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Neutralized electrolyzed water efficiently reduces *Fusarium* spp. *in vitro* and on wheat kernels but can trigger deoxynivalenol (DON) biosynthesis

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ABSTRACT

Fusarium Head Blight (FHB), caused by a blend of *Fusarium* species, is a destructive fungal disease of wheat and other small grain cereals. FHB has become an important issue in food and feed industry. Moreover, the majority of FHB pathogens have the ability to synthesize a range of mycotoxins. Although several physical and chemical control measures can be taken to control these fungi in the field, research is needed to provide new techniques for control during storage and transport of cereals. Mounting evidence shows that electrolyzed oxidizing water (EOW) has antimicrobial activity and might be a useful alternative for conventional control measures. The objective of the present work, was to investigate the influence of EOW on outgrowth and germination of *Fusarium* spp. and deoxynivalenol (DON) production. Both an *in vitro* and *in vivo* approach were pursued. In a first approach, a screening of the main FHB causing species was conducted. Secondly, the effect of EOW on *Fusarium graminearum* and the effect on DON biosynthesis was investigated using a trichothecene knockout mutant. These experiments showed an increase in DON levels upon sub lethal amendments of EOW to *F. graminearum* spores. In addition, the reactive oxygen species H_2O_2 was shown to govern this induction.

Finally, the work was validated on a laboratory scale via an *in vivo* assay using wheat grains in which the *Fusarium* outgrowth was measured. The present work demonstrates that EOW has potential to control *Fusarium* spp. in wheat grains during transport and storage although sub lethal concentrations can result in increased DON biosynthesis.

1. Introduction

Fusarium Head Blight (FHB), the most important ear disease in wheat, is caused by a complex of up to 17 *Fusarium* species of which *Fusarium graminearum, Fusarium poae, Fusarium culmorum* and *Fusarium avenaceum* are predominantly present in Europe (Xu et al., 2005). Although FHB symptoms have a classical impact on yield at the level of thousand-kernel weight and on baking/brewing quality (Prange et al., 2005; Wang, Pawelzik, Weinert, Zhao, & Wolf, 2008; Wang et al., 2005), the major concern referred to FHB is the presence of mycotoxins. *Fusarium* spp. are able to produce mycotoxins with diverse biological features (Bottalico & Perrone, 2002). Amongst these mycotoxins, the type B trichothecene deoxynivalenol (DON) is world-wide the *Fusarium* toxin most frequently detected.

In order to control *Fusarium* in the field, a diversified portfolio of active compounds such as triazoles and strobilurins are available. In general, these products guarantee a good control of *Fusarium* spp. Nevertheless, the short vulnerable period of wheat i.e. during anthesis, can pose a serious impediment for an efficient *Fusarium* targeted control in the field. In addition, several lines of evidence illustrate elevated DON biosynthesis when fungicides are applied sub optimally (Audenaert, Callewaert, Hofte, De Saeger, & Haesaert, 2010; Covarelli, Turner, & Nicholson, 2004)

Although *Fusarium* is a typical field pathogen which is essentially controlled in the growing crop, it is hard to completely abrogate the fungus from crude wheat kernels and both fungus and concomitant mycotoxins remain a serious issue during storage and

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processing of wheat derived products. Therefore, several post-harvest measures are available to control mycotoxins and fungi. Firstly, the prevailing water activity (a_w) and temperature during storage are unequivocal parameters determining the outgrowth of fungi and their metabolite production (Birzele, Prange, & Kramer, 2000; Jouany, 2007). Secondly, physical treatments of contaminated grains are hurdles that can be included in post-harvest control strategies against outgrowth of fungi and accumulation of their metabolites. Elimination of damaged kernels (Balzer, Tardieu, Bailly, & Guerre, 2004), removal of the bran fraction in a dehuller (Wilson et al., 2004), gamma irradiation (Aziz & Moussa, 2004) and milling (Saunders, Meredith, & Voss, 2001) can contribute to lower the fungal and mycotoxin risks in grains as well. A third strategy to reduce fungal and/or mycotoxin levels in grains are chemicals. Treatments of wheat grains with ozone (Wu, Doan, & Cuenca, 2006), propionic acid, formic acid, sodium hypochlorite, hydrochloric acid, or gaseous ammonia (Jouany, 2007). Formaldehyde gas and glutaraldehyde are reported to be used as infrastructural disinfectors but intensive use of these chemicals is hampered by their noxious effects on humans and animals. Finally, some newer trends such as mycotoxin detoxifiers and absorbents are currently under research (Fuchs, Binder, Heidler, & Krska, 2002; Jouany, 2007; Molnar, Schatzmayr, Fuchs, & Prillinger, 2004).

Mounting evidence shows that electrolyzed oxidizing water (EOW) has antimicrobial activity and might be a useful alternative for classical control measures. It has the advantage of being non-toxic and non-corrosive to organic matter compared to many other control strategies (Huang, Hung, Hsu, Huang, & Hwang, 2008).

