2011

# Co-culture of Yeast Antagonists of Fusarium Head Blight and their Effect on Disease Development in Wheat

D. A. Schisler
*USDA-ARS*

P. J. Slininger
*USDA-ARS*

Michael J. Boehm
*University of Nebraska-Lincoln*, mboehm3@unl.edu

P. A. Paul
*Ohio State University*

Plant Pathology Journal 10 (4): 128-137, 2011
ISSN 1812-5387 / DOI: 10.3923/ppj.2011.128.137




# Co-culture of Yeast Antagonists of Fusarium Head Blight and their Effect on Disease Development in Wheat

[1]D.A. Schisler, [1]P.J. Slininger, [2]M.J. Boehm and [3]P.A. Paul
[1]USDA-ARS, National Center for Agricultural Utilization Research,
1815 N. University, Peoria, IL 61604
[2]Department of Plant Pathology, The Ohio State University, Columbus, OH 43210
[3]Department of Plant Pathology, The Ohio State University/OARDC, Wooster, OH 44691

**Abstract:** Multistrain mixtures of biocontrol agents which can reduce plant disease to a greater extent than the individual strains of the mixture, commonly, are prepared by blending separately produced fermentation products. Co-cultivation of strains to equivalent biomass yields would provide mixture advantages without incurring the cost disadvantages of multiple fermentation and processing protocols. Fusarium Head Blight (FHB) antagonists *Cryptococcus flavescens* OH 182.9 (NRRL Y-30216), *C. aureus* OH 71.4 (NRRL Y-30213) and *C. aureus* OH 181.1 (NRRL Y-30215), were grown in two- and three-strain co-cultures to assess the quality and efficacy of the fermentation end products produced. Final cell counts of component strains of all co-cultures produced were equivalent when plated on a medium that contained the trisaccharide melezitose as a sole carbon source and produced colonies of strain-distinguishable sizes. Co-cultures of *C. flavescens* OH 182.9 and *C. aureus* OH 71.4 significantly reduced FHB disease severity (32%, p = 0.05, Dunnett's t-test) when averaged across four greenhouse studies. In wheat field trials, biomass from co-cultures of these two strains reduced FHB incidence in some cases but rarely other FHB disease parameters (p = 0.05, Bonferoni mean separation). Relative Performance Index (RPI) analysis of the overall effect of treatments at both field sites revealed that treatment with the OH 71.4 and OH 182.9 co-culture significantly reduced FHB, as evidence by a higher RPI value than for the control, while the individual strains did not. The potential for obtaining superior efficacy and cost benefits with multi-strain cultures of biocontrol agents justifies additional research effort.

**Key words:** Biocontrol, biological control, co-culture, microbial mixtures, Fusarium head blight, *Cryptococcus flavescens, Cryptococcus aureus, Fusarium graminearum, Gibberella zeae*

## INTRODUCTION

Losses due to Fusarium head blight (FHB), a devastating disease of wheat and barley, are considered to be among the greatest known for a plant disease (McMullen *et al*., 1997; Goswami and Kistler, 2004). In North America, FHB is caused predominantly by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein Petch) (Aoki and O'Donnell, 1999), a phylogenetically diverse species (Akinsanmi *et al*., 2006). The pathogen not only reduces grain yield (Paul *et al*., 2010) but produces mycotoxins including deoxynivalenol (DON), a trichothecene mycotoxin that can reduce the market value of grain (Snijders, 1990; Pirgozliev *et al*., 2003) and be harmful to animals that consume the contaminated grain (Rocha *et al*., 2005; Awad *et al*., 2006).

A variety of approaches to limiting disease such as disease forecasting, resistant varieties, fungicides, cultural controls and biocontrol agents can contribute to reducing FHB (Dill-Macky and Jones, 2000; Schisler *et al*., 2002a; Khan *et al*., 2004; Beyer *et al*., 2006; Paul *et al*., 2008; Pereyra and Dill-Macky, 2008; Khan and Doohan, 2009; Xue *et al*., 2009; Skinnes *et al*., 2010) but significant reduction of FHB and DON contamination of wheat and barley on a consistent basis has not been achieved.

Utilizing an integrated pest management approach against the disease, such as combining resistant wheat varieties with fungicides, has been beneficial (Willyerd *et al*., 2010). Using biological control as part of the integrated management of FHB remains understudied yet has considerable potential for aiding in the reduction of FHB and DON (Schisler *et al*., 2002a).

Discovery, fermentation and formulation are crucial steps in the process of developing biological control

**Corresponding Author:** D.A. Schisler, USDA-ARS, National Center for Agricultural Utilization Research, 1815 N. University, Peoria, IL 61604

products, with each step contributing to final product performance (Schisler and Slininger, 1997). Though fermentation as it pertains to the production of pharmaceuticals has been extensively studied (Walsh *et al*., 2001), fermentation research has been limited in regards to factors that impact the quantity and quality of biocontrol biomass produced. Fermentation protocols can be optimized to enhance strain efficacy, as demonstrated in selecting fungicide tolerant variants of yeast *Cryptococcus flavescens* OH 182.9, that exhibit enhanced efficacy in reducing FHB of wheat (Schisler and Boehm, 2011). Decreasing the fermentation temperature by 10°C as cells of strain OH 182.9 transition from logarithmic to stationary phase growth resulted in the production of cells with enhanced tolerance to hyperosmotic shock and increased membrane wall spring constants, survival during formulation and biocontrol effect compared to non-adapted cells (Zhang *et al*., 2006; Dunlap *et al*., 2007). Including broth supernatants in formulations of biocontrol agents can be beneficial or harmful to agent survival and should be considered when optimizing fermentation schemes. The polysaccharide marginal, produced in liquid culture by cells of biocontrol agent *Pseudomonas fluorescens* S11:P:12, improved cell tolerance to rapid drying when used in cell formulations (Slininger *et al*., 2010a).

Work in our laboratory and others has documented that combinations of separately produced biocontrol agents can improve the level and consistency of plant disease control obtained compared to treatment with a single strain (Janisiewicz, 1996; Schisler *et al*., 1997; Kolombet *et al*., 2005; Domenech *et al*., 2006; Karthikeyan *et al*., 2008; Karthikeyan and Gnanamanickam, 2008; Johnson *et al*., 2009). Formulations consisting of mixes of biocontrol agents also have been tailored to increase efficacy based on combining strains with different pathogen targets (Kim *et al*., 2008). However, a draw-back to this approach is that, compared to a single large volume process, running several smaller volume cultivations of pure strains and then blending the cells produced represents significantly higher manufacturing costs due to loss of the economy of scale (Searcy and Flynn, 2009) and, potentially, increased registration costs to prospective commercial manufacturers. An approach to avoiding these disadvantages would be to produce multiple strains of antagonists in a single, co-culture. The study of Slininger and co-workers (Slininger *et al*., 2010b), is the only example where mixtures of microbial antagonists were cultivated to near equivalent populations and evaluated for efficacy in reducing plant disease. Slininger and co-workers produced a tripartite culture of *Psuedomonas* spp., that reduced disease on stored potatoes to a greater extent than mixtures of the same strains produced individually.

