Selection and evaluation of the potential of choline-metabolizing microbial strains to reduce Fusarium head blight

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Received 7 April 2006; accepted 10 August 2006
Available online 15 August 2006

Abstract

Choline and betaine are found in wheat flower tissues and have been implicated in stimulating hyphal growth of the primary causal agent of Fusarium head blight (FHB), Gibberella zeae. Choline metabolizing strains (CMS) from wheat anthers may therefore be a useful source of antagonists of G. zeae. One-hundred twenty-three of 738 microbial strains that were recovered from wheat anthers collected from plants grown in Illinois and Ohio were CMS as determined by growth in a liquid medium containing choline as a sole carbon and nitrogen source and a colorimetric, choline oxidase-based assay of culture filtrate. Thirty-one out of 123 CMS reduced FHB disease severity by at least 25% in greenhouse tests on wheat and 17 reduced FHB severity by at least 50%. All five CMS selected for field testing in 2003 reduced disease severity compared to the untreated check at both field locations on moderately resistant cultivar Freedom. Freedom wheat treated with Pseudomonas sp. AS 64.4 had 63% and 46% less FHB severity than untreated wheat at the two sites. Three of five CMS reduced severity at both locations on susceptible cultivar Pioneer Brand 2545. Disease control was comparable to that obtained using the fungicide Folicur 3.6F. Selection of wheat anther colonists for ability to utilize choline as a sole carbon and nitrogen source has utility as a screening tool in the search for efficacious antagonists of G. zeae although choline utilization does not insure that an isolate will be an effective biocontrol agent against Fusarium head blight.

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Keywords: Fusarium graminearum; Wheat scab; Erwinia; Pantoea; Aureobasidium; Arthrobacter

1. Introduction

Fusarium head blight (FHB), primarily caused by Fusarium graminearum Schwabe Group 2 (Aoki and O'Donnell, 1999) (perfect state = Gibberella zeae (Schwein.) Petch) is a devastating disease of wheat and barley throughout the semi-humid and humid cereal producing regions of the world (McMullen et al., 1997; Muthomi et al., 2002; Yu Gagkaeva and Yli-Mattila, 2004). Reducing the impact of FHB on grain production and quality remains an intractable problem. Fungicides can be effective in reducing FHB (Jones, 1999; Suty and Mauler-Machnik, 1997; Wilcoxson, 1996), but residues, reports of fungicide resistance and instances of increases in the mycotoxin deoxynivalenol in the grain of treated wheat can be concerns with fungicide use (Chen et al., 2000; Gale et al., 2002; Mauler-Machnik and Zahn, 1994; Ramirez et al., 2004). Although the development of resistant cultivars of small grains holds promise in reducing FHB, highly resistant cultivars with ideal agronomic traits have not been developed (Bai and Shaner, 2004; Bushnell et al., 1998; Johnston, 1994). The genetic diversity of G. zeae (Cumagun et al., 2004; McCallum et al., 2004; O'Donnell et al., 2004; Walker et al., 2001) raises concerns regarding how durable the efficacy of fungicides and individual genes for resistance will be. Conventional tillage
of fields is partially effective in reducing local production of pathogen inoculum and, concomitantly, FHB (Dill-Macky and Jones, 2000; Miller et al., 1998; Pereyra et al., 2004), but minimum tillage is the preferred agricultural practice for soil conservation. The potential of ascospores of G. zeae dispersing over long distances (Schmale et al., 2005) and the diverse crops that can act as alternative hosts of the pathogen (Chongo et al., 2001), reduce the potential of crop rotation being effective in reducing FHB.

Biological control of FHB has attracted considerable research interest since the mid 1990s with laboratory and field reduction of FHB being demonstrated (Bujold et al., 2001; Gilbert and Fernando, 2004; da Luz et al., 2003; Perondi et al., 1996; Schisler et al., 2002b). Public acceptance, compatibility with other disease management measures, and durability are all factors in support of developing strategies for biologically controlling FHB.

