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Management of *Fusarium* Head Blight of Wheat and Deoxynivalenol Accumulation Using Antagonistic Microorganisms

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Abstract: Laboratory and green house studies were conducted at the Faculty of Agriculture, University of Nairobi, to evaluate the efficacy of *Epicoccum* sp., *Alternaria* sp., *Trichoderma* sp. and *Bacillus* sp. in control of *Fusarium* head blight of wheat caused by *F. graminearum*. Fungicides folicur® and copper oxychloride were used as standard checks. Laboratory assay was carried out by paired cultures and antagonism was measured as reduction in pathogen colony diameter. Green house experiments involved dual inoculation of pathogen and antagonist onto wheat ears and head blight severity and grain yield determined. Doxynivalenol content in the resulting grain was determined by competitive direct ELISA. The antagonists and fungicides significantly reduced the growth of *Fusarium graminearum* colonies in culture. Folicur® and copper oxychloride completely inhibited the growth of the pathogen while *Trichoderma* sp. showed 64% colony growth reduction. However, the antagonists showed limited reduction in head blight severity in green house trials. *Trichoderma* sp. reduced head blight severity by 18% while folicur® reduced the disease by 28%. All the antagonists had little or no significant effect on grain yield. Only folicur®, copper oxychloride and *Alternaria* sp. reduced DON in grain by 76 to 93%. Obtained results indicate that microbial antagonists may offer potential benefit in FHB management and screening of more antagonists both under controlled and field conditions is necessary.

Key words: Antagonists, *Fusarium* head blight, fungicides, wheat

INTRODUCTION

Fusarium Head Blight (FHB) is a serious disease of small grain cereals and has caused severe and repeated epidemics resulting in enormous losses (Bateman, 2005; Kolombet *et al*., 2005; Brennan *et al*., 2007). In addition to grain yield reduction, FHB can result in the reduction of grain quality, either by affecting grain processing qualities or by producing a range of toxic metabolites that have adverse effects on humans and livestock (Dohlman, 2004; Grosjean and Barrier-Guillot, 2004; Goyarts *et al*., 2007). *Fusarium graminearum,* one of the major causal organisms of FHB, produces mycotoxin Deoxynivalenol (DON), which may accumulate to unacceptable levels in harvested grain (Demeke *et al*., 2005; Paul *et al*., 2005; Browne, 2007). Levels of DON above 2 ppm may render grain and their by-products unfit for commercialization and consumption.

Efforts to minimize the impact of FHB and DON have been centred on the use of management strategies such as crop rotation, host plant resistance, tillage and fungicides application (Heier *et al*., 2005; Wisniewska and Kowalczyk, 2005; Kriel, 2006; Browne, 2007). An integrated approach to management of FHB that includes chemical, cultural and host plant resistance seems the most logical way to reduce losses (Pirgozliev *et al*., 2003; Pereyra and Dill-Macky, 2004). Several studies on chemical control of FHB have been reported but conflicting evidence exists regarding the effect of fungicides on the development of FHB and accumulation of trichothecene mycotoxins in grain (Halley *et al*., 2005; Ramirez *et al*., 2004). Fungicides have been found to affect DON concentrations indirectly by influencing the inoculum of *Fusarium* species in the grain (Suty *et al*., 1996; Pirgozliev *et al*., 2003). Fungicide application has been reported to reduce FHB severity without significant reduction of DON (Edwards *et al*., 2001). However, Draper *et al*. (2005) found no effect on both DON and the disease severity after using folicur® and metconazole. Ramirez *et al*. (2004) tested four azoles and one strobulurin and found that while all of them reduced *F. graminearum* growth *in vitro*, there was reduced efficacy on the same pathogen when applied in the field. The fungicides had a varied effect on production and accumulation of DON with some resulting in stimulation of DON production.

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All labelled systemic fungicides appear to increase yield, but those that contain a triazole, instead of a strobilurin active ingredient are more effective in reducing mycotoxin (DON) levels in infected grain (Hollingsworth, 2004; Ramirez *et al*., 2004; Müllenborn *et al*., 2007). However, use of fungicides on wheat ears has the disadvantage of accumulation of residues in the resulting grain. The use of biological control would lead to reduction, if not elimination of the possible chemical residues in grain, environmental pollution and potential hazards to humans. Therefore, this study was carried out with the objective of evaluating the efficacy of fungal and bacterial antagonists in management of FHB caused by *F. graminearum*.