We have demonstrated the efficacy of three different yeast strains of the genus *Cryptococcus* in reducing FHB (Schisler *et al*., 2002a; Khan *et al*., 2004) but have not determined whether these strains can be co-cultured to near equivalent cell counts and, if so, whether the resultant co-cultured strain mixture would reduce FHB on wheat. The objectives of this study were (1) to develop methodologies for quantifying cell growth of two- and three-strain co-cultures of *Cryptococcus* spp.; (2) to produce near equivalent cells numbers of each component strain of a co-cultivated mixture in shake flasks; (3) to evaluate the efficacy of individual strains and co-cultivated strain mixtures against FHB on wheat in greenhouse tests and (4) to evaluate in field tests those co-cultured strain mixtures that were effective in reducing FHB in greenhouse tests.

## MATERIALS AND METHODS

**Growth and quantification of cells in co-culture:** When needed, pure cultures of *C. flavescens* OH 182.9 (NRRL Y-30216), *C. aureus* OH 71.4 (NRRL Y-30213) and *C. aureus* OH 181.1 (NRRL Y-30215) were initiated on 1/5 strength Tryptic soy broth agar (TSBA, Difco Laboratories, Detroit, MI) from 10% glycerol stocks of the strains stored at -80°C. After growth at 25°C for 24 h, cells from plates were used to inoculate precultures of each of the strains. Precultures were produced in non-baffled, 125 mL Erlenmeyer flasks containing 25 mL of liquid medium (SDCL) (Slininger *et al*., 2010a) that was incubated at 250 rpm, 2.5 cm orbit and 25°C for 24 h. Prior to evaluating the amenability of the strains to being co-cultured, methods for quantifying the number of cells of each strain in cell mixtures had to be developed due to cells of the 3 strains producing colonies of similar size, color and morphology on standard laboratory media. To identify a carbon source that the three strains utilized to differing degrees, cells of the three strains were grown on plates of BUY™ Agar medium (Biolog Inc., Hayward, CA) overnight. Cells were harvested from agar plates, washed twice in sterile distilled water and applied to YT Microplate™ plates that enabled a wide variety of carbon sources to be assayed for utilization as sole carbon sources by the yeast strains. Color intensity and turbidity of wells were read after 48 h incubation at 26°C using a Biolog Model 21907 turbidimeter. The trisaccharide melezitose was identified as differentially supporting growth of the 3 strains. A minimal medium containing the carbohydrate as a sole carbon source was then prepared

and included: melezitose (14 g $L^{-1}$), $KH_2PO_4$ and $K_2HPO_4$ (2 g $L^{-1}$ each), $MgSO_4$ (0.3 g $L^{-1}$), $CaCl_2$ (0.4 g $L^{-1}$), $FeSO_4$ (0.10 g $L^{-1}$), $(NH_4)_2SO_4$ (0.7 g $L^{-1}$), vitamins (0.001 g $L^{-1}$ each of thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine and thioctic acid) and agar (18 g $L^{-1}$). All ingredients were autoclaved, except vitamins which were filter sterilized.

Co-cultures using the three yeast antagonists were then initiated using the SDCL liquid medium. Precultures of each strain first were established as described above. For all two-strain and the three-strain co-cultures, growth rate inequalities between strains were overcome by adjusting strain precultures in late log growth to produce equivalent (OD) seed inocula of all strains and using weighted ratios (Table 1) of each of the inocula to seed test cultures to an initial OD of approximately 0.100 at 620 nm wavelength light (A620). Populations of all cultures were determined every 12 h for 72 h by plating duplicate samples on the melezitose minimal medium. Co-culture component populations in shake flasks were compared at regular intervals using ANOVA and means separated using Tukey's HSD (p = 0.05). Shake flask experiments were conducted three times and results of all trials were pooled prior to statistical analysis.

**Assay of efficacy of co-cultures in reducing fusarium head blight on wheat in the greenhouse:** To produce wheat for experimental use, two plants of FHB susceptible hard red spring wheat cultivar Norm were grown per 19-cm-diameter plastic pot. Each pot contained air-steam pasteurized (60°C for 30 min) potting mix (Terra-lite Redi-earth mix, W.R. Grace, Cambridge, MA) and plants were grown in a growth chamber (25°C, 14 h photoperiod, 600 ρ mol/[$m^2$/s]) for 7-8 weeks prior to transfer to greenhouse benches. Pots were fertilized after 1 week and weekly thereafter with 50 mL of a solution containing 1.25 g $L^{-1}$ Peters 20-20-20 (Grace-Sierra Horticultural Products, Milpitas, CA) and 0.079 g $L^{-1}$ iron chelate (Sprint 330, Becker Underwood, Inc., Ames, IA). Each experiment to determine the efficacy of co-cultures and individual strains of yeasts was conducted in a climate-controlled greenhouse where temperatures ranged from 17 to 20°C at night and 25 to 28°C during the day. Natural sunlight was supplemented by high-pressure sodium lights for 14 h $day^{-1}$.

Biomass of strains OH 182.9, OH 181.1 and OH 71.4 was produced in single or mixed strain co-cultures as described (above and Table 1), harvested at 48 h and diluted to ¼ strength with weak $PO_4$ buffer before use. Macroconidia of *G. zeae* isolate Z-3639 were produced on clarified V8 juice agar (CV8 agar) under a regime of 12 h $day^{-1}$ fluorescent light for seven days at 24°C (Schisler *et al*., 2002b). Colonized plates were flooded with weak $PO_4$ buffer to obtain conidial suspensions of the pathogen (Schisler *et al*., 2002b).

