up column (Mycosep 225,Romer). Then, 2 ml of the filtrated was evaporated to dryness ($N_2$, 50 °C). DON concentration was determined by liquid chromatography as previously described (Palazzini et al., 2007).

### 2.4. Biocontrol of F. graminearum sensu stricto inoculum on wheat stubble

In order to ensure high inoculum pressure availability in the field, the stubble assays were done in the same area where the biocontrol on wheat anthers assays took place. No extra *F. graminearum* inoculum was sprayed over the stalks. Remained wheat stalks (20–25 cm long) attached to the soil after harvest were used as the experimental plots. Each plot consisted of 5 rows (1 m/row, 0.2 m between rows; 400 stalks per plot). *Streptomyces* sp. RC 87B ($1 \times 10^7$ cfu/ml) was applied by using a commercial sprayer at a rate of 15 ml per linear meter (75 ml/plot), plots inoculated with sterile distilled water plus Tween 80 (0.05%) served as positive control plots. Treatments were done by quintuplicate. The experiments were done in a random block design with uninoculated plots serving as buffer plots.

A total of 20 wheat stalks per plot (100 per treatment) were sampled at the beginning of the experiments and after 45, 90 and 180 days. Stalks were dried at 105 °C (to determine the dry weight) and pulverized in a mill with a 1 $mm^2$ mesh (Cyclotech, Foss Tecator). DNA was extracted from 10 mg of pulverized stalks by using the DNeasy 96 plant kit (Qiagen) according to the manufacturer's instruction. The *F. graminearum* inoculum on wheat stalks was followed by using TaqMan real-time PCR (qPCR).

TaqMan qPCR quantifications were done on an ABI Prism 7500 Sequence Detection System (Applied Biosystems). TaqMan reactions were performed in 25 µl, using 12.5 µl of TaqMan universal PCR master mix (Applied Biosystems) and 100 nM of FAM-labeled target and internal control probe and 400 nM of forward and reverse primer for both the target *F. graminearum* (Waalwijk et al., 2004, 2008) as well as the internal positive control (Klerks et al., 2004). TaqMan reactions were performed on 2 µl of DNA preparations from samples. Thermal cycling conditions consisted on a single cycle of 2 min to 50 °C to degrade uracil containing DNA and 10 min to 95 °C to inactivate uracil-N-glicosidase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curve was generated by using 10-fold serial dilutions of pure DNA in the range of 0.1 pg/µl to $1 \times 10^4$ pg/µl. Quantifications were always done by duplicate.

### 2.5. Statistical analysis

FHB incidence data from individual years were subjected to Kruskal-Wallis ANOVA on ranks. Severity data were subjected Kruskal-Wallis ANOVA on ranks while means were separated by Dunn's test. Deoxynivalenol content was subjected to a one-way ANOVA. Means were separated by Holm-Sidak method ($P < 0.001$) and expressed as the plot means ± one standard deviation.

Wheat stubble experiments data were subjected to analyses of variance (ANOVA). Mean DNA concentration of *F. graminearum* for the different treatments and sampling dates were separated with Holm-Sidak method ($P \leq 0.001$). All statistical analyses were done using SigmaStat for Windows Version 3.5 (SPSS Inc.).

## 3. Results

### 3.1. Biocontrol of F. graminearum sensu stricto at anthesis stage

The antagonist *Streptomyces* sp. RC 87B was applied on wheat spikes during anthesis and heads were evaluated for FHB symptoms during two consecutive harvest seasons (2014/15 and 2015/16). Disease severity was diminished by 29.8% under the *Streptomyces* sp. 87B treatments during the 2014 field trial, meanwhile a better disease reduction was observed during the second year of evaluation (39.5%). DON accumulation on grains was also statistically reduced under the *Streptomyces* sp. 87B treatments by 69–85% (Table 1). *Streptomyces* sp. RC 87B treatment was not able to significantly reduce FHB incidence, but during the 2015 field trial it was arithmetically reduced from 26% for the control to 20.6% for the biocontrol treatment.

