USDA-ARS/
U.S. Wheat and Barley Scab Initiative
FY09 Final Performance Report
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Cover Page

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Fiscal Year:	2009
USDA-ARS Agreement ID:	59-0790-6-072
USDA-ARS Agreement Title:	Enhancing Biological Strategies to Control Fusarium Head Blight and Evaluating Biological Control Agents in Uniform Tests against FHB.
FY09- USDA-ARS Award Amount:	$ 34,309

USWBSI Individual Project(s)

USWBSI Research Category*	Project Title	ARS Adjusted Award Amount
MGMT	Control of DON Production in Grain with Non-Toxigenic Strains of Fusarium.	$ 12,683
MGMT	Evaluation of Biological Agents for FHB Control.	$ 11,430
MGMT	Effects of Defense Peptides on Fusarium Head Blight.	$ 10,196
	Total Award Amount	**$ 34,309**

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Principal Investigator Date

* MGMT – FHB Management
FSTU – Food Safety, Toxicology, & Utilization of Mycotoxin-contaminated Grain
GDER – Gene Discovery & Engineering Resistance
PBG – Pathogen Biology & Genetics
BAR-CP – Barley Coordinated Project
DUR-CP – Durum Coordinated Project
HWW-CP – Hard Winter Wheat Coordinated Project
VDHR – Variety Development & Uniform Nurseries – Sub categories are below:
SPR – Spring Wheat Region
NWW – Northern Winter Wheat Region
SWW – Southern Sinter Wheat Region

Project 1: *Control of DON Production in Grain with Non-Toxigenic Strains of Fusarium.*

1. **What major problem or issue is being resolved relevant to Fusarium head blight (scab) and how are you resolving it?**

While host genetic resistance and fungicides have proven to be useful in managing Fusarium head blight (FHB) and deoxynivalenol (DON) accumulation, new methods are needed to augment these strategies, particularly in controlling DON. We tested the concept that pre-application of a non-toxigenic (Tox-) hypovirulent strain of *Fusarium graminearum* to wheat heads can inhibit floret infection by toxigenic (Tox+) virulent pathogen strains and, ultimately, reduce DON accumulation in the grain. This concept is based on the theory that the Tox- strain can compete with the Tox+ pathogen for niches and substrates on florets and potentially can induce host resistance mechanisms. A wild, Tox- isolate (WG-9) provided by L Gale was tested against the standard Tox+ strain PH-1. Seven greenhouse experiments are completed by G. Yuen in which WG-9 and PH-1 were co-inoculated onto flowering heads of scab susceptible spring wheats 'Bobwhite' and 'Wheaton'. Inoculation was performed in one set of experiments by was spray inoculating WG-9 first and PH-1 1 day later, both at either 10^5 or 10^4 spores/ml. In another set of experiments, the two strains were simultaneously point inoculated in the center spikelet of individual heads. In both sets of experiments, disease severity was determined 1 week after inoculation. Seed were harvested at maturity for determination of Fusarium diseased kernels (FDK). Diseased and asymptomatic seed were assayed for DON by University of Minnesota Diagnostic Lab. In addition, the identity of the *Fusarium* strain infesting individual seed were determined in first set of experiments by L. Du. A multiplex PCR system with primers based on TRI3 and TRI12 gene sequences was used for the identification procedure (Figure 1).