MATERIALS AND METHODS

Laboratory and green house studies were conducted in 2006 at the Faculty of Agriculture, University of Nairobi, to evaluate the efficacy of *Epicoccum* sp., *Alternaria* sp., *Trichoderma* sp. and *Bacillus* sp. to control *Fusarium* head blight of wheat caused by *F. graminearum*.

Isolation and multiplication of pathogen and antagonists: Isolates of *Fusarium graminearum*, *Epicoccum*, *Alternaria* and *Trichoderma* sp. were isolated from wheat kernels by plating on low strength PDA amended with mineral salts and antibiotics (Muthomi, 2001; PDA 17 g, KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4$ 0.5 g and Agar 10 g). The fungi were identified based on cultural and morphological characteristics like colony colour, pigment production, presence of aerial mycelium in addition to morphological characteristics like conidia shape, septation and conidiophores. *Bacillus* sp. was also isolated from wheat seeds plated on Nutrient Agar (NA) and identified based on cultural characteristics.

Inoculum of three highly pathogenic isolates of *F. graminearum* was produced in mung bean broth (Bai and Shaner, 1994). Mung bean (40 g) was cooked in 1000 mL of water for 10 min and the extract was filtered through double layer cheesecloth, the volume completed and the extract sterilized. The sterilized mung bean extract was inoculated with mycelial agar discs cut from 14 day old pathogen cultures and placed on a mechanical shaker (50-70 cycles min^{-1}) for 4 days followed by 10 day incubation under stationary conditions. *Epicoccum*, *Alternaria* and *Trichoderma* sp. were raised on PDA for 14 days at 25°C in cycles of 12 h daylight and 12 h darkness while *Bacillus* sp. was grown on nutrient agar for 2 days. Pathogen inoculum was harvested by passing the liquid culture through double layer cheesecloth while that of the antagonists was by flooding the cultures with distilled water and passing the solution through a double layer of cheese cloth. The fungal inoculum was adjusted to 1×10^5 spores mL^{-1} using a haemocytometer while *Bacillus* sp. inoculum was adjusted to about 1×10^4 cfu mL^{-1}.

Determination of efficacy of antagonists to suppress growth of *F. graminearum* in culture: Antagonism was determined by paired cultures method, where the pathogen agar disc was inoculated at the middle of plate and the antagonist at 4 equidistant points located 2 cm from plate edge. Each of the antagonists *Epicoccum*, *Alternaria*, *Trichoderma* and *Bacillus* sp. was tested separately. Fungicides folicur® (1000 ppm) and copper oxychloride (1000 ppm) were used as standard checks while negative control consisted of *F. graminearum* cultured alone. Each treatment was replicated four times and the plates arranged in a completely randomized design on laboratory benches and incubated at 25°C for 7 days in cycles of 12 h daylight and 12 h darkness. Degree of antagonism was determined by measuring the pathogen colony diameters and percentage inhibition calculated:

$$\text{Inhibition (\%)} = \text{Colony diameter of pathogen alone(control)} - \frac{\text{Colony diameter of pathogen + Antagonist}}{\text{Colony diameter of pathogen alone}} \times 100$$

Efficacy of antagonists to reduce FHB under greenhouse conditions: Highly susceptible wheat variety Mbuni, was planted in 22 cm diameter pots in greenhouse following recommended fertilization regimes. Inoculation was done at 50% flowering (GS 65; Zadoks *et al*., 1974) and treatments consisted of inoculation of ears with antagonist together with *F. graminearum*, *F. graminearum* together with fungicide, antagonist alone and *F. graminearum* alone. The antagonists tested were *Epicoccum*, *Alternaria*, *Trichoderma* and *Bacillus* sp. Folicur®, a systemic fungicide and copper oxychloride, a contact fungicide, were used as standard checks at the rate of 1.5 and 6 g L^{-1} of water, respectively. Control plants were sprayed with sterile distilled water. The antagonists and the fungicides were sprayed two days before and after inoculation with *F. graminearum*. Inoculation with *F. graminearum* was repeated 5 days after the first inoculation. Each treatment was replicated four times and arranged in a randomised complete block design. The treated heads were covered with polythene bags for 48 h to maintain humidity conducive for infection.

