

ORIGINAL ARTICLE

Imidazolium salts with antifungal potential for the control of head blight of wheat caused by *Fusarium graminearum*A.D. Ribas^{1,2}, E.M. Del Ponte³, A.M. Dalbem², D. Dalla-Lana⁴, C. Bündchen⁵, R.K. Donato², H.S. Schrekker² and A.M. Fuentefria^{1,2,4}

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Keywords

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Abstract

Aims: Evaluate the *in vitro* effect of imidazolium salts (IMS) on the conidia germination and mycelial growth of *Fusarium graminearum* and their *in vivo* efficacy for suppressing the symptoms of the disease and infection of kernels in wheat plants.

Methods and Results: The minimum inhibitory concentrations (MIC) of three IMS ($C_{16}MImCl$, $C_{16}MImMeS$ and $C_{16}MImNTf_2$) were determined for four *F. graminearum* isolates using serial broth dilution method. The MICs found for all IMS were either 3·12 or 6·25 $\mu g ml^{-1}$ across the isolates, with the former as the most frequent. In the mycelial growth assay on potato dextrose agar media, only the $C_{16}MImCl$ among the IMS reduced 50% of mycelial growth of one isolate at an estimated concentration of 0·32 $mg ml^{-1}$. The time-kill curves showed a strong fungicidal effect starting 1 h after incubation at a concentration of 12·5 $\mu g ml^{-1}$, representing a fourfold increase in the most frequent MIC. The $C_{16}MImCl$ sprayed onto the spikes of potted wheat plants during the flowering stage reduced disease intensity at levels comparable to the commercial fungicide when applied preventatively (1 h prior to fungal inoculation), rather than curatively, and at the higher dosage (2 $mg ml^{-1}$) rather than lower dosage (0·5 $mg ml^{-1}$).

Conclusions: $C_{16}MImCl$ proved to be a potent inhibitor of *F. graminearum* growth and provided good levels of control of the disease at levels comparable to a commercial fungicide, in wheat plants treated prior to fungal infection during flowering stages.

Significance and Impact of the Study: This study suggests the potential of using IMS as alternative to the hazardous standard fungicides in the management of *Fusarium* head blight of wheat.

Introduction

Fusarium graminearum species complex is the main cause of *Fusarium* head blight (FHB), an emerging disease of small grains that poses a serious threat to food safety due to the production of dangerous mycotoxins by the pathogenic fungus (McMullen *et al.* 2012). The use of resistant

cultivars and fungicides applied during flowering stages of wheat crops are the most used practices for controlling the disease (Gilbert and Haber 2013). The most commonly used fungicides to combat FHB belong to the demethylation inhibitors (DMI) group, and are formulated either as a solo activity ingredient (AI) or as a mixture of two AI such as two triazoles (tebuconazole and metconazole) and

triazolinthione (prothioconazole) (Paul *et al.* 2008). Additionally, benzimidazole fungicides (BMC), such as carben-dazim, have been used in FHB management (Yu *et al.* 2011). Currently, wheat varieties with good levels of resistance are not available and the efficacy of triazole and triazolinthione fungicides is quite variable and rarely exceeds 65% (Paul *et al.* 2008). In addition, reduced sensitiveness to triazole and carben-dazim fungicides have been reported with consequences in the efficacy and sustainability of chemical control (Chen and Zhou 2009; Becher *et al.* 2010; Spolti *et al.* 2014).

In order to minimize the risks to the environment, as well as human and animal health, associated with the increasing use of chemical fungicides, alternative products have been tested with promising results for controlling *Fusarium* diseases of cereals. These include the use of bio-control agents (BCAs) (e.g. *Bacillus* and *Trichoderma*) (Dunlap *et al.* 2011; Schöneberg *et al.* 2015), hormones (Petti *et al.* 2012), chitosan and algae extracts (Ferrer *et al.* 2014; Pagnussatt *et al.* 2014).