Wheat heads first become susceptible to infection by G. zeae at anthesis (Paulitz, 1999). In a study designed to identify potential antagonists of FHB, we isolated more than 700 microbial strains from wheat anthers in order to obtain strains with enhanced potential for colonizing wheat heads and to serve as a source of potential antagonists of FHB. Three Gram positive bacterial and four yeast strains were obtained from this collection that reduced symptoms of FHB on hard red spring, durum and soft red winter wheats (Khan et al., 2004; Milus et al., 2001; Schisler et al., 2002b,c). Biocontrol efficacy and reliability have been enhanced in other pathosystems by combining two or more biocontrol strains, especially when combinations are made based on distinct mechanisms of action and/or nutrient utilization profiles (Duffy et al., 2004; Ji et al., 2006; Lutz et al., 2004; Schisler et al., 1997). While this approach to enhancing biocontrol reliability has received research attention in other pathosystems, it is a relatively untouched area in the biological control of FHB.

Microorganisms that utilize choline may be useful as antagonists of FHB and as coinoculants with OH 182.9 in 50 ml Erlenmeyer flasks. Choline chloride (1 g/L) served as the sole carbon and nitrogen source in MDL. Cultures were incubated in a shaker incubator (Inova 4230, New Brunswick Scientific, Edison, NJ) at 25°C with a throw of 2.5 cm and 250 rpm for 72 h. Two milliliters of each culture broth were then centrifuged (5000 rpm or approximately 2000g relative centrifugal force for 10 min at 4°C) and the supernatants retained. Supernatants were analyzed for the presence of choline using a modification of a colorometric procedure of Takayama et al. (1977) (Fig. 1). If choline is not fully metabolized by a microbial strain during growth, the addition of choline oxidase to spent broth produces hydrogen peroxide (H₂O₂) which, in the presence of phenol, 4-aminoantipyrine, and peroxidase produces 3H-pyrazol-3-one, a dye which colors the reaction fluid red (Fig. 1). Conversely, complete utilization of choline by a strain during the 72 h growth period would result in a clear reaction fluid. One liter of colorizing reagents (CR) was composed of 1000 units choline oxidase (from Alcaligenes species, Sigma-Aldrich, St. Louis, MO), 2200 units of peroxidase (Type XII from horseradish, Sigma-Aldrich,

2. Materials and methods

2.1. Isolation of wheat anther colonists

Procedures utilized to acquire a collection of microbial strains that were isolated from anthers are described elsewhere (Khan et al., 2001). Briefly, wheat anthers from winter wheat sampled from locations scattered across the states of Illinois and Ohio. Anthers from four to six wheat heads that were obtained from a single location in a field were placed in vials containing 10% (v/v) glycerol at 5°C immediately after collection and stored frozen at −80°C until use. More than 400 vials of anthers were obtained. To isolate individual strains of microorganisms from anthers, vials were thawed until the glycerol suspension reached 4°C. Vials were then mixed using a vortex mixer for 30 s to liberate microorganisms from anther surfaces. Suspensions containing microorganisms were then serially diluted using a sterile pH 7 phosphate buffer (0.00023 M K₂HPO₄, 0.000147 M KH₂PO₄, and 0.002 M MgCl₂). Samples were plated onto a variety of solidified media (Khan et al., 2001). Single colonies of antagonists showing distinct growth morphology were streaked for purity on one-fifth strength Tryptic soy broth agar (TSBA/5, pH 6.8) (Difco Laboratories, Detroit, MI). Seven hundred thirty-eight microbial isolates were purified and preserved in 10% w/v glycerol at −80°C until needed.

