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ORIGINAL ARTICLE

Tolerance of triazole-based fungicides by biocontrol agents used to control *Fusarium* head blight in wheat in Argentina

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Significance and Impact of the Study: This study evaluates the possibility to use biocontrol agents (*Bacillus velezensis* RC 218, *Brevibacillus* sp. RC 263 and *Streptomyces* sp. RC 87B) in combination with triazole-based fungicides to control *Fusarium* head blight in wheat. The evaluation of biocontrol agents' growth under *in vitro* conditions was carried out in Petri dishes containing either prothioconazole, tebuconazole or metconazole. Viability studies demonstrated that *B. velezensis* RC 218 and *Streptomyces* sp. RC 87B were more tolerant to the fungicides evaluated. Results obtained reflect the possibility to use fungicides at low doses combined with biocontrol agents.

Keywords
Bacillus, biocontrol, fungicide tolerance, *Fusarium* head blight, integrated management, *Streptomyces*.

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Abstract

Fusarium head blight (FHB) caused by *Fusarium graminearum* species complex is a devastating disease that causes extensive yield and quality losses to wheat around the world. Fungicide application and breeding for resistance are among the most important tools to counteract FHB. Biological control is an additional tool that can be used as part of an integrated management of FHB. *Bacillus velezensis* RC 218, *Brevibacillus* sp. RC 263 and *Streptomyces* sp. RC 87B were selected by their potential to control FHB and deoxynivalenol production. The aim of this work was to test the tolerance of these biocontrol agents to triazole-based fungicides such as prothioconazole, tebuconazole and metconazole. Bacterial growth was evaluated in Petri dishes using the spread plating technique containing the different fungicides. *Bacillus velezensis* RC 218 and *Streptomyces* sp. RC 87B showed better tolerance to fungicides than *Brevibacillus* sp. RC 263. Complete growth inhibition was observed at concentrations of 20 μg ml^{-1} for metconazole, 40 μg ml^{-1} for tebuconazole and 80 μg ml^{-1} for prothioconazole. The results obtained indicate the possibility of using these biocontrol agents in combination with fungicides as part of an integrated management to control FHB of wheat.

Introduction

Fusarium head blight (FHB) or scab is a widespread and destructive disease of small grains crops like wheat and barley. Within the *Fusarium graminearum* species complex, the main pathogen associated with the disease is *F. graminearum sensu stricto* (McMullen *et al.* 2012). The pathogen can not only reduce grain yield and quality but also produce potent mycotoxins such as deoxynivalenol (DON), which is toxic for humans and other animals (Champeil *et al.* 2004). Reduction in the impact of FHB has been a major challenge to grain growers all over the world. Chemical control, cultural practices and the use of less susceptible cultivars are partially effective to control FHB, but no single strategy ensure effective control (Hollins *et al.* 2003; Palazzini *et al.* 2016a). The benefit of crop rotation as a control measure is reduced by the wide host range of *F. graminearum* (Dill-Macky and Jones 2000); meanwhile plant breeding has not been successful in developing high resistant cultivars (Steiner *et al.* 2017). Chemical control, mainly triazole-based fungicides, is a complementary control measure when weather conditions are conducive to infection. The control of this disease in the field has not always been highly effective or consistent

through years, mainly due to dose and application time, spike coverage and the increase in pathogen resistance to the fungicides applied (Mesterházy *et al.* 2011). In addition, it has been reported that some fungicides induced DON accumulation in grains under *in vitro* assays (Simpson *et al.* 2001; Ramirez *et al.* 2004). Triazoles are among the most successful in controlling FHB (Wegulo *et al.* 2015). Tebuconazole, metconazole and prothioconazole are included in this category, being the former one of the most recommended and probed against FHB (Edwards and Godley 2010; Mesterházy *et al.* 2011).