Head blight severity was assessed five days after the last inoculation and after every 5 days thereafter until ripening (GS 87). Proportion of ear bleached was determined based on a 1-9 scale, where 1% = no symptoms, 2 = <5%, 3 = 5-15%, 4 = 16-25%, 5 = 26-45%, 6 = 46-65%, 7 = 66-85%, 8 = 86-95%, 9 = 96-100% of spikelet bleached. Assessment was done on ten average-sized ears per pot. Mean disease severity and the area under disease progress curve (AUDPC; Shaner and Finney, 1977) were calculated from single ratings recorded over the assessment period. At maturity (GS 95) the ten heads assessed for disease were harvested and threshed separately for weight determination. The kernels harvested (100 kernels per treatment) were plated on culture media for re- isolation of the causal pathogen. The experiment was conducted over two greenhouse cycles.

Analysis of Deoxynivalenol (DON) content: Deoxynivalenol in the grain from greenhouse experiments was analyzed by direct competitive Enzyme-Linked Immunosorbent Assay (ELISA; Gathumbi *et al*., 2001). Each sample was homogenized and 100 g ground to fine powder. Five grams of the ground sample was extracted with 25 mL of methanol:water (50:50 v/v). The extract was de-fatted with 10 mL hexane and 4 mL of the methanolic layer taken and diluted to 10% using Phosphate Buffer Solution (PBS). Microtitre polystyrene plates were coated with 100 μL of anti-deoxynivalenol antiserum DON143/16 (Usleber *et al*., 1992) in bicarbonate buffer (pH 9.6) per well. Absorbance was determined using spectrophotometer Elisa reader at 450 nm wavelength. A calibration curve for the standard dilutions was plotted using $\log_{10}$ of standard concentration against the percentage inhibition of the standard.

Data analysis: All data was subjected to Analysis of Variance (ANOVA) using the PROC ANOVA procedure of Genstat (Lawes Agricultural Trust Rothamsted Experimental station 2006, Version 9) and differences among the treatment means were compared using the Fisher's protected LSD test at 5% probability level.

RESULTS

All the antagonists and fungicides tested significantly ($p \leq 0.05$) reduced colony diameters of *F. graminearum* (Table 1). Folicur® and copper oxychloride completely inhibited the growth of *F. graminearum in vitro*. The highest colony diameter reduction (64%) was observed in the treatment with *Trichoderma* sp. while the least reduction (45%). was observed in paired cultures with *Epicoccum* sp.

Table 1: Colony diameter (cm) and percentage reduction in colony diameter of *F. graminearum* in paired cultures

	Experiment 1		Experiment 2	
Treatments	Colony diameter	Reduction (%)	Colony diameter	Reduction (%)
Fusarium+Alternaria	1.7	55.0	1.8	48.0
Fusarium+Bacillus	1.9	51.0	1.6	53.0
Fusarium+Epiccocum	2.0	49.0	1.9	45.0
Fusarium+Folicur	0.0	100.0	0.0	100.0
Fusarium+Trichoderma	1.4	64.0	1.2	65.0
Fusarium+Copper	0.0	100.0	0.0	100.0
Control	3.8	0.0	3.4	0.0
Mean	1.4	60.0	1.4	59.0
LSD ($p \leq 0.05$)	0.6		0.4	
CV (%)	21.6		14.4	

LSD: Least Significant Difference; CV: Coefficient of Variation

Table 2: Severity percentage of FHB over time, mean severity, AUDPC and re-isolation rate for the plants inoculated with *F. graminearum* and the respective antagonists

	Days after inoculation						
Treatments	5	10	15	20	Mean	AUDPC	Re-isolation (%)
Fusarium+ *Alternaria*	49.9	73.5	84.7	95.9	76.0	1153.5	48.0
Fusarium+ *Epicoccum*	46.8	68.6	81.1	92.6	72.3	1096.5	50.0
Fusarium+ *Trichoderma*	34.2	61.3	68.2	75.1	59.7	948.5	58.5
Fusarium+ *Bacillus*	55.4	77.3	90.1	99.0	80.5	1221.5	73.5
Fusarium+ Folicur	32.9	38.7	38.8	49.1	39.9	539.5	41.5
Fusarium+ Copper	18.8	35.9	73.5	83.2	52.9	904.5	41.5
Alternaria	12.4	15.8	23.3	33.1	21.2	321.5	24.5
Epicoccum	12.6	12.7	18.8	27.2	17.4	274.5	24.5
Trichoderma	13.4	15.7	21.8	31.3	20.6	301.0	30.5
Bacillus	11.6	14.0	18.6	26.5	17.7	258.0	24.5
Fusarium	52.6	74.2	87.9	96.9	77.9	1185.5	83.0
Control	12.0	12.7	18.5	26.7	17.5	248.5	26.5
LSD ($p \leq 0.05$)	7.0	16.0	10.0	19.0	9.5	94.1	31.0
CV (%)	14.0	25.5	12.5	19.5			23.0