2.2. Choline utilization test

Samples of cultures frozen at −80°C in 10% glycerol were transferred to TSBA/5 and streaked for purity. Single colonies were then grown on TSBA/5 for 24 h at 28 °C and used to heavily inoculate (optical density (OD) of approximately 0.5 at 620 nm wavelength light (A₆₂₀)) 10 ml of a minimal defined liquid medium (MDL, Slivinger et al., 1994) in 50 ml Erlenmeyer flasks. Choline chloride (1 g/L) served as the sole carbon and nitrogen source in MDL. Cultures were incubated in a shaker incubator (Inova 4230, New Brunswick Scientific, Edison, NJ) at 25°C with a throw of 2.5 cm and 250 rpm for 72 h. Two milliliters of each culture broth were then centrifuged (5000 rpm or approximately 2000g relative centrifugal force for 10 min at 4°C) and the supernatants retained. Supernatants were analyzed for the presence of choline using a modification of a colorometric procedure of Takayama et al. (1977) (Fig. 1).
St. Louis, MO), 0.12 g 4-aminoantipyrine, 0.20 g phenol, 0.08 g calcium chloride dihydrate, 6.99 g Tris–HCl, and 2 ml Triton X-100 wetting agent. Individual tests of spent culture broths were conducted by combining 6.7 ml of spent broth with 1 ml CR, incubating for 15 min at 37 °C and assessing the presence or absence of color development using a spectrophotometer. Uninoculated broth served as a control to ascertain the maximum color intensity that would be obtained when a test strain utilized none of the 1 g/L choline chloride present in the growth medium.

2.3. Greenhouse testing of choline-metabolizing strains (CMS)

Two seedlings of hard red spring wheat (cultivar Norm) per 19-cm diameter pot were grown in an air-steam pasteurized (60 °C for 30 min) potting mix (Terralite Redearth mix, W.R. Grace, Cambridge, MA) in a growth chamber (25 °C, 14 h light/day, 600 mol/(m²/s)) for approximately 8 weeks prior to transfer to greenhouse benches for use in biocontrol bioassays. Conidial inoculum of *G. zeae* isolate Z-3639 was produced on clarified V8 juice agar (CV8 agar) under 12 h per day fluorescent light for 7 days at 24 °C (Schisler and Slininger, 1994). Suspensions of macroconidia were obtained by flooding the surface of colonized CV8 agar with PO₂ buffer, dislodging conidia using a sterile inoculating loop and adjusting the concentration of conidia to 5 × 10⁵ conidia/ml. To produce CMS biomass, cells were grown on TSBA/5 for 24 h as described above. These cells were then used to inoculate (OD of approximately 0.2, A₆₂₀) 10 ml of a semi-defined liquid medium (SDCL, Slininger et al., 1994) in 50-ml Erlenmeyer flasks. Flasks were incubated at 25 °C and shaken at 250 rpm for 48 h. At wheat anthesis (Feekes growth stage 10.5.1 (Large, 1954)), colonized broth of a CMS was diluted 1:3 (v/v) with an aqueous solution of 0.04% Tween 80 in phosphate buffer (approximately 2 × 10⁹ CFU/ml and 5 × 10⁸ CFU/ml for bacteria and yeast strains, respectively). Cell suspensions were then misted until run-off onto 12–14 wheat heads, or four plants, using a Crown Spra-tool air assist sprayer (Aerove Industries Inc., Gardnerville, NV) followed immediately by a mist application of a suspension of conidia of *G. zeae* Z-3639 (5 × 10⁵ conidia/ml). Heads treated with the buffer/Tween solution followed by the conidial suspension served as “pathogen only” controls. Plants were placed in plastic humidity chambers (22–26 °C) consisting of a PVC pipe frame covered with clear plastic, removed after 3 days, and scored for disease severity after 16 days using a 0–100% scale (Stack and McMullen, 1995). Due to experimental size constraints, multiple experiments were conducted in order to test 116 full and 7 partial CMS. More than 30 greenhouse experiments were conducted in the screening of CMS. In cases where disease pressure was excessive across treatments for an individual experiment, results are presented from an experimental repeat where more moderate disease pressure was obtained. In order to compare the influence of CMS from different experiments using a normalized scale, disease severity values for heads treated with a specific CMS were expressed as a percent change in severity compared to the disease severity of the control for the same experiment.

