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Rising atmospheric CO_2 concentration may imply higher risk of *Fusarium* mycotoxin contamination of wheat grains

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Abstract

Increasing atmospheric CO_2 concentration not only has a direct impact on plants but also affects plant-pathogen interactions. Due to economic and health-related problems, special concern was given thus in the present work to the effect of elevated CO_2 (750 μmol mol^{-1}) level on the *Fusarium culmorum* infection and mycotoxin contamination of wheat. Despite the fact that disease severity was found to be not or little affected by elevated CO_2 in most varieties, as the spread of *Fusarium* increased only in one variety, spike grain number and/or grain weight decreased significantly at elevated CO_2 in all the varieties indicating that *Fusarium* infection generally had a more dramatic impact on the grain yield at elevated CO_2 than at the ambient level. Likewise, grain deoxynivalenol (DON) content was usually considerably higher at elevated CO_2 than at ambient level in the single-floret inoculation treatment, suggesting that the toxin content is not in direct relation to the level of *Fusarium* infection. In the whole-spike inoculation, DON production did not change, decreased or increased depending on the variety × experiment interaction. Cooler (18°C) conditions delayed rachis penetration while 20°C maximum temperature caused striking increases in the mycotoxin contents, resulting in extreme high DON values and also in a dramatic triggering of the grain zearalenone contamination at elevated CO_2. The results indicate that future environmental conditions, such as rising CO_2 levels, may increase the threat of grain mycotoxin contamination.

Keywords: deoxynivalenol, elevated CO_2, *Fusarium culmorum*, mycotoxin, *Triticum aestivum*, zearalenone

List of abbreviations*:*

AUDPC= area under the disease progress curve
DON= deoxynivalenol
FHB= *Fusarium* head blight
SFI = single-floret inoculation
ZEN = zearalenone
WSI= whole-spike inoculation

Introduction

Global atmospheric CO_2 concentration has increased above 400 μmol mol^{-1} , while its annual rise has grown to the highest rate ever, almost 3 μmol mol^{-1} since 2015 (Dlugokencky and Tans, online reference). The impact of high CO_2 level on plants is well documented (e.g. DaMatta et al. 2010, Xu et al. 2015), less attention has, however, been paid to the interaction between plants and diseases at elevated CO_2 (Newton et al. 2011, Pangga et al. 2011).

Fusarium species play a major role in the success of food and feed production as they may result in severe yield losses (Murray et al. 1998, Bai et al. 2003, Mesterházy 2003, Stack 2003, Jansen et al. 2005) and fusariotoxins in the grains making them unsuitable for human or animal consumption (Reddy et al. 2010). The toxic effects of trichothecenes (such as e.g. deoxynivalenol, DON), include gastrointestinal effects (vomiting, diarrhea, bowel inflammation) and feed refusal, while zearalenone (ZEN), which can accumulate, is significantly toxic to the reproductive system due to its estrogenous action (Milićević et al. 2010, Reddy et al. 2009, Sobrova et al. 2010). Although the severity of infections caused by fungi is influenced by a variety of environmental factors, future growth conditions with slightly elevated CO_2 concentrations interacting with temperature and water availability have been proposed to favour certain mycotoxigenic species, including *Fusarium* spp. (Magan et al. 2011, Juroszek and Tiedemann 2013).

Research on maize found that elevated CO_2 level increased susceptibility to *Fusarium verticillioides* proliferation with no change in mycotoxin levels (Vaughan et al. 2014). In wheat, an increase in *Fusarium pseudograminearum* biomass and/or in stem browning was found in response to elevated CO_2 in some situations, depending on the varieties (Melloy et al. 2010, 2014). Fungal biomass increased significantly at elevated CO_2 in two of the three reported studies. Increased disease levels were also reported for *F. graminearum* in wheat (Váry et al. 2015). Infection severity was, however, in no close relation with the grain yield (Mesterházy 1995). A study on *Fusarium* crown rot revealed that increased *Fusarium* biomass in wheat tissue at elevated CO_2 matched raised DON content of the stem base under continuous cropping (Khudhair et al. 2014). A clear relationship was, however, not found between disease severity in the case of natural infection and DON content as high disease pressure of *Fusarium* resulted in a higher DON content or no correlation depending on the year (Paterson and Lima 2010). High variability indicated that no single environmental variable is sufficient for prediction of disease or mycotoxin contamination, though significant semi-partial Spearman correlations were determined between DON content and relative humidity variables, while there was correlation between nivalenol and temperature and relative humidity variables only from the start of anthesis (Kriss et al. 2012).

Despite the work done so far, there is still uncertainty regarding the food safety issues of future crop production. In many cases, *Fusarium* head blight (FHB) symptoms arise as the outcome of more infections caused by various *Fusarium* species, many of which are not known how they act under the changing climatic and environmental conditions; or even how their toxin profile and production alters. Besides, resistance to FHB in wheat includes several components, such as e.g. resistance to initial infection of one spikelet, resistance to the spread of *Fusarium* along the spike from an initially infected spikelet (type I and type II resistance, respectively, Schroeder and Christensen 1963) and resistance to DON accumulation in the infected kernels (Mesterházy 2003). These resistant components might, however, be influenced by the environmental change in different ways. Various QTLs linked to these traits will play a very important role in future prevention (Buerstmayr et al. 2003, 2009). Thus, the aim of the present work was to reveal the impact of elevated atmospheric CO_2 level on the resistance of wheat to *Fusarium culmorum* and on the mycotoxin contamination of the grains.

Materials and Methods

Plant material and experimental design

Winter wheat varieties with different genetic background and various resistance levels to FHB were chosen for the tests: Mv Regiment, Mv Mambo, Mv Emma, Apache and Ukrainka. As a general rule for all the experiments, four seedlings were planted to each 2.7 L pot after vernalization at 4°C for 42 days. The pots were placed randomly in the growth chambers and rearranged at the heading period. Daily irrigation was provided for the plants in each case, occasionally replaced with a nutrient supply as indicated below. The atmospheric CO_2 concentration was maintained at ambient (400 μmol mol^{-1}) or elevated levels (750 μmol mol^{-1}) in parallel chambers (both in the greenhouse and in the phytotron) where all the rest of the growth parameters were the same.