Approximately one week after transferring plants to the greenhouse, wheat heads were inoculated at anthesis by spraying 25% freshly harvested liquid cultures of individual antagonist strains and co-cultures ($5\times10^7$ cfu $mL^{-1}$) in $PO_4$ buffer and a final concentration of 0.036% Tween 80 (Sigma Chemical Co., St. Louis, MO). Antagonist cells were applied in the greenhouse at a field equivalent rate of approximating 375 L $h^{-1}$a in replicated experiments. For each treatment application, 50 mL of inoculum was used to treat four plants representing a total of 12 to 16 heads. Heads were then challenged immediately by spraying 12 mL of a conidial suspension of *G. zeae* ($1.5\times10^4$ conidia $mL^{-1}$) in $PO_4$ buffer with 0.036% Tween 80. Wheat heads treated only with a conidial suspension of *G. zeae* served as a disease control and untreated pots were used to insure pathogen inoculum was not spreading between treatments. Treated plants were misted lightly with distilled water and incubated in a plastic humidity chamber at 17 to 20°C at night and 25 to 28°C during the day for 3 days before being transferred to greenhouse benches. Treatments were arranged in a completely randomized design with four replicate plants for each treatment. FHB severity was visually estimated using a 0 to 100% scale (Stack and McMullen, 1995) at 10 to 14 days after inoculation. All greenhouse experiments were conducted 4 times. Disease severity data from repeated experiments were combined and analyzed using one-way analysis of variance (ANOVA) after preliminary analysis revealed that experiment by treatment interactions were not significant (p = 0.05). Means were separated using Dunnett's t-test (p = 0.05).

**Biocontrol assay on fusarium head blight in field grown wheat:** Soft red winter wheat cultivars Freedom (moderately FHB resistant) and Pioneer Brand 2545 (FHB susceptible) were utilized for field trials conducted at Peoria, IL and Wooster, OH. Methods for conducting field trials in both locations have been described in detail elsewhere (Schisler *et al*., 2006). In summary, for trials conducted in Peoria, IL, alternating rows of both wheat cultivars were planted in the fall with a length of 1.8 and 0.3 m between rows. The following spring, 2 to 3 weeks before the anticipated date of wheat flowering, 25 to 35 kernels $m^{-2}$ of yellow dent corn colonized by *G. zeae* Fg 111-B (Schisler *et al*., 2006) were applied uniformly by hand to the site. Perithecia appeared on the kernels after about 12 days and were releasing ascospores at the time of wheat anthesis. Biomass of *C. flavescens* OH 182.9,

Table 1: Description of single strain liquid cultures and multiple strain co-cultures of antagonists of Fusarium head blight of wheat and culture influence on symptoms of Fusarium head blight of wheat in greenhouse tests

| Treatment[1] | Fermentation type | Ratio of seed inoculum[2] | Disease severity (%)[3] |
|---|---|---|---|
| OH 181.1 (A) | One strain | NA | 51 |
| OH 182.9 (B) | One strain | NA | 51 |
| OH 71.4 (C) | One strain | NA | 59 |
| A + C | Two strain | 14:1 | 53 |
| B + C | Two strain | 1:4 | 44* |
| A + B + C | Three strain | 45:1:4 | 49 |
| *G. zeae* only | NA | NA | 65 |

[1] OH 181.1 (A), OH 182.9 (B) and OH 71.4 (C) are abbreviations for strains *Cryptococcus aureus* OH 181.1 (NRRL Y-30215), *C. flavescens* OH 182.9 (NRRL Y-30216) and *C. aureus* OH 71.4 (NRRL Y-30213), respectively. [2] Varying ratios of seed inoculum for co-cultures were utilized to insure near equivalent final cell counts of each strain utilized in a co-culture. NA= Not applicable. [3]Within the column values followed by * differ significantly from the *Gibberella zeae* control (Dunnett's t-test, p = 0.05)

*C. aureus* OH 71.4 and a co-culture of the two strains was produced in B Braun Biostat B fermentors (B. Braun Biotech Inc., Allentown, PA) charged with 1.5 L of SDCL medium. Antifoam 204 (Sigma, St Louis, MO) was added prior to medium sterilization and cultures were not pH controlled after inoculation at pH 7.0. Log-growth cells of strains OH 182.9 and OH 71.4 served as 5% total seed inoculum when produced individually and the co-culture was inoculated at a ratio of 1:4, respectively (Table 1). Fermentors were operated at 25°C, 1.5 L $min^{-1}$ aeration and 200 rpm agitation. After dissolved oxygen had recovered to saturation at 48 h, cells were harvested for use in field trials. The three biological treatments were applied at early wheat flowering (Feekes 10.5.1 (Large, 1954) with two adjacent rows (one each of Freedom and Pioneer 2545) receiving treatment and the next row serving as a buffer row throughout the plot. Adjacent rows of the two varieties were also randomly assigned as untreated controls. Biomass was applied at a final spray concentration of ~$1.5\times10^8$ cfu $mL^{-1}$ and all treatments were applied at 375 L $ha^{-1}$. Treatment suspensions were applied using a $CO_2$-pressurized backpack sprayer charged at 2.8 kg $cm^{-2}$ and attached to a boom equipped with 2, #6 Cone-jet® nozzles (R and D Sprayers, Opelousas, LA) spaced 30 cm apart and mounted pointing downward and toward the center of the boom at 45°. Treatment suspensions were charged with $CO_2$ just prior to application. Treatments were applied in the late afternoon and there were 5 replicates per treatment arranged in a randomized block design. From the morning after treatment application until midmilk kernel development (Feekes 11.1), wheat heads were misted during the night with city water at a rate of approximately 1 cm water/day to supplement rainfall events. Field assessments of FHB severity and incidence were made by evaluating 75 heads per replicate (375 heads/treatment) using a 0 to 100% scale when heads were between mid-milk and soft dough development (Feekes 11.1 to 11.2). Wheat heads were harvested by hand and threshed using an Almaco single plant and head thresher (Almaco, Nevada, IA) when grain reached full maturity. Grain samples obtained from each replicate row were evaluated for 100 kernel weight and for deoxynivalenol content using GC/MS and published methods (Mirocha *et al*., 1998; Fuentes *et al*., 2005). Disease severity, incidence, 100 kernel weights and DON data were subject to analysis of variance (ANOVA) and the Bonferroni mean comparison test (p = 0.05) was used to compare treatment means (Statistix 7.0, Tallahassee, FL).

