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Comparison of quinone outside inhibitor fungicide-resistant and -sensitive isolates of *Cercospora sojina*

Guirong Zhang [1], Carl A. Bradley*

Department of Crop Sciences, University of Illinois, 1102. S. Goodwin Ave., Urbana, IL 61801, USA

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ABSTRACT

Isolates of *Cercospora sojina*, causal agent of frogeye leaf spot of soybean (*Glycine max*), that are resistant to quinone outside inhibitor (QoI) fungicides have been reported in the United States. Radial mycelial growth and sporulation of QoI-resistant and -sensitive *C. sojina* isolates were compared in the laboratory, and virulence of QoI-resistant and -sensitive isolates on a susceptible soybean cultivar (Blackhawk) and on a cultivar (Davis) with the *Rcs3* resistance gene that confers resistance to frogeye leaf spot were compared. No differences in amount of sporulation between QoI-resistant and -sensitive isolates were observed. On cultivar (cv.) Blackhawk, frogeye leaf spot caused by QoI-resistant isolates was more severe than disease caused by QoI-sensitive isolates 7–8 days after inoculation, but no differences in disease between QoI-resistant and -sensitive isolates were observed 9 days after inoculation. On cv. Davis, QoI-resistant isolates caused significantly greater disease severity than sensitive isolates 8–14 days after inoculation. Although cv. Davis was affected more severely by QoI-resistant isolates, disease severity was less on cv. Davis than cv. Blackhawk, indicating that the *Rcs3* gene was still effective against the *C. sojina* isolates tested. Our findings indicate that no fitness costs associated with QoI resistance in *C. sojina* were observed in the characteristics measured in our research.

1. Introduction

Frogeye leaf spot (FLS) caused by *Cercospora sojina* reduces soybean yields in the major soybean growing regions in the United States (Koenning and Wrather, 2010). Symptoms caused by FLS include circular to angular lesions with light-colored centers and dark margins that can occur on leaves, stems, and pods (Wise and Newman, 2015). FLS can be managed by applying foliar fungicides, planting resistant soybean cultivars, rotating to non-host crops, and tilling to bury infested soybean debris (Wise and Newman, 2015). Despite the multiple tactics available to manage FLS, some growers relied heavily on quinone outside inhibitor (QoI) fungicide application to control this disease. As a result, QoI fungicide-resistant mutants were selected, and were subsequently detected in North America for the first time in 2010 (Zhang et al., 2012). Characterization of the *C. sojina* mutant cytochrome b gene showed that a glycine to alanine substitution at codon 143 (G143A mutation) allowed for QoI resistance (Zeng et al., 2015). Studies on G143A mutations in *Saccharomyces cerevisiae*, *Venturia inaequalis*, *Ustilago maydis*, and *Plasmopara viticola* showed decreased fitness because of functionally impaired mitochondria characterized by reduced electron flow through the cytochrome bc1 complex (Heaney et al., 2000; Köller et al., 2001; Zheng et al., 2000; Ziogas et al., 2002). However, no fitness penalty was found with G143A mutations in *Blumeria graminis* (Heaney et al., 2000), *Magnaporthe grisea* (Avila-Adame and Köller, 2003) and *Magnaporthe oryzae* (Ma and Uddin, 2009). In *M. grisea*, G143A mutants were significantly less virulent in rice than sensitive strains based on disease severity assessments; however, researchers observed no differences in colony size and conidia formation. (Avila-Adame and Köller, 2003).

Pathogen fitness is loosely linked with aggressiveness. In a study with *Tapesia yallundae* and *T. acuformis* (causal agents of eyespot on wheat), a 50% stable coexistence of carbendazim resistant and sensitive strains was observed (Bierman et al., 2002). However, dicarboximide fungicide resistant *Botrytis cinerea* completely replaced the sensitive strain after two applications of vinclozolin,

* Corresponding author. Present address: Department of Plant Pathology, University of Kentucky Research and Education Center, 1205 Hopkinsville St., Princeton, KY 42445, USA.
E-mail address: carl.bradley@uky.edu (C.A. Bradley).
[1] Present address: National Center for Agricultural Utilization Research, United States Department of Agriculture – Agricultural Research Service, 1815 N. University St., Peoria, IL 61604, USA.