2.4. Field testing of CMS

CMS that were superior in reducing FHB disease severity compared to controls in the greenhouse bioassay were selected for field trials. An isolate of an *Arthrobacter* sp. was also selected for field testing due to strains in this genus characteristically possessing desiccation tolerance and competitiveness due to breadth of carbon-source utilization. Soft red winter wheat cultivars Freedom (moderately FHB resistant) and Pioneer Brand 2545 (FHB susceptible) were utilized for field trials conducted at Peoria, Illinois and...
Wooster, Ohio. Field trials using 6 CMS (AS 52.5, AS 52.8, AS 54.4, AS 57.1, AS 64.4, and AS 58.2 (Table 1)) were conducted in 2003. In 2003 strain AS 64.4, the only strain that significantly reduced FHB severity and incidence in 2002 field trials was tested along with 4 additional CMS strains not tested in 2002 (Table 2). The fungicide Folicur 3.6F (38.7% tebuconazole, Bayer Crop Science, Kansas City, MO), which in recent years has received yearly exemption (38.7% tebuconazole, Bayer Crop Science, Kansas City, MO), which in recent years has received yearly exemption, was included as a treatment in the field trials and applied at the recommended label rates.

2.4.1. Antagonist and pathogen inoculum production

Cells of antagonists were taken from ~80°C cold storage and grown on TSBA/5 for 24 h as described above. Cells of individual strains then were harvested and used to seed precultures consisting of 100 ml of SDCL in 500-ml flasks (OD of 0.10 at A620). Flasks were incubated at 25°C at 250 rpm (2.5 cm eccentricity) for 24 h. Pre-cultures then were used to seed 3-L Fernbach flasks containing 1.5 L of SDCL to an OD of 0.1. Cultures were incubated for 48 h at 25°C and 250 rpm. Colonized broth was then transferred to sterile containers, transported to the field on ice and used within 24 h. Inoculum of G. zeae was prepared on sterile, yellow dent corn as described by Khan et al. (2004). Briefly, yellow dent corn kernels were soaked in water for 24 h, drained, placed in an autoclavable vessel, and autoclaved for 1 h on two consecutive days. Kernels were then inoculated with G. zeae strain Fg 111-B for the Peoria, Illinois field trial and G. zeae Fg 3-93 and Fg 6-93 for the Wooster, Ohio field trial. Pathogen strains were isolated from diseased wheat grown in the local area in previous years. Vesicles were shaken periodically during 2 weeks’ incubation at 25°C. Infested corn kernels were then removed from vesicles, individual kernels separated, and used immediately to inoculate field plots.

2.4.2. Field test in Peoria, Illinois

In Peoria, Illinois, an Orthents complex, with a silty loam surface layer of approximately 25 cm and underlying silty clay loam was conventionally cultivated in the fall after application of 1120 kg/ha of Parker’s Super Soilife 10-10-10 (Pursell Industries, Inc., Sylacauga, AL) (3.92% ammonium nitrate, 6.68% urea nitrogen (1.7% slow release)). In the fall, rows of both wheat cultivars were planted by hand with a length of 2.1 m and 0.3 m between rows. Rows of a cultivar plant were separated from rows of the other cultivar by 0.3 m walkways. A border row of Pioneer Brand 2545 surrounded the experiment site and was not treated. The following spring, 2–3 weeks before the anticipated date of wheat flowering, 20 kernels per m² of yellow dent corn colonized by G. zeae were applied uniformly by hand to the site. Perithecia appeared on the kernels after about 10 days and were releasing ascospores at the time of wheat anthesis. Prior to application to flowering wheat heads, colonized culture broths were diluted to 50%

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Table 1 (continued)

<table>
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<th>CMS strain</th>
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<th>Greenhouse experiment number</th>
<th>% Change in FHB severity vs pathogen control</th>
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<td>−17</td>
</tr>
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</table>

a Fully colonized liquid cultures (48 h) of microbial strains were diluted to one-quarter strength with a solution containing 0.004% phosphate buffer, 0.019% MgCl₂, and 0.036% tween 80 and misted onto 12–14 wheat heads at flowering (Feekes' growth stage 10.5.1). After 5 min, heads were then misted with a suspension of conidia of *Gibberella zeae* Z-3639 (5 × 10⁷ coincubated in a humidity tent for 3 days, and scored for FHB disease development after 16 days.  

b Best trial performance is presented if a strain was tested more than once due to excessive disease development in the control treatment.  

c Negative or positive values represent the percentage decrease or increase in FHB severity compared to the pathogen control (wheat heads treated with conidia of *Gibberella zeae*). Average FHB severity for control = 89%.  

d Strain partially utilized choline in liquid culture bioassay.  