AUDPC: Area Under Disease Progress Curve; LSD: Least Significant Difference; CV: Coefficient of Variation

Head blight severity and the Area Under Disease Progress Curve (AUDPC) were significantly different ($p \leq 0.05$) among the antagonists (Table 2). Folicur® reduced the disease severity by up to 47% while copper oxychloride reduced disease severity by up to 36%. Among the antagonists, *Trichoderma* sp. was the most effective with a significant reduction of FHB of up to 25%. *Epicoccum* and *Alternaria* sp. had minimal effect on FHB and *Bacillus* sp. had no significant effect on head blight severity. Minimal amounts of disease were observed on plants inoculated with antagonist alone and the control. *Fusarium graminearum* was re-isolated at very high levels from kernels harvested from ears inoculated with the pathogen alone but the re-isolation rate differed for the kernels from ears inoculated with different antagonists.

Table 3: Ten-ear weight (g) and weight per pot (g) for plants treated with *F. graminearum* and respective antagonists

	10 ear kernel weight		Kernel weight per pot	
Treatments	Weight (g)	Reduction (%)	Weight (g)	Reduction (%)
Fusarium+Alternaria	9.3	41.1	18.2	44.7
Fusarium+Epiccocum	10.1	37.4	18.6	43.5
Fusarium+Trichoderma	10.2	36.7	19.4	41.0
Fusarium+Bacillus	9.5	40.8	18.2	44.7
Fusarium+Folicur	14.6	9.1	26.5	19.5
Fusarium+Copper	13.3	17.3	24.3	26.1
Alternaria	16.2	0.0	30.7	6.7
Epicoccum	15.2	5.7	30.8	6.4
Trichoderma	15.4	4.3	29.5	10.3
Bacillus	16.3	0.0	32.0	2.7
Fusarium	9.6	40.3	17.2	47.7
Control	16.1		32.9	
LSD ($p \leq 0.05$)	2.1		5.60	
CV (%)	11.4		20.4	

LSD: Least Significant Difference; CV: Coefficient of Variation

Table 4: Deoxynivalenol (DON) content ($\mu g\ kg^{-1}$) of wheat grain harvested from ears inoculated with different antagonistic microorganisms

Treatments	Trial 1	Trial 2	Mean	Reduction (%)
Fusarium+Alternaria	75.0	145.0	125.0	91.4
Fusarium+Epiccocum	1,500.0	900.0	1,250.0	15.5
Fusarium+Trichoderma	1,200.0	5,200.0	3,100.0	+113.8
Fusarium+Bacillus	900.0	4,000.0	2,450.0	+69.0
Fusarium+Folicur	0.0	200.0	100.0	93.1
Fusarium+Copper	425.0	250.0	337.5	76.7
Alternaria	50.0	250.0	125.0	91.4
Epicoccum	50.0	400.0	225.0	84.5
Trichoderma	50.0	300.0	175.0	87.9
Control (uninoculated)	59.0	350.0	204.0	85.9
Fusarium alone	1,500.0	1,400.0	1,450.0	-
Mean	528.1	1,336.0		
LSD ($p \leq 0.05$)	1,118.0	1,313.9		
CV (%)	88.5	44.7		

LSD: Least Significant Difference; CV: Coefficient of Variation

Table 5: Correlation coefficients ($p<0.05$) among disease severity, Area Under Disease Progress Curve (AUDPC), grain weight and Deoxynivalenol (DON) content

	Disease severity	AUDPC	10 ear weight	Ear weight/pot
AUDPC	0.97**			
10 ear weight	−0.89**	−0.86**		
Ear weight / pot	−0.86**	−0.86**	0.80**	
DON	0.64*	0.67	−0.61	−0.45

*: Indicates significant correlation; **: Indicates highly significant correlation at $p \leq 0.05$

The antagonists had little or no significant effect on grain weight (Table 3). However, folicur® and copper oxychloride significantly ($p \leq 0.05$) increased grain weight by between 47 and 94%, respectively, compared to ears inoculated with *F. graminearum* alone. Folicur®, copper oxychloride and *Alternaria* sp. reduced DON content in the grain by between 76 and 93% but *Trichoderma* and *Bacillus* sp. increased DON compared to treatments with *F. graminearum* alone (Table 4). Head blight severity, kernel weight and DON content in grain were significantly ($p \leq 0.05$) correlated (Table 5).

