J. Ky. Acad. Sci. 67(2):117–124. 2006.

Inhibition of *Pyricularia oryzae*, the Gray Leaf Spot Pathogen of Perennial Ryegrass (*Lolium perenne*), by AH010, a Novel Fungicidal Material

Norman E. Strobel[1]

Division of Natural Sciences, Bluegrass Community and Technical College, 470 Cooper Drive, Lexington, Kentucky 40506-0235

ABSTRACT

Mycelial growth of *Pyricularia oryzae* on amended potato-dextrose agar was inhibited to 50% of controls by ≥1250 ppm AH010 (40% benzalkonium chloride, 60% urea) and almost completely prevented by 10,000 ppm AH010. *P. oryzae* spore germination was completely inhibited by ≥18.6 ppm AH010 in sterile deionized water, and inhibition at 18.6 and 46.5 ppm AH010 was abolished by potato-dextrose broth. Sub-inhibitory concentrations of salicylic acid (SA; 50, 75, and 100 μM) negated the delaying effect of 9.3 ppm AH010 on spore germination. Maximal protection of perennial ryegrass plants from gray leaf spot disease (70–80% symptom reduction) was obtained with 2000 ppm foliar-applied AH010. Foliar-applied SA (2 mM) provided modest disease suppression that was dependent upon time of application and light regime. However, 2 mM SA antagonized disease suppression by AH010. Phytotoxicity of AH010 to *L. perenne* was observed, and this was conditioned by AH010 concentration and light regime.

KEY WORDS: *Pyricularia oryzae, Lolium perenne,* benzalkonium chloride, salicylic acid, light

INTRODUCTION

At present most American farmers protect their crop plants from diseases caused by fungal pathogens with synthetic fungicides that are thought to pose threats to human health and the environment. The utility of many of these fungicides also can be limited by the tendency of fungi to develop genetically transmissible resistance that renders these chemicals less effective, or ineffective in some cases. For these reasons, alternative means of plant disease suppression are under intensive investigation. A novel fungicidal material, AH010, is under development by United Promotions Incorporated (UPI, Atlanta,GA) as a possible replacement for standard fungicides. The active ingredient of AH010 is alkylbenzyldimethylammonium chloride (benzalkonium chloride), a quaternary ammonium compound that has been widely used as a surface disinfectant, primarily for the elimination of harmful bacteria in medical and food processing facilities. AH010 contains 40% benzalkonium chloride by weight, with 60% urea added as a stabilizer. AH010 is non-carcinogenic and biodegradable and is currently registered with the EPA for use as a disinfectant and algaecide for greenhouse floors and benches (UPI product, Timsen®) and for the control of diseases of ornamental crops under greenhouse production (UPI product, PronTech®).

The primary purpose of this research was to evaluate the ability of AH010 to suppress the *in vitro* and *in planta* development of *Pyricularia oryzae* Cav., a fungus that is the causal agent of the gray leaf spot disease of perennial ryegrass (*Lolium perenne* L.). This disease can cause serious reductions in turfgrass quality. Cultural practices are alone insufficient to control the disease. Although new cultivars of *L. perenne* with substantial resistance to the pathogen have been developed recently, it will be many years before they are widely planted, and it remains necessary to employ fungicides to obtain satisfactory disease control on existing susceptible turf (Paul Vincelli, Department of Plant Pathology, University of Kentucky, pers. comm.). Several of the most effective fungicides for control of this disease are at risk for developing resistance in the pathogen (Vincelli 1999), and the development of *P. oryzae* strains resistant to strobilurin fungicides has resulted in the failure of these fungicides to control the disease in some instances (Vincelli and Dixon 2002). The potential for salicylic acid (SA), an inducer of heightened disease resistance in many plant species (Delaney et al. 1994), to interact with

[1] Corresponding e-mail: Norman.strobel@kctcs.edu

AH010 in an additive or synergistic manner with respect to the suppression of *P. oryzae* was also investigated. SA was previously shown to possess direct antifungal activity and to synergistically enhance the activity of diverse other antifungal agents (Strobel and Porter 2005). Although it has been most intensively studied in dicotyledonous species, exogenous SA has been reported to enhance the heat tolerance of Kentucky bluegrass (*Poa pratensis* L.; He et al. 2005), activate the expression of genes related to disease resistance in wheat (*Triticum aestivum* L; Gorlach et al.1996), and protect barley (*Hordeum vulgare* L.) from oxidative damage incited by paraquat (Ananieva et al. 2004).