Among the new chemical compounds, imidazolium salts (IMS), which are constituted of an organic imidazolium cation together with an inorganic or organic anion (Riduan and Zhang 2013), have been tested against fungi and bacteria that are pathogenic to humans with promising results (Docherty and Kulpa 2005; Liu *et al.* 2013). The cation and/or anion constituents modify the chemical and physical properties (Plechko and Seddon 2008), which may result in interesting properties including an amphiphilic nature and low toxicity to human cells (Schrekker *et al.* 2013; Dalla Lana *et al.* 2015). When tested as plant protection agent, IMS formulations were developed for two fungicides commonly used for controlling potato tuber disease in Northern Ireland, imazalil and thiabendazole. The authors found an increased hydrophobicity and an enhanced activity against three *Fusarium* species (*Fusarium coeruleum*, *Fusarium culmorum* and *Fusarium sambucinum*) and *Phoma* spp. (Bica *et al.* 2011).

The main objective of this study was to evaluate the effect of three IMS that have shown promising antifungal properties against pathogenic fungi (Schrekker *et al.* 2013; Dalla Lana *et al.* 2015) for suppressing *in vitro* growth of *F. graminearum* isolates. In addition, the efficacy of IMS performing best was evaluated for the control of the disease in inoculated wheat plants in the greenhouse.

Material and methods

Imidazolium salts, fungicide and fungal strains

Three IMS that showed an excellent fungicide activity against human pathogenic fungi were selected from

previous studies (Schrekker *et al.* 2008; Dalla Lana *et al.* 2015). Three compounds: 1-*n*-hexadecyl-3-methylimidazolium chloride (C₁₆MImCl), 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate (C₁₆MImMeS) and 1-*n*-hexadecyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide (C₁₆MImNTf₂) with molecular weights of 342.99, 402.32 and 587.71 g mol⁻¹, respectively, (Fig. 1) were synthesized (final purity ≥98%) as previously described (Cassol *et al.* 2006; Schrekker *et al.* 2008). A commercial triazole fungicide recommended for FHB control (tebuconazole Nortox 200 EC; Nortox S.A., São Paulo, Brazil) with molecular weight of 307.92 g mol⁻¹, was included in several assays for comparison. In total, five isolates representative of *F. graminearum sensu stricto*, the main member of the complex commonly found to be associated with wheat kernels in Brazil (Del Ponte *et al.* 2015) were selected for evaluation in the experiments (Table 1). The fungal inoculum used in the experiments consisted of a macroconidial suspension obtained from 5-day-old cultures grown on SNA media (Synthetic low-Nutrient-Agar) media and adjusted to 2 × 10⁵ macroconidia ml⁻¹ using a haemocytometer (Spolti *et al.* 2012a).

Inhibition of conidia germination and mycelial growth

MIC, or the lowest concentration of the compound in which fungal spores do not germinate (no visible growth), was determined using a broth microdilution method (CLSI 2008) in the RPMI-MOPS media (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate (Sigma-Aldrich Co., St. Louis, MO) buffered to pH 7.0 with 0.165 mol l⁻¹ MOPS buffer – Sigma). Ten increasing concentrations of the compounds were tested: 0.09; 0.19; 0.39; 0.78; 1.56; 3.12; 6.25; 12.5; 25.0 and 50.0 µg ml⁻¹. Thereafter, an aliquot (100 µl) was dispensed in a flat-bottom 96-well microtitre plate. The negative control (sterility control) consisted of noninoculated (no fungus) RPMI-MOPS media not amended with any antifungal. The positive control consisted of the media inoculated with the fungus. The microtitre plates were incubated at 25°C for 48 h and the MIC readings interpreted visually. Three readings (triplicates) were taken for each antifungal-concentration combination and the whole experiment was repeated once. Data on the MICs for each combination of isolate and antifungal were summarized based on the median of three readings per treatment for each run of the experiment.