Biological control by antagonistic micro-organisms, including yeasts, fungi and bacteria, appears to be a promising tool for preventing FHB and can be used as part of an integrated management of FHB (Khan and Doohan 2009; Schisler *et al.* 2014; Palazzini *et al.* 2016a, 2017). In addition, several bacteria and fungi are being evaluated for commercial development as biopesticides (Khan *et al.* 2004; Zhang *et al.* 2005). Biological control appears to be a useful tool when integrated with fungicide applications and host resistance (Khan *et al.* 2004; Köhl *et al.* 2011). The use of lower amounts of fungicide in integrated systems, either through a combination of a lower dose applied simultaneously with a biocontrol agent, or by alternating treatments of chemicals and biocontrol agents reduces the environmental risks pressure caused by fungicides. To achieve a fully integrated control program, however, it is important to ascertain if selected natural biocontrol agents are tolerant to other fungicides, insecticides, selective herbicides and antibiotics used within the same crop.

In previous studies three bacteria, *Bacillus velezensis* RC 218, *Brevibacillus* sp. RC 263 and *Streptomyces* sp. RC 87B were selected by their potential to control *F. graminearum* growth and DON production under *in vitro* conditions (Palazzini *et al.* 2007). Also, it was demonstrated that these physiologically improved micro-organisms were able to control FHB and DON production under greenhouse trials (Palazzini *et al.* 2009). Furthermore, field evaluations revealed the true potential of *B. velezensis* RC 218 and *Streptomyces* sp. RC 87 as biocontrol agents controlling *F. graminearum* on heads at flowering and on stubble after wheat harvest (Palazzini *et al.* 2016a,b, 2017). The aim of this work was to evaluate the tolerance of *B. velezensis* RC218, *Streptomyces* sp, RC87B and *Brevibacillus* sp. RC263 to triazole-based fungicides commonly used to control FHB in wheat to be used as an integrated pest management.

Results and discussion

Great effort is been carried out to reduce the impact of FHB all over the world, being chemical, biological control, crop rotation and breeding for resistance the most promising tools to achieve it (McMullen *et al.* 2012; Wegulo *et al.* 2015; Legrand *et al.* 2017). An integrated strategy based on the combination of biocontrol agents (BCAs) with fungicides (at lower doses) appears to be one a reliable option for large-scale utilization of microbial antagonists in the control of FHB. This combination will provide the utilization of reduced concentrations of fungicides to control pathogens under a more environmental friendly condition. In addition, the widespread diffusion of fungal pathogen isolates that have become resistant to fungicides used for a long time in the field has led to the need of assessing the compatibility and efficacy of BCAs with new and recently developed fungicides. In previous studies, we have demonstrated the effectiveness of *B. velezensis* RC 218, *Brevibacillus* sp. RC 263 and *Streptomyces* sp. RC 87B on the reduction in FHB and DON accumulation at field level (Palazzini *et al.* 2016a,b, 2017). In this study, differences in the viability of the three bacteria evaluated were observed in the presence of the different fungicides. *B. velezensis* RC 218 and *Streptomyces* sp. RC 87B showed better tolerance to the fungicides than *Brevibacillus* sp. RC 263, who grew only in the lowest dose evaluated of the fungicide (0·5 μg ml^{-1}; Fig. 1a). When compared at the same dose, prothioconazole had the lowest effect on bacterial growth, followed by tebuconazole and metconazole.

Bacillus velezensis RC 218 was the most tolerant strain to the fungicides evaluated. No statistical differences were observed when grown in the lowest dose of the three fungicides; prothioconazole had the lowest effect on the bacteria viability, with no statistical differences in relation to the control at doses of 5, 10 and 20 μg ml^{-1}. Tebuconazole and metconazole at 10 μg ml^{-1} reduced 22% viability in comparison with the control (Fig. 1b). *Streptomyces* sp. RC 87B showed high growth tolerance to prothioconazole at a concentration up to 10 μg ml^{-1}; and only at the highest dose (80 μg ml^{-1}) a complete growth inhibition was observed. The fungicides tebuconazole and metconazole at 10 μg ml^{-1} reduced growth by 60–85% (Fig. 1c).