MATERIALS AND METHODS

Chemicals and Fungal Culture Materials

Deionized water (DIW) was used in the preparation of culture media for maintenance and preparation of spore inoculum of *P. oryzae*. AH010 was supplied by UPI (2030 Powers Ferry Road SE, Suite 136, Atlanta GA 30339). Salicylic acid (SA) was of USP grade. The fungicide Daconil 2787 (12.9% active ingredient chlorothalonil, manufactured by ISK Biotech Corporation, distributed by Dragon Corporation, Roanoke, VA 24019) was obtained locally.

P. oryzae Source, Maintenance, and Inoculum Preparation

P. oryzae isolate KY96, obtained from Dr. Paul Vincelli, University of Kentucky Department of Plant Pathology, was originally isolated from diseased *L. perenne*. For studies of *in vitro* mycelial growth, the fungus was maintained by serial mycelial transfer on potato-dextrose agar (PDA) incubated at room temperature (20–22 °C) in the dark. PDA was prepared by the addition of 2% non-nutritive agar to potato-dextrose broth (PDB). For studies of chemical effects on *P. oryzae* spore germination *in vitro*, spore production was accomplished by maintenance (via serial streaking of spores) on one-tenth strength oatmeal agar amended with non-nutritive agar to yield 2% agar content (DOA), with incubation at room temperature under continuous fluorescent lighting. Typically, spores produced within 6–8 d of transfer were employed for these studies. To harvest spores, culture plates were flooded with 5 ml DIW, their surfaces scraped with a sterile transfer loop, and the resultant suspension filtered through autoclaved cheesecloth to remove hyphal fragments and conidiophores. Spore density in filtered suspensions was determined with a haemocytometer. For studies of chemical effects on disease development in *L. perenne* plants, spore inoculum of *P. oryzae* was prepared each time by inoculating DOA with KY96 mycelia and spores that had been stored at −20 °C on colonized filter paper discs after routine re-isolation from *L. perenne* for the purpose of maintaining pathogenicity. Although maintenance of fungal pathogenicity was not crucial to the *in vitro* studies, a subsequent bioassay on *L. perenne* plants revealed that *P. oryzae* had not lost pathogenicity as a result of serial transfer on DOA.

In Vitro Inhibition of *P. oryzae* Mycelial Growth by AH010

The effect of AH010 on mycelial growth of *P. oryzae* was evaluated in polystyrene Petri plates (15 × 100 mm) containing PDA amended after autoclaving (but while still molten) with aliquots of filter-sterilized stock solutions of AH010. Inoculum plugs (approximately 4–6 × 4–6 mm) were cut from the actively growing margins of fungal colonies on PDA and placed in the centers of amended PDA plates (4 replicate plates per treatment in each experiment). Colony diameters were measured 12–14 d after inoculation. PDA not amended with AH010 served as the experimental control. Experiment 1 (conducted twice) employed six final concentrations of AH010 in PDA: 0, 250, 625, 1250, 1875, and 2500 ppm of formulated product. Experiment 2 (conducted twice) employed higher final concentrations of AH010 in PDA (0, 2500, 3000, 4000, 5000, and 10,000 ppm).