DISCUSSION

All the antagonists inhibited the growth of *F. graminearum* in culture, indicating a possible release of extracellular volatile metabolites that diffused through the media. However, folicur® and copper oxychloride were the most effective, inhibiting the growth of the pathogen completely. In the two greenhouse trials, the fungicides reduced the disease severity by between 28-58%, although no complete control was found, therefore confirming earlier findings (Chala *et al*., 2003; Müllenborn *et al*., 2007). The low disease levels observed on plants inoculated with antagonist alone and the control could have been caused by pathogen conidia spread by wind from the inoculated ears. Among the antagonists, *Trichoderma* sp. reduced disease severity and this is in agreement with the finding by Perello *et al*. (2002), Lutz *et al*. (2003) and Müllenborn *et al*. (2007). Perello *et al*. (2002) reported that among the antagonists tested, *Bacillus* sp. was the one with the highest inhibitory effect to pathogens in culture. *Epicoccum purpurascens* (*E. nigrum*) produces antifungal compounds, which may increase its effectiveness (Brown *et al*., 1987). This has also been reported to be true for *Trichoderma* and *Bacillus* sp. (Schunmacher *et al*., 2007; Seddon, 2007). Production of antifungal secondary metabolites by *Trichiderma atroviride* can induce resistance of plants against infection by pathogenic microorganisms.

Application of folicur® and copper oxychloride led to an increase in yield thus confirming earlier findings by Pirgozliev *et al*. (2003) and Mesterhazy *et al*. (2003). The effect of fungicides on *Fusarium* sp. is dependent on timing and frequency of applications (Parry *et al*., 1995) and treatments at anthesis seems to be the best time for reduction of *Fusarium* infection (Mesterhazy *et al*., 2003; Hollingsworth, 2004). In the current study, *Trichoderma* sp. was found to reduce both disease severity and slightly increase the grain yield. This shows that biological control has considerable promise for reducing FHB as has been reported by Kolombet *et al*. (2005) and Seddon (2007). Bateman (1979) demonstrated biological control of *Microdochium nivale* with *Sporobolomyces* sp. which significantly reduced grain contamination. Rodemann (2007) reported a 40 and 30% reduction of FHB severity and DON, respectively on wheat treated with the antagonist *Plectosporium tabacinum* at anthesis under greenhouse conditions. Inoculations before anthesis with *Cladosporium* sp. were effective when applied before *M. nivale*, while *Alternaria* sp. were effective whether applied before or after. *Pseudomonas fluorescens* biov1,

B. subtilis and *Streptomyces* sp. were found to be antagonistic to *F. graminearum*. *Fusarium graminearum* mycelial growth was reduced by cell free and volatile metabolites of the bacterial antagonists by 37-97%. However, Draper *et al.* (2005) and Jochum *et al.* (2004) found no yield benefits when using *Lysobacter enzymogenes*, *Bacillus* sp. and *Pseudomonas fluorescens* alone but dual application with folicur® led to an increase in test kernel weight. A recent trial by Bacon and Hinton (2007) using *Bacillus mojavensis* showed that several strains of the bacterium were antagonistic to *F. graminearum* and *F. verticillioides in vitro* and *in vivo*. *Fusarium* is capable of suppressing the expression of the genes that control the antagonistic activity of *Trichoderma harzianum* but not that of *Pseudomonas fluorescens* (Brion, 2001; Lutz *et al*., 2003). A recent study by Seddon (2007) reported susceptibility of *Fusarium* sp. to *Bacillus brevis*.

The antagonist effect could be explained by two mechanisms. Firstly, through production of antifungal metabolites which inhibits conidial germination and subsequent growth of the pathogen (Brunner *et al*., 2003; Seddon, 2007), Secondly, by indirect action through production of a biosurfactant, which modifies the plant surface reducing surface wetness. From the results obtained in this study, it is clear that all the antagonists reduced the growth of *F. graminearum* in culture indicating that they produced antifungal compounds. The results also indicate that three antagonists reduced FHB severity although not very pronounced. Testing of a broader spectrum of other possible antagonists under field conditions and determination of their mechanisms of action would be necessary.

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