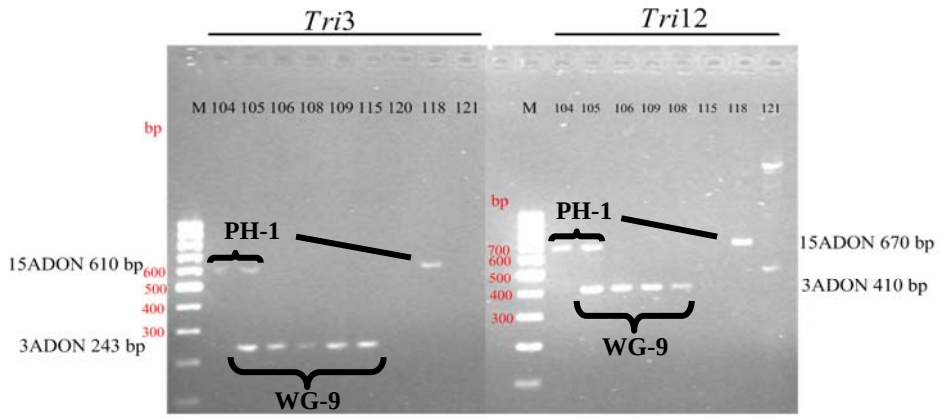

Figure 1. Representative electrophoresis results from *TRI3* (left) and *TRI12* (right) multiplex PCR of DNA extracted from plated seed and mycelia. *Fg* PH-1 (TOX+) and *Fg* WG-9 (TOX-) are indicated by amplicons of different size in each multiplex**.**

2. **List the most important accomplishment and its impact (i.e. how is it being used) to minimize the threat of Fusarium head blight or to reduce mycotoxins. Complete both sections (repeat sections for each major accomplishment):**

Accomplishment:

- Experiments revealed ***inoculation with Tox- WG-9 can reduce DON concentrations in the seed*** (Tables 1 and 2). The Tox- isolate, however, did not suppress scab symptom development.

- PCR analysis of fungi from individual seed revealed preinoculation with WG-9 reduced the frequency of seed infection by PH-1. In one experiment for example, less than 50% of the seeds from heads preinoculated with WG-9 prior to inoculation with PH-1 were infected with PH-1, as compared to 100% infection of seeds by PH-1 in heads with no WG-9 preinoculation.

Table 1. Results from 4 representative experiments testing coinoculation of wheat heads with Tox- WG-9 and Tox+ PH-1, both at 10^4 spores/ml. Experiments 1&2: WG-9 spray inoculated 1 day before spray inoculation with PH-1. Experiments 3&4: WG-9 and PH-1 simultaneously inoculated into center floret of each head.

Experiment & cultivar	Treatment	% infected spikelets	% FDK	DON (ppm) in diseased seed	DON (ppm) in asymptomatic seed
1 'Bobwhite'	WG-9/PH-1	70.7 A	79.3 AB	24.9 B	0.8 B
	Water/PH-1	34.4 B	65.6 B	176.0 A	3.1 A
	WG-9/water	78.0 A	93.2 A	0#	0#
	Water/water	3.4 C	4.6 C	Not tested	Not tested
2 'Wheaton'	WG-9/PH-1	64.8 A	91.7 A	12.1 B	2.8 B
	Water/PH-1	12.0 B	86.4 A	97.9 A	16.5 A
	WG-9/water	78.5 A	96.5 A	1.7 C	0#
	Water/water	8.0 B	7.5 B	Not tested	Not tested
3 'Bobwhite'	PH-1 only	38.4 A	32.9 A	202.4 A	2.5 A
	WG-9 + PH-1	53.9 A	46.7 A	119.8 A	0.5 B
	WG-9 only	4.6 B	4.5 B	0#	0#
	Water	0.4 B	0.3 B	Not tested	Not tested
4 'Wheaton'	PH-1 only	68.0 A	75.1 A	48.4 A	1.0 A
	WG-9 + PH-1	56.7 A	81.1 A	18.7 B	0.3 B
	WG-9 only	23.3 B	46.5 B	0#	0#
	Water	2.8 C	9.4 C	Not tested	Not tested

Letters indicate significant differences at P=0.05.
Below detection level of 0.5 ppm. Values were not used in statistical analysis.

Impact:

This study has demonstrated a novel strategy to manage DON worthy of further research. The results support the hypothesis that a Tox- isolate of *Fusarium graminearum* might be used as a biological control agent to compete with or exclude Tox+ pathogens strains and, thus, reduce DON levels in the harvest grain. The high disease severity and expected yield loss caused by the Tox- strain alone is an obvious drawback to immediate use of the strategy. But we anticipate this problem could be overcome by 1) applying WG-9 at much lower spore concentrations; 2) using scab resistant cultivars; or 3) using Tox- strains of *F. graminearum* with lower virulence than WG-9. Another alternative would be to identify the mechanisms by which WG9 exerts its competitive/exclusive effects.