Three experiments were carried out in recent years, with slight differences in the growth conditions. The first test (Exp1) was done in 2012/13 in the greenhouse (Global Glasshouse Venlo), on 8 pots (max. 32 plants) per treatment and variety (Mv Regiment and Mv Mambo). The plants were raised at a day/night temperature of 12/10°C (tillering period) and 18/15°C (heading period) with 16-h day length under 200 μmol m^{-2} s^{-1} light intensity. The plants received 0.15L of 1g L^{-1} nutrient solution (Volldünger Linz Classic, Gartenhilfe GmbH., Austria) once a week between week 4 and 8. To prevent powdery mildew infection, a fungicide treatment with Amistar (containing azoxystrobin) was applied after planting, followed by two later elementary sulphur applications (Thiovit).

The second experiment (Exp2) was performed during 2013/14 in the phytotron, in Conviron PGB-96 growth chambers (Winnipeg, Canada). There were 20 pots (80 plants) for the tests per treatment and variety (Mv Regiment, Mv Emma, Apache, Ukrainka). However, the actual number of successful infections in all the experiments depended on how many spikes could be inoculated, being in the right phenological phase. The plants were supplied with nutrients twice a week up till heading, in the form of 0.4g·L^{-1} Volldünger nutrient solution (0.1L/pot). Fungicide control was applied with Bumper 25EC (propiconazole) on all plants in week 1 and with Amistar on week 5 after planting. The photosynthetic photon flux density was 280 μmol m^{-2} s^{-1} while the temperature regime changed weekly according to the Spring 2–Summer 2 (T2-Ny2) climatic programme (Tischner et al. 1997) until it reached 14/18°C, before the period of infection. Two weeks after inoculation the temperature started to increase gradually until it reached 19/23°C by maturation. Relative humidity of the air was 80% from inoculation till maturation.

The third trial (Exp3) was carried out in 2015/16 in the phytotron, in Conviron PGB-96 growth chambers (Winnipeg, Canada). There were 12 pots (48 plants) per treatment and variety (Mv Regiment, Mv Emma). A fungicide treatment with Cherokee (co-formulation of chlorothlonil, cyproconazole and propiconazole) was applied, followed by three elementary sulphur applications preceding heading. The plants were supplied with nutrients in tap-water (0.1L/pot) twice a week, from week 2 till week 9 after planting, in the form of 1g L^{-1} nutrient solution (Volldünger Linz Classic, Gartenhilfe GmbH., Austria). The temperature regime changed weekly according to the Spring 2–Summer 2 (T2-Ny2) climatic programme (Tischner et al. 1997) beginning with a min/max of 10/12ºC during the first week and increasing until it reached 16/20ºC by week 9, before the period of infection. From week 13, the temperature increased with 1ºC (night and day) every week, until it reached 20/24°C. The maximum photosynthetic photon flux density was 250 μmol m^{-2} s^{-1}). Relative humidity of the air was 80% from inoculation till maturation.

Inoculation with Fusarium

The *Fusarium culmorum* strain IFA 104 used in the experiment was kindly provided by Prof. Hermann Buerstmayr (IFA, Tulln, Austria) and maintained in Martonvásár since then. The inoculum was produced using a sterilized 3:1 mixture of wheat and oat grains for the propagation of fungal conidia, which were suspended with deionised water and diluted to the required concentration of spores (see below).
Two kinds of tests were performed either to examine the resistance against the spread of the fungus from an inoculated spikelet (Type II resistance) or to test the combined resistance to the penetration and spread of the pathogen (Type I+II resistance, Schroeder and Christensen 1963). In the case of single-floret inoculation, one spikelet (usually the 6^{th}–8^{th} from the top of each spike) was injected with a syringe dispenser at anthesis with 2×5 μL inoculum (500,000 conidia mL^{-1}), while in the latter type of infection (whole-spike inoculation), a suspension of spores (50,000 conidia mL^{-1}) was sprayed along the entire surface of each spike at anthesis. Inoculated spikes were covered with plastic bags for 48 hours to promote the successful establishment of the fungus. Whole-spike inoculation treatment in Mv Regiment in Exp2 resulted in a low number of infected individuals so these data were excluded from the analyses. The disease progress was monitored from day7 till day25 (Exp1) or day30 (Exp2 and Exp3) after inoculation, via the visual determination of the number of infected spikelets in each inoculated spike. The area under the disease progress curve (AUDPC) was calculated from the data till day25 and day30 according to Campbell and Madden (1990). The day of successful penetration into the rachis by the fungus was also recorded for each spike in Exp2 and 3. In these experiments, the following parameters were determined after maturation for each infected spike: spike weight, grain number, grain yield and thousand kernel weight. Due to the characteristics of the disease, the yield parameters and the infection data were not normally distributed, so they were statistically evaluated with the non-parametric Mann–Whitney U test (SPSS 16.0, SPSS Inc., Chicago, IL, USA).

Fusarium toxin determination

The *Fusarium* toxin measurements were performed on Mv Regiment in Exp1, on Mv Regiment (only in single-floret inoculation), Mv Emma, Apache and Ukrainka in Exp2 and on Mv Regiment and Mv Emma in Exp3. The combined grain yield of individual spikes successfully infected was used for the analyses, which were carried out by the service laboratory of Soft Flow Hungary Ltd. (Gödöllő, Hungary). The grain samples were milled to fine powder and homogenized, from which 5g wholemeal was used for the tests in three replications for each toxin. The DON content was quantified using Toxi-Watch DON ELISA test kit (Catalogue No: 3000031, Lot No: 090014), while the zearalenone (ZEN, toxin F2) concentration was determined with Toxi-Watch ZEARALENONE ELISA test kit (Catalogue No: 3000071, Lot No: 070024). Both kits were developed by Soft Flow Hungary Ltd, based on the method of competitive enzyme immuno-assay (EIA), using antigen-antibody reactions. According to the data provided by the manufacturer, the sensitivity (lowest detectable amount) of the assay is 0.65 ng mL^{-1} and 0.13 ng mL^{-1} (μg kg^{-1}), the range of detection is 125 – 4000 μg kg^{-1} and 28.125– 225 μg kg^{-1} for DON and ZEN, respectively. For ZEN, cross reactions for the anti-body used were: zearalanone (138%), α-zearalenol (91%), β-zearalenol (21%), α-zearalanol (69%), β-zearalanol (6 %), intra -assay: 8.91 CV% at validation, inter-assay 9.63 CV%, recovery% of the Trilogy reference standard samples (Trilogy Analytical Laboratories, Washington, USA, ISO 17025 accreditation): 97.8+/-6.51% at validation. For DON: intra-assay was 7.53 CV% at validation, inter-assay 9.87 CV%, recovery of the Trilogy reference samples: 96.1+/-4.12% at validation.

