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Effect of antagonistic microorganisms on severity of *Fusarium* head blight of wheat and grain yield

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Abstract: Laboratory and green house studies were conducted to evaluate the efficacy of *Epicoccum* sp, *Alternaria* sp., *Trichoderma* sp., and *Bacillus* sp. in control of *Fusarium* head blight of wheat. Fungicides folicur® and copper oxychloride were used as standard checks. Laboratory assay was carried out by paired cultures where, a pathogenic isolate of *Fusarium. graminearum* was grown together with antagonist. Antagonism was measured as reduction in pathogen colony diameter. Green house experiments involved co-inoculation of pathogen and antagonist onto wheat ears and data collected included head blight severity and grain yield. Area under disease progress curve was derived from the severity data. The antagonists and fungicides significantly reduced the growth of *F. graminearum* colonies in culture. Fungicides folicur and copper oxychloride completely inhibited pathogen growth while *Trichoderma* sp. Reduced pathogen colonies by 64%. The least effective was *Epicoccum* sp. However, the antagonists showed limited reduction in head blight severity in green house trials. Among the antagonists, *Trichoderma* sp. showed the highest disease severity reduction (18%) while fungicide folicur was most effective with a reduction of 28%. All the antagonists had little or no significant effect on grain yield. Although application of antagonists does not result in reduction in head blight severity it would be important to determine whether it has any significant effect on mycotoxin accumulation.
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 (Nganje *et al*., 2004; Kolombet *et al*., 2005; Windels, 2000; Wood, 2002). In addition to causing significant reductions in grain yield, 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 (Bottallico and Perrone, 2002; Pirgozliev *et al.,* 2003). *Fusarium graminearum* is one of the major causal agents of FHB and produces mycotoxin deoxynivalenol (DON), which may accumulate to unacceptable levels in harvested grain (Paul *et al*., 2005). DON levels above 2 ppm may render grain and their by-products unfit for human and animal consumption.

Efforts to minimize the impact of FHB and DON have been centred on the use of management strategies such as crop rotation, host resistance tillage, and fungicide application (Pirgozliev *et al.,* 2003). An integrated approach to management of FHB includes chemical, cultural and host resistance. Host resistance is the most economical method of reducing losses due to the disease (Jones, 2000; Pirgozliev *et al*., 2002). Several studies on chemical control of FHB have been reported but conflicting evidence however exists regarding the effect of fungicides on the development of FHB and on the concentration of trichothecene mycotoxins in grain (Edwards *et al.,* 2001; Halley *et al*., 2005; Henriksen and Elen, 2005). Pirgozliev *et al.,* (2002) reported that fungicides affected the deoxynivalenol (DON) concentrations indirectly by influencing the amount of *Fusarium* species in the grain. Edwards *et al* (2001) reported that Azoxystrobin did not affect levels of trichothecene-producing *Fusarium* compared with those of untreated controls. Metconazole and tebuconazole significantly reduced the amount of trichothecene-producing *Fusarium* in harvested grain but did not alter rate of DON production. However, Suty, (1996) reported that tebuconazole reduced both the severity and DON concentration. A more recent study by Draper *et al.* (2005) found no effect on both DON and the disease severity after using Folicur®. Hollingsworth *et al*., (2005) and Ruden *et al*. (2005) tested more fungicides including Folicur and metconazole and did not find any reduction in disease or the DON concentration. Therefore, unlike other diseases, complete FHB control is not possible with today's fungicides. All labeled systemic fungicides appear to increase yield, but those that contain a 'triazole', instead of a 'strobilurin' active ingredient have been more effective in reducing mycotoxin (DON) levels in infected grain (Hollingsworth, 2004).

The use of fungicides on wheat ears has the disadvantage of accumulation 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 people using the fungicides. Therefore, this study was carried out to evaluate the efficacy of fungal and bacterial antagonists in management of FHB caused by *F. graminearum*.

Materials and Methods

Isolation and multiplication of pathogen and antagonists.