For field work conducted in Wooster, OH, Pioneer Brand 2545 and Freedom wheat were planted at a rate of approximately 79 seeds $m^{-1}$ of row in Ravenna silt loam using a Hege 1000 Series plot planter (Wintersteiger, Inc., Salt Lake City, UT) at the Ohio Agricultural Research and Development Center near Wooster, OH. Prior to planting, the field was mold-board plowed and 84.2 kg $ha^{-1}$ of ammonium nitrate was broadcast over the field and incorporated with a disc. The experimental treatments were arranged in a randomized block design with 6 replicate plots. Each experimental unit consisted of a 7-row plot that measured 1.7×4.6 m. Additional nitrogen was applied the following spring as 109 kg $ha^{-1}$ of ammonium nitrate. Plots were inoculated by broadcasting corn kernels infested with a mixture of 10 *G. zeae* strains approximately 3 weeks prior to wheat flowering. Plots were mist irrigated each day from 1 week prior to flowering to 2 weeks after flowering at a volume of approximately 2.5 cm of water/day. Experimental treatments were made at early wheat flowering (Feekes 10.5.1) as described for the Peoria, IL trial with the following exceptions. Identical amounts of total active ingredients or colony forming units were applied at 188 L $ha^{-1}$ with a $CO_2$-pressurized back pack sprayer that used 2 Twinjet XR8001VS nozzles were mounted 38 cm apart and at 60° inwards to allow simultaneous coverage of each side of heads in individual rows of wheat. Field assessments of FHB severity and incidence were made by evaluating 75 heads per replicate (450 heads/treatment) when plants were between mid-milk and soft dough development. When grain reached full maturity, wheat heads were harvested using a Hege plot combine (Hege Maschinen, Waldenburg, Germany) set on the lowest blower setting to retain lighter weight kernels infected by *G. zeae*. Harvested wheat was evaluated for yield, test weight and DON. Data were subject to ANOVA and the Bonferroni mean comparison test (p = 0.05) was used to compare treatment means (Statistix 7.0, Tallahassee, FL).

The overall efficacy of treatments at the Peoria, IL and Wooster, OH field sites was determined by calculating a Relative Performance Index (RPI) (Slininger *et al*., 2010b) for the treatment efficacy observed for each combination of site (Peoria, IL and Wooster, OH), wheat variety (Pioneer 2545 and Freedom) and disease parameter (disease severity, disease incidence and DON) measured. The 12 RPI's obtained for each treatment (2 sites×2 varieties×3 disease parameters) were then subject to ANOVA and the Bonferroni mean comparison test ($p = 0.05$) was used to compare overall $RPI_{efficacy}$ treatment means. RPI's allow treatment data obtained on dissimilar population types (such as identical treatments applied at different locations or to wheat varieties that differ in susceptibility to *G. zeae*) using different types of quantitative measures of disease intensity (such as disease severity, diseases incidence and DON data) to be compared directly using a standardized scale. RPI's are dimensionless and theoretically range in value from 0 to 100 for normally distributed data. In the present study, $RPI_{efficacy}$ was calculated by: $|(F-2)|*25$ where $F = (X-X_{avg})/s$, X is a single disease measurement and $X_{avg}$ and s are the average and standard deviation, respectively, of the data obtained for each unique combination of field location, wheat variety and disease intensity variable. Treatments with higher $RPI_{efficacy}$ values have enhanced efficacy in reducing FHB disease parameters.

## RESULTS AND DISCUSSION

**Growth and quantification of cells in co-culture:** Cells of the three biocontrol strains (*C. flavescens* OH 182.9 (NRRL Y-30216), *C. aureus* OH 71.4 (NRRL Y-30213) and *C. aureus* OH 181.1 (NRRL Y-30215)) utilized in producing co-cultures in liquid medium did not produce colonies that were distinguishable from each other when grown on common nutrient media. Results from studies using Biolog YT Microplate™ plates revealed that the trisaccharide melezitose supported 3 distinct levels of growth for the three yeast strains (Fig. 1). Growing individual strains and known ratios of mixtures of the three strains on a solid medium containing melezitose as a sole carbon source resulted in three distinguishable colony sizes and counts which agreed with expected counts from mixing known cell numbers of the three strains. After 4 days' incubation at 28°C, strains OH 182.9, OH 71.4 and OH 181.1 produced the largest, intermediate and smallest colonies, respectively, on the melezitose medium. Slininger and co-workers relied on differential growth rates, colony morphology and colony color produced on two types of media to separate counts of three strains of Pseudomonas biocontrol agents produced in mixed strain fermentations (Slininger *et al*., 2010a).

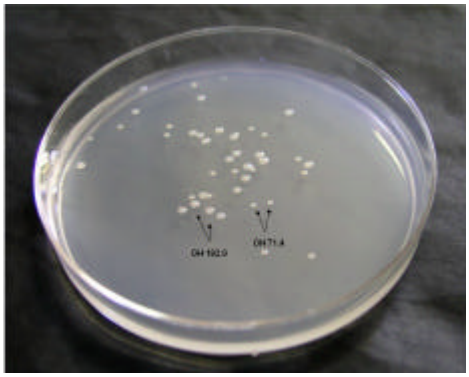

Fig. 1: Distinguishable colony sizes of strains *Cryptococcus flavescens* OH 182.9 and *C. aureus* OH 71.4 on a medium containing melezitose as a sole carbon source. Under identical growth conditions, *C. aureus* OH 181.1 produces smaller colonies than either of the strains pictured

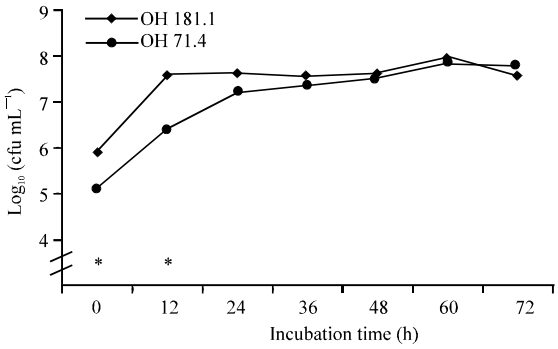


Fig. 2: Growth of component strains of a co-culture of *Cryptococcus aureus* strains OH 181.1 and OH 71.4 in SDCL medium in shake flasks when inoculated at an initial ratio of 14:1, respectively. An "*" above an incubation time indicates that the populations of individual strains of the co-culture differed significantly at the time point (Tukey HSD, $p = 0.05$). Means represent averages from three separate shake flask experiments

All possible combinations of three yeast antagonists were co-cultivated in shake flasks to produce near equivalent levels of each component strain after 48, 60 and 72 h (Fig. 2-4; data not shown for the co-culture of OH 181.1 and OH 182.9) demonstrating for the first time, the feasibility of producing multiple strains of yeast biocontrol agents in a single batch fermentation, thereby reducing the manufacturing and possibly registration costs associated with conducting separate fermentations of each component strain. While populations of