In Vitro Inhibition of *P. oryzae* Spore Germination in Sterile Deionized Water Amended With AH010 and/or SA

These experiments were conducted to more closely simulate the natural conditions of low nutrient availability under which *P. oryzae* spores normally germinate to initiate infection on perennial ryegrass leaves. Polystyrene 24-well culture plates received sterile deionized water and appropriate aliquots of filter-steril-

ized AH010 and SA to yield a volume of 400 μL in each well (four replicate wells per AH010 concentration were used in each experiment). Wells were then inoculated with 30 μL aliquots of *P. oryzae* spore suspension (1–2 × 10^4 spores per ml), and germination was assessed microscopically 16–18 hr after inoculation and again at 3–5 d after inoculation to detect delayed germination of spores. The purpose of these experiments was to identify concentrations of chemicals that resulted in 100% inhibition of spore germination. Wells in which germination appeared to be completely inhibited were thoroughly examined to ensure that no germinated spores were present. In wells in which germination had occurred, several microscope fields, each containing 10–20 spores, were examined, and percent germination estimated. Experiment 3 (conducted twice) employed final concentrations of 0, 465, 930, 1860, 2790, and 3720 ppm AH010 in each of four replicate wells. Experiment 4 (conducted twice) employed final concentrations of 0, 4.65, 9.3, 18.6, 46.5, and 93.0 ppm AH010 in each of four replicate wells per treatment. Preliminary experiments were conducted in well plates to assess the sensitivity of *P. oryzae* spore germination to SA. SA concentrations of 0, 100, 200, and 300, or 0, 50, 75, and 100 μM were selected for experiments 5a and 5b, respectively, which evaluated the interactive effects of SA and AH010 (0, 0.93, 1.86, 4.65, 9.3, and 18.6 ppm) on spore germination. Experiments 5a and 5b were conducted once, with a total of two replicate wells per treatment combination in each experiment.

Inhibition by AH010 of Gray Leaf Spot Disease Caused by *P. oryzae* on *L. perenne*

The ability of AH010 alone or in combination with 2 mM SA to protect perennial ryegrass plants from gray leaf spot disease was investigated in a wick-culture system according to the materials and methods of Vincelli and Dixon (2002) except where noted below. Experiment 6 employed foliar applications of 0, 500, 1000, 2000, 3000, or 4000 ppm of AH010 and was conducted twice. Positive controls were treated with a commercial protectant fungicide, Daconil, at the concentration recommended for control of gray leaf spot under field conditions. One day before chemical treatment pots containing approximately 40–50 two-week old *L. perenne* plants were trimmed to a height of 4–5 cm to provide uniform plant size and to ensure proper fit during the three-day incubation period in moist chambers. Chemicals were applied by spraying foliage to the point of runoff. After air-drying for one hour, plants in four replicate pots per treatment were inoculated by spraying with a suspension of *P. oryzae* spores (2–3 × 10^5 spores per ml) and sealed in moist chambers maintained in dim light for 24 hr followed by 48 hr under the normal fluorescent-lighting regime. After removal from moist chambers, plants were incubated for an additional 3–4 d under the normal light regime to allow the disease to develop. For disease assessment plant material in each pot was removed by gathering it together and clipping with scissors approximately 5 mm above the growth-medium surface. Twenty individual plants per pot were drawn randomly from each aggregate sample, and the percent of plant leaf area bearing necrotic lesions incited by *P. oryzae* was estimated visually. These data were summed and then divided by 20 to derive an average percent disease value for each pot. In the first trial of the experiment, plants in two additional replicate pots per chemical treatment were sprayed with deionized water free of *L. perenne* spores and then incubated as described to determine whether any of the AH010 concentrations employed were phytotoxic to *L. perenne*.