### 3.2. Biocontrol of F. graminearum sensu stricto on wheat stubble

At the beginning of the 2013/14 trial, similar amounts of *F. graminearum* DNA were observed between the *Streptomyces* sp. 87B treated plots and the control plots (263.69 pg and 273.71 pg DNA/mg, respectively, Fig. 2). After 45 days, a significant reduction on *F. graminearum* DNA was observed in the *Streptomyces* sp. 87B treated plots (reduction of 82%, $P \leq 0.001$) and after 90 days, *F. graminearum* inoculum was reduced to undetectable levels, meanwhile in the control plot the pathogen DNA remained in low amounts (9.85 pg DNA/mg). At 180 days, no pathogen DNA was detected either in the control or treated plots. During the 2014/15 trial (conducted after the second year of wheat anthesis trial), lower amounts of *F. graminearum* inoculum was observed (169.7 pg DNA/mg in the control plots) when compared with the previous trial. At the beginning and 45 days, no significant differences were observed when comparing treated and control plots ($P = 0.047$). After 90 days, a significant reduction of *F. graminearum* DNA was observed in the biocontrol plots (46% reduction). A remaining

**Table 1**
Biocontrol activity of *Streptomyces* sp. RC 87B.

| Treatments | Fusarium head blight | | | | | |
|---|---|---|---|---|---|---|
| | Incidence (%)[a] | | Severity (%)[b] | | DON (ppm)[c] | |
| | 2014 | 2015 | 2014 | 2015 | 2014 | 2015 |
| *F. graminearum* mixture + *Streptomyces* sp. RC 87B | 19.05a | 20.6a | 26.1a | 33.7a | 0.23a | 0.54a |
| *F. graminearum* mixture | 21.63a | 26a | 37.2b | 55.7b | 1.59b | 1.78b |

[a] Incidence data were subjected Kruskal-Wallis ANOVA on ranks, no statistical differences were observed ($P = 0.821$).
[b] Severity data were subjected Kruskal-Wallis ANOVA on ranks, means were separated by Dunn's test. On each year, different letters indicate significant differences ($P < 0.05$).
[c] Deoxynivalenol content was subjected to One way ANOVA, means were separated by Holm-Sidak method ($P < 0.001$).

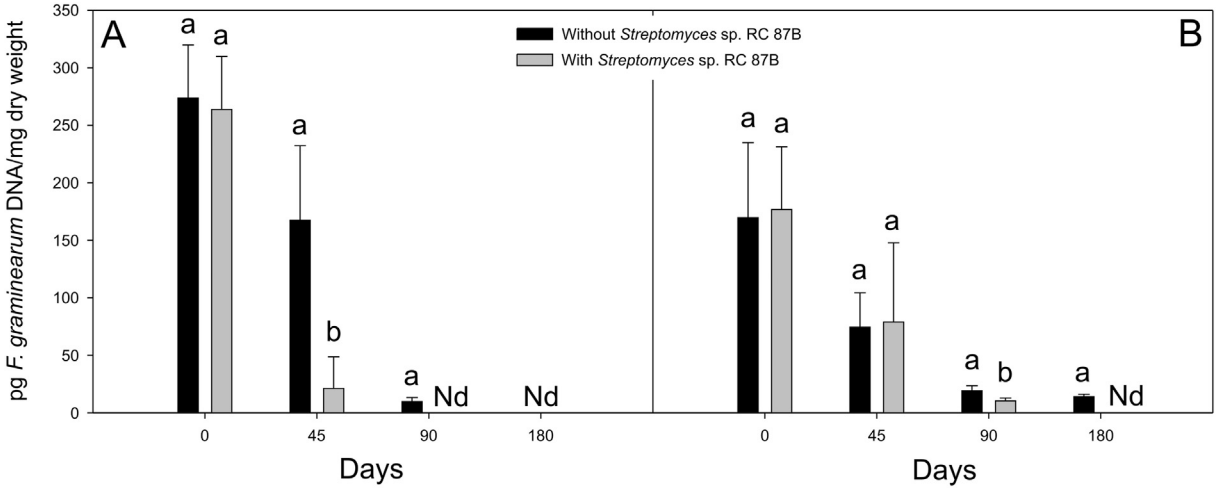


**Fig. 2.** Effect of treatment with *Streptomyces* sp. 87B on *F. graminearum* DNA on wheat stalks under two field trials (A: 2014 and B: 2015).
For each sampling date, different letters indicate significant differences according to Holm–Sidak test ($P < 0.001$). Error bars represent standard deviations. Nd: not detected (no amplification, detection limit 0.1 pg DNA/mg dry weight).