e Field tested in 2002 with only AS 64.4 demonstrating biocontrol activity.  

f Bold highlight = Strain selected for field trials in 2003.

of full strength using phosphate buffer. Tween-80 was added to microbial suspensions to a final concentration of 0.036% (v/v). Final CFU per ml counts for antagonist treatments were approximately 1 × 10⁹ CFU/ml and 6 × 10⁹ CFU/ml for bacteria and yeast strains, respectively. Treatment suspensions were applied at flowering using a CO₂ backpack sprayer charged at 2.8 kg per cm² and attached to a boom equipped with two, #6 Cone-jet® nozzles (R&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 CO₂ just prior to application. Treatments were applied at 750 L/ha just prior to and continuing after sunset to minimize potential UV degradation of antagonist cells. There were 5 replicates per treatment which were arranged in a

Table 2

<table>
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<th>CMS designatora</th>
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<tr>
<td>AS 35.5</td>
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<td><em>Pantoea agglomerans</em>b,d</td>
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<td>AS 55.2</td>
<td><em>Aureobasidium pullulans</em>e,f</td>
</tr>
<tr>
<td>AS 64.4</td>
<td><em>Pseudomonas</em> sp.b,a</td>
</tr>
<tr>
<td>OH 221.3</td>
<td><em>Arthrobacter</em> sp.b,h</td>
</tr>
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</table>

a CMS, choline-metabolizing strain.  
b Identification by MIDI Labs, Newark, DE, based on 16S rDNA 500 base-pair sequence homologies.  
c Closest species match of *E. amylovora* differed from AS 35.5 by 1.52%.  
d Species match differed from AS 52.2 by 0.09%.  
e Identification by MIDI Labs, Newark, DE, based on the variable D2 region of the large sub-unit rDNA 300 base-pair sequence homologies.  
f Species match differed from AS 55.2 by 0.31%.  
g Closest species matches of *Pseudomonas syringae*, *P. amygdali*, *P. savastanoi* and *P. fluorescens* F differed from AS 64.4 by 0.00%, 0.00%, 0.00% and 0.38%, respectively.  
h Closest species match of *A. histidinolovorans* differed from OH 221.3 by 2.39%.
randomized block design. The primary control treatment consisted of plants treated with a solution of buffer/Tween. A second control consisted of untreated plants. Treatment applications were confined to individual rows using two PVC pipe frames covered by plastic that were placed on either side of the row being treated. The PVC pipe frames were thoroughly rinsed after each treatment application. From the morning after treatment application until mid-milk kernel development (Feekes 11.1), wheat heads were misted with city water for 10 min/h from 5:00 P.M. until 12:00 A.M. and 2 min/h from 12:00 A.M. until 7:00 A.M. (approximately 2 cm water/day). Rainbird nozzles (15H, 15° 180° and 15Q, 15: 90° nozzles and PA-8S plastic shrub adapters) (Azusa, CA) mounted on 1.2-m risers were spaced to provide equivalent water coverage across the entire plot.

Field assessments of FHB severity and incidence were made by evaluating 60 heads per replicate (300 heads/treatment) when plants were between mid-milk and soft dough development (Feekes 11.1–11.2). Wheat heads were harvested by hand and threshed using an Almaco single plant and head thresh (Almaco, Nevada, IA) when grain reached full maturity. Grain samples obtained from each replicate row were evaluated for 100-kernel weight. Disease severity, incidence, and 100 kernel weight data were analyzed using one-way analysis of variance (ANOVA) after preliminary analysis revealed significant block by treatment interactions for the majority of disease parameters. Means were separated from the untreated and the buffer/Tween controls at P ≤ 0.05 using Fisher’s protected LSD test (Statistix 7.0, Tallahassee, FL).