Interactive Effects of AH010 and SA on Development of Gray Leaf Spot Disease

Methods described above were employed to conduct a preliminary dose-response study with SA to identify a suitable concentration for use in the interaction studies with AH010. Two to three replicate pots per treatment were sprayed with unbuffered aqueous solutions containing 0, 0.5, 1, or 2 mM SA. The interactive effects of AH010 and SA on gray leaf spot disease development were examined in experiment 7 (conducted twice) in which methods differed in several details from those described above. Experimental plants were sprayed with chemical solutions (DIW, SA (2 mM), AH010 (2000 ppm), or SA (2 mM) + AH010 (2000 ppm) either 1 or 24 hr before inoculation with *P. oryzae*. The purpose of the

Table 1. Influence of 250–2500 ppm AH010 on mycelial growth of *Pyricularia oryzae* on potato-dextrose agar. Data presented are mean colony diameters (mm), standard errors, and percent relative to controls, for four replicate Petri plates per treatment in each of two experimental trials.

	AH010 (ppm)					
	0	250	625	1250	1875	2500
Trial I	70.9	63.5	49.5	41.9	30.8	23.5
	0.33	0.50	0.64	0.77	0.60	0.61
	(100)	(81.6)	(69.8)	(59.1)	(43.4)	(33.1)
Trial II	70.4	61.4	45.5	38.8	26.5	20.8
	0.94	1.11	0.61	0.32	1.06	0.52
	(100)	(87.2)	(64.6)	(55.1)	(37.6)	(29.5)

Table 2. Influence of 2500–10,000 ppm AH010 on mycelial growth of *Pyricularia oryzae* on potato-dextrose agar. Data presented are mean colony diameters (mm), standard errors, and percent relative to controls, for four replicate Petri plates per treatment in each of two experimental trials.

	AH010 ($\times 10^3$ ppm)					
	0	2.5	3.0	4.0	5.0	10.0
Trial I	69.3	21.0	18.0	11.4	10.8	7.8
	0.48	0.48	0.20	0.13	0.14	0.97
	(100)	(30.3)	(26.0)	(16.5)	(15.6)	(4.3)
Trial II	78.8	23.4	20.4	13.0	11.4	5.9
	0.43	0.13	0.38	0.20	0.22	0.31
	(100)	(29.7)	(25.9)	(16.5)	(14.5)	(7.5)

24-h pretreatment was to allow sufficient time for plant tissues to respond (e.g., via activation of antipathogen defense mechanisms) to chemical(s) that may have been absorbed through the cuticle. Plants were trimmed immediately before inoculation with the pathogen, rather than 24 hr before inoculation, to minimize excessive localized leaf wetness associated with contact of elongating shoots with the plastic covers of moist chambers. Plants in both trials of the experiment were inoculated by spraying with a spore suspension containing 1×10^5 spores/ml and were inadvertently maintained in dim light during the entire 3-d period of incubation within moist chambers, rather than the 1-d period of dim light in the dose-response studies. Significant water-soaking of older leaf tissues of all plants treated with 2000 ppm AH010 (regardless of SA treatment) was observed upon removal of plants from the moist chambers. Water-soaked tissues developed a bleached appearance (ranging from very pale tan to white) upon subsequent incubation under light. In most instances, this apparent phytotoxicity was readily distinguishable from the gray-green to brown coloration associated with necrosis induced by *P. oryzae*. In cases where the distinction was unclear, affected tissues were rated as necrotic due to *P. oryzae* infection. Estimates of percent necrosis reflected the extent of necrosis attributed to *P. oryzae* relative to the total leaf area (including portions damaged by AH010). Following harvest of plant material for disease assessment, the clipped pots were returned to the standard lighting regime for an additional 10 d to allow regrowth of surviving plants to occur. Plant tissues in each pot were cut 7–10 mm above the growth-medium surface to minimize contamination with necrotic plant material and fresh weight (mg) determined.

RESULTS

In Vitro Inhibition of *P. oryzae* Mycelial Growth by AH010

AH010 inhibited the mycelial growth of *P. oryzae* in a dose-dependent manner (Table 1). In both trials, concentrations in excess of 1250 ppm AH010 were required to inhibit mycelial growth by 50% relative to controls not exposed to AH010, and none of the concentrations employed abolished mycelial growth. Higher concentrations of AH010 resulted in greater inhibition of *P. oryzae* mycelial growth (Table 2). The onset of *P. oryzae* mycelial growth was substantially delayed at 5000 and 10,000 ppm, and after 14 d mycelial growth on PDA amended with 5000 ppm AH010 extended only a short distance into the amended agar. Short felt-like mycelium developed on the surfaces of the original inoculum plugs in Petri plates containing PDA amended with 10,000 ppm AH010. Mycelia did not extend into the amended agar in these plates.