In the case of DON, usually 1000× sample dilution was applied while the standard 50× dilution was sufficient for ZEN determination, in Exp3, however, 5000 or 8000× dilution for DON and 100× dilution for ZEN was needed in some cases. The toxin results were statistically evaluated using linear mixed model, split into varieties (SPSS 16.0). Fixed effects were the CO_2 level and the type of *Fusarium* infection.

Results and Discussion

Disease progress and grain yield of the infected spikes at elevated CO_2 concentration

In general, the area under the disease progress curve exhibited little variation in response to elevated CO_2 (Table 1 and Table S1, Online Resource). In the single-floret inoculation, the severity of *F. culmorum* infection was not or very little affected by the atmospheric CO_2 concentration as AUDPC increased significantly due to elevated CO_2 only in one variety, Ukrainka (Exp2). Though there were some differences between the CO_2 levels in the number of infected spikelets at certain stages of the disease progress, the trends were similar to the AUDPC values in most varieties. This suggests that Type II resistance was little affected by the CO_2 concentration as the spread of *F. culmorum* was similar in four of the five varieties at both CO_2 levels.

In the whole-spike inoculation treatment, disease severity depended more on the conditions of the experiment as a decline in disease levels was only found in response to elevated CO_2 when the initial infection of the spikelets was lower (in Mv Regiment in Exp3). In Mv Emma, the penetration of *Fusarium* into the rachis was delayed at elevated CO_2 under the cooler (18ºC) conditions of Exp2 (Table S1, Online Resource). Infection was also poorer at the early stage (day11) in Apache at elevated CO_2 but the counteracting, slightly faster rate of *Fusarium* spread (which was only significant on day18; Table S1, Online Resource) resulted in no significant change in the overall infection level (AUDPC value) in response to elevated CO_2.

The change in the yield components in response to elevated CO_2 was usually more pronounced than the change in the infection scores (Table 1, Table S2, Online Resource). In the single-floret inoculation, either the grain number or the thousand kernel weight was significantly less in Mv Regiment in all the experiments at elevated CO_2, compared to the ambient level, despite the fact that there was no significant change in the disease symptoms. There was also a decline in one or more yield components of Ukrainka which also had higher infection level at elevated CO_2, Mv Mambo and, to a lesser extent, in Mv Emma.

In the whole-spike inoculation treatment, yield components of Mv Regiment did not change in response to elevated CO_2 even under less severe disease levels (Table 1, Table S2, Online Resource). The lower infection rate in Mv Emma in Exp2 resulted in larger spike grain number and thousand kernel weight at elevated CO_2, while there was a decrease in yield in Exp3 where, however, no apparent change in infection was observed. The grain number or thousand kernel weight was reduced at elevated CO_2 in Mv Mambo and Ukrainka, while Apache responded principally to elevated CO_2, slightly increasing its grain number, irrespective of the *Fusarium* treatment.

The above results suggest that *F. culmorum* infection usually had a more dramatic influence on the yield components at elevated CO_2 compared to the ambient level, even though in most cases there were only slight (or no) changes in the infection scores at elevated CO_2. This was in accordance with previous research, finding no close relation between infection severity and grain yield (Mesterházy 1995).

DON and ZEN concentrations of the grains in response to elevated CO_2 level

Due to the fact that only the grains of the infected spikes were analysed for toxin content, the DON concentration was very high in the grain samples (Fig.1-3). In the single-floret inoculation, DON content was usually considerably higher at elevated CO_2 than at the ambient level, despite the fact that infection severity seemed to be less influenced by the CO_2 level. This confirms the hypothesis that the grain toxin content is not in direct relation with the level of *Fusarium* infection as was suggested before (Paterson and Lima 2010, Vaughan et al. 2014). However, the highest rate of increase was recorded in Ukrainka, the only variety, which exhibited higher rate of infection at elevated CO_2. Similar increases in mycotoxin production due to elevated CO_2 were also found in wheat point-inoculated with different chemotypes of *F. graminearum* (Salgado et al. 2014).

In the whole-spike inoculation treatment, when *Fusarium* had to penetrate first, DON production decreased (Mv Regiment, Exp1) increased (Apache, Exp2, Mv Emma Exp3) or did not change at elevated CO_2, depending on the variety × experiment interaction (Table1, Fig. 1-3). DON content had, however, no association with the infection severity.

Grain ZEN content, compared to DON, was of minor importance in Exp1 and Exp2 with 18°C maximum temperatures (Fig.1-2). In certain cases, its values were even below detectable levels (0.500 ng g^{-1}). In the single-floret inoculation, ZEN increased significantly in response to elevated CO_2 in Mv Emma (Exp2), while in the whole-spike inoculation, there was a drop in ZEN content in Mv Regiment (Exp1) and Ukrainka (Exp2) due to CO_2 enrichment. In Exp3, where the maximum temperature was 20°C, ZEN production was significant and had greatly increased levels at elevated CO_2 in both Mv Regiment and Mv Emma in both *Fusarium* treatments (Fig.3).

In Exp3, DON and ZEN levels were considerably increased in the whole-spike inoculation treatment compared to single-floret inoculation, even at the ambient CO_2 level (Fig.3). This might be due to that initial infection was established in more than one spikelet simultaneously, starting infections at several points of the spike (Day7, Table S1, Online Resource).

DON production was reported to promote the spread of *F. graminearum* to neighbouring florets, compared to the trichothecene knockout mutant (Proctor et al. 1995). Previous research also pointed out that the production of trichothecens by *F. graminearum* in wheat inhibited the host defence process of cell wall fortifications against rachis penetration (Jansen et al 2005). As the production of protective compounds may be stimulated by elevated CO_2 (McElrone et al. 2005), the increase in mycotoxins might suggest a similar situation.

DON was also reported to promote H_2O_2 accumulation resulting in programmed host cell death (Desmond et al. 2008). This was confirmed by the present work as a considerably higher number of dead (uninfected) spikelets was found where the toxin content was increased in response to elevated CO_2 (Exp2, in all the varieties tested in the single-floret inoculation and in the variety Apache in the whole-spike inoculation, Table S3, Online Resource). Highly increased ZEN concentrations at elevated CO_2, however, could not be associated with the number of dead spikelets.