pathogen DNA (mean of 14.1 pg) was observed after 180 days in the control plots, meanwhile in the *Streptomyces* sp. 87B treated plots no pathogen DNA was quantified (detection limit 0.1 pg DNA/mg).

## 4. Discussion

Since individual control measures have not succeeded in consistently reducing FHB, an integrated management seems the best option to reduce the impact of this disease. Conservative agriculture, despite conserving the environment by minimal tillage, could favor inoculum growth or maintenance on crop residues left after harvest. In addition, *Fusarium* species have the ability to survive on gramineous weeds, which could also contribute to the primary inoculum (Landschoot et al., 2011; Pereyra and Dill-Macky, 2008). Crop rotation with non-hosts favors inoculum reduction, as it was observed in wheat-corn/wheat-soybean systems (Dill-Macky and Jones, 2000; Schaafsma et al., 2005). However, outside field inoculum can also contribute to FHB development since it was observed that ascospores can be wind-transported and deposited in cereal heads by precipitation or gravitational settling (Maldonado-Ramirez et al., 2005; Schmale et al., 2006). Fungicide application is another option to control FHB, being demethylation inhibitor class fungicides the most widely used, although complete control is not always achieved (McMullen et al., 2012). Biological control, as an additional strategy, can be used under an integrated FHB management.

Biocontrol of *F. graminearum* at anthesis stage was effectively achieved in several studies (Khan and Doohan, 2009; Palazzini et al., 2016a; Schisler et al., 2014; Zhao et al., 2014) and the utilization of biocontrol strains on crop debris to control *Fusarium* species was also evaluated (Luongo et al., 2005; Palazzini et al., 2013; A. G. Xue et al., 2014b). In the present study, it was demonstrated that *Streptomyces* sp. 87B is an effective biocontrol agent against *F. graminearum* reducing both FHB severity and DON accumulation on grains. Also, this biocontrol agent was able to reduce *F. graminearum* inoculum in the stubble left after harvest. In a previous study, *Streptomyces* sp. 87B reduced DON production by 70–100% on irradiated wheat grains and by 100% on entire spikes under greenhouse experiments (Palazzini et al., 2007). This is in agreement with other authors who found that lab selections have been successfully correlated with field experiments (Khan and Doohan, 2009; Palazzini et al., 2007; Schisler et al., 2014).

Effectiveness of *Streptomyces* sp. 87B in reducing FHB ranged from 30 to 40% meanwhile DON reduction on harvested grains ranged from 69 to 85%. Disease severity was higher during the 2015 field trial since relative humidity and precipitations were more favourable for the disease (data not shown). Nevertheless, biocontrol effectiveness was higher during the 2015 harvest season, which reflects *Streptomyces* sp. 87B biocontrol performance under different environmental conditions. Other studies also focused on controlling both FHB development and DON reduction. For example, He et al. (He et al., 2009) found that two *Paenibacillus polymyxa* strains were able to reduce FHB severity by 56.5% and DON production by 89.4%, but only under greenhouse conditions. Zhao and co-workers found that *Bacillus subtilis* SG6 was able to suppress both FHB incidence and severity (72.6 and 77.5%, respectively) and to reduce DON by 69.1% under field trials (Zhao et al., 2014). An *in vitro* study, showed the isolation and characterization of 13 novel bacteria, being able to degrade DON from 100 ppm to below 0.5 ppm on agarized media (Sato et al., 2012). Since food safety has become a major global concern, the European Union has established a maximum level permitted for DON of 0.75 ppm (mg/kg) in cereals intended for direct human consumption (European Commission, 2007). Management of FHB is required to meet these tolerance levels.