Three highly pathogenic isolates of *Fusarium graminearum* were isolated from wheat kernels and tested

for capacity to induce head blight during an earlier study. *Epicoccum*, *Alternaria* and *Trichoderma spp*. were also isolated from wheat by plating on low strength PDA amended with mineral salts and antibiotics (Muthomi, 2001) (PDA 17g, KH_2PO_4 1.0g, KNO3 1.0g, $MgSO_4$ 0.5g, Agar10g). The fungi were identified based on cultural, morphological and physiological characteristics like colony colour, pigment production, presence of aerial mycelium in addition to morphological characteristics (conidia shape, septation, and sporophores). *Bacillus* sp. was isolated also isolated from wheat seeds plated on nutrient agar (NA) and identified based on cultural characteristics mainly endospore production.

Inoculum of the three *F. graminearum* isolates was multiplied separately in mung bean broth (Bai and Shaner, 1994). Mung bean (40 grams) was cooked in 1000 ml of water for 10 minutes and the extract was filtered through double layer cheesecloth. Twenty millilitre aliquots of the extract were autoclaved in 250ml Erlenmeyer flasks. After cooling, each flask was inoculated with mycelial agar discs cut from 14 day-old pathogen cultures and incubated on mechanical shaker (50-70 cycles per minute) for 4 days followed by 10-day incubation under stationery conditions. *Epicoccum, Alternaria and Trichoderma* spp. were multiplied on PDA for 14 days at 25^{o}c in cycles of 12 hr daylight and 12 hr 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 cheesecloth. The inoculum was adjusted to $1x10^5$ spores/ml using a haemocytometer. The *Bacillus sp.* inoculum density was assessed by serial dilution and plating 0.2 ml of the solution on nutrient agar. The average inoculum density was 1 x1 0^4 cfu/ml.

Determination of the 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 the edge. Each of the antagonists *Epicoccum, Alternaria, Trichoderma* and *Bacillus sp.* was tested separately. Fungicides Folicur® (3000ppm) and copper oxychloride (2500ppm) were used as standard checks while negative control consisted of *F. graminearum* cultured alone. The fungicide was dissolved in 10ml of distilled water and added onto liquid media cooled to 45^{o}C. Each treatment was replicated four times and the plates arranged in a completely randomized design on laboratory benches and incubated at 25 ^{0}C for 7 days in cycles of 12hr daylight and 12hr darkness. Degree of antagonism was determined by measuring the pathogen colony diameters and percentage inhibition calculated according to Bora and Ozaktan (1998):

$$\text{Percent inhibition} = \frac{\text{diameter of pathogen alone} - \text{diameter of pathogen + antagonist}}{\text{diameter of pathogen alone}} \times 100$$

Efficacy of antagonists to reduce FHB under greenhouse conditions.

Certified seeds 'Mbuni', a highly susceptible wheat variety, were planted in 22 cm diameter pots containing forest soil/manure (2:1v/v) growth media. The plants were allowed to grow outside the green house until flowering to simulate field conditions. The plants were fertilised at germination (GS10; Zadoks *et al.*, 1974), at tillering (GS 22) and at booting (GS 41) with 5g/pot of NPK (20-20-0), NPK (20-20-0) and Urea (46%N), respectively. Aphids and other insect pests were controlled with 1000ppm dimethoate (Danadim ®). Inoculation was done at 50% flowering (GS 65) and treatments consisted of inoculation with each of the antagonists 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 at the rate of 3000ppm and 2500ppm, respectively. Control plants were sprayed with sterile distilled water. The antagonists and the fungicides were sprayed two days before and 2 days after inoculation with *F. graminearum*. Re-inoculation with *F. graminearum* was done 5 days after the first inoculation. Each treatment was replicated four times. The treated heads were covered with polythene bags for 48 hours to maintain high humidity for infection. The inoculated plants were placed in the greenhouse and arranged in a randomised complete block design.