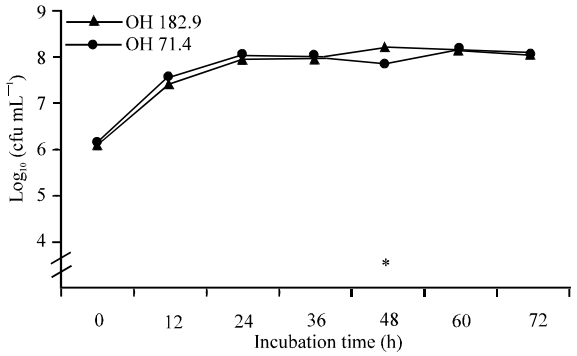


Fig. 3: Growth of component strains of a co-culture of *Cryptococcus flavescens* strain OH 182.9 and *C. aureus* OH 71.4 in SDCL medium in shake flasks when inoculated at an initial ratio of 1:4, respectively. An "*" above an incubation time indicates that the populations of individual strains of the co-culture differed significantly at the time point (Tukey HSD, p = 0.05). Means represent averages from three separate shake flask experiments

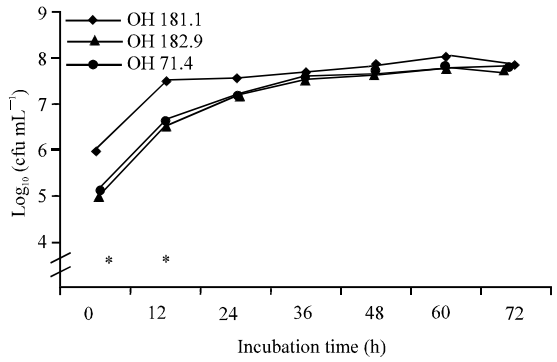


Fig. 4: Growth of component strains of a co-culture of *Cryptococcus aureus* OH 181.1, *C. flavescens* OH 182.9 and *C. aureus* OH 71.4 in SDCL medium in shake flasks when inoculated at an initial ratio of 45:1:4, respectively. An "*" above an incubation time would indicate that the populations of individual strains of the co-culture differed significantly at the time point (Tukey HSD, p = 0.05). Means represent averages from three separate shake flask experiments

component strains in co-cultures were frequently statistically different during the early stages of culture growth, in nearly every instance, populations of each component of the two- and three-strain co-cultures were not statistically separable after 36 h of incubation. Significant strain population differences in the early incubation stages of some co-cultures were associated with disparities in the ratio of seed inocula used to initiate the cultures. The inoculum of strain OH 182.9 was reduced compared to the other strains used to initiate co-cultures because strain OH 182.9 has the fastest doubling time of the three strains studied.

**Efficacy of co-cultures in reducing Fusarium head blight on wheat in the greenhouse:** The co-culture of *C. flavescens* OH 182.9 and *C. aureus* OH 71.4 was the only treatment that reduced FHB disease severity significantly compared to the control (32%, p = 0.05, Dunnett's t-test) when averaged across four greenhouse studies, though all of the antagonist treatments reduced disease severity arithmetically. The co-culture of strains OH 182.9 and OH 71.4 reduced FHB severity by 10% to 25% more than the single antagonist cultures and other co-cultures. Disease severity averaged 65% for the control and 44 to 59% for wheat treated with the various strain cultures.

Our work and that by others has reported that mixtures of strains can enhance and/or improve the consistency of biological control (Janisiewicz, 1996; Schisler *et al*., 1997; Johnson, 2010). Published reports on this subject have dealt with applications of strains that have been mixed after cultivation. In a recent study Slininger *et al*., (2010b), co-cultures of strains proved to be more efficacious and, significantly, consistent in inciting biological control of various maladies of potatoes than were mixtures of the same strains produced individually. Additional work on co-culturing microorganisms to develop biocontrol products is limited to one study and involves solid substrate, rather than liquid culture, fermentation (Olivain *et al*., 2004). The limited literature on multi-strain liquid cultures concerns producing biochemical products or the degradation of toxic compounds (Hanly and Henson, 2011; Nachiappan *et al*., 2011; O-Thong *et al*., 2011; Zhong *et al*., 2011). Interestingly, Johnson (2010), regarding the topic of pathogen refuge, postulates that some potentially infectious pathogen propagules are not susceptible to the influence of an antagonistic microbial agent but that the size of the pathogen refuge can be reduced via a number of measures including antagonist mixtures. Combinations of antagonist strains, such as produced via co-culturing in this study and that of Slininger *et al*. (2010a, b) could owe their enhanced efficacy to the co-cultured strains possessing compatible abilities for reducing pathogen refuge size.

In the present study, assays were conducted to determine how to produce equivalent numbers of antagonist cells in co-cultures and this was accomplished by varying the ratio of cells of the strains used in a particular co-culture. In previous studies with FHB antagonist OH 182.9, we have demonstrated the

Table 2: 2008 field trial results at Peoria, IL: Influence of *Cryptococcus flavescens* OH 182.9, *C. aureus* OH 71.4 and a co-culture of strains OH 182.9 and OH 71.4 on winter wheat cultivar Freedom

| | Wheat cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Pioneer brand 2545 | | | Freedom | | |
| Treatment[1,2,3] | DS (%) | DI (%) | DON (ppm) | DS (%) | DI (%) | DON (ppm) |
| Untreated control | 8.0$^{A}$ | 41.3$^{A}$ | 11.8$^{A}$ | 4.1$^{A}$ | 28.0$^{A}$ | 3.5$^{A}$ |
| OH 182.9 | 7.6$^{A}$ | 31.2$^{AB}$ | 13.6$^{A}$ | 4.9$^{A}$ | 24.8$^{AB}$ | 2.8$^{A}$ |
| OH 71.4 | 5.2$^{A}$ | 20.3$^{C}$ | 6.9$^{A}$ | 3.1$^{A}$ | 21.3$^{AB}$ | 4.1$^{A}$ |
| OH 71.4 + OH 182.9 co-culture | 5.4$^{A}$ | 25.1$^{BC}$ | 6.6$^{A}$ | 2.9$^{A}$ | 18.7$^{B}$ | 3.0$^{A}$ |

[1] OH 182.9 and OH 71.4 are abbreviations for strains *C. flavescens* OH 182.9 (NRRL Y-30216) and *C. aureus* OH 71.4 (NRRL Y-30213), respectively. OH 71.4 + OH 182.9 co-culture was produced by inoculating culture broth with both strains to produce a culture at harvest with near equivalent cell numbers of each strain (see Methods). [2]DS = Disease severity (= Average % of individual heads visually diseased), DI = Disease incidence, DON = Deoxynivalenol. [3] Within a column, means not followed by the same letter are significantly different (p = 0.05, Bonferoni mean separation)