Additionally, BCAs may be focused on controlling more than one disease caused by the same pathogen. The control of FHB and *Fusarium* crown root caused by *F. graminearum* at greenhouse and field level was observed using *Pseudomonas fluorescens* LY1–8 (Wang et al., 2015). *Chlonostachys rosea* ACM941 reduced FHB index by 30–46%, being more effective when applied over resistant wheat cultivars and at the highest concentration ($10^8$ cfu/ml) (Xue et al., 2014a) and also reduced perithecial production on several crop debris (Xue et al., 2014b).

Biocontrol with *Clonostachys rosea* strains over several *Fusarium* species was previously demonstrated on wheat stalks (Palazzini et al., 2013). In addition, biocontrol with *Chlonostachys* species was also achieved for other crops debris such as maize (Luongo et al., 2005), wheat and soybean debris (Xue et al., 2014b) and floral crops as rose (Morandi et al., 2003, 2008). In our previous study, stubble assays were done on 10–12 cm wheat stalk pieces obtained from the harvested wheat, but in the present study, the bioassay was done on the remained wheat stalks attached to the soil after harvest, which could retain better humidity in the stem base for pathogen survival. This aspect was observed in the dynamics of *Fusarium* species, whose survival was superior in stem base rather than in nodes and internodes (Köhl et al., 2007). By using qPCR to quantify *F. graminearum* DNA, we observed an 82 and 46% reduction after 45 and 90 days in the *Streptomyces* sp. 87B treated plots during the first and second year experiments, respectively. No detection of DNA pathogen was observed after 90 and 180 days for the same years (below 1 pg DNA/mg). In 2012, in the same area of the field trials and after a severe FHB outbreak, the inoculum of *F. graminearum* was quantified on wheat stubble by up to 2344 pg DNA/mg (mean

1869 pg DNA/mg) and after 180 days, mean levels of 313 pg DNA/mg were quantified (*unpublished results*). Six months later, when starting the biocontrol at wheat stubble experiment, the pathogen level was 273.71 pg DNA/mg. During 2010 and 2011, and without a previous FHB outbreak, lower amounts of *F. graminearum* DNA was detected (4–20 pg), but it was present in the stubble for the following crop at high levels (Palazzini et al., 2013). It is therefore possible that sufficient amounts of inoculum remained viable until the next year, or wind transported spores arrived at the field, which is strongly supported by several studies (Maldonado-Ramirez et al., 2005; Palazzini et al., 2013; Pereyra et al., 2004; Schmale et al., 2006). Since the biocontrol trials on wheat stubble were done in the same area than biocontrol trials on wheat head, it is likely possible that the inoculum applied on head treatments remained viable on the ground during the wheat stubble trials. Further trials could be done in separated areas to avoid cross-inoculum interactions.

Despite the success of other methods that are used to quantify *Fusarium* species in host tissues, qPCR seems a more adequate and accurate tool to monitor *Fusarium* pathogens compared to culturing or observing perithecia production microscopically (Luongo et al., 2005; Xue et al., 2014b). Finally, since residues of previous crops such as maize, barley, sunflower or weeds in the field are an important reservoir for *Fusarium* species (Landschoot et al., 2011; Pereyra and Dill-Macky, 2008), the application of *Streptomyces* sp. 87B to other crop debris could also contribute to reducing *Fusarium* species in this reservoirs of inoculum (Xue et al., 2014b).

A mayor challenge still remains regarding the optimization and formulation technology necessary to develop a biofungicide based on *Streptomyces* sp. 87B ready to the market. As observed in previous studies, Kaolin, alginate beads, talcum powder, starch granules and skimmed milk could be good carriers. The addition of nutrients substrates such as chitin, glucans, different sugars or aminoacids to the formulation could be also important to improve shelf life (Crane et al., 2014; Sabaratnam and Traquair, 2002).

The biocontrol strain *Streptomyces* sp. 87B showed high potential for contributing to the reduction of FHB on wheat at different points along the life cycle of *F. graminearum*.

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