Only a few studies reveal the interactions between BCAs and fungicides; but many others compare the effectiveness of these chemicals in relation with the pathogens in *in vitro*, greenhouse and field studies. For example Lima *et al.* (2011) tested the compatibility of two yeasts with four novel developed fungicides to control blue mould on apples in *in vitro* and *in situ* experiments. The authors observed reductions up to 100% in combined treatments, where two fungicides were utilized at 25% of the label dose, and also observed reduction in patulin production by *Penicillium expansum*. Schisler *et al.* (2006) reported that Folicur 3.6 (tebuconazole as active ingredient) had the same effect in reducing FHB disease in field tests than the biocontrol strain *Pseudomonas* sp. AS 64.4, but they did

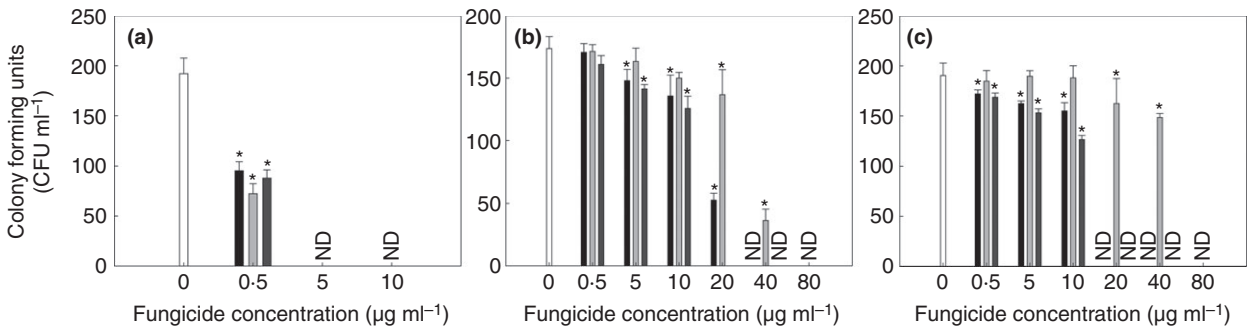

Figure 1 Viability of biocontrol strains in the presence of fungicides. (a): *Brevibacillus* sp. RC 263; (b): *Bacillus velezensis* RC 218 and (c): *Streptomyces* sp. RC 87B. Means were subjected to ANOVA and separated according to Holm–Sidak test ($P < 0{\cdot}001$). On each graph, means with an asterisk indicates significant differences compared with Control treatment. ND: No growth (■) Tebuconazole; (▒) Prothioconazole; (▓) Metconazole and (□) Control.

not evaluate a combination of both strategies. In contrast, Jochum *et al.* (2006) showed the effectiveness of combining *Lysobacter enzymogenes* C3 and the fungicide tebuconazole in several field trials, and observed inconsistency in controlling FHB alone or in combinations. The authors concluded that this inconsistency could be attributed to the application technology, and better methods to achieve a more uniform delivery of BCAs to spikelets are required to increase efficacy and obtain a more disease-suppresive product; and proposed the combination of strategies as an useful tool for FHB disease management.