In Vitro Inhibition of *P. oryzae* Spore Germination in Sterile Deionized Water Amended With AH010 and/or SA

In both trials of experiment 3, approximately 90% of spores germinated within 16–18 hr after their introduction to control wells containing only sterile DIW. No spore germination was detected in either trial at 16–18 hr or after 3–5 d in wells amended with AH010

(465–3720 ppm; data not shown). The effects of lower concentrations of AH010 (4.65–93.0 ppm) on spore germination were examined in Experiment 4. At 16–18 hr after inoculation, approximately 90% germination was observed in wells that contained either sterile DIW or 4.65 ppm AH010, but the germ tubes of spores in the latter wells were stunted when compared with those of water controls. No spore germination was observed at 16–18 hr after inoculation in wells containing 9.3, 18.6, 46.5, or 93.0 ppm of formulated product. When plates were observed 3–5 d after introduction of spores to wells, delayed germination of *P. oryzae* spores was observed the 9.3 ppm concentration of AH010 (germ tubes were stunted), whereas no germination was observed in wells that contained 18.6, 46.5, or 93.0 ppm of AH010.

A preliminary dose-response study with SA indicated that a concentration of 100 μM SA had no apparent effect on spore germination (which was approximately 90%, as in the DIW controls), whereas spore germination was strongly inhibited by 200 μM SA (the 2–5% of spores that germinated had stunted germ tubes) and completely inhibited by 300 μM SA. In experiments 5a and 5b, germination at 16–18 hr was comparable to that of DIW controls (approximately 90%) in wells containing 0.93, 1.86, or 4.65 ppm AH010. Germination at 9.3 ppm AH010 was delayed by 12–24 hr and resulted in relatively stunted germ tubes, and germination rarely occurred in wells containing 18.6 ppm AH010. Little or no germination occurred in any wells amended to contain 200 or 300 μM SA. Inclusion of 50, 75, or 100 μM SA in wells containing 0, 0.93, or 1.86 ppm AH010 did not affect germination relative to that of DIW controls. Inclusion of 100 μM SA in wells containing 4.65 or 9.3 ppm AH010 diminished percent germination to approximately 70–80% of DIW controls. SA concentrations of 50, 75, and 100 μM overcame the delay in germination otherwise observed at 19.3 ppm AH010 (germination occurred within 8 hr rather than 24–36 hr), and 100 μM SA sometimes enabled delayed germination (55–75%) in the presence of 18.6 ppm AH010. Thus, sub-inhibitory concentrations of SA appeared to antagonize the inhibitory effects of 9.3 and 18.6 ppm AH010 on spore germination.

Table 3. Influence of AH010 on severity of gray leaf spot disease in perennial ryegrass (*L. perenne*) inoculated with *Pyricularia oryzae*. Data presented are mean necrotic leaf area, standard error, and percent disease relative to controls, of four replicate pots per treatment in each of two trials.

	AH010 ($\times 10^3$ ppm)						
	0	0.5	1.0	2.0	3.0	4.0	Daconil
Trial I	63.4	48.5	18.8	12.3	11.5	17.8	0.0
	3.3	4.3	1.4	0.8	0.3	2.7	0.0
	(100)	(76.5)	(29.7)	(19.4)	(18.1)	(28.1)	(0.0)
Trial II	66.6	34.6	19.8	18.4	18.3	20.6	0.0
	3.2	3.4	1.9	2.7	1.2	1.0	0.0
	(100)	(52.0)	(29.7)	(27.6)	(27.5)	(30.9)	(0.0)