Table 3: 2008 field trial results at Wooster, OH: Influence of *C. flavescens* OH 182.9, *C. aureus* OH 71.4 and a co-culture of strains OH 182.9 and OH 71.4 on winter wheat cultivar Freedom

| | Wheat cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Pioneer brand 2545 | | | Freedom | | |
| Treatment[1,2,3] | DS (%) | DI (%) | DON (ppm) | DS (%) | DI (%) | DON (ppm) |
| Untreated control | 11.9$^{A}$ | 34.4$^{A}$ | 8.1$^{A}$ | 1.0$^{A}$ | 11.8$^{A}$ | 2.8$^{A}$ |
| OH 182.9 | 12.6$^{A}$ | 28.0$^{A}$ | 6.8$^{A}$ | 0.9$^{A}$ | 9.6$^{A}$ | 3.0$^{A}$ |
| OH 71.4 | 13.1$^{A}$ | 34.2$^{A}$ | 8.1$^{A}$ | 0.6$^{A}$ | 7.3$^{A}$ | 2.8$^{A}$ |
| OH 71.4 + OH 182.9 co-culture | 7.8$^{A}$ | 23.6$^{A}$ | 7.6$^{A}$ | 1.4$^{A}$ | 10.7$^{A}$ | 2.7$^{A}$ |

[1] OH 182.9 and OH 71.4 are abbreviations for strains *Cryptococcus flavescens* OH 182.9 (NRRL Y-30216) and *C. aureus* OH 71.4 (NRRL Y-30213), respectively. OH 71.4 + OH 182.9 co-culture" was produced by inoculating culture broth with both strains to produce a culture at harvest with near equivalent cell numbers of each strain (see Methods). [2]DS = Disease severity (= Average % of Individual heads visually diseased), DI = Disease incidence, DON = Deoxynivalenol. [3] Within a column, means not followed by the same letter are significantly different (P=0.05, Bonferoni mean separation)

importance of optimizing carbon loading, C:N ratio (Zhang *et al*., 2005) and reducing incubation temperature in the late log growth stage of the strain (Zhang *et al*., 2006; Dunlap *et al*., 2007) in order to enhance the efficacy and stress tolerance of the antagonist. We have not attempted to alter growth conditions to optimize cell biocontrol efficacy and/or stress tolerance in yeast co-cultures but previous results indicate such studies have the potential to increase the efficacy of the product produced.

**Influence of strains OH 182.9, OH 71.4 and a co-culture of both on FHB in field grown wheat:** For studies on Pioneer Brand 2545 and Freedom wheat in Peoria, IL, high variability in field data precluded statistically separating disease data obtained for individual disease parameters in most cases. On both wheat varieties, single antagonist and the co-culture treatments did not differ significantly from the control regarding disease severity though antagonist OH 71.4 alone or in co-culture with strain OH 182.9 arithmetically reduced FHB severity to the greatest extent (Table 2). On wheat variety Pioneer 2545, the same two treatments significantly reduced disease incidence (p = 0.05, Bonferoni mean separation) while only the co-culture of OH 71.4 and OH 182.9 reduced incidence on wheat variety Freedom. None of the treatments significantly altered DON (Table 2) or 100 kernel weights (data not shown). Data variability similarly limited statistical separation of treatment means in the trial conducted in Wooster, Ohio. On Pioneer Brand 2545, the co-culture of strains OH 71.4 and OH 182.9 reduced disease severity and incidence arithmetically but not significantly, compared to the other treatments and the control (Table 3). On wheat cultivar Freedom, treatments had no significant effect on disease severity, incidence, DON (Table 3) or test weight (data not shown).

RPI analysis of the overall effect of treatments across both field sites, wheat varieties and disease parameters used revealed that treatment with the co-culture of strains OH 71.4 and OH 182.9 significantly reduced disease parameters overall, as evidence by a higher RPI value for this treatment, compared to the untreated control (p = 0.05, Bonferoni mean separation) (Table 4). The RPIs calculated for treatment with strain OH 182.9 alone and OH 71.4 alone indicated these treatments reduced FHB disease parameters arithmetically, but not significantly, compared to the control. Interestingly, in field work conducted in four States of the U.S. over two years, treatment with the fungicide Prosaro® (Bayer CropScience, Research Triangle PK, NC) at flowering and a co-culture of strains OH 182.9 and OH 71.4 five days later significantly reduced the mycotoxin deoxynivalenol (DON) by an average of 36% (Yuen *et al*., 2010). Treatment with the co-culture alone at flowering

Table 4: Overall relative performance index values for treatment efficacy ($RPI_{efficacy}$) in reducing FHB disease parameters in 2008 Peoria, IL and Wooster, OH field trials conducted to determine the influence of *C. flavescens* OH 182.9, *C. aureus* OH 71.4 and a co-culture of strains OH 182.9 and OH 71.4 on Fusarium head blight

| Treatment[1] | $RPI^{efficacy2,3}$ |
|---|---|
| Untreated control | $34.6^{B}$ |
| OH 182.9 | $44.9^{AB}$ |
| OH 71.4 | $56.3^{AB}$ |
| OH 71.4 + OH 182.9 co-culture | $64.2^{A}$ |

[1]OH 182.9 and OH 71.4 are abbreviations for strains *Cryptococcus flavescens* OH 182.9 (NRRL Y-30216) and *C. aureus* OH 71.4 (NRRL Y-30213), respectively. OH 71.4 + OH 182.9 co-culture" was produced by inoculating culture broth with both strains to produce a culture at harvest with near equivalent cell numbers of each strain. [2] Overall $RPI_{efficacy}$ value is an average of 12 individual RPI values obtained for each combination of site (Peoria, IL and Wooster, OH), wheat variety (Pioneer 2545 and Freedom) and disease parameter (disease severity, disease incidence and DON) measured in 2008 field trials. [3] $RPI_{efficacy}$ means not followed by the same letter are significantly different (p = 05, Bonferoni mean separation)

significantly reduced disease severity across all locations compared to the control both years of the study. Because co-cultured inoculum for the studies of Yuen *et al.* (2010) was produced in 100 L, rather than 1.5 L fermentors and was separated from spent broth before use, it is possible that improvements in inoculum efficacy could have been achieved in the present study if a similar scale and handling of the co-cultured inoculum was utilized.