Tebuconazole has been widely used for many years; meanwhile metconazole has been introduced in 1994, with similar effects than tebuconazole (Edwards and Godley 2010). Prothioconazole entered the market in 2004 and it was probed to have inhibitory effects on *F. graminearum in vitro* (Klix *et al.*, 2007) and at field level (Paul *et al.* 2008). The effect of tebuconazole and metconazole on *F. graminearum* growth and DON production has been also studied by Ramirez *et al.* (2004), who found that concentrations higher than 15 μg ml^{-1} were inhibitory for *F. graminearum* growth. Edwards and Godley (2010) observed that prothioconazole application before wheat flowering reduced total FHB severity and DON accumulation In addition, Pirgozilev *et al.* (2002) reported that metconazole (at a quarter of label dose) reduced *Tri5* DNA concentration (68–79%) and DON production (75–98%) by *F. graminearum* and *F. culmorum* under field experiments. In our study, *B. velezensis* RC 218 and *Streptomyces* sp. RC 87B showed a viability of approximately 60–85% at a concentration of 10 μg ml^{-1} for both tebuconazole and metconazole, so, a possible combination of these BCAs' with sub lethal doses of metconazole or tebuconazole (8–10 μg ml^{-1}) could be a good strategy to evaluate under greenhouse or field experiments. In the case of prothioconazole, it is noticeable that the tolerance of both *B. velezensis* RC 218 and *Streptomyces* sp. RC 87B was higher (up to 40 μg ml^{-1}) when compared with tebuconazole and metconazole. Bradley and McMullen (2008) reported prothioconazole as good as tebuconazole in the control of FHB under field experiments and a better reduction in DON. So, as proposed before, it is possible to combine prothioconazole at sublethal doses (e.g. 20–40 μg ml^{-1}) and these two BCAs' to evaluate the performance in reducing FHB parameters and DON accumulation in wheat. *Brevibacillus* sp. RC 263 showed a severe decrease in viability with the three fungicides assayed at the lowest concentration, concluding that it is not possible to use this BCA in combination with the fungicides evaluated in this study.

Understanding the behaviour of BCAs in the presence of common used fungicides to control FHB is mandatory to define the effective dose-combination to be applied at greenhouse or field experiments. Results obtained in this study indicate the possibility to use *B. velezensis* RC 218 and *Streptomyces* sp. RC 87B in combination with fungicides at lower doses as part of an integrated management of FHB in wheat. Further studies at field level are necessary to validate this hypothesis.

Materials and methods

Bacterial strains and culture media

Bacillus velezensis RC 218, *Brevibacillus* sp. RC 263 and *Streptomyces* sp. RC 87B were originally isolated from

Table 1 Active ingredients, product name and company of the fungicides utilized during the *in vitro* studies

Active ingredient	Product name	Company
Prothioconazole, 480 g l^{-1}	Pucara	Bayer
Tebuconazole, 250 g l^{-1}	Folicur	Bayer
Metconazole, 90 g l^{-1}	Caramba	Basf

wheat anthers during the 2004 harvest season (Palazzini *et al.* 2007). The bacterial strains were stored at −80°C in 10% glycerol (w v^{-1}), thawed at room temperature, streaked for purity on nutrient agar and incubated at 28°C for 24 h to obtain single colonies. The growth medium (basal medium) consisted of sucrose (10 g l^{-1}) and yeast extract (5 g l^{-1}), pH of 6·7 and had a_W of 0·995 (Costa *et al.*, 2002). A single colony was used to inoculate 100 ml of the basal liquid medium in a 250 ml Erlenmeyer flask and incubated for 12 h (overnight culture, o/n) at 28 ± 0·5°C on a rotatory shaker (150 rev min^{-1}) to obtain mid-log phase cells (*c.* 10^6 cells per ml). After the o/n culture, cell counting was performed in a haemocytometer chamber and adjusted to 2×10^3 cells per ml.

Fungicide assays

Three different fungicides were used in the bioassay (prothioconazole, tebuconazole and metconazole, Table 1). These fungicides were chosen based on the efficacy on field trials reported in previous studies (Mesterházy and Bartók 2001; Bradley and McMullen 2008; Mesterházy *et al.* 2011) and because they are the most common fungicides utilized to control FHB in Argentina (Simón *et al.* 2013).

The experiment was carried out on nutrient agar. The media were autoclaved at 121°C for 15 min and after cooling at 50°C fungicides were added to obtain the required concentrations (0·5; 5; 10; 20; 40 and 80 μg ml^{-1}). An aliquot of 0·1 ml of each bacterial strain (2×10^3 cells per ml) was inoculated in Petri dishes using the spread plating technique containing nutrient agar amended with the different fungicides at the concentrations described above. Inoculated plates were incubated at 28°C for 48 h and colony counting was done. Plates without fungicides were used for each strain and served as control. The experiment was carried out in triplicates and repeated once. Colony counting was subjected to a one-way ANOVA and means were compared by Holm–Sidak's method ($P \leq 0{\cdot}001$). The statistical analyses were performed using Sigma Stat for Windows ver. 3.5 (SPSS Inc.).