EOW is generated by electrolysis of a dilute solution of sodium chloride in an electrolysis chamber where anode and cathode are separated by a non-selective membrane resulting in either neutralized electrolyzed water or acidic electrolyzed water. The sterilization mechanisms are categorized according to three germicidal factors: pH, oxido reduction potential (ORP) and available chlorine concentrations (Cl_2, HOCl and OCl^-). The antifungal effect has been illustrated against numerous plant pathogens such as *Fusarium oxysporum, Botrytis cinerea, Alternaria* spp, *Colletotrichum* spp. and *Monilinia longicolla* (Abbasi & Lazarovits, 2006; Bonde et al., 1999; Buck, van Iersel, Oetting, & Hung, 2002; Mueller, Hung, Oetting, van Iersel, & Buck, 2003). Although EOW seems to be a potentially interesting new technology, some drawbacks such as the quick loss of activity in presence of organic matter are a serious impediment for the implementation of this technology in control strategies against fungi and their toxins.

The objective of the present work was (i) to disentangle the influence of EOW on several Fusarium spp. Using an *in vitro* approach, the susceptibility of *F. graminearum, F. culmorum , Fusarium verticilloides* and *F. poae* was evaluated in a comparative study with the fungicide prothioconazole + fluoxastrobin. (ii) to elucidate the effect of EOW on DON biosynthesis by *F. graminearum*. For this purpose, a new analytic methodology was developed using a UPLC/MSMS (Ultraperformance liquid chromatography-tandem mass spectrometry) approach to distinguish DON from its acetylated derivatives. (iii) to analyze the short- and long-term effects of EOW application on the outgrowth of *F. graminearum* in wheat grains.

2. Material and methods

2.1. Fungal strains, induction of conidia, harvest of conidia, H_2O_2 measurements

The *Fusarium* isolates used in the present work are presented in Table 1. These isolates were grown on potato dextrose agar (PDA) for 7 days at 20 °C and kept at 4 °C upon use. Conidia of *Fusarium* spp. were obtained by incubating a mycelium plug on a PDA plate for 7 days under a light regime of UV/darkness (12 h(365 nm 10 W)/12 h). Conidia were harvested by adding distilled water amended with 0.01% of Tween80 (Merck, Germany) to the fully grown PDA plates and by rubbing the conidia-bearing mycelium with a spatula. Conidia were counted with a Bürker counting chamber and diluted to a final concentration of 10^6 conidia/ml. In the germination assays, fungal conidia were visualised using a 0.02% cotton blue solution prepared in lactic acid (85%). H_2O_2 measurements were performed as previously described by Audenaert et al. (2010).

Table 1
Fusarium isolates used in the present study.

Species	Isolate	Original Substrate	Reference/origin
F. culmorum	MUCL 555	*Secale cereale*	W.G. Smith Saccardo
F. graminearum	MUCL 11946	*Triticum, sp.*	Kinnard A.
	MUCL 42841	*Triticum aestivum*	MUCL[b] collection
	isolate 8/1, GFP	NK[a]	(Jansen et al., 2005)
	Isolate 8/1, GFP, Tri5$^-$	NK[a]	(Jansen et al., 2005)
F. poae	MUCL 6114	*Hordeum vulgare*	MUCL[b] collection
	MUCL 15926	*Hordeum vulgare*	MUCL[b] collection
	MUCL 8279	*Hordeum vulgare*	MUCL[b] collection
F. avenaceum	MUCL6130	NK	MUCL[b] collection
F. sporotrichioides	MUCL 6133	*Nicotiana tabacum*	MUCL[b] collection

[a] NK: not known.
[b] MUCL: Mycothèque Université Louvain-la-Neuve.

2.2. Production of EOW

Neutralized EOW was prepared using a prototype EOW generator by hydrolysis of a 100 mM solution of NaCl. The electric current passing the generator was fixed at 60 A and the water kept at a constant pressure of 0.11 MPa. Before use, the EOW flow was allowed to run for at least 5 min in order to stabilise. The pH value was set at 7.2 (pH 301. GLP Bench, Hanna Instruments, Portugal) and the ORP at 889 ± 10 mV. The amount of hypochlorite was determined by a volumetric iodometric hypochlorite determination with a 0.1 N $Na_2S_2O_3$ solution. This hypochlorite solution was the start for the used dilution series and was kept at 4 °C upon use for maximum three days.

2.3. In vitro assay EOW

The efficiency of EOW to inhibit spore germination and fungal growth was investigated in an *in vitro* assay previously described by Audenaert et al. (2010). A dilutions series of fluoxastrobin + prothioconazole was included in the experiment with concentrations ranging from 12.5 mg/l up to 200 mg/l and served as a positive fungicidal control. EOW was diluted with distilled water to concentrations of 200 mg/l, 100 mg/l, 25 mg/l and 12.5 mg/l OCl^-. The *in vitro* assay was carried out in a 24-well plate. In these wells, 250 μl of conidial suspension was added and amended with 250 μl of the fungicide dilution or the EOW dilution. These wells were incubated at 20 °C for 48 h. Each treatment consisted of 3 repetitions and the experiment was repeated twice independently in time. Control treatments consisted of 250 μl of spore suspension and 250 μl of distilled water.

2.4. DON quantification using UPLC/MSMS and fungal quantification in vitro

In order to discriminate between DON and its acetylated forms 15-acetyl-DON (15-ADON) and 3-acetyl-DON (3-ADON), an UPLC/MSMS methodology was optimized which was able to analyze DON and to distinguish 3-ADON and 15-ADON in a single run. Although, we worked only with a 15-ADON chemotype in present work, it