The EC₅₀, or the effective concentration leading to a 50% reduction of mycelial growth, of the 09Tr130 isolate, was quantified using an agar dilution method as described (Spolti *et al.* 2012b). A control (no antifungal) and five increasing concentrations (0.2, 0.4, 0.8, 1.6 and

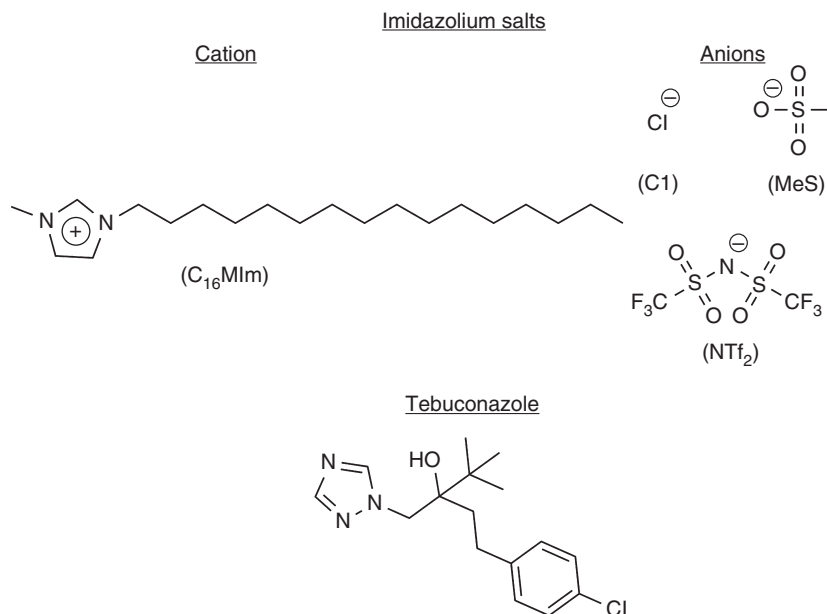


Figure 1 Molecular structures of imidazolium salts (cations and anions) and Tebuconazole, used in this study.

Table 1 Information for the five *Fusarium graminearum* isolates obtained from diseased wheat spiked from previous studies and used in the experiments

Isolate code	Species	Experiment	Reference
09Tr110	<i>F. graminearum</i>	MIC	Del Ponte <i>et al.</i> (2015)
09Tr130	<i>F. graminearum</i>	MIC, EC ₅₀ , TKA	Del Ponte <i>et al.</i> (2015)
09Tr26	<i>F. graminearum</i>	MIC	Del Ponte <i>et al.</i> (2015)
09Tr107	<i>F. graminearum</i>	MIC	Del Ponte <i>et al.</i> (2015)
08Tr044	<i>F. graminearum</i>	PB	Spolti and Del Ponte (2013)

MIC, minimal inhibitory concentration; EC₅₀, effective concentration that results in 50% mycelial growth inhibition; TKA, time-kill assay; PB, plant bioassay with the MIC and EC₅₀ determined separately.

3.2 mg ml⁻¹) were tested for the three IMS and tebuconazole. The experiment was conducted twice and the data combined for analysis. The EC₅₀ values for each anti-fungal were estimated by a linear regression model for relationship between per cent inhibition and logarithmic concentrations of the antifungals (Yuan *et al.* 2013).

Time-kill assay

The time-kill assay (Natesan *et al.* 2008) was conducted for C₁₆MImCl, the IMS that best suppressed the fungal growth in the two previous assays. Fungal inocula of the 09Tr130 isolate were dispensed in a microtube with solutions amended with increasing concentrations of the MIC (3.12 µg ml⁻¹) as follows: 1 × MIC, 2 × MIC and 4 × MIC. Aliquots of the conidial suspension were removed at different incubation times (1, 6, 12, 24 and 48 h), and diluted to 10⁻¹ to 10⁻³. A 0.10 ml aliquot was

spread onto PDA plates and incubated at 25°C for 48 h to determine the number of CFU ml⁻¹. Time-kill curves were constructed by plotting the log₁₀ CFU ml⁻¹ against the time of exposure to various concentrations of the IMS. The assay was performed in triplicate and repeated once.