Project 2: *Evaluation of Biological Agents for FHB Control.*

1. **What major problem or issue is being resolved relevant to Fusarium head blight (scab) and how are you resolving it?**

Although more effective fungicides and host resistance in some wheat market classes are now available for scab and DON management, these strategies are not available for all cereal crops nor completely effective. Biological control measures that can be effective in diverse environments are needed to augment current strategies. The Yuen laboratory conducted field experiments were conducted in two Nebraska locations (Lincoln and Mead) as part of the multistate Uniform Biocontrol Trials. The biocontrol agents in this study were Taegro (Novozymes Biologicals, Salem, VA), a commercial product containing *Bacillus amyloliquefaciens* FZB24, and "double yeast" treatment, which consisted of *Cryptococcus flavescens* OH 182.9 (NRRL Y-30216) and *C. aureus* OH 71.4 (NRRL Y-30213) cultured together, supplied by D. Schisler, USDA ARS NCAUR. One application of each agent was made at anthesis and were compared with non-treated controls and the fungicide Prosaro 421 SC alone. Other treatments included Taegro alone at late bloom; a tank mix of Taegro and Prosaro applied at anthesis; and Prosaro at anthesis followed by Taegro at late bloom. Although a seventh treatment, Prosaro at anthesis followed by the 'double yeast' at late bloom, was planned, the treatment was eliminated due to difficulties with the late application. A susceptible hard red winter wheat 2137 was used in both locations, as was artificial inoculation with Fusarium-infested grain and mist irrigation. Scab severity, incidence, and index were determined in the field. Percent Fusarium diseased kernels, DON levels, and test weights were measured after harvest.

2. **List the most important accomplishment and its impact (i.e. how is it being used) to minimize the threat of Fusarium head blight or to reduce mycotoxins. Complete both sections (repeat sections for each major accomplishment):**

Accomplishment:

Scab-conducive environmental conditions occurring at anthesis resulted in moderate incidence in both locations. The tank mix of Taegro and Prosaro reduced the incidence of scab at one location below the control and below Taegro and Prosaro applied alone (Table 2). Subsequent environmental conditions, however, arrested scab development such that Index, % FDK, and DON levels were too low to allow comparison of treatments.

Impact :

The results from the experiments conducted in Nebraska provide further evidence that combinations of biocontrol agents with fungicides have the potential to provide better control of scab than fungicide alone. In context of the Uniform Biocontrol Trials, the Nebraska results are in line with those from other states in which the Taegro-Prosaro combinations were effective in control scab in the field, and, in one location, reduced DON.

Table 2. Results from experiment conducted in at two Nebraska locations in 2009.

	INCIDENCE (%)		INDEX (%)		FDK (%)		Range of DON (ppm)	
Treatment	Mead	Lincoln	Mead	Lincoln	Mead	Lincoln	Mead	Lincoln
Control	55	66	4	9	1	1	<0.5	<0.5
Prosaro alone at anthesis	50	58	4	5	1	1	<0.5 - 0.5	<0.5
Taegro alone at anthesis	58	59	6	5	2	2	<0.5	<0.5 - 0.7
Taegro alone at late bloom	46	63	3	6	2	2	<0.5 - 0.6	<0.5
Prosaro + Taegro tank mix at anthesis	38*#	59	3	6	2	<1	<0.5	<0.5
Prosaro at anthesis/Taegro at late bloom	49	54	4	7	1	2	<0.5 - 0.5	<0.5
Double yeast alone at anthesis	47	52	4	5	1	1	<0.5	<0.5 - 0.5
P	0.025	Ns	0.048	0.081	Ns	0.099	-	-
$LSD_{0.05}$	11	-	2	-	-	-	-	-

* = Value is significantly lower than the control at the 95% confidence level
# = Value is significantly lower than Prosaro at the 95% confidence level
Ns = not significant, i.e., *P*>0.1
- = not applicable

Project 3: *Effects of Defense Peptides on Fusarium Head Blight.*

1. What major problem or issue is being resolved and how are you resolving it?

In this project, we are testing the concept that antifungal peptides can be used to suppress infection of wheat by macroconidia of *Fusarium graminearum* or ascospores of the sexual pathogen form, *Gibberella zeae*. This should ultimately lead to reduced DON accumulation. Two groups of peptides are being investigated, the first being native and derivative forms of mating pheromones from *F. graminearum* and *Neurospora* discovered by the Leslie lab. A second group includes those recently identified in the English lab and derived from combinatorial phage-display peptide libraries.