Plant resistance to *Fusarium* and mycotoxin production is a highly complex mechanism, including also antioxidant secondary metabolites, which can be toxic to the fungi, participate in cell wall reinforcement as well as interfere with toxin biosynthesis (Atanasova-Penichon et al. 2016). Fast activation of the antioxidant defence system was also matched with better resistance levels of plants (Khaledi et al. 2016, Spanic et al. 2017). Plant growth at elevated CO_2 was, however, reported to result in a low level of the antioxidant system in wheat (with very low activities of glutathione reductase, glutathione-S-transferase, guaiacol peroxidase and ascorbate peroxidase, Bencze et al. 2014). This feature might also have contributed to the weaker resistance of plants to DON production increased at elevated CO_2 in the case of single-floret inoculation (in the absence of Type I resistance).

Interaction of factors influencing DON and ZEN levels in the grain

The results presented here indicate that the reaction of plants to *Fusarium* infection at elevated CO_2 was not a single response, but the outcome of various interacting factors, similarly to previous findings on the impact of other environmental factors, such as e.g. temperature and air relative humidity (Kriss et al. 2012, Lemmens et al. 2004). In the present work, one factor affecting DON and ZEN toxin levels also proved to be the growth temperature, 20°C was more favourable for toxin production than 18°C, which was similar to what was found for *Fusarium langsethiae* and *F. sporotrichioides* in durum wheat (Nazari et al. 2014).
Not surprisingly, differences between the varieties were also observed in the present work, similarly to what was found in wheat in the case of *Fusarium pseudograminearum, F. culmorum and F. graminearum* (Melloy et al. 2010, Melloy 2015, Mesterházy et al. 2015, Lemmens et al. 2004). DON concentration tended to be the lowest of all the varieties (especially for whole-spike inoculation) in Apache, which did not have any detectable ZEN concentrations under any infection type or CO_2 level, in contrast with the other varieties (Fig. 2). This suggests that Apache has a certain level of resistance to *Fusarium* mycotoxin production.

In conclusion, the interaction between plants and pathogens is a rather complex area to which the present work provided a better view, taking into account, besides elevated CO_2, also the differences in environmental factors, which influence the start, process and result of *Fusarium culmorum* infection. Although the genotypic differences can be contrasting, the present findings indicate that the changing environmental factors, including rising temperature and atmospheric CO_2 level, may enhance the risk of conditions favouring *Fusarium* infection. An increase in mycotoxin contamination with rising CO_2 levels might also be expected under favourable conditions. More investigations are needed to reveal the underlying processes.

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Tables

Table 1. Change in the infection level, grain yield parameters, grain deoxynivalenol and zearalenone content in wheat varieties in response to elevated CO_2 level.

	Single floret inoculation							Whole-spike inoculation						
	AUDPC	No of infected spikelets*	Spike grain number	Spike grain yield (g)	TKW (g)	DON	ZEN	AUDPC	No of infected spikelets*	Spike grain number	Spike grain yield (g)	TKW (g)	DON	ZEN
Mv Regiment														
Exp1	NS	NS	↓	↓	NS	↑	NS	NS	NS	NS	NS	NS	↓	↓
Exp2	NS	↑D14	NS	NS	↓	↑	NS	-	-	-	-	-	-	-
Exp3	NS	↓D7	↓	NS	NS	NS	↑	↓	↓ D7-D30	NS	NS	NS	NS	↑
Mv Mambo														
Exp1	NS	↑ D16, D18	NS	↓	↓	-	-	NS	NS	↑	NS	↓	-	-
Mv Emma														
Exp2	NS	NS	↓	NS	NS	↑	↑	↓	↓ D11-D30	↑	↑	↑	NS	n.d
Exp3	NS	↓D9	NS	NS	NS	NS	↑	NS	NS	↓	↓	NS	↑	↑
Apache														
Exp2	NS	↑D18	↑	NS	NS	NS	n.d.	NS	↓D11	↑	NS	NS	↑	n.d
Ukrainka														
Exp2	↑	↑D11-D25	NS	↓	NS	↑	n.d.	NS	NS	↓	↓	NS	NS	↓

*only dates with significant differences are indicated.

Exp1-3= experiments 1-3, with 18, 18 and 20ºC maximum growth temperatures during infection, respectively. AUDPC= area under the disease progress curve, TKW= thousand kernel weight, deoxynivalenol (DON), zearalenone (ZEN), D7-D30= Days 7-30 after infection, NS= no significant change, ↑, ↓= significant increase or decrease in response to elevated CO_2 level ($p<0.05$), n.d= not detectable.

Figures

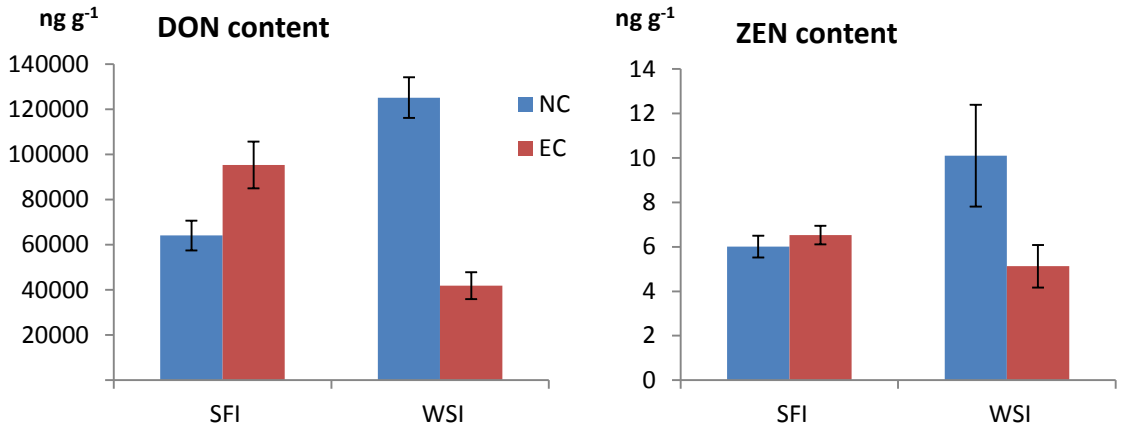

Fig. 1
Deoxynivalenol (DON) and zearalenone (ZEN) content of the grains of Mv Regiment, infected by *Fusarium culmorum*, at ambient and elevated CO_2 levels in Exp1.