Plant inoculation experiment

Seeds of the wheat cultivar BRS Guamirim, which exhibits a moderate resistance to FHB (Spolti *et al.* 2012a), were sown in 5L pots (10 seeds per pot) containing substrate 1 : 1 : 1 (humus, soil and vermiculite) with NPK fertilizer (10 : 10 : 10) and kept in a greenhouse from planting to physiological maturity of grain. The average temperature in the greenhouse during the experiment was 23°C and relative humidity ≥60%. A single *F. graminearum* isolate (08Tr044) previously identified as aggressive (Spolti and Del Ponte 2013) was used in the experiment. The MIC and EC₅₀ of this isolate for the C₁₆MImCl was determined prior to the experiment and both values were similar to the 09Tr130 isolate (*data not shown*). Inoculum was produced as previously described with its concentration adjusted to 2 × 10⁵ macroconidia ml⁻¹. The experiment was a 2 × 2 × 2 factorial, with two antifungals (C₁₆MImCl and tebuconazole), two doses (0.5 and 2 mg ml⁻¹) and two application times (preventative and curative). The experiments were conducted in a completely randomized design with five replications, each replication consisting of one pot with 7–10 spikes. A negative check treatment consisted of mock-inoculated nontreated plants and a positive check consisted of inoculated nontreated plants. The experiment was repeated once.

Plants were inoculated around a week after the beginning of flowering by spraying wheat spikes (1 ml per spike) with a spore suspension (2×10^5 macroconidia ml^{-1}) until run-off. All spikes of a single pot were covered with a plastic bag and moved into a growth chamber set at 23°C for 48 h to promote wetness for infection. For the preventative application, the macroconidial suspension was spray-inoculated 1 h after the application of the antifungal treatment, followed by 24 h of incubation under the wetness conditions as described. For the curative application, inoculated plants were sprayed with the antifungal treatment 24 h of incubation, thus allowing the fungus to infect the plant. An extra 24 h incubation period followed. After 48 h, plants were moved back to the greenhouse and monitored daily.

For both inoculation times, the disease variables evaluated were incidence (% diseased spikes) and severity (% diseased spikelets per spike) recorded at 7 and 15 days after inoculation (Spolti and Del Ponte 2013). When plants reached physiological maturity (≈ 30 days after flowering), the spikes were manually threshed and the kernels dried to 13% humidity. *Fusarium*-damaged kernels (FDK, %) was determined based on a standard seed health test after kernel surface disinfection. The Levene test indicated data from the variables were homoscedastic. A three-way ANOVA was conducted and the interaction and main effects estimated at a 5% significance level. Means of the treatments were compared using the LSMEANS package of R (The R Project for Statistical Computing, Vienna, Austria).

Results

Inhibition of spore germination and mycelial growth

The MIC could be determined for all but the $\text{C}_{16}\text{MImNTf}_2$ IMS, for which all isolates were able to germinate in all tested concentrations. For the other two IMS, the MICs ranged from 3.12 to 6.25 $\mu\text{g ml}^{-1}$ (Table 2). The most frequent MIC of 3.12 $\mu\text{g ml}^{-1}$ is equivalent to 7.75, 9.09 and 10.13 nmol ml^{-1} for $\text{C}_{16}\text{MImMeS}$, $\text{C}_{16}\text{MImCl}$ and tebuconazole respectively. In the EC_{50} bioassay, $\text{C}_{16}\text{MImMeS}$ and $\text{C}_{16}\text{MImNTf}_2$ did not reduce mycelial growth in their maximum concentration (Fig. 2). The EC_{50} values estimated for $\text{C}_{16}\text{MImCl}$ and tebuconazole were 0.32 and 0.0008 mg ml^{-1} respectively.

Time-kill curves

CFU counting reached maximum value in the check treatment (no antifungal) during the first hour of the experiment, there being no decline until the end of the experiment. For the $1 \times \text{MIC}$ (3.12 $\mu\text{g ml}^{-1}$) and $2 \times \text{MIC}$ (6.24 $\mu\text{g ml}^{-1}$) concentration, CFU values tended

Table 2 Minimum inhibitory concentration (MIC) for two imidazolium salts and a commercial triazole fungicide determined in a spore germination assay for four *Fusarium graminearum* isolates

Antifungal*	MIC ($\mu\text{g ml}^{-1}$)†			
	09Tr026	09Tr107	09Tr110	09Tr130
$\text{C}_{16}\text{MImCl}$	3.12	6.25	3.12	3.12
$\text{C}_{16}\text{MImMeS}$	6.25	6.25	3.12	6.25
Tebuconazole	3.12	3.12	6.25	3.12

* $\text{C}_{16}\text{MImCl}$ = 1-*n*-hexadecyl-3-methylimidazolium chloride; $\text{C}_{16}\text{MImMeS}$ = 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate.