A first question is whether all small peptides are equally effective. The Leslie lab tested a number of single amino-acid-substituted derivatives of the *Neurospora* and *Fusarium* mating hormone peptides. The peptides were tested for inhibition of macrocondium germination by an in vitro assay in which spores were mixed with each peptide (1 to 20 µM) on microscope slides.

Further efforts in testing the concepts have focused on using the *Pichia pastoris* fermentation system, in use in the English lab, to generate large amounts of mating hormone or combinatorially derived peptides attached to scaffolds that are secreted from the yeast into the culture medium. The scaffold is a secretable plant protein, maize cytokinin oxidase/dehydrogenase (ZmCKX1). Scaffold-peptides purified from the culture extracts will then be used for application to wheat in greenhouse experiments.

A third focus has been the evaluation of a selection of chemically synthesized peptides for effects on *F. graminearum*/*G. zeae* in vitro (English lab) and in planta (Yuen lab). Within the in vitro bioassay, ascospores (10^5 per ml) of *G. zeae* were exposed to individual mating hormone or combinatorially selected peptides (0.2 to 4 µM) in drops mounted on microscope slides.

Two in planta bioassays were developed for this project specifically to test small amounts of peptides. One assay examined effects of peptides on growth of the fungus on the plant surface; the other evaluated peptides for inhibition of scab symptoms. Peptides diluted in 2% DMSO were mixed with to ascospores (10^5 per ml). In the first assay, 10µl drops were deposited on the surface of detached spikelets supported on water agar in Petri dishes. Growth of pathogen was examined daily and rated on a 0-4 scale (0=no visible growth; 4=profuse mycelia covering the spikelet. In the second in planta assay, 10µl volumes of peptide-spore mixtures were inoculated (without damaging tissues) into the middle floret of detached heads maintained in water-filled floral tubes. After the inoculated heads were incubated in a moisture chamber for 2 weeks, the percentage of spikelets exhibiting scab symptoms was determined.

2. **List the most important accomplishment and its impact (i.e. how is it being used)**

Accomplishment:

1. In testing amino-acid-substituted derivatives of the *Neurospora* and *Fusarium* mating hormone peptides, some substitutions were less-efficient at inhibiting macroconidum germination and further growth than the native pheromone peptide, and in some cases more efficient. Patterns included that increasing the pI of the peptide generally increased inhibition. However, these increases usually were acquired through the addition of additional arginine residues, which could make the peptide more susceptible to bacterial and other enzymatic degradation. Although the peptides in hand certainly can inhibit growth and spore

germination, additional changes in peptide sequence need to be tested to identify those that are even better inhibitors of spore germination and fungal growth than the 10 already tested.

2. We have we have constructed transformation vectors for expression of 4 peptides on the ZmCKX1 scaffold in *Pichia*. Liter amounts of yeast fermentation culture fluid have been produced. Logistical difficulties have hindered the final purification of peptides from the culture fluids, but alternative purification equipment recently has been found, so purified peptides will be available shortly for greenhouse experiments.
3. Chemically synthesized mating pheromone peptide, Pgz, and two combinatorially derived peptides, F3A and F8B, were found to inhibit the germination of ascospores in vitro.
4. Pzg and F8B inhibited growth of *F. graminearum* when mixtures of peptides and ascospores were spotted onto detached on spikelets (Fig. 2 left). This effect was concentration-dependent; Pgz at 20µM completely suppressed mycelial growth while lower concentrations acted in slowing fungal growth (Fig. 2 right). The same peptides inhibited scab development, also in a concentration-dependent manner, when peptide-ascospore mixtures were inoculated into florets on intact heads (Table 3). The effect of Pzg at 20µM on scab severity was the same as that of Prosaro fungicide. Another combinatorial peptide F3A had no effect on growth of the pathogen or scab severity.