Inhibition by AH010 of Gray Leaf Spot Disease caused by *P. oryzae* on *L. perenne*

In experiment 6, control plants (sprayed with DIW one hour before inoculation with *P. oryzae* spores) were severely diseased, with an average percent necrotic leaf area of 63.4 and 66.6 in trials I and II, respectively (Table 3). Substantial reductions in disease development were observed for all concentrations of AH010 employed in these trials. In trial I, 2000 and 3000 ppm AH010 provided 80% reduction in symptomatic leaf area, relative to controls. A lesser degree of disease suppression by 2000 and 3000 ppm AH010 was observed in trial II (72% reduction, relative to controls). Interestingly, the highest concentration of AH010 employed, 4000 ppm, tended to provide somewhat less disease control (relative to 2000 and 3000 ppm) in both trials I and II. Plants not inoculated with *P. oryzae* showed similar patterns of growth for AH010 concentrations of 0, 500, 1000, 2000, and 3000 ppm, whereas plants treated with 4000 ppm AH010 were moderately stunted and exhibited a white to light tan chlorosis at the tips of some leaf blades. Daconil, a conventional synthetic fungicide that is registered with the EPA for use in controlling gray leaf spot, completely prevented disease development and was not phytotoxic in these trials.

Interactive Effects of AH010 and SA on Disease Development and Plant Regrowth

Exogenous SA affected disease development in a dose-dependent manner. Percent necrotic leaf area (mean ± SE) for plants sprayed with SA 0 (DIW controls), 0.5, 1, or

Table 4. Influence of AH010, salicylic acid (SA), and the timing of their application on severity of gray leaf spot disease and regrowth of perennial ryegrass (*L. perenne*) inoculated with *Pyricularia oryzae*. Data presented are mean necrotic leaf area (percent) and regrowth (mg fresh weight), standard errors, and percent disease and regrowth relative to controls. Trials I and II employed four and two replicate pots per treatment, respectively. 1 hr and 24 hr denote the number of hours between application of chemicals and inoculation with *P. oryzae*.

	AH010 (0 ppm) SA (0 mM)		AH010 (2000 ppm) SA (0 mM)		AH010 (0 ppm) SA (2 mM)		AH010 (2000 ppm) SA (2 mM)	
	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Trial I Disease	48.2	50.6	18.4	15.4	41.8	55.4	25.2	23.6
	2.7	3.4	0.9	2.6	3.4	4.0	2.2	2.6
	(100)	(105.0)	(38.2)	(32.0)	(86.7)	(114.9)	(52.3)	(49.0)
Trial I Regrowth	43.5	31.0	84.0	93.3	82.5	34.5	74.0	76.5
	9.5	8.4	11.6	8.5	10.0	6.8	14.0	14.8
	(100)	(71.3)	(193.1)	(214.5)	(189.7)	(79.3)	(170.1)	(175.9)
Trial II Disease	69.5	70.4	30.3	38.6	48.3	67.9	30.9	38.5
	1.2	4.4	10.0	3.2	0.0	4.9	7.4	11.0
	(100)	(101.3)	(43.6)	(55.5)	(69.5)	(97.7)	(44.5)	(55.4)
Trial II Regrowth	11.5	24.0	91.5	37.5	42.5	31.5	68.0	80.5
	5.5	7.0	0.5	7.5	6.5	9.5	3.0	23.5
	(100)	(208.7)	(795.7)	(326.1)	(369.6)	(273.9)	(591.3)	(700.0)