Head blight severity was assessed five days after the last inoculation and after every 5 days until ripening stage (GS 87). Proportion of head 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 (Miedaner *et al*., 1996). Assessment was done on ten average-sized heads per pot. Mean disease severity and the area under disease progress curve (AUDPC) were calculated from single ratings according to Shaner and Finney (1977):

$$AUDPC = \sum[(0.5)\ (Y_{i+1} + Y_i)\ (T_{i+1} + T_i)]$$

Where, Y = disease severity at time i and T = time (days) of the assessment

Harvesting was done at physiological maturity (GS 95) and the ten heads that were assessed for disease were threshed separately. The grain was dried to about 15% moisture content before determination of the ten-ear weight and total grain weight per pot. Seed samples (100 kernels) from each treatment were randomly sampled for re- isolation of the pathogen. The experiment was repeated over two greenhouse cycles.

Data analysis

All data were 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 treatments means were compared using the Fisher's protected LSD test at 5% probability level.

Results

All the antagonists reduced colony diameters of *F. graminearum* (Table 1). Folicur and copper oxychloride completely inhibited the growth of *F. graminearum.* There were significant differences (P=0.05) among the pathogen colony diameters with different antagonists and the fungicides. *Trichoderma* spp showed the highest

reduction (64%) in pathogen colony diameter while *Epicoccum* sp. had the least reduction (45%).

Table 1: The colony diameter (cms) and the percentage reduction in colony diameter of *F. graminearum* in paired cultures.

Treatment	Experiment 1		Experiment 2	
	Colony diameter	% reduction	Colony diameter	% reduction
Fusarium +Alternaria	1.7bc	55.0	1.8c	48.0
Fusarium+Bacillus	1.9bc	51.0	1.6c	53.0
Fusarium+Epiccocum	2.0c	49.0	1.9c	45.0
Fusarium+Folicur	0.0a	100.0	0.0a	100.0
Fusarium+Trichoderma	1.4b	64.0	1.2b	65.0
Fusarium +Copper	0.0a	100.0	0.0a	100.0
Control	3.8d	0.0	3.4d	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	

The severity overtime, mean severity and the area under disease progress curve (AUDPC) were significantly different (P= 0.05) among the antagonists (Table 2 and 3). Folicur significantly reduced head blight in the two trials by up to 47% but *Bacillus* sp. had no significant effect on disease severity. Copper oxychloride also significantly 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 spp.* had minimal effect on FHB. Similar results were observed for the AUDPC. 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 of ears inoculated with the pathogen alone but the re-isolation rate differed for the kernels from ears inoculated with different antagonists.

Table 2: The percentage severity overtime, mean severity, AUDPC and re-isolation rate for the plants inoculated with *F. graminearum* and the respective antagonists in 1st experiment.

Treatment	Days after inoculation						
	5	10	15	20	Mean	AUDPC	Re-isolation
Fusarium +Alternaria	61.8	86.0	90.4	95.7	83.5d	1276f	50
Fusarium+Epicoccum	56.6	80.2	92.1	92.1	80.2d	1233f	50
Fusarium+Trichoderma	56.3	66.6	72.4	73.1	67.1c	1019e	57
Fusarium+Bacillus	70.7	84.6	91.3	99.0	86.4d	1304f	67
Fusarium+Folicur	32.7	33.3	34.6	49.2	37.4b	545c	43
Fusarium+copper	25.2	46.7	82.9	87.4	60.6c	877d	40
Alternalia	12.7	18.5	23.5	37.1	23.0a	336b	23
Epicoccum	12.2	13.3	15.5	26.4	16.8a	265ab	23
Trichoderma	13.8	17.4	18.5	31.6	20.3a	294ab	25
Bacillus	11.1	14.9	15.2	27.0	17.0a	246a	24
Fusarium	64.1	86.3	93.8	95.7	84.9d	1300f	73
Control	11.9	13.3	14.9	25.3	16.3a	235a	30
Mean	35.7	46.7	53.8	61.6			42
LSD	10.0	10.0	7.0	8.0	10.0	86.7	17
CV%	18.0	15.0	9.0	9.0	8.0		26

AUDPC = Area Under Disease Progress Curve.