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while the resistant isolate remained at the initial level of the population in absence of fungicides (Vali and Moorman, 1992). Populations of *Erysiphe graminis* f.sp. *tritici* from wheat that were resistant to QoI fungicides increased in numbers slowly and remained steady on nontreated host tissue over three generations (Chin et al., 2001). However, QoI-resistant *Plasmorphara viticola* failed to increase in the population because of less fitness than sensitive wild types (Heaney et al., 2000). In another report, a *M. graminicola* isolate from chlorothalonil fungicide sprayed plots was more aggressive in wheat than isolates from non-treated plots, which indicated that more frequent fungicide applications and higher dosages were associated with pathogen aggressiveness (Kema et al., 1996).

Cercospora sojina is a dynamic pathogen with extensive diversity. Bradley et al. (2012) reported that a worldwide collection of *C. sojina* isolates had a high level of genetic diversity with no clear association of isolate origin with similarity, and Kim et al. (2013) reported that *C. sojina* populations in Arkansas had high levels of genetic diversity. Mian et al. (2008) proposed 12 soybean differential lines and 11 *C. sojina* races, demonstrating the diversity among populations in the U.S. Races 2, 3, 4 and 5 of *C. sojina* make up the majority of populations that cause FLS in the U.S. (Athow et al., 1962; Ross, 1968; Phillips and Boerma, 1980). Three resistant genes have been shown to condition resistance to these races; the *Rcs1* gene in cultivar (cv.) Lincoln confers resistance to race 1 of *C. sojina* (Athow and Probst, 1952), the *Rcs2* gene in cv. Kent confers resistance to race 2 (Athow et al., 1962), and the *Rcs3* gene from cv. Davis confers resistance to race 5 and to all other known races in the U.S (Boerma and Phillips, 1984; Cruz and Dorrance, 2009; Phillips and Boerma, 1982). and Brazil (Yorinori, 1992). The virulence and host adaptation of QoI fungicide-resistant *C. sojina* isolates are unknown.

Because of the importance of FLS to soybean production in the U.S. and the potential FLS management issues in light of QoI-resistant strains of *C. sojina*, it is important to gain a better understanding of the biology of these QoI-resistant strains. Therefore, the objective of our study was to compare QoI-resistant and -sensitive *C. sojina* isolates for: (i) sporulation in culture; (ii) radial growth of mycelium in culture; and (iii) aggressiveness on a susceptible and a resistant soybean cultivar (*Rcs3* resistance) in the greenhouse.

2. Materials and methods

2.1. Sporulation and radial growth studies

Twenty-four (11 QoI-resistant and 13 QoI-sensitive) *C. sojina* isolates were used in sporulation and radial growth studies (Table 1). Each isolate was cultured from a single spore that was obtained from a lesion on a soybean leaf, and maintained on soybean stem lima bean agar (SSLBA) (Phillips and Boerma, 1980) with rifampicin (25 mg/L). The cultures were incubated for 5 days under alternating light (34 W fluorescent with 40 W black lights for 12 h) and dark (12 h) at 25 ± 2 °C until the agar surface was covered with mycelia and conidia.

Conidia were washed with 5 μl of sterilized water with a pipette and transferred onto SSLBA in petri dishes (100 mm diameter), then spread across the agar surface using a sterile bent glass rod. After 16 h of incubation, six germinated spores were individually transferred onto SSLBA using a scalpel to cut the media around the spores and a small laboratory spatula to move the cut media. Plates were arranged in a completely randomized design on a laboratory bench at room temperature (approximately 21°–23 °C).