Exp1= experiment 1, with 18ºC maximum growth temperature during infection. SFI= single floret inoculation, WSI= whole spike inoculation, NC= ambient (400 µmol mol^{-1}), EC= elevated (750 µmol mol^{-1}) CO_2 level.
The graphs show the means and the standard deviations (n=3).

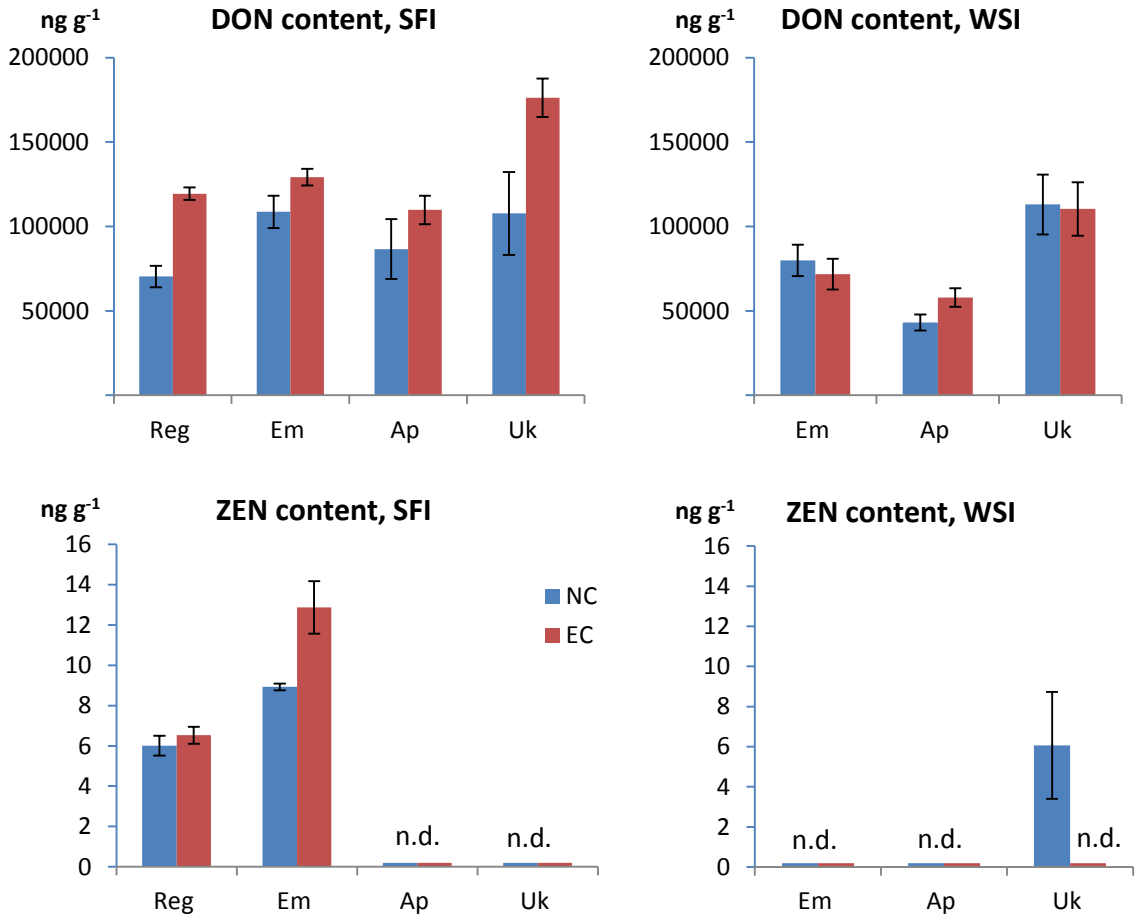

Fig. 2
Deoxynivalenol (DON) and zearalenone (ZEN) content of the grains of winter wheat varieties, infected by *F. culmorum*, at ambient and elevated CO_2 levels in Exp2

Exp2= experiment 2, with 18ºC maximum growth temperature during infection. Varieties: Reg= Mv Regiment, Em= Mv Emma, Ap= Apache, Uk= Ukrainka. SFI= single floret inoculation, WSI= whole spike inoculation, n.d.= not detectable – below the limit of detection (0.500 ng g^{-1}). NC= ambient (400 µmol mol^{-1}), EC= elevated (750 µmol mol^{-1}) CO_2 level.
The graphs show the means and the standard deviations (n=3)

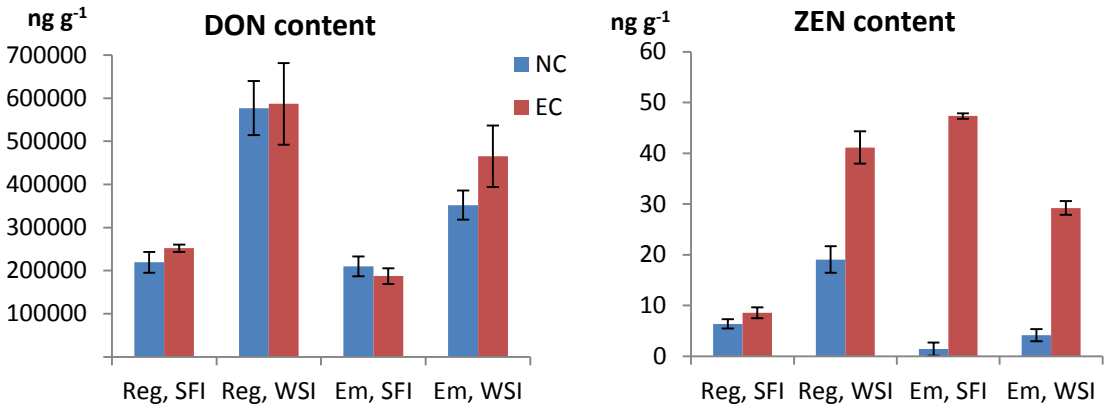

Fig. 3
Deoxynivalenol (DON) and zearalenone (ZEN) content of the grains of Mv Regiment and Mv Emma, infected by *F. culmorum*, at ambient and elevated CO_2 levels in Exp3.

Exp3= experiment 3, with 20ºC maximum growth temperature during infection. SFI= single-floret inoculation, WSI= whole-spike inoculation. Reg= Mv Regiment, Em= Mv Emma, NC= ambient (400 µmol mol^{-1}), EC= elevated (750 µmol mol^{-1}) CO_2 level. The graphs show the means and the standard deviations (n=3).