The potential for obtaining superior efficacy with co-cultures, such as one produced using strains *C. flavescens* OH 182.9 and *C. aureus* OH 71.4, compared to the individual component strains and cost benefits in producing, processing and registering a co-cultured biocontrol product justifies additional research effort on this concept. Genomic and proteomic research tools should foster further understanding of the complexity of relationships between host, biocontrol agents and pathogen. Concomitantly, research of this nature should provide additional guidance on how to improve the quality of biomass produced in liquid culture and its efficacy when deployed in field applications alone or in combination with other control measures.

## ACKNOWLEDGMENTS

The expertise of Jennifer Sloan, Kelly Hall, Todd Hicks, Joe Rimelspach, Matthew Wallhead, William Bardall and Karlten Austin in conducting experiments is greatly appreciated as are the efforts of Dr. Yanhong Dong, University of Minnesota, in conducting the DON analysis. Studies were made possible, in part, by a grant from the U.S. Wheat and Barley Scab Initiative. Salaries and additional research support for P.A. Paul and M.J. Boehm were provided by state and federal funds to the Ohio Agricultural Research and Development Center, The Ohio State University.

## REFERENCES

Akinsanmi, O., D. Backhouse, S. Simpfendorfer and S. Chakraborty, 2006. Genetic diversity of Australian *Fusarium graminearum* and *F. pseudograminearum*. Plant Pathol., 55: 494-504.

Aoki, T. and K. O'Donnell, 1999. Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the group I population of *F. graminearum*. Mycologia, 91: 597-609.

Awad, W.A., J. Bohm, E. Razzazi-Fazeli and J. Zentek, 2006. Effects of feeding deoxynivalenol contaminated wheat on growth performance, organ weights and histological parameters of the intestine of broiler chickens. J. Anim. Physiol. Anim. Nutr. (Berl), 90: 32-37.

Beyer, M., M.B. Klix, H. Klink and J.A. Verreet, 2006. Quantifying the effects of previous crop, tillage, cultivar and triazole fungicides on the deoxynivalenol content of wheat grain: A review. J. Plant Dis. Protec., 113: 241-246.

Dill-Macky, R. and R.K. Jones, 2000. The effect of previous crop residues and tillage on Fufarium head blight of wheat. Plant Dis., 84: 71-76.

Domenech, J., M.S. Reddy, J.W. Kloepper, B. Ramos and J. Gutierrez-Manero, 2006. Combined application of the biological product LS213 with *Bacillus, Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. BioControl, 51: 245-258.

Dunlap, C.A., K.O. Evans, B. Theelen, T. Boekhout and D.A. Schisler, 2007. Osmotic shock tolerance and membrane fluidity of cold-adapted *Cryptococcus flavescens* OH 182.9, previously reported as Cr. nodaensis, a biocontrol agent of Fusarium head blight. FEMS Yeast Res., 7: 449-458.

Fuentes, R.G., H.R. Mickelson, R.H. Busch, R. Dill-Macky and C.K. Evans *et al*., 2005. Resource allocation and cultivar stability in breeding for fusarium head blight resistance in spring wheat. Crop Sci., 45: 1965-1972.

Goswami, R.S. and H.C. Kistler, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol. Plant Pathol., 5: 515-525.

Hanly, T.J. and M.A. Henson, 2011. Dynamic flux balance modeling of microbial co-cultures for efficient batch fermentation of glucose and xylose mixtures. Biotechnol. Bioeng., 108: 376-385.

Janisiewicz, W., 1996. Ecological diversity, niche overlap and coexis-tence of antagonists used in developing mixtures for biocontrol of post-harvest diseases of apples. Phytopathology, 86: 473-479.

Johnson, K.B., T.L. Sawyer, V.O. Stockwell and T.N. Temple, 2009. Implications of pathogenesis by *Erwinia amylovora* on rosaceous stigmas to biological control of fire blight. Phytopathology, 100: 128-138.

Johnson, K.B., 2010. Pathogen refuge: a key to understanding biological control. Annu. Rev. Phytopathol., 48: 141-160.

Karthikeyan, M., K. Radhika, R. Bhaskaran, S. Mathiyazhagan, R. Sandosskumar, R. Velazhahan and D. Alice, 2008. Biological control of onion leaf blight disease by bulb and foliar application of powder formulation of antagonist mixture. Arch. Phytopathol. Plant Protec., 41: 407-417.

Karthikeyan, V. and S.S. Gnanamanickam, 2008. Biological control of Setaria blast (*Magnaporthe grisea*) with bacterial strains. Crop Protec., 27: 263-267.

Khan, M.R. and F.M. Doohan, 2009. Bacterium-mediated control of Fusarium head blight disease of wheat and barley and associated mycotoxin contamination of grain. Biol. Control, 48: 42-47.

Khan, N.I., D.A. Schisler, M.J. Boehm, P.E. Lipps and P.J. Slininger, 2004. Field testing of antagonists of Fusarium head blight incited by *Gibberella zeae*. Biol. Control Theory Appl. Pest Manage., 29: 245-255.

Kim, Y.C, H. Jung, K.Y. Kim and S.K Park, 2008. An effective biocontrol bioformulation against Phytophthora blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions. Eur. J. Plant Pathol., 120: 373-382.

Kolombet, L.V., A.A. Starshov and D.A. Schisler, 2005. Greenhouse testing *Trichoderma asperellum* GJS 03-35 and yeast *Cryptococcus nodaensis* OH 182.9 as biocontrol agents against *Fusarium* head blight of wheat. Mycol. Phytopathol., 5: 80-88.

Large, E.C., 1954. Growth stages in cereals. Illustration of the feeks scale. Plant Pathol., 3: 128-129.

McMullen, M.P., R. Jones and D. Gallenberg, 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Dis., 81: 1340-1348.

Mirocha, C.J., E. Kolaczkowski, W. Xie, H. Yu and H. Jelen, 1998. Analysis of deoxynivalenol and its derivatives (batch and single kernel) using gas chromatography/mass spectrometry. J. Agric. Food Chem., 46: 1414-1418.

Nachiappan, B., Z. Fu and M.T. Holtzapple, 2011. Ammonium carboxylate production from sugarcane trash using long-term air-lime pretreatment followed by mixed-culture fermentation. Bioresour. Technol., 102: 4210-4217.

O-Thong, S., A. Hniman, P. Prasertsan and T. Imai, 2011. Biohydrogen production from cassava starch processing wastewater by thermophilic mixed cultures. Int. J. Hydrogen Energy, 36: 3409-3416.

Olivain, C., C. Alabouvette and C. Steinberg, 2004. Production of a mixed inoculum of *Fusarium oxysporum* Fo47 and *Pseudomonas fluorescens* C7 to control Fusarium diseases. Biocontrol Sci. Technol., 14: 227-238.