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Conflict of Interest

The authors declare no conflict of interest.

References

Bradley, C. and McMullen, M. (2008) Fungicides for FHB management: past, present and future. In *Proceeding of the 2008 National Fusarium* Head Blight Forum ed. Canty, S., Clark, A., Walton, E., Ellis, D., Mundell, J. and Van Sanford, D. December 2–4, 2008 Indianapolis, USA, p. 12. Indianapolis, IN, Lexington, KY: University of Kentucky.

Champeil, A., Doré, T. and Fourbet, J.F. (2004) *Fusarium* head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. *Plant Sci* **166**, 1389–1415.

Costa, E., Usall, J., Teixidó, N., Delgado, J. and Viñas, I. (2002) Water activity, temperature and pH effects on growth of the biocontrol agent Pantoea agglomerans CPA-2. *Can J Microbiol* **48**, 1082–1088.

Dill-Macky, R. and Jones, R.K. (2000) The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. *Plant Dis* **84**, 71–76.

Edwards, S. and Godley, N. (2010) Reduction of *Fusarium* head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole. *Food Add Cont A* **27**, 629–635.

Hollins, T.W., Ruckenbauer, P. and De Jong, H. (2003) Progress towards wheat varieties with resistance to *Fusarium* head blight. *Food Cont* **14**, 239–244.

Jochum, C.C., Osborne, L.E. and Yuen, G.Y. (2006) *Fusarium* head blight biological control with *Lysobacter enzymogenes* strain C3. *Biol Control* **39**, 336–344.

Khan, M. and Doohan, F. (2009) Bacterium-mediated control of *Fusarium* head blight disease of wheat and barley and associated mycotoxin contamination of grain. *Biol Control* **48**, 42–47.

Khan, N.I., Schisler, D.A., Boehm, M.J., Lipps, P.E. and Slininger, P.J. (2004) Field testing of antagonist of *Fusarium* head blight incited by *Gibberella zeae*. *Biol Control* **29**, 245–255.

Klix, M., Verreet, J.-A. and Beyer, M. (2007) Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop Prot* **26**, 683–690.

Köhl, J., Postma, J., Nicot, P., Ruocco, M. and Blum, B. (2011) Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biol Control* **57**, 1–12.

Legrand, F., Picot, A., Cobo-Díaz, J., Chen, W. and Le Floch, G. (2017) Challenges facing the biological control strategies for the management of *Fusarium* head blight of cereals caused by *F. graminearum*. *Biol Control* **113**, 26–38.

Lima, G., Castoria, R., De Curtis, F., Raiola, A., Ritieni, A. and De Cicco, V. (2011) Integrated control of blue mould using new fungicides and biocontrol yeasts lowers levels of fungicide residues and patulin contamination in apples. *Postharvest Biol Tec* **60**, 164–172.

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G. and Van Sanford, D. (2012) A unified effort to fight an enemy of wheat and barley: *Fusarium* head blight. *Plant Dis* **96**, 1712–1728.

Mesterházy, Á. and Bartók, T. (2001). Fungicide control of *Fusarium* head blight in wheat. In *2001 National Fusarium Head Blight Forum* eds. Canty, S.M., Lewis, J., Siler, L., Ward, R.W. December 8–10, 2001 Erlanger, USA, pp. 70–74. Erlanger, KY: Michigan State University.

Mesterházy, A., Tóth, B., Varga, M., Bartók, T., Szabó-Hebér, A., Farády, L. and Lehoczki-Krsjak, S. (2011) Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of *Fusarium* head blight and deoxynivalenol. *Toxins* **3**, 1453–1483.

Palazzini, J.M., Ramirez, M.L., Torres, A.M. and Chulze, S.N. (2007) Potential biocontrol agents for *Fusarium* head blight and deoxynivalenol production in wheat. *Crop Prot* **26**, 1702–1710.

