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

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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.

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

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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 wheat in greenhouse and field tests (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 our most efficacious strain studied to date, *Cryptococcus nodaensis* OH 182.9 (NRRL Y-30216). While there is agreement that choline and betaine can stimulate hyphal growth of *G. zeae*, there is some disagreement as to the role this phenomenon plays in FHB development (Engle et al., 2004; Nkongolo et al., 1993; Strange and Smith, 1978). Choline and betaine are found in wheat anthers and other wheat head tissues susceptible to infection by *G. zeae* (Strange and Smith, 1971, 1978), suggesting that some choline metabolizing strains (CMS) from our anther culture collection may be effective competitors of the pathogen and useful as coinoculants with OH 182.9.

The objectives for this study were, therefore, to identify CMS from among strains obtained from wheat anthers, to evaluate the FHB biocontrol efficacy of CMS via greenhouse bioassays, and to field test CMS that reduced FHB in greenhouse bioassays to identify field-effective CMS biocontrol agents to formulate with *C. nodaensis* OH 182.9 in future studies. An abstract describing a portion of this work has been published previously (Schisler et al., 2004).

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 K_2HPO_4, 0.000147 M KH_2PO_4, and 0.002 M $MgCl_2$). 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 (A_{620})) 10 ml of a minimal defined liquid medium (MDL, Slininger 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 2000*g* 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 colormetric 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 (H_2O_2) 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,