2 mM were 65.4 ± 4.2, 39.2 ± 3.4, 56.1 ± 4.7, and 22.2 ± 5.9, respectively. 2 mM SA was selected for use in interaction studies with AH010 (experiment 7). The interactive effects of AH010 and SA, and the timing of their application, on disease development and plant regrowth were complex (Table 4). Despite several differences in methodology between this experiment and experiment 6, disease development in water-sprayed controls was roughly comparable to that in experiment 6, ranging from 48.2 ± 2.7% to 70.4 ± 4.4% in experiment 7 as compared with 63.4 ± 3.3% to 66.6 ± 3.2% in experiment 6 (Table 3). Disease suppression by 2000 ppm AH010 ranged from 44–68%, relative to water controls. The timing of treatment applications (either 1 or 24 hr prior to inoculation with *P. oryzae* spores) had no significant effect on disease development in water controls, or in plants sprayed with 2000 ppm AH010. The effects of SA on disease development were significantly affected by the timing of SA application to plants. When SA was applied 1 hr before inoculation with *P. oryzae*, the chemical had a slight inhibitory effect on disease development in the first trial, and suppressed disease development by 30% in the second trial, relative to water controls. Application of SA 24 hr prior to inoculation with *P. oryzae* resulted in disease development comparable to that in water controls, and significantly more disease than observed in plants sprayed with SA 1 hr before inoculation with the pathogen. A consistent pattern in the relative regrowth of plants after clipping to remove fungus-inoculated tissues was observed for both trials, although the trials differed in absolute amounts of regrowth (measured as mg fresh weight). Regrowth was least in pots that had sustained the greatest disease development (water controls and plants sprayed with SA 24 hr before inoculation with *P. oryzae*) and greatest in pots that had sustained the least amount of disease (those treated with AH010 at either 1 or 24 hr before inoculation with *P. oryzae*, and those treated with SA 1 hr before inoculation with the fungus).

DISCUSSION

Mycelial growth of *P. oryzae* on AH010-amended PDA (a nutritive medium) was far less sensitive to AH010 than was spore germination in AH010-amended DIW (the ED_{50} was 1250–1875 ppm for mycelial growth on PDA and 5–10 ppm for spore germination in DIW). The mechanism(s) underlying the greater sensitivity of spores to AH010 in DIW remain to be determined. Preliminary experiments, however, revealed that *P. oryzae* spores were able to germinate in the presence of 50 and 200 ppm AH010 in half-strength PDB. Thus the presence of nutrients in PDA may have contributed to the lesser sensitivity of *P.*

oryzae towards AH010 observed in studies of mycelial growth as compared with spore germination. Because a hazy appearance developed when >500 ppm AH010 was combined with half-strength PDB or with PDA, it is also possible that direct physical interactions of AH010 with one or more components of these media may have reduced the availability of the substance to the fungus.

Antagonistic interactions (*sensu* Kosman and Cohen 1996) of SA with AH010 were observed *in vitro* (spore germination assays) and *in planta* (disease suppression studies). In contrast, SA was previously reported to synergistically enhance the *in vitro* activity of several other antifungal agents (Strobel and Porter 2005). The mechanism(s) underlying the antagonism of AH010 by SA are unknown. The SA concentrations employed did not appear to interact physically with AH010 (no haze formation was observed) and did not reduce the pH of AH010 solutions below that indicated by the manufacturer to be suitable for antifungal activity of AH010 (pH 3.0). SA has been reported to activate an efflux pump in the plasma membrane of *Burkholderia cepacia* that confers resistance to multiple antibiotics (Nair et al. 2004). Increased activity of similar efflux pumps has been implicated in the tolerance of diverse bacteria to benzalkonium chloride (Poole 2005) and the pathogenicity of rice blast isolates of *P. oryzae* (Urban et al. 1999). It remains to be determined whether activation of a similar efflux pump contributes to the increased benzalkonium chloride tolerance of perennial ryegrass isolates of *P. oryzae* afforded by SA and constituents of PDA and PDB.