Paul, P.A., M.P. McMullen, D.E. Hershman and L.V. Madden, 2010. Meta-analysis of the effects of triazole-based fungicides on wheat yield and test weight as influenced by fusarium head blight intensity. Phytopathology, 100: 160-171.

Paul, P.A., P.E. Lipps, D.E. Hershman, M.P. McMullen, M.A. Draper and L.V. Madden, 2008. Efficacy of Triazole-Based Fungicides for fusarium head blight and deoxynivalenol control in wheat: A multivariate meta-analysis. Phytopathology, 98: 999-1011.

Pereyra. S.A. and R. Dill-Macky, 2008. Colonization of the residues of diverse plant species by *Gibberella zeae* and their contribution to Fusarium head blight inoculum. Plant Disease, 92: 800-807.

Pirgozliev, S.R., G.E. Simon, G.H. Martin and P. Jenkinson, 2003. Strategies for the control of *Fusarium* head blight in cereals. Eur. J. Plant Pathol., 109: 731-742.

Rocha, O., K. Ansari and F.M. Doohan, 2005. Effects of trichothecene mycotoxins on eukaryotic cells: A review. Food Addit. Contam., 22: 369-378.

Schisler, D.A. and P.J. Slininger, 1997. Microbial selection strategies that enhance the likelihood of developing commercial biological control products. J. Ind. Microbiol. Biotechnol., 19: 172-179.

Schisler, D.A., P.J. Slininger and R.J. Bothast, 1997. Effects of antagonist cell concentration and two strain mixtures on biological control of *Fusarium* dry rot of potatoes. Phytopathology, 87: 177-183.

Schisler, D.A., M.J. Boehm, T.E. Hicks and P.E. Lipps, 2002a. USDA-ARS, Ohio State University Cooperative Research on Biologically Controlling Fusarium Head Blight 2: 2002 Field Tests of Antagonist and Antagonist/Fungicide Mixtures. In: The 2002 National Fusarium Head Blight Forum, Canty, S.M., J. Lewis, L. Siler and R.W. Ward (Eds.). Michigan State University, Erlanger, KY, pp: 119-122.

Schisler, D.A., N.I. Khan, M.J. Boehm and P.J. Slininger, 2002b. Greenhouse and field evaluation of biological control of Fusarium head blight on durum wheat. Plant Dis., 86: 1350-1356.

Schisler, D.A., N.I. Khan, M.J. Boehm, P.E. Lipps, P.J. Slininger and S. Zhang, 2006. Selection and evaluation of the potential of choline-metabolizing microbial strains to reduce Fusarium head blight. Biol. Control, 39: 497-506.

Schisler, D.A. and M.J. Boehm, 2011. Prothioconazole tolerant *Cryptococcus flavescens* strains for biological control of Fusarium head blight. Patent Application No. 20110027233, Patent Pending February 2011. http://www. faqs. org/patents/app/20110027233

Searcy, E. and P. Flynn, 2009. The impact of biomass availability and processing cost on optimum size and processing technology selection. Applied Biochem. Biotechnol., 154: 92-107.

Skinnes, H., K. Semagn, Y. Tarkegne, A.G. Maroy and T. Bjornstad, 2010. The inheritance of anther extrusion in hexaploid wheat and its relationship to Fusarium head blight resistance and deoxynivalenol content. Plant Breeding, 129: 149-155.

Slininger, P.J., C.A. Dunlap and D.A. Schisler, 2010a. Polysaccharide production benefits dry storage survival of the biocontrol agent *Pseudomonas fluorescens* S11:P:12 effective against several maladies of stored potatoes. Biocontrol Sci. Technol., 20: 227-244.

Slininger, P.J., D.A. Schisler, M.A. Shea-Andersh, J.M. Sloan, L.K. Woodell, M.J. Frazier and N.L. Olsen, 2010b. Multi-strain co-cultures surpass blends for broad spectrum biological control of maladies of potatoes in storage. Biocontrol Sci. Technol., 20: 763-786.

Snijders, C.H.A., 1990. Fusarium head blight and mycotoxin contamination of wheat. Plant Pathol., 96: 187-198.

Stack, R.W. and M.P. McMullen, 1995. A visual scale to estimate severity of Fusarium head blight in wheat. North Dakota State University Extension Service Bulletin, pp: 1095, http://www. ag. ndsu. edu/pubs/plantsci/smgrains/pp1095w.htm.

Walsh, U.F., J.P. Morrissey and F. O'gara, 2001. *Pseudomonas* for biocontrol of phytopathogens: From functional genomics to commercial exploitation. Curr. Opin. Biotechnol., 12: 289-295.

Willyerd, K., L. Madden, M. McMullen, S. Wegulo and W. Bockus *et al*., 2010. Inoculated field trials for evaluating FHB/Don integrated management strategies. Proceedings of the 2010 National Fusarium Head Blight Forum, December 7-9, 2010, University of Kentucky, Milwaukee, WI, pp: 109-110.

Xue, A.G., H.D. Voldeng, M.E. Savard and G. Fedak, 2009. Biological management of Fusarium head blight and mycotoxin contamination in wheat. World Mycotoxin J., 2: 193-201.

Yuen, G.Y., C.C. Jochum, S.A. Halley, L.E. Sweets, W. Kirk and D.A. Schisler, 2010. 2010 uniform biological control trials-preliminary results. Proceedings of the 2010 National Symposium on Fusarium Head Blight Forum, December 7, 2010, University of Kentucky, Milwaukee, WI, pp: 112-115.

Zhang, S., D.A. Schisler, M.J. Boehm and P.J. Slininger, 2005. Carbon-to-nitrogen ratio and carbon loading of production media influence freeze-drying survival and biocontrol efficacy of *Cryptococcus nodaensis* OH 182.9. Phytopathology, 95: 626-631.

Zhang, S., D.A. Schisler, M.A. Jackson, M.J. Boehm, P.J. Slininger and Z.L. Liu, 2006. Cold shock during liquid production increases storage shelf-life of *Cryptococcus nodaensis* OH 182.9 after air-drying. Biocontrol Sci. Technol., 16: 281-293.

Zhong, Y., T. Luan, L. Lin, H. Liu and N.F.Y. Tam, 2011. Production of metabolites in the biodegradation of phenanthrene, fluoranthene and pyrene by the mixed culture of *Mycobacterium* sp. and *Sphingomonas* sp. Bioresour. Technol., 102: 2965-2972.