2.4.3. Field test in Wooster, Ohio

In the full of 2002, seeds of Pioneer Brand 2545 and Freedom wheat were planted at a rate of approximately 24 seeds per ft 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, Ohio. Prior to planting, the field was mold-board plowed and 84.2 kg/ha of ammonium nitrate was broadcast over the field and incorporated with a disc. The experimental treatments were arranged in a randomized block design with 4 and 5 replicate plots for Freedom and Pioneer Brand 2545, respectively. Each experimental unit consisted of a 7-row plot that was 1 m x 1 m. Additional nitrogen was applied the following spring as 109 kg of ammonium nitrate. Plots were inoculated by broadcasting corn kernels infested with G. zeae over the plot surface approximately 3 weeks prior to wheat flowering. Plots were mist irrigated each day from 1 week prior to stem extension (Feekes 11.1), wheat heads were harvested by hand and threshed using an Almaco thresher. Two grain samples obtained from each replicate plot were evaluated for 100-kernel weight. Disease severity, incidence, and 100 kernel weight data were analyzed using one-way ANOVA analysis of variance after preliminary analysis revealed significant block by treatment interactions for the majority of disease parameters. Means were separated from the untreated and the buffer/Tween controls at P ≤ 0.05 using Fisher’s protected LSD test (Statistix 7.0, Tallahassee, FL).

3. Results

3.1. Choline utilization test

A total of 738 microbial strains were recovered from wheat anthers collected across the states of Illinois and Ohio. Of this total, 123 choline metabolizing strains (CMS) were identified (16.7%) when choline chloride was supplied as a sole carbon and nitrogen source in liquid culture and culture filtrates were analyzed for choline using a colorimetric, choline oxidase-based bioassay. Seven of these CMS only partially utilized choline during 72 h growth (Table 1) as determined by the production, in test supernatants, of measurable color of less intensity than produced when testing uninoculated control broths. 

3.2. Greenhouse testing of choline-metabolizing strains (CMS)

Thirty-one out of 123 CMS (116 full, 7 partial) (25% of all strains tested) reduced FHB disease severity by at least 25% compared to “disease only” controls in greenhouse tests (Table 1) and 17 (14% of all strains tested) reduced FHB severity by at least 50%. Several strains which substantially reduced FHB severity compared to the control were not selected for field testing due to the strain possessing similar colony morphology to other strains from the same anther sample or possessing growth characteristics...
that would limit its utility for commercial development. Ten CMS with FHB biocontrol activity in greenhouse assays were selected for field testing in 2002 and/or 2003 (Table 1). Of the strains tested at Wooster, Ohio in 2002, AS 64.4 was the only antagonist that reduced both disease severity and incidence on Pioneer Brand 2545 (38% and 17%, respectively, versus the untreated control) though it did not reduce FHB symptoms on cultivar Freedom. The other strains tested in the 2002 field trials were rarely effective at either field location. Therefore, strain AS 64.4 and 4 strains that had not been field tested previously were selected for replicate 2003 field trials in Peoria, Illinois and Wooster, Ohio. These CMS were identified as members of the genera Erwinia, Pantoea, Aurobasidium, Pseudomonas, and Arthrobacter (Table 2) by MIDI Labs (Newark, DE) using 16S or large subunit rDNA gene sequence homologies with known strains as determined by Applied Biosystems MicroSeqTM microbial analysis software and database (Foster City, CA).

3.3. Field testing of CMS

3.3.1. 2003 Field test in Peoria, Illinois

In plots of cultivar Freedom, FHB severity was low (treatment averages were less than 2% in all experimental plots). Wheat treated with any of the five CMS had lower FHB severity and incidence compared to the untreated control (P ≤ 0.05) with AS 64.4 and AS 35.5 treated wheat at 63% and 58% of the severity level of the control, respectively (Table 3). The buffer/Tween control and the buffer treated with the fungicide Folicur 3.6F also had less severity and incidence than the untreated control (Table 3). The 100 kernel weights of wheat harvested from treated plots did not differ significantly.

In Peoria plots of cultivar Pioneer Brand 2545, Folicur 3.6F treated wheat had lower disease severity than in either control plot while wheat treated with 3 of the 5 CMS had reduced disease severity compared to the “untreated” but not the “buffer/Tween” control. Wheat treated with the same 3 CMS or Folicur 3.6F had less FHB incidence than the untreated control and treatments AS 55.2, AS 64.4 and Folicur 3.6F also reduced incidence compared to the buffer/Tween control. Strain AS 64.4 and Folicur 3.6F treated wheat had 41% and 38% of the incidence level of the untreated control (Table 3). Wheat treated with CMS or fungicide did not differ in 100 kernel weight compared to the untreated control though most treated wheat had lower 100 kernel weights than that of the buffer/Tween control.