Although 20 ppm AH010 completely inhibited germination of *P. oryzae* spores *in vitro*, 500–4000 ppm AH010 afforded only partial protection of perennial ryegrass seedlings from *P. oryzae*. The reasons for this discrepancy are unknown. The maximum degree of disease suppression afforded by AH010 (80%) was significantly less than that afforded by Daconil (100%), a commercial fungicide commonly employed to suppress the disease under field conditions. The extent of disease suppression by AH010 was not enhanced by applying the chemical 24 hr prior to fungal inoculation or by combining AH010 with SA, although insufficient light availability during the early part of the incubation period in those experiments may have contributed to this finding (Chandra-Shekara et al. 2005; Genoud et al. 2002; Molina et al. 1998). Further testing is required to evaluate the effects of light on the interactions of AH010, SA, and *P. oryzae* with perennial ryegrass seedlings in controlled environments, and to evaluate the efficacy of AH010 for suppression of gray leaf spot disease under field conditions.

ACKNOWLEDGMENTS

This work was supported by grants from United Promotions Inc. and the Bluegrass Community and Technical College (Innovation Grant, 2005-2006). The author thanks Dr. Paul Vincelli (Professor) and Edward Dixon (Research Support Specialist) for providing cultures of *Pyricularia oryzae*, technical assistance and guidance, and the use of laboratory and plant culture facilities in the Department of Plant Pathology, University of Kentucky, Lexington. Thanks also to Larry Porter, Instructional Specialist, at Bluegrass Community and Technical College, for editorial assistance.

LITERATURE CITED

Ananieva, E. A., K. N. Christov, and L. P. Popova. 2004. Exogenous treatment with salicylic acid leads to increased antioxidant capacity in leaves of barley plants exposed to paraquat. Journal of Plant Physiology 161: 319–328.

Chandra-Shekara, A. C., M. Gupte, D. Navarre, S. Raina, R. Raina, D. Klessig, and P. Kachroo. 2006. Light-dependent hypersensitive response and resistance signaling against Turnip Crinkle Virus in *Arabidopsis*. The Plant Journal 45:320. Accessed from the Blackwell Publishing database (8/23/06) www.blackwell-synergy.com/doi/abs/10.1111/j.1365-313X.2005.02618.

Delaney, T. P., S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessman, E. Ward, and J. Ryals. 1994. A central role of salicylic acid in disease resistance. Science 266:1247–1250.

Genoud, T., A. J. Buchala, N.-H. Chua, and J.-P. Metraux. 2002. Phytochrome signaling modulates the SA-perceptive pathway in *Arabidopsis*. The Plant Journal 31:87–95.

Gorlach, J., S. Volrath, G. Knauf-Beiter, G. Hengy, U. Beckhove, K.-H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessman, and J. Ryals. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. The Plant Cell 8:629–643.

He, Y., Y. Liu, W. Cao, M. Huai, B. Xu, and B. Huang.

2005. Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in Kentucky bluegrass. Crop Science 45:988–995.

Kosman, E., and Y. Cohen. 1996. Procedures for calculating and differentiating synergism and antagonism in action of fungicide mixtures. Phytopathology 86:1263–1272.

Molina, A., M. D. Hunt, and J. A. Ryals. 1998. Impaired fungicide activity in plants blocked in disease resistance signal transduction. Plant Cell 10:1903–1914.

Nair, B. M., K. J. Cheung, A. Griffith, and J. L. Burns. 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (*B. cenocepacia*). Journal of Clinical Investigations 113:464–473.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. Journal of Antimicrobial Chemotherapy 56:20–51.

Strobel, N. E., and L. A. Porter. 2005. Salicylate inhibits growth of plant-pathogenic fungi and synergistically enhances the activity of other antifungal materials *in vitro*. Journal of the Kentucky Academy of Science 66:118–128.

Urban, M., T. Bhargava, and J. E. Hamer. 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. The EMBO Journal 18:512–521.

Vincelli, P. 1999. Efficacy of fungicides for controlling gray leaf spot of perennial ryegrass. University of Kentucky Cooperative Extension Factsheet PPFS-OR-T-3. http://www.ca.uky.edu/agcollege/plantpathology/ext_files/PPFShtml/PPFS-OR-T-3.pdf. Date accessed (8/23/06).

Vincelli, P., and E. Dixon. 2002. Resistance to Q_o I (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Disease 85: 235–240.