Values followed by the same letters within columns are not significantly different

Table 3: The percentage severity overtime, mean severity, re-isolation and AUDPC for the plants inoculated with *F. graminearum* and the respective antagonists in 2nd experiment.

Treatment	Days after inoculation					AUDPC	Re-isolation
	5	10	15	20	Mean		
Fusarium + Alternaria	38	61	79	96	68de	1031d	46
Fusarium Epicoccum	37	57	70	93	64d	960cd	50
Fusarium+Trichoderma	12	56	64	77	52c	878c	60
Fusarium+Bacillus	40	70	88	99	74e	1139e	80
Fusarium+Folicur	33	44	43	49	42b	534b	40
Fusarium+copper	12	25	64	79	45bc	932cd	43
Alternalia	13	13	23	29	19a	307a	26
Epicoccum	13	12	22	28	18a	284a	26
Trichoderma	13	14	25	31	20a	304a	36
Fusarium	41	62	82	98	70de	1071de	23
Control	12	12	22	28	18a	262a	93
Mean	22	35	48	58	41	641.8	33
LSD	4	22	13	30	9	101.5	45
CV%	10	36	16	30	14		20

AUDPC = Area Under Disease Progress Curve.

Values followed by the same letters within columns are not significantly different.

Inoculation of FHB infected ears with the antagonists had little or no significant effect on grain weight (Table 4 and 5). However, application of fungicides Folicur and copper oxychloride significantly ($P<0.05$) increased grain weight compared to ears inoculated with *F. graminearum* alone. Folicur was the most effective with a ten-ear weight increase of between 47 and 94%. *Bacillus, Alternaria* and *Epicoccum spp.* showed no significant effect on grain yield. Among the antagonists *Trichoderma* sp. showed the least reduction in yield, though not significant.

Table 4: Ten-ear weight (g), 100-kernel weight (g) and weight per pot (g) for plants treated with *F. graminearum* and respective antagonists in 1st trial

	10 ear kernel weight		Kernel weight per pot	
Treatment	Weight (g)	%Reduction	Weight (g)	%Reduction
Fusarium +Alternalia	8.78a	51.86	6.35ab	52.72
Fusarium+Epiccocum	9.83a	46.11	5.20a	61.28
Fusarium+Trichoderma	9.91a	45.67	5.63a	58.08
Fusarium+Bacillus	8.37a	54.11	5.11a	61.95
Fusarium+Folicur	17.49c	2.47	10.99b	16.30
Fusarium +copper	14.86b	18.53	9.61b	28.44
Alternaria alone	17.89c	1.92	13.43bc	0.00
Epicoccum alone	18.35c	0.00	12.49b	7.00
Trichoderma alone	18.09c	0.82	13.20bc	1.71
Bacillus alone	19.12c	0.00	12.60b	6.18
Fusarium alone	9.45a	48.19	5.11a	61.95
Control	18.24c	0.00	13.43bc	0.00
Mean	14.00		9.07	
LSD (P=0.050	2.26		3.07	
CV%	11.10		16.90	

Values followed by the same letters within columns are not significantly different.

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 (Fiddaman and Rossal, 1993, Brown *et al.,* 1987). However, Folicur and copper oxychloride were most effective, completely inhibiting the growth of the pathogen. In the two greenhouse trials, the fungicides reduced the disease severity by between 28-58%, although complete control was not achieved, therefore, confirming earlier findings by Chala *et al.* (2003). Among the antagonists there was reduction in disease severity by *Alternaria, Epicoccum* and *Trichoderma.* This was in line with findings by Perello *et al*. (2002), Gonzalez *et al.* (1999) and Lutz *et al*., (2003). Perello *et al*. (2002) reported that among the antagonists tested *Bacillus* sp. was the one with the highest interference to pathogens in culture. *Epicoccum purpurascens* (*E. nigrum*) produces antifungal compounds, which may increase its effectiveness (Brown *et al*., 1987).