Impact:

The results provide evidence towards proving the concept that application of specific peptides to wheat heads can affect infection by *F. graminearum*. The spikelet and whole head bioassays developed in this study will be used to screen a number of candidate peptides and to optimize application parameters prior to testing of peptides produced by fermentation for scab and DON control in large scale greenhouse experiments.

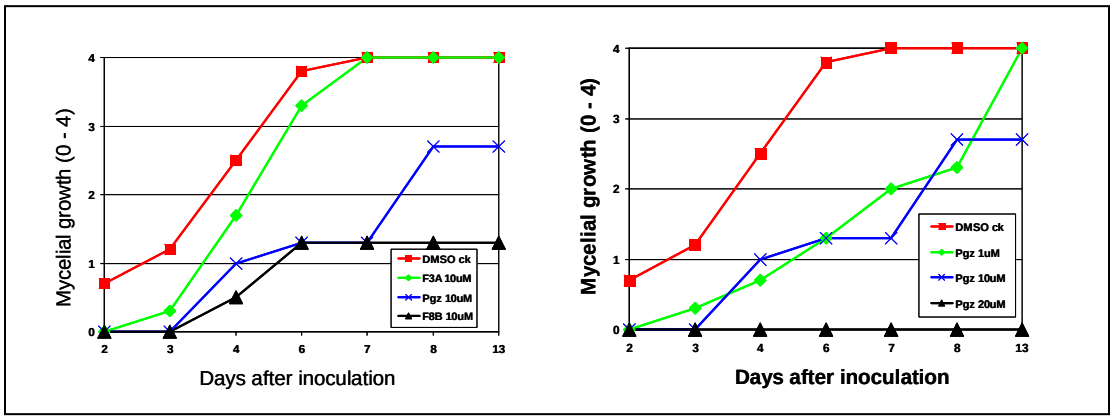

Figure 2. Effects of peptides on growth of *Fusarium graminearum* on detached spikelets: left) comparison of mating hormone peptide Pzg and combinatorial peptides F3A and F8B, all at 10µM; right) comparison of different concentrations of hormonal peptide Pzg.

Table 3. Effects of mating hormone Pzg and combinatorial peptides F3A and F8B at different concentrations on severity of scab caused by *Fusarium graminearum*.

Treatment & concentration	Percent infected spikelets
Combinatorial peptide F3A 1µM	100 A#
F3A 10µM	100 A
Combinatorial peptide F8B 1µM	98 A
F8B 10µM	64 B
F8B 20µM	35 B
Hormonal peptide Pgz 1µM	100 A
Pgz 10µM	83 AB
Pgz 20µM	1 C
Prosaro fungicide	1 C
DMSO 2% (diluent)	100 A

Letters denote significant differences at P=0.05

Include below a list of the publications, presentations, peer-reviewed articles, and non-peer reviewed articles written about your work that resulted from all of the projects included in the grant. Please reference each item using an accepted journal format. If you need more space, continue the list on the next page.

Published articles and abstracts

Gary Y. Yuen, C. Christy Jochum, Liangcheng Du, Isis Arreguin, and Liane R. Gale. 2009. Inhibition of deoxynivalenol accumulation by preinoculation with nontoxigenic *Fusarium graminearum* - Preliminary tests of a novel strategy. Proceedings of the 2009 National Fusarium Head Blight Forum, page 100.

G.Y. Yuen, C.C. Jochum, S.A. Halley, K. Misek, L.E. Sweets, W. Kirk and D.A. Schisler. 2009. Results of 2009 Uniform Biological Control Trials. Proceedings of the 2009 National Fusarium Head Blight Forum, page 101-105.

Presentations

Inhibition of Deoxynivalenol Accumulation by Preinoculation with Nontoxigenic *Fusarium graminearum* - Preliminary Tests of a Novel Strategy. Gary Y. Yuen, C. Christy Jochum, Liangcheng Du, Isis Arreguin, and Liane R. Gale. Poster presented at 2009 National Fusarium Head Blight Forum, Dec. 7-9, Orlando, FL.

Results of 2009 Uniform Biological Control Trials. G.Y. Yuen, C.C. Jochum, S.A. Halley, K. Misek, L.E. Sweets, W. Kirk and D.A. Schisler. Poster presented at 2009 National Fusarium Head Blight Forum, Dec. 7-9, Orlando, FL.