3.3.2. 2003 Field test in Wooster, Ohio

Disease pressure in 2003 was high in Wooster, Ohio, on cultivars Freedom and Pioneer Brand 2545. In Freedom wheat, FHB severity was less in plots treated with all CMS and Folicur 3.6F than in the untreated check and wheat treated with 3 of the 5 CMS and Folicur 3.6F had less FHB severity than the buffer/Tween control wheat (P ≤ 0.05). Reductions in severity for wheat treated with AS 64.4 and

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<th>DI (%)</th>
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* DS, % disease severity; DI, % disease incidence; 100-KW, 100 kernel weight. Within a column, means followed by “#” and “*” are significantly different from the untreated check and buffer/Tween check, respectively (FPLSD, P ≤ 0.05). Significant interactions complicate interpretation of pooled 100-KW data.

Folicur 3.6F were 46% and 54%, respectively, compared to untreated wheat and 33% and 43%, respectively, compared to the “buffer/Tween” treated wheat. The incidence of FHB was also less in plots treated with CMS AS 64.4 and Folicur 3.6F compared to either control. Regardless of the treatment used, 100 kernel weights in treated plots did not differ from the control plots.

In Wooster plots of cultivar Pioneer Brand 2545, wheat treated with any of the five CMS or Folicur 3.6F had lower FHB severity than at least one of the controls (P ≤ 0.05). Wheat treated with AS 55.2 had the lowest disease severity of any of the wheat treatments though damage was still severe on treated plots. All treated wheat had near 100% incidence and the 100 kernel weights of wheat from treated plots did not differ significantly.

4. Discussion

Choline metabolizing strains tested in 2003 moderately reduced FHB disease under field conditions in Wooster, Ohio but were not as effective in tests conducted in Peoria, Illinois (Tables 3 and 4). The best performing CMS, Pseudomonas sp. AS 64.4 often reduced FHB disease in field tests to approximately the same extent as Folicur 3.6F, the only fungicide that has received approval for use against
FHB on wheat heads in the United States. Strain AS 64.4 was also the only CMS that reduced FHB symptoms in the 2002 field tests. In this investigation, screening antagonist colonists for ability to utilize choline as a sole source of carbon and nitrogen had some utility as an initial step in identifying antagonists of G. zeae though choline utilization alone was not in itself predictive of strain biocontrol efficacy. The modes of action for the CMS evaluated have not been investigated though none of the modes of action for the CMS evaluated have not been was not in itself predictive of strain biocontrol efficacy seen for CMS tested in greenhouse versus field environments. However, experiment size and labor constraints necessitates utilizing greenhouse bioassays as a primary screening tool for evaluating the potential of a biocontrol strain slated for field use. Clearly, our results demonstrate that greenhouse discoveries of putative antagonists must subsequently be proven under field conditions. Ultimately, field testing of biocontrol strains is the only bioassay which provides adequate exposure of biocontrol strains to the diversity of environments and pathogen strain complexes under which they must perform if strains are to be considered for commercial development.

Table 4
2003 Wooster, Ohio, field test of the efficacy of choline-metabolizing microbial strains against Fusarium head blight on wheat cultivars Freedom and Pioneer Brand 2545\textsuperscript{a,b}

<table>
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<th>DI (%)</th>
<th>100-KW (g)</th>
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\textsuperscript{a} DS, % disease severity; DI, % Disease incidence; 100-KW, 100 kernel weight. Within a column, means followed by “\#” are significantly different from the untreated check and buffer/Tween check, respectively (FPLSD, P \leq 0.05).

\textsuperscript{b} Significant interactions complicate interpretation of pooled 100-KW data.

\textsuperscript{c} Treatments were applied at 750 L/ha at the time of wheat flowering (Feekes’ growth stage 10.5.1). Buffer/Tween consisted of a solution containing 0.004% phosphate buffer, 0.019% MgCl, and 0.036% Tween 80. Folicur 3.6F was applied at 292 ml/ha as per label instructions. Fully colonized microbial broths of antagonists were diluted 1:1 with Buffer/Tween before application.