Application of Folicur and copper oxychloride led to an increase in yield gauged on the weight per pot and this is in agreement with findings by Masterhazy *et al*. (2003) and Pirgozliev (2002). The effect of fungicides on *Fusarium is* dependent of timing and frequency of applications (Parry *et al.,* 1995) and treatments after flowering seems to be the best time for reduction of *Fusarium* infection (Masterhazy *et al*., 2003, Hormdock *et al*., 2000; Hollingsworth, 2004). Fungicide application is recommended for susceptible spring wheat if weather conditions favour *Fusarium* spore production, and if the cost of the treatment is economically justified.

In the current study, *Trichoderma* sp. was found to reduce disease severity and increase the grain yield. This shows that biological control has considerable promise for reducing FHB, as it has been reported by Bateman (1979), Draper *et al*. (2005), Jochum *et al*., (2004), Kolombet *et al.*, (2005) and Nourozian (2006). Bateman (1979) attempted biological control of M*icrodochium. nivale* inoculated on wheat ears in the greenhouse. Subsequent inoculation at anthesis with *Sporobolomyces* spp. significantly reduced grain contamination. Inoculation before anthesis with *Cladosporium* spp. were effective when applied before *M. nivale*, whereas *Alternaria* spp. were effective whether applied before or after the pathogen. *Pseudomonas fluorescens* biov1, *B. subtilis* and *Streptomyces* sp. were found to be antagonistic to *F. graminearum* (Nourozian, 2006). *Fusarium graminearum* mycelial growth was reduced by cell free and volatile metabolites of the bacterial antagonists by 37%-97%. Draper *et al*., (2005) and Jochum *et al*., (2004) found no effect on yield by the use of *Lysobacter enzymogenes*, *Bacillus* spp. and *Pseudomonas fluoresce*alone but co application with Folicur led to an increase in test weight. Fusaric acid has been shown to modulate fungus-bacterium interactions that affect plant health (Duffy and Défago, 1997). In two different studies Duffy (2001) and Lutz, *et al*., (2003) demonstrated that *Fusarium* was able to suppress the expression of the genes that control the antagonistic activity of *Trichoderma harzianum* but not of *Pseudomonas fluorescens.*

From the results 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 Trichoderma sp. reduced disease severity although no very pronounced. Therefore, further research is needed to determine the efficacy of the effect of the antagonists and the fungicides on mycotoxin deoxynivalenol under controlled and field conditions. In addition further testing of a broader spectrum of other possible antagonists and determination of their mechanisms of action would be necessary.

Table 5: Ten-ear weight (g), 100-kernel weight (g) and weight per pot (g) with *F. graminearum* and respective antagonists in 2nd trial.

	10 ear weight		Weight /pot	
Treatment	Weight (g)	%Reduction	Weight (g)	%Reduction
Fusarium +*Alternaria*	9.74a	30.33	11.45a	43.08
Fusarium+*Epiccocum*	10.35ab	25.97	11.89a	40.87
Fusarium+*Trichoderma*	10.48ab	25.04	12.84ab	36.18
Fusarium+*Bacillus*	10.71ab	23.39	12.34ab	38.63
Fusarium+Folicur	11.79b	15.67	12.89ab	35.90
Fusarium +Copper	11.79b	15.67	12.30ab	38.87
Alternaria alone	14.47c	0.00	15.60b	26.14
Epicoccum alone	12.03bc	13.95	18.69c	7.11
Trichoderma alone	12.73bc	8.94	15.09b	28.55
Fusarium alone	9.80a	29.90	10.17a	49.42
Control	13.98c	0.00	20.12c	0.00
Mean	11.60		14.00	
LSD (P=0.050	1.95		3.97	
CV%	11.70		23.90	

Values followed by the same letters within columns are not significantly different.

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