†Median values of three replicates in a repeated experiment testing ten concentrations (0.09; 0.19; 0.39; 0.78; 1.56; 3.12; 6.25; 12.5; 25.0 and 50.0 $\mu\text{g ml}^{-1}$) of the antifungal amended to a RPMI-MOPS liquid media.

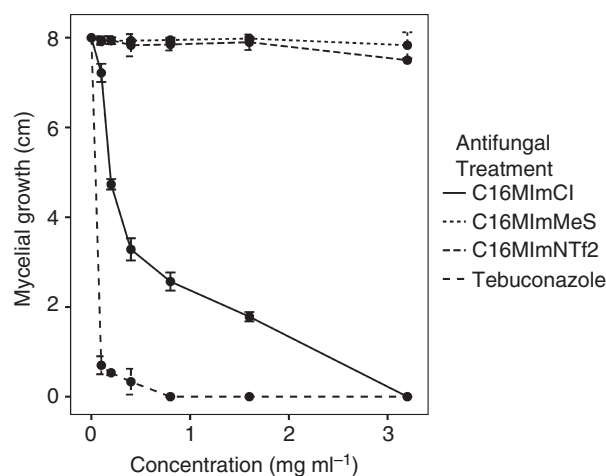


Figure 2 Mycelial growth rate of a *Fusarium graminearum* isolate (09Tr130) influenced by increasing concentration of three imidazolium salts $\text{C}_{16}\text{MImCl}$ (1-*n*-hexadecyl-3-methylimidazolium chloride); $\text{C}_{16}\text{MImMeS}$ (1-*n*-hexadecyl-3-methylimidazolium methanesulfonate) and $\text{C}_{16}\text{MImNTf}_2$ (1-*n*-hexadecyl-3-methylimidazolium bis (trifluoromethylsulfonyl)imide) and a commercial fungicide (tebuconazole) amended to a potato dextrose agar media at five increasing concentrations 0.2, 0.4, 0.8, 1.6 and 3.2 mg ml^{-1} of the antifungal. The estimated EC_{50} was 0.32 mg ml^{-1} and <0.001 for the $\text{C}_{16}\text{MImCl}$ was and tebuconazole, which were able to reduce 50% of the mycelial growth rate. Error bars represent the standard deviation.

to peak and decline at the end of the experiment, suggesting a sublethal concentration level. The strongest fungicide effect was observed 1 h after inoculation for the $4 \times \text{MIC}$ (12.5 $\mu\text{g ml}^{-1}$) concentration, and no further growth was observed during the course of the experiment (Fig. 3).

Disease control assay

FHB symptoms were noticed in the majority of plants of the inoculated and nontreated check, reaching incidence

