Research

Identification and Characterization of *Rhizoctonia* Species Associated with Soybean Seedling Disease

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Abstract

In an effort to identify the *Rhizoctonia* spp. associated with seedling diseases of soybean, *Rhizoctonia* isolates were recovered from soybean seedlings with damping off and root and hypocotyl rot symptoms from Arkansas, Illinois, Kansas, Michigan, Minnesota, and the Canadian province of Ontario between 2012 and 2014. Based on cultural morphology, polymerase chain reaction restriction fragment length polymorphism, and phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA genes, 80 isolates were confirmed to be *Rhizoctonia solani*, 24 were binucleate *Rhizoctonia* spp., and 10 were *R. zae*. Of the 80 *R. solani* isolates, one belonged to anastomosis group (AG) 2-1, 52 belonged to AG-2-IIIB, five belonged to AG-3 PT, three belonged to AG-4 HGI, two belonged to AG-4 HGIII, nine belonged to AG-7, and eight belonged to AG-11. Bayesian inference of

phylogeny using the ITS region revealed two clades of *R. solani* AG-7 that possibly correspond to different AG-7 subgroups. Phylogenetic analysis also provided evidence for genetic relatedness between certain binucleate *Rhizoctonia* and some *R. solani* isolates. On 'Williams 82' soybean, isolates of AG-2-IIIB were the most aggressive, followed by isolates of AG-7, AG-4, and AG-11. On 'Jubilee', a sweet corn cultivar, AG-2-IIIB and AG-4 isolates caused significant stunting and root damage, whereas the damage caused by the AG-11 isolates was mostly restricted to the mesocotyl. Isolates of *R. zae* and the binucleate *Rhizoctonia* spp. were not pathogenic on soybean or corn. Our results indicate that soybean and corn are hosts to the predominant and aggressive AG of *R. solani*, implying that rotation between these two crops may not be an effective management practice.

Seedling diseases have a history as a major constraint to soybean (*Glycine max* (L.) Merr.) production in North America. In the north-central United States, the estimated impact of seedling diseases on soybean yields was first noted between 1989 and 1991 (Doupnik 1993), and between 2003 and 2005, a nationwide estimated suppression in yield was reported, with the greatest impact occurring in Illinois, Ohio, Minnesota, Kansas, and North Dakota (Wrather and Koenning 2006). From 2006 to 2009, seedling diseases ranked third among diseases and pests that reduced soybean yields in the United States and Canada (Koenning and Wrather 2010).

Among the myriad of plant pathogens associated with the seedling disease complex of soybean, *Rhizoctonia solani* (Kühn) (syn. *Thanatephorus cucumeris* (A. B. Frank) Donk) represents an important and intractable group. In addition to having a necrotrophic and saprophytic lifestyle, this plant pathogen exhibits significant genetic diversity, often evident as differences in colony morphology, biochemical and molecular properties, and pathogenicity on different hosts. As a consequence, members of this species complex have been classified into 14 anastomosis groups (AG) (AG 1 to 13 and AG-BI) (Carling 1996; Carling et al. 1999; Ogoshi 1987), which are considered to be noninterbreeding populations (Anderson 1984) with distinct evolutionary origins (Vilgalys and Cubeta 1994). On the basis of characteristics other than hyphal fusion, several AG have been further divided into subgroups, with the exception of AG-2, which is subdivided on the basis of the frequency of fusion between isolates. Within AG-1, six subgroups (AG-1-1A, AG-1-1B, AG-1-1C, AG-1-1D, AG-1-1E, and AG-1-1F) have been identified (Kuninaga et al.

2002; Ogoshi 1972; Parmeter et al. 1969; Priyatmojo et al. 2001; Richter and Schneider 1953; Watanabe and Matsuda 1966), and members of this AG can attack corn (*Zea mays* L.), soybean, bean (*Phaseolus vulgaris* L.), cabbage (*Brassica oleracea* L. Capitata group), lettuce (*Lactuca sativa* L.), and sorghum (*Sorghum bicolor* (L.) Moench) (Grosch et al. 2004; Herr 1992; Jones and Belmar 1989; Martin and Lucas 1984a; Mukou et al. 1975; Priyatmojo et al. 2001; Yang et al. 1990). Two subgroups are known within AG-2: AG-2-1 (crucifer type) and AG-2-2 (canker pathogen of sugar beet [*Beta vulgaris* L.] and carrot [*Daucus carota* L. subsp. *sativus* (Hoffm.) Arcang.]). AG-2-1 isolates are autotrophic for thiamine, while AG-2-2 isolates are thiamine auxotrophic (Ogoshi 1987). Three subgroups exist within AG-2-1: AG-2-1, AG-2t (tulip isolate), and AG-2Nt (tobacco isolate) (Kuninaga et al. 2000; Nicoletti et al. 1999; Schneider et al. 1997), while five subsets can be differentiated within AG-2-2: AG-2-IIIB, AG-2-2IV, AG-2-2LP, AG-2-3, and AG-2-4 (Liu and Sinclair 1992; Stevens Johnk et al. 1993). Host crops of AG-2 include soybean, sugar beet, turf grass (tall fescue: *Festuca arundinacea*; creeping bent grass: *Agrostis palustris*), corn, canola (*Brassica napus* L.), wheat (*Triticum aestivum* L.), tulip (*Tulipa gesneriana* L.), and tomato (*Lycopersicon esculentum* Mill) (Dorrance et al. 2003; Engelkes and Windels 1996; Liu and Sinclair 1991; Misawa and Kuninaga 2010; Muyolo et al. 1993a; Nelson et al. 1996; Paulitz et al. 2006; Schneider et al. 1997; Sumner and Bell 1982; Watanabe and Matsuda 1966; Windels and Nabben 1989). Members of AG-3 are pathogens of solanaceous crops—tomato, tobacco (*Nicotiana tabacum* L.), and potato (*Solanum tuberosum* L.) (Meyer et al. 1990; Misawa and Kuninaga 2010; Nelson et al. 1996; Windels et al. 1997; Woodhall et al. 2007)—and three subgroups have been reported: AG-3 PT (potato type), AG-3 TB (tobacco type), and AG-3TM (tomato type) (Kuninaga et al. 2000, 2007; Ogoshi 1972; Parmeter et al. 1969; Richter and Schneider 1953; Stevens Johnk et al. 1993; Watanabe and Matsuda 1966). Three homogeneous groups (HG) have been identified in AG-4 (HG-I, HG-II, and HG-III), and host range of this AG include soybean, tomato, dry bean, cotton (*Gossypium hirsutum* L.), peanut (*Arachis hypogaea* L.), potato, melon (*Cucumis melo* L.), broccoli (*B. oleracea* L.), and spinach (*Spinacia oleracea* L.) (Balali et al. 1996; Brenneman 1996; Fenille et al. 2002; Kuramae et al. 2003;