Because CMS Pseudomonas sp. AS 64.4 was the most consistently efficacious CMS in field tests, field testing of this strain as a coinoculant with C. nodaensis OH 182.9 to determine if additive, synergistic, or more consistent biocontrol performance against FHB can be achieved is justified. C. nodaensis (nomen nudem) OH 182.9 has been successfully tested in a number of field trials (Khan et al., 2004; Milus et al., 2001; Schisler et al., 2002c). Arthrobacter sp. OH 221.3 may also warrant field testing as a coinoculant with C. nodaensis OH 182.9 based on its moderate field efficacy and its enhanced potential for being stabilized and formulated (Kieft et al., 1994) compared to the other bacterial strains tested in this study. Combining biocontrol agents in a single application can be an effective method for enhancing the efficacy and reliability of biocontrol (Duffy et al., 2004; Lutz et al., 2004; Schisler et al., 1997). The fermentation environment utilized to produce biomass of biocontrol agents and culture age influence the efficacy, stability, and desiccation tolerance of many biocontrol agents including spore forming fungi (Jackson et al., 2003; Montazeri and Greaves, 2002; Schisler et al., 1991), yeasts (Zhang et al., 2003), and bacteria (Slininger et al., 1996). Production protocols would have to be evaluated and developed for each component of any proposed biocontrol mixture before the feasibility of utilizing the mixture commercially could be determined. Combinations of biocontrol agents and reduced levels of fungicides also have potential to improve the level and consistency of disease reduction (Elmer and McGovern, 2004; Schisler et al., 2002a). This integration of diverse control measures also warrants further investigation for use against FHB.

The field efficacy of Arthrobacter sp. OH 221.3, despite its lack of performance in the greenhouse bioassay, and the large number of CMS that were effective in the greenhouse but not in the field, demonstrates that even bioassays based on whole plants have limitations in predicting biocontrol performance of a strain in the field. In work with antagonists of the causal agent of dry rot G. pulicaris, we demonstrated that biocontrol strain efficacy can vary significantly depending on the cultivar and pathogen strain utilized in a bioassay (Schisler et al., 1997). Different in greenhouse versus field testing regarding pathogen strains present, the cultivars tested, environmental differences including temperature, rainfall, humidity, UV exposure, and the resident microflora on wheat heads could all contribute to the differences in efficacy seen for CMS tested in greenhouse versus field environments. However, experiment size and labor constraints necessitates utilizing greenhouse bioassays as a primary screening tool for evaluating the potential of a biocontrol strain slated for field use. Clearly, our results demonstrate that greenhouse discoveries of putative antagonists must subsequently be proven under field conditions.
The role of choline and betaine in the pathogenesis of *G. zeae* on wheat remains an interesting question. Strange and Smith (1978) implicated choline and betaine in stimulating early germ tube elongation by conidia of *G. zeae*. Engle and coauthors (2004) determined that hyphal growth of *G. zeae* from agar plugs was modestly but significantly stimulated in the presence of low concentrations of choline, betaine, or equivalent amounts of both. Our method for selecting choline metabolizing strains required microbial utilization of choline as a carbon and nitrogen source, minimizing the possibility of isolating strains that only oxidized choline to betaine. In our tests under controlled greenhouse conditions where confounding environmental and microbial diversity factors were reduced compared to field conditions, 25% of CMS were effective in reducing FHB severity by at least 25%. Determining the impact on biocontrol efficacy of site directed mutagenesis and restoration of choline oxidase activity in CMS antagonists could clarify the role of choline in *G. zeae* pathogenesis.

Acknowledgments

This material is based upon work supported by the U.S. Department of Agriculture and is, in part, a cooperative project with the U.S. Wheat & Barley Scab Initiative. Expert technical support was provided by Jennifer Sloan, Todd Hicks and Shama Khan. We thank Jim VanCauwenberge and Audrey Johnston for their crucial role in field plot preparation and maintenance in Peoria, IL and Wooster, OH, respectively.

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