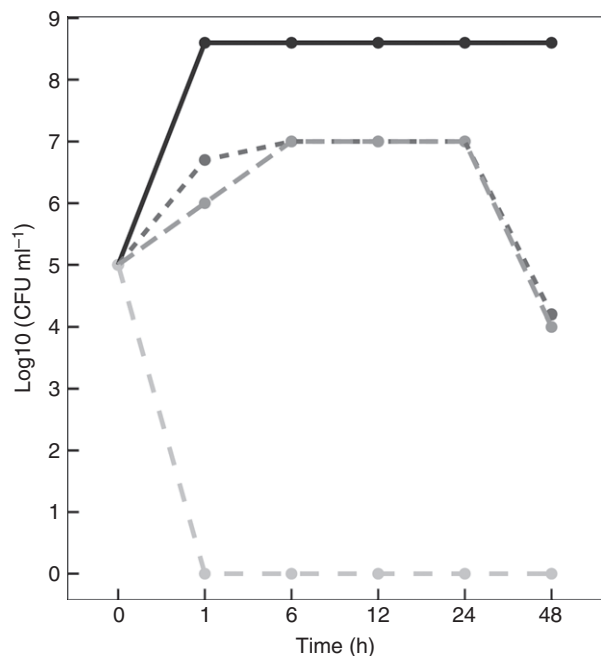


Figure 3 Time-kill curve for one *Fusarium graminearum* (09Tr130) based on the antimicrobial activity of one-, two- and fourfold increase of the minimum inhibitory concentrations (3–12 $\mu\text{g ml}^{-1}$) determined for the imidazolium salt $\text{C}_{16}\text{MImCl}$ (1-*n*-hexadecyl-3-methylimidazolium chloride). (—●—) 0 – Control, (—●—) 1 \times MIC, (—●—) 2 \times MIC, (—●—) 4 \times MIC.

and severity levels of 96.4 and 100% respectively. ANOVA showed significance for the triple interaction ($P < 0.05$), suggesting that the response of all variables were dependent on application timing, antifungal and dosage. Overall, preventative applications provided better control than curative applications for all three disease-related variables evaluated (Fig. 4). The highest disease reductions relative to the positive check treatment (75–95%) for all disease variables were found for both fungicides at the highest dose (2 mg ml^{-1}) applied preventatively, not differing between them. However, means of these two treatments differed at the lowest dose (0.5 mg ml^{-1}), with higher disease suppression for the tebuconazole treatment (Fig. 4). For the curative applications, the highest dose of both anti-fungal treatments still provided good control efficacy levels, but only a slight reductions in disease levels were observed for $\text{C}_{16}\text{MImCl}$, applied curatively in the lower concentration (Fig. 4).

Discussion

This study further demonstrates the potential of IMS as a new class of agricultural fungicides corroborating previous studies using plant pathogenic fungi (Bica *et al.* 2011). To our knowledge, this is the first report on the

efficacy of an IMS for suppressing the *in vitro* growth of *F. graminearum* as well the disease in inoculated wheat plants. We showed that $\text{C}_{16}\text{MImCl}$, an IMS with a long hexadecyl chain (C_{16}) and chloride (Cl) anion, outperformed two other IMS, all previously tested for the control of fungi of medical importance (Bergamo *et al.* 2015; Dalla Lana *et al.* 2015). Apart from providing the best *in vitro* control, $\text{C}_{16}\text{MImCl}$ reduced disease severity at levels comparable to tebuconazole fungicide when applied prior to plant inoculation.

The reasons for the failure of two IMS to inhibit mycelial growth at the tested concentrations, also presenting the same cation and a long hexadecyl chain (C_{16}), may be related to the selective anion effect. Recently, Wang *et al.* (2011) investigated the effects of five IMS on the growth of anaerobic bacterium, *Clostridium* sp., and found a strong influence of the anion type, with NTf_2 performing best among the compounds. Contrariwise, Schrekker *et al.* (2013) reported stronger effect of the Cl anion IMS against pathogenic yeasts, outperforming the other anions (BF_4^- , NTf_2^- , MeS, PF_6^-). Dalla Lana *et al.* (2015) found that less bulky anions, such as Cl and MeS, exhibited stronger antifungal effect against filamentous fungi. Although the $\text{C}_{16}\text{MImMeS}$ was able to inhibit spore germination in this study, mycelial growth was not suppressed at the highest tested concentration. Although the role of the anion in the molecule is yet to be elucidated, it is hypothesized that a low mass chloride anion favours the interaction of the compound with the fungal cells by promoting the antifungal ability of the hexadecyl chain (Dalla Lana *et al.* 2015).