Electronic Supplementary Material

RISING ATMOSPHERIC CO_2 CONCENTRATION MAY IMPLY HIGHER RISK OF *FUSARIUM* MYCOTOXIN CONTAMINATION OF WHEAT GRAINS

Mycotoxin Research

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Table S1 (*Part 1*)

Spike *Fusarium* infection in Exp1-Exp3 at ambient and elevated CO_2 levels

			Mean number of infected spikelets							
Exp1	N	$AUDPC_{D25}$	D7	D11	D14	D16	D18	D21	D23	D25
Mv Regiment										
Single floret inoculation										
NC	16	102		3.38	4.56		5.56	7.31	8.13	8.81
EC	23	111		3.09	5.17		7.22	7.96	8.57	9.17
*Asymp. Sig. (2-tailed)**		*0.408*		*0.692*	*0.463*		*0.139*	*0.349*	*0.544*	*0.515*
Whole spike inoculation										
NC	21	204		8.62	10.05		11.40	12.38	12.24	12.95
EC	18	164		7.00	8.11		9.06	9.50	10.11	10.89
*Asymp. Sig. (2-tailed)**		*0.242*		*0.336*	*0.308*		*0.198*	*0.061*	*0.109*	*0.186*
Mv Mambo										
Single floret inoculation										
NC	18	44.81	1.72	1.83	1.89	2.11	2.28			2.78
EC	24	63.08	2.08	2.42	2.79	3.25	3.58			4.04
*Asymp. Sig. (2-tailed)**		*0.158*	*0.877*	*0.202*	*0.079*	***0.020***	***0.016***			*0.054*
Whole spike inoculation										
NC	21	129.64	4.33	5.38	6.10	6.57	7.10			8.45
EC	23	167.39	5.65	6.70	7.17	7.78	9.22			10.83
*Asymp. Sig. (2-tailed)**		*0.080*	*0.171*	*0.136*	*0.247*	*0.186*	*0.068*			*0.06*

* Mann-Whitney test. Values in bold represent significant differences at the $p \leq 0.05$ probability level

Exp1-3= experiments 1-3, with 18, 18 and 20ºC maximum growth temperatures during infection, respectively.

NC = ambient (400 µmol mol^{-1}), EC = elevated (750 µmol mol^{-1}) CO_2 level. N= number of samples, AUDPC= area under the disease progress curve calculated from the data till the day indicated. D7-D30= Days 7-30 after infection

Table S1 continued **(*Part 2*).** *For explanations see Table S1. Part1.*

			Mean number of infected spikelets											Day of rachis penetration
Exp2	N	$AUDPC_{D30}$	D7	D9	D11	D14	D16	D18	D21	D23	D25	D28	D30	
Mv Regiment														
Single floret inoculation														
NC	62	139	0.82	1.29	2.18	2.97	4.58	5.79	7.06	8.43	9.81	10.46	11.81	9.7
EC	61	147	0.69	1.33	2.46	3.84	4.77	5.90	7.46	8.89	10.05	11.26	11.95	9.3
*Asymp. Sig. (2-tailed)**		*0.540*	*0.127*	*0.961*	*0.384*	***0.040***	*0.682*	*0.869*	*0.561*	*0.575*	*0.893*	*0.468*	*0.593*	*0.304*
Mv Emma														
Single floret inoculation														
NC	59	178	1.05	2.37	3.97	5.19	6.03	7.32	8.90	9.66	11.27	12.78	13.41	10.34
EC	52	207	1.48	2.62	4.47	5.88	7.38	8.79	10.12	11.71	13.35	14.25	14.62	9.42
*Asymp. Sig. (2-tailed)**		*0.178*	*0.079*	*0.520*	*0.301*	*0.407*	*0.119*	*0.133*	*0.292*	*0.098*	*0.294*	*0.972*	*0.842*	*0.401*
Whole spike inoculation														
NC	47	246			3.77	7.77	9.47	11.26	12.53	13.83	14.36	16.60	17.09	13.24
EC	43	150			1.66	2.66	4.78	5.98	7.44	8.98	10.86	12.33	12.98	16.70
*Asymp. Sig. (2-tailed)**		***0.001***			***0.003***	***0.000***	***0.001***	***0.001***	***0.001***	***0.001***	***0.012***	***0.001***	***0.001***	***0.003***
Apache														
Single floret inoculation														
NC	45	173	0.87	1.84	2.89	3.96	5.18	6.73	8.42	10.27	12.44	13.71	14.31	10.03
EC	59	198	1.02	2.00	3.71	4.78	6.15	8.93	10.27	12.31	13.69	13.98	14.03	8.63
*Asymp. Sig. (2-tailed)**		*0.256*	*0.416*	*0.684*	*0.113*	*0.271*	*0.219*	***0.046***	*0.134*	*0.130*	*0.407*	*0.979*	*0.763*	*0.053*
Whole spike inoculation														
NC	33	173			3.45	5.42	6.64	7.03	8.06	8.88	9.91	11.76	12.39	15.96
EC	31	108			0.56	1.65	2.58	3.61	5.58	6.58	8.97	10.16	11.23	16.41
*Asymp. Sig. (2-tailed)**		*0.416*			***0.002***	*0.074*	*0.176*	*0.523*	*0.668*	*0.579*	*0.756*	*0.299*	*0.34*	*0.495*
Ukrainka														
Single floret inoculation														
NC	63	184	1.19	2.39	3.97	5.03	6.26	8.11	9.40	10.35	12.02	13.39	13.68	10.76
EC	53	247	1.49	3.32	5.41	7.48	9.25	10.92	12.08	13.87	15.98	16.38	16.47	8.23
*Asymp. Sig. (2-tailed)**		***0.007***	*0.221*	*0.056*	***0.013***	***0.001***	***0.001***	***0.007***	***0.037***	***0.011***	***0.005***	*0.097*	*0.160*	*0.244*
Whole spike inoculation														
NC	14	216			3.14	5.00	6.21	8.79	11.71	13.21	14.43	15.57	16.00	12.21
EC	32	169			2.16	4.25	5.88	7.50	8.94	10.00	10.84	12.22	13.25	14.55
*Asymp. Sig. (2-tailed)**		*0.107*			*0.563*	*0.514*	*0.782*	*0.374*	*0.105*	*0.08*	*0.07*	*0.09*	*0.13*	*0.571*