Palazzini, J.M., Ramirez, M.L., Alberione, E., Torres, A.M. and Chulze, S.N. (2009) Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents on *Fusarium* head blight in wheat. *Biol Control* **51**, 370–376.

Palazzini, J.M., Alberione, E., Torres, A., Donat, C., Köhl, J. and Chulze, S. (2016a) Biological control of *Fusarium graminearum* sensu stricto, causal agent of *Fusarium* head blight of wheat, using formulated antagonists under field conditions in Argentina. *Biol Control* **94**, 56–61.

Palazzini, J.M., Dunlap, C.A., Bowman, M.J. and Chulze, S.N. (2016b) *Bacillus velezensis* RC 218 as a biocontrol agent to reduce *Fusarium* head blight and deoxynivalenol accumulation: genome sequencing and secondary metabolite cluster profiles. *Microbiol Res* **192**, 30–36.

Palazzini, J.M., Yerkovich, N., Alberione, E., Chiotta, M. and Chulze, S.N. (2017) An integrated dual strategy to control *Fusarium graminearum* sensu stricto by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions. *Plant Gene* **9**, 13–18.

Paul, P.A., Lipps, P.E., Hershman, D.E., McMullen, M.P., Draper, M.A. and Madden, L.V. (2008) Efficacy of triazole based fungicides for *Fusarium* head blight and deoxynivalenol control in wheat: a multivariate meta-analysis. *Phytopathology* **98**, 999–1011.

Pirgozilev, S.R., Edwards, S.G., Hare, M.C. and Jenkinson, P. (2002) Effect of dose rate of azoxystrobin and metconazole on the development of *Fusarium* head blight and the accumulation of deoxynivalenol (DON) in wheat grain. *Eur J Plant Pathol* **108**, 469–478.

Ramirez, M.L., Chulze, S. and Magan, N. (2004) Influence of environmental factors and fungicides on growth and deoxynivalenol production by *Fusarium graminearum* isolates from Argentinian wheat. *Crop Prot* **23**, 117–125.

Schisler, D.A., Khan, N.I., Boehm, M.J., Lipps, P.E. and Zhang, S. (2006) Selection and evaluation of the potential of choline-metabolizing microbial strains to reduce *Fusarium* head blight. *Biol Control* **39**, 497–506.

Schisler, D.A., Core, A., Boehm, M.J., Horst, L., Krause, C., Dunlap, C. and Rooney, A. (2014) Population dynamics of the *Fusarium* head blight biocontrol agent *Cryptococcus flavescens* OH 182.9 on wheat anthers and heads. *Biol Control* **70**, 17–27.

Simón, M., Fleitas, M. and Schalamuk, S. (2013) Recent advances on integrated foliar disease management with special emphasis in Argentina wheat production. In *Fungicides - Showcases of Integrated Plant Disease Management from Around the World* ed. Nita, M., pp. 3–34. London, UK: InTech. https://doi.org/10.5772/51950.

Simpson, D.R., Weston, G.E., Turner, J.A., Jennings, P. and Nicholson, P. (2001) Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain. *Eur J Plant Pathol* **107**, 421–431.

Steiner, B., Buerstmayr, M., Michel, S., Schiweiger, W., Lemmens, M. and Buerstmayr, H. (2017) Breeding strategies and advances in line selection for *Fusarium* head blight resistance in wheat. *Trop Plant Pathol* **42**, 165–174.

Wegulo, S.N., Baenziger, P.S., Hernandez Nopsa, J., Bockus, W.W. and Hallen-Adams, H. (2015) Management of *Fusarium* head blight of wheat and barley. *Crop Prot* **73**, 100–107.

Zhang, S., Boehm, M.J., Schisler, D.A. and Slininger, P.J. (2005) Carbon to-nitrogen ratio and carbon loading of production media influence freeze-drying survival and biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9. *Phytopathology* **95**, 626–631.