The MICs determined for the $\text{C}_{16}\text{MImCl}$ and $\text{C}_{16}\text{MImMeS}$ ranged between two adjacent concentrations. Previous studies testing the antimicrobial activity of *N*-substituted IMS showed much larger MIC variation (0.014–100.00 $\mu\text{g ml}^{-1}$) suggesting that the MIC depends not only the IMS but on the micro-organism under evaluation (Liu *et al.* 2013; Bergamo *et al.* 2015). The fungicidal effect of $\text{C}_{16}\text{MImCl}$ occurred within the first hour of exposure, which is an advantage over tebuconazole, for example, which exhibit a fungistatic effect (Yin *et al.* 2009). In fact, a temporary suppression of FHB severity progress has been reported for tebuconazole-treated wheat plants (Becher *et al.* 2010; Spolti and Del Ponte 2013).

The EC_{50} value determined based on the mycelial growth assay was much higher for $\text{C}_{16}\text{MImCl}$ compared with tebuconazole. For the latter, the effective concentration could not be estimated with confidence because a very large reduction in mycelial growth was found in the first concentration of the fungicide. Nevertheless, previous studies with *F. graminearum* isolates from Brazil showed median EC_{50} value of 0.001 $\mu\text{g ml}^{-1}$ (<0.001–0.152) in a sample of 50 isolates, agreeing with our