Table S1 continued **(*Part 3*).** *For explanations see Table S1. Part1.*

			Mean number of infected spikelets											Day of rachis penetration
Exp3	N	$AUDPC_{D30}$	D7	D9	D11	D14	D16	D18	D21	D23	D25	D28	D30	
Mv Regiment														
Single floret inoculation														
NC	44	170	1.52	2.64	3.55	4.80	6.07	7.34	8.59	9.82	10.50	11.05	11.07	7.50
EC	43	176	1.16	2.58	3.86	4.77	6.35	8.16	9.26	10.28	10.74	11.28	11.65	7.47
*Asymp. Sig. (2-tailed)**		*0.348*	***0.02***	*0.864*	*0.406*	*0.797*	*0.231*	*0.053*	*0.148*	*0.400*	*0.710*	*0.598*	*0.261*	*0.798*
Whole spike inoculation														
NC	39	306	7.18	8.79	10.03	10.77	11.82	12.87	13.28	13.59	13.87	13.87	13.87	9.41
EC	48	252	5.10	6.79	7.73	8.58	9.98	11.15	11.77	12.31	12.60	12.75	12.77	9.81
*Asymp. Sig. (2-tailed)**		***0.001***	***0.005***	***0.010***	***0.003***	***0.004***	***0.010***	***0.006***	***0.014***	***0.025***	***0.019***	***0.035***	***0.040***	*0.302*
Mv Emma														
Single floret inoculation														
NC	40	199	1.38	2.33	3.38	4.85	5.88	7.55	9.25	11.45	13.80	15.65	16.60	8.13
EC	45	183	1.11	1.73	2.78	4.13	5.18	6.49	8.44	10.49	12.00	13.73	15.89	9.36
*Asymp. Sig. (2-tailed**		*0.275*	*0.138*	***0.049***	*0.187*	*0.158*	*0.352*	*0.270*	*0.632*	*0.605*	*0.175*	*0.127*	*0.536*	***0.049***
Whole spike inoculation														
NC	44	305	4.48	6.66	9.07	11.86	12.86	13.66	14.39	14.93	15.45	15.93	15.93	8.66
EC	43	309	4.88	7.53	9.53	11.56	12.79	13.67	14.65	15.47	15.70	15.81	15.93	8.67
*Asymp. Sig. (2-tailed)**		*0.990*	*0.824*	*0.316*	*0.615*	*0.841*	*0.782*	*0.789*	*0.908*	*0.815*	*0.990*	*0.559*	*0.664*	*0.803*

Table S2
Yield parameters of the infected spikes of wheat varieties

	Single floret inoculation					Whole spike inoculation				
	N	spike weight (g)	grain number	grain yield (g)	TKW (g)	N	spike weight (g)	grain number	grain yield (g)	TKW (g)
Exp1										
Mv Regiment										
NC	16	1.07	32.63	0.65	21.71	21	0.69	24.05	0.28	10.28
EC	23	0.75	23.39	0.35	15.33	18	0.83	22.44	0.42	17.19
*Asymp. Sig. (2-tailed)**		***0.007***	***0.000***	***0.012***	*0.145*		*0.464*	*0.554*	*0.397*	*0.172*
Mv Mambo										
NC	17	1.14	19.06	0.70	37.27	21	0.64	18.33	0.28	14.58
EC	21	0.75	18.10	0.33	19.69	23	0.47	22.43	0.14	6.79
*Asymp. Sig. (2-tailed)**		***0.013***	*0.332*	***0.038***	***0.013***		***0.048***	***0.021***	*0.072*	***0.028***
Exp2										
Mv Regiment										
NC	62	1.39	32.29	0.90	25.60					
EC	61	1.35	34.44	0.76	21.09					
*Asymp. Sig. (2-tailed)**		*0.905*	*0.265*	*0.246*	***0.046***					
Mv Emma										
NC	59	1.40	37.58	0.88	23.49	46	1.10	27.98	0.59	18.91
EC	52	1.49	32.29	0.83	23.88	42	1.70	42.35	1.08	24.56
*Asymp. Sig. (2-tailed)**		*0.525*	***0.013***	*0.279*	*0.795*		***0.000***	***0.000***	***0.000***	***0.013***
Apache										
NC	45	1.43	31.71	0.88	26.39	33	1.45	30.00	0.94	28.41
EC	58	1.34	36.25	0.85	22.67	31	1.59	37.23	1.10	28.66
*Asymp. Sig. (2-tailed)**		*0.371*	***0.013***	*0.806*	*0.091*		*0.234*	***0.016***	*0.221*	*0.941*
Ukrainka										
NC	63	1.15	32.43	0.65	18.42	14	0.94	31.71	0.47	14.18
EC	53	1.05	28.36	0.48	15.36	32	1.23	40.19	0.74	17.79
*Asymp. Sig. (2-tailed)**		*0.233*	*0.060*	***0.020***	*0.102*		*0.052*	***0.007***	***0.050***	*0.233*
Exp3										
Mv Regiment										
NC	44	1.17	34.11	0.70	20.20	39	0.76	28.13	0.25	9.66
EC	43	1.11	29.60	0.64	20.49	15	0.75	29.80	0.30	9.72
*Asymp. Sig. (2-tailed)**		*0.312*	***0.024***	*0.310*	*0.909*		*0.369*	*0.908*	*0.954*	*0.602*
Mv Emma										
NC	40	1.73	48.55	1.11	21.73	44	1.01	42.39	0.44	10.07
EC	45	1.62	44.73	1.09	24.08	42	0.80	35.50	0.31	8.13
*Asymp. Sig. (2-tailed)**		*0.309*	*0.124*	*0.751*	*0.666*		***0.001***	***0.001***	***0.006***	*0.151*

* Mann-Whitney test. Values in bold represent significant differences at the $p \leq 0.05$ probability level

Exp1-3= experiments 1-3, with 18, 18 and 20ºC maximum growth temperatures during infection, respectively. NC, = ambient (400 µmol mol^{-1}), EC = elevated (750 µmol mol^{-1}) CO_2 level. N= number of samples, TKW= thousand kernel weight

Table S3 ***(Part 1)***

Number of dead spikelets in wheat varieties infected by *Fusarium culmorum* at ambient and elevated CO_2 levels, Exp1-Exp3