After five days, three sporulating colonies were cut into approximately 1 cm^2 pieces and transferred separately into 1-ml microcentrifuge tubes containing 500 μl sterilized water and homogenized using sterilized toothpicks and agitated with a vortex mixer. A hemacytometer was used to determine the concentration of spores in a volume of 10 μl. The remaining three colonies were used to measure radial growth. After 6 and 12 days of growth, the colonies were scanned using a flatbed scanner (Expression 1000XL; Epson America Inc. Long Beach, CA), and mycelia growth area per colony was calculated using Assess 2.0 software (American Phytopathological Society, St. Paul, MN). The experimental design for the sporulation and mycelium growth experiments was a completely randomized design (CRD) with three replication, and experiments were repeated.

Table 1
Comparison of QoI fungicide-resistant and -sensitive *Cercospora sojina* isolates for sporulation (number of conidia) 5 days after single conidia were placed on soybean stem lime bean agar.

Isolate	QoI sensitivity	Geographic origin	Number of conidia[a]
CS1036	Resistant	Lauderdale Co., TN	11,083 a[b]
CS1091	Sensitive	Caldwell Co., KY	7421 b
CS10127	Sensitive	Gibson Co., TN	6854 bc
CS1065	Resistant	Gallatin Co., IL	6583 bcd
CS1090	Resistant	Caldwell Co., KY	6554 bcd
CS10110	Sensitive	Gibson Co., TN	6533 bcd
CS10190	Sensitive	Pope Co., IL	6196 bcde
CS1084	Resistant	Caldwell Co., KY	6088 bcde
CS1054	Sensitive	DeKalb Co., IL	5779 cdef
CS1076	Resistant	Gallatin Co., IL	5442 cdefg
CS10117	Resistant	Gibson Co., TN	5146 defgh
CS10187	Resistant	Pope Co., IL	4954 efghi
CS1068	Sensitive	Gallatin Co., IL	4925 efghi
CS1093	Resistant	Caldwell Co., KY	4788 efghi
CS10186	Sensitive	Pope Co., IL	4688 fghi
CS10116	Sensitive	Gibson Co., TN	4613 fghi
CS1049	Sensitive	DeKalb Co., IL	4271 ghij
CS1082	Sensitive	Gallatin Co., IL	4258 ghij
CS1031	Resistant	Lauderdale Co., TN	4038 ghijk
CS107	Sensitive	Warren Co., IL	3792 hijk
CS1013	Sensitive	Warren Co., IL	3604 ijk
CS1044	Resistant	Lauderdale Co., TN	3504 ijk
CS1097	Sensitive	Caldwell Co., KY	2788 jk
CS1033	Resistant	Lauderdale Co., TN	2654 k
Overall mean of QoI resistant isolates			**5530 A[c]**
Overall mean of QoI sensitive isolates			**5055 A**

[a] Number of conidia produced by a single-spore culture on a 100 mm diameter petri dish with soybean stem lima bean agar.
[b] Means followed by the same lowercase letter are not significantly different from each other according to Fisher's least significant difference test ($\alpha = 0.05$).
[c] Overall means followed by the same uppercase letter are not significantly different from each other according to Fisher's least significant difference test ($\alpha = 0.05$).

2.2. Comparison of isolates for aggressiveness in the greenhouse

Ten *C. sojina* isolates, five QoI-resistant (CS10187, CS10117, CS1036, CS1065, CS1093) and five QoI-sensitive (CS10190, CS10116, CS1054, CS1082, CS1091) (Table 1), were used to inoculate soybean plants in the greenhouse. Cultures of each isolate were maintained on SSLBA as described previously. Conidia were collected from 5-day old colonies by placing mycelia plugs bearing conidia into 150 ml sterilized water and agitating with a vortex mixer for 2 min. The suspension was passed through four layers of cheesecloth to remove large mycelial fragments. Conidial suspensions were adjusted to approximately 6×10^4 conidia/ml prior to inoculating plants in the greenhouse.

Seeds of soybean cultivars Blackhawk (susceptible to FLS) and Davis (containing the *Rcs3* resistant gene) were planted in 5 × 5 cm pots containing Sunshine Mix 1 (Sun Gro Horticulture Inc. Bellevue, WA) and placed in 20 × 30 cm trays. Each tray contained 12 pots of cv. Davis or cv. Blackhawk plants. Plants were grown under 1000-W