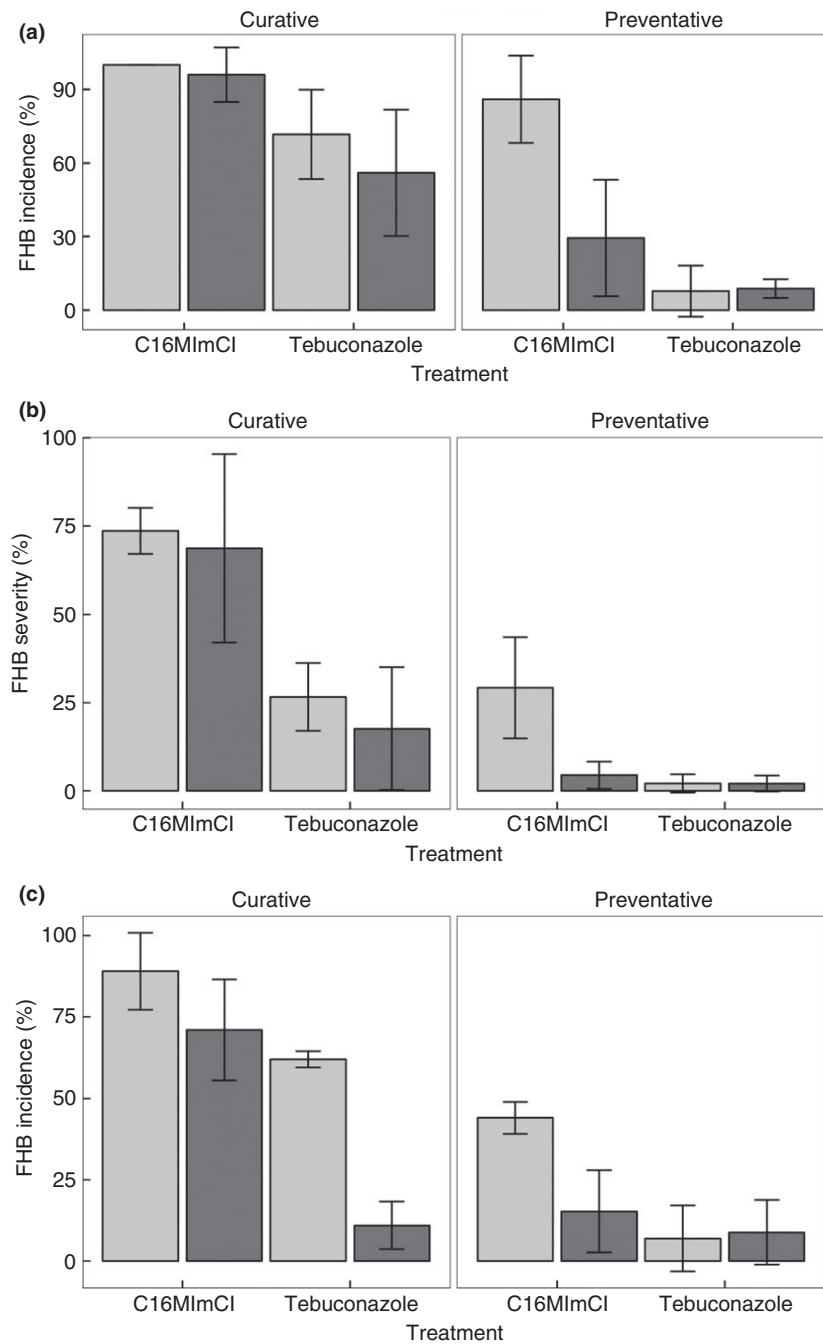


Figure 4 Means and respective 95% confidence interval ($n = 5$) of FHB incidence (a), severity, factor (dose) (□) 0.5, (■) 2 (b) and kernel infection (c) in wheat spikes treated with the C₁₆MImCl (1-*n*-hexadecyl-3-methylimidazolium chloride) and tebuconazole fungicide and applied either after pathogen inoculation (curative) or before pathogen inoculation (preventative) at two dosage rates (0.5 or 2 mg ml⁻¹).

findings (Spolti *et al.* 2012b). On the other hand, MIC values determined based on conidial germination were similar between these two compounds. Such difference may be related to the regulating system of the fungi (Harris 2005) and the mode of action of the compounds. Mycelial cells seem to exhibit higher resistant to antifungal agents than conidia, but contradictory results of susceptibility to antifungal agents between hyphal and conidial inoculum have been reported for *Fusarium* spp.

(Lionakis *et al.* 2003) and *Aspergillus* spp. (Guarro *et al.* 1997). The mode of action of the IMS needs to be better elucidated, as there is no evidence of systemic translocation in plants. Previous studies reported similarities in the mode of action between C₁₆MImCl IMS and imidazoles and triazoles, which act by inhibiting the enzyme lanosterol 14 α -demethylase, which is necessary to convert lanosterol to ergosterol (Schrekker *et al.* 2013) or interference with the cell's osmotic balance (Hirayama *et al.*

2012). The better results obtained for more than one IMS in the germination inhibition test compared to the mycelial growth assay as well as the best control of the disease when C₁₆MImCl was applied preventatively (prior to spore landing), suggests that the IMS may act primarily by inhibiting spore germination. Macroconidial spores are composed of chitin in apical spore cells and the cortical regions of macroconidial cells, which later produce the germ tube. A functional microtubule is required for transporting polarity key factors to specific sites that have a regulating system that indicate sites of emergence in the germ tube (Harris 2005). We speculate that in some way the C₁₆MImCl IMS disturbs this system and inhibits the germination process, thus preventing further invasion of the plant tissue. Interference in the germination *F. graminearum* spores may also play a role in the reduction of deoxynivalenol (DON) levels, a trichothecene mainly synthesized on the tips of the hyphae, which move forward along the intercellular space and inhibiting host plant defences (Brown *et al.* 2011).

In general, DMI (sterol biosynthesis inhibitors) fungicides, such as triazoles, are considered the most effective chemical fungicides against FHB pathogens (Paul *et al.* 2008) but they are also harmful to the environment and human health (Menegola *et al.* 2006). One of the advantages of IMS includes the low toxicity to humans. For the C₁₆MImCl, specifically, no damage to human DNA was found in the concentrations tested using on human leucocyte cells in the Comet test (Schrekker *et al.* 2013). Recent studies showed absence of cytotoxicity, mutagenicity and genotoxicity in leucocyte human cell at concentrations 100× higher than the MIC determined for pathogenic fungi (Dalla Lana *et al.* 2015). The ecotoxicity of IMS has been questioned and, in some cases, the negative effect is at least equivalent to those of common organic solvents (Wells and Coombe 2006). Moreover, the toxicity seems to be highly dependent on the concentration of IMS and the anion type (Biczak *et al.* 2014). Further studies should focus on the understanding of the mode of action and possible structural changes in these molecules, in order to impart desirable characteristics to the fungicide. It would be also advisable to perform field tests and check whether disease reduction is accompanied by mycotoxin reduction in mature kernels.

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Conflict of Interest

No conflict of interest declared.

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