		Mean number of dead spikelets						
Exp1	N	D11	D14	D16	D18	D21	D23	D25
Mv Regiment								
Single floret inoculation								
NC	16	1.81	3.13	3.69	3.44	3.75	3.63	4.13
EC	23	1.48	2.96	3.57	4.13	4.61	4.48	4.74
*Asymp. Sig. (2-tailed) **		*0.588*	*0.964*	*0.824*	*0.221*	*0.111*	*0.163*	*0.180*
Whole-spike inoculation								
NC	21	0.33	1.95	2.24	2.48	2.76	2.71	2.71
EC	18	0.11	0.94	1.28	1.39	1.56	2.17	2.06
*Asymp. Sig. (2-tailed) **		*0.604*	*0.176*	*0.181*	*0.127*	*0.180*	*0.729*	*0.624*
Mv Mambo								
Single floret inoculation								
NC	18	0.56	0.61	0.94	0.89			1.39
EC	24	0.63	0.83	1.29	1.46			1.92
*Asymp. Sig. (2-tailed) **		*0.781*	*0.607*	*0.622*	*0.389*			*0.494*
Whole-spike inoculation								
NC	21	0.14	0.19	0.24	0.50			0.30
EC	23	0.09	0.13	0.83	1.70			2.57
*Asymp. Sig. (2-tailed) **		*0.648*	*0.383*	*0.375*	*0.058*			***0.002***

* Mann-Whitney test. Values in bold represent significant differences at the $p \leq 0.05$ probability level

Exp1-3= experiments 1-3, with 18, 18 and 20ºC maximum growth temperatures during infection, respectively. NC = ambient (400 μmol mol^{-1}), EC = elevated (750 μmol mol^{-1}) CO_2 level. N= number of samples, D7-D30= days 7-30 after infection

Table S3 continued. ***(Part 2).*** *For explanations see Table S3. Part1.*

		Mean number of dead spikelets								
Exp2	N	D11	D14	D16	D18	D21	D23	D25	D28	D30
Mv Regiment										
Single floret inoculation										
NC	62	0.14	0.68	1.39	2.18	2.48	2.49	2.70	2.82	2.82
EC	61	0.43	1.23	1.89	2.13	2.49	2.84	2.92	3.07	3.18
*Asymp. Sig. (2-tailed)**		*0.104*	***0.042***	*0.133*	*0.777*	*0.753*	*0.129*	*0.215*	*0.114*	***0.039***
Mv Emma										
Single floret inoculation										
NC	59	0.76	1.27	1.47	1.71	1.75	1.95	2.15	2.15	2.17
EC	52	1.69	1.83	2.48	2.69	2.83	2.92	3.15	3.25	3.25
*Asymp. Sig. (2-tailed)**		***0.007***	*0.055*	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
Whole-spike inoculation										
NC	47	0.02	0.36	0.64	1.32	2.47	2.89	3.09	3.09	3.11
EC	43	0.05	0.16	0.77	2.21	2.44	2.84	3.72	4.07	4.16
*Asymp. Sig. (2-tailed)**		*0.937*	*0.289*	*0.715*	*0.217*	*0.852*	*0.847*	*0.369*	*0.115*	*0.078*
Apache										
Single floret inoculation										
NC	45	0.22	0.71	1.24	1.53	1.89	2.07	2.13	2.27	2.38
EC	59	1.19	1.61	1.64	2.00	2.49	2.42	2.51	2.51	2.51
*Asymp. Sig. (2-tailed)**		***0.000***	***0.003***	*0.172*	*0.111*	*0.059*	*0.171*	***0.038***	*0.124*	*0.288*
Whole-spike inoculation										
NC	33	0.06	0.33	0.42	0.88	1.06	0.94	1.55	2.45	2.45
EC	31	0.16	0.19	0.65	0.68	1.03	2.00	2.42	2.94	2.94
*Asymp. Sig. (2-tailed)**		*0.521*	*0.345*	*0.595*	*0.815*	*0.723*	***0.017***	***0.040***	*0.237*	*0.237*
Ukrainka										
Single floret inoculation										
NC	63	0.92	1.63	1.76	1.89	1.90	2.05	2.13	2.52	2.61
EC	53	1.68	2.11	2.38	2.34	2.57	2.75	2.79	2.79	2.77
*Asymp. Sig. (2-tailed)**		***0.006***	*0.119*	***0.032***	*0.174*	***0.023***	***0.009***	***0.010***	*0.403*	*0.751*
Whole-spike inoculation										
NC	14	0.36	1.36	2.00	2.36	3.21	3.93	4.21	4.64	4.64
EC	32	0.28	0.75	0.97	2.50	3.00	3.22	3.28	3.53	3.66
*Asymp. Sig. (2-tailed)**		*0.985*	*0.336*	*0.119*	*0.960*	*0.734*	*0.312*	*0.170*	*0.115*	*0.145*

Table S3 continued. ***(Part 3).*** *For explanations see Table S3. Part1.*

		Mean number of dead spikelets								
Exp3	N	D11	D14	D16	D18	D21	D23	D25	D28	D30
Mv Regiment										
Single floret inoculation										
NC	44	2.41	2.80	3.93	4.18	4.27	4.30	4.30	4.36	4.36
EC	43	2.77	2.81	3.51	3.81	3.72	3.95	4.26	4.47	4.60
*Asymp. Sig. (2-tailed)**		*0.439*	*0.979*	*0.255*	*0.078*	***0.043***	*0.129*	*0.617*	*0.942*	*0.462*
Whole-spike inoculation										
NC	39	1.77	2.15	2.41	3.67	3.85	3.92	3.85	3.97	4.03
EC	48	1.38	2.31	3.56	3.96	4.06	4.40	4.75	4.94	4.96
*Asymp. Sig. (2-tailed)**		*0.056*	*0.824*	***0.017***	*0.532*	*0.510*	*0.287*	***0.046***	***0.039***	***0.046***
Mv Emma										
Single floret inoculation										
NC	40	1.83	3.50	3.83	4.10	4.33	4.25	4.30	4.43	4.53
EC	45	2.04	3.07	3.42	3.84	4.04	4.11	4.47	4.56	4.62
*Asymp. Sig. (2-tailed)**		*0.690*	*0.235*	*0.326*	*0.372*	*0.810*	*0.845*	*0.331*	*0.277*	*0.358*
Whole-spike inoculation										
NC	44	1.50	3.39	6.34	6.50	7.09	7.14	7.11	7.11	7.20
EC	43	1.42	3.60	4.63	6.23	7.56	7.47	7.58	7.65	7.65
*Asymp. Sig. (2-tailed)**		*0.760*	*0.478*	***0.046***	*0.782*	*0.418*	*0.600*	*0.466*	*0.382*	*0.442*