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Accepted for publication 26 November 2016.

Muyolo et al. 1993b; Rothrock 1996; Windels and Nabben 1989). AG-5 isolates are associated with potato, turf grass, bean, soybean, and sugar beet (Balali et al. 1995; Martin and Lucas 1984a; Nelson et al. 1996; Windels et al. 1997). The nonpathogenic AG-6, which is mostly associated with mycorrhiza and orchid (Carling et al. 1999; Pope and Carter 2001), has been divided into two groups, the HG (HG-I) and isolates with remarkable genetic variation (Kuninaga and Yokosawa 1984). AG-8 isolates are pathogens of small grains, including barley (*Hordeum vulgare* L.) and wheat (Neate and Warcup 1985; Roberts and Sivasithamparam 1986), and they have been divided into at least five pectic zymogram groups (ZG): ZG1-1, ZG1-2, ZG1-4, ZG1-4, and ZG1-5 (MacNish and Sweetingham 1993). Two subgroups, thiamine prototrophic and thiamine auxotrophic, are evident in AG-9 (Carling and Kuninaga 1990), and potato, lettuce, and carrot are common hosts (Carling et al. 1987). Subgroups within AG-7 (host crops: soybean, potato, cotton, and water melon), AG-10 (nonpathogenic), AG-11 (host crops: cotton, wheat, soybean, potato, lupine [*Lupinus luteus* L.], and radish [*Raphanus sativus* L.]) (Carling et al. 1994; Kumar et al. 1999), AG-12 (host crops: mycorrhiza [Mycorrhiza], orchid [family Orchidaceae], radish, and cauliflower [*B. oleracea* L. Botrytis group]) (Carling et al. 1999; Pope and Carter 2001), AG-13 (nonpathogenic), and AG-BI (nonpathogenic) have not been reported.

Around the globe, isolates of the subgroup AG-2-IIIB (Fenille et al. 2002; Liu and Sinclair 1991; Muyolo et al. 1993a; Sneh et al. 1991) and of those of AG-4 (Bolkan and Ribeiro 1985; Fenille et al. 2002; Muyolo et al. 1993a; Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996; Zhao et al. 2005) and AG-5 (Nelson et al. 1996) have been identified as the major culprits causing seedling diseases of soybean. Other less aggressive AG associated with soybean seedling diseases include AG-3 (Nelson et al. 1996), AG-7 (Baird et al. 1996; Rothrock et al. 1993), and AG-11 (Carling et al. 1994).

In the United States, *Rhizoctonia* root and hypocotyl rot is an important seedling disease of soybean, especially in the north-central region (Doupnik 1993), where more than 80% of the total U.S. soybean production occurs (American Soybean Association 2015). Reports of *Rhizoctonia* root rot epidemics on soybean first emerged during the 1967 growing season in Iowa, where a reduction in stand was observed (Tachibana 1968). In 1971, as high as a 48% reduction in yield was reported in small research plots (Tachibana et al. 1971). Causal AG or species of *Rhizoctonia* were unknown at the time the epidemic occurred; however, in 1996, Rizvi and Yang (1996) identified AG-2-2 and AG-4 isolates as the primary AG of *R. solani* associated with soybean seedling disease in Iowa. In 1993, Rothrock et al. (1993) reported the occurrence of AG-7 isolates on soybean seedlings from field sites at Colt, AR. A survey of the prevalent species of *Rhizoctonia* that attack soybean also was conducted in Ohio (Muyolo et al. 1993a), the Red River Valley of Minnesota and North Dakota (Nelson et al. 1996), and the Canadian province of Ontario (Zhao et al. 2005). Given that the population of *R. solani* affecting soybean seedlings in other major soybean-producing states in the United States has not been extensively investigated, information regarding pathogen variability and distribution across the country is scarce. Cropping systems differ across soybean-growing locations, and these differences can significantly enhance the prevalence of certain species of *Rhizoctonia* over others (Garbeva et al. 2006; Gudmestad et al. 1989; Hua et al. 2014). Moreover, *R. solani* AG and subgroups differ not only in their aggressiveness on specific host crops but also in their sensitivity to different fungicide seed treatments (Barnes et al. 1990; Carling et al. 1990; Kataria et al. 1991; Martin et al. 1984b). Therefore, information on the predominant groups, together with knowledge of their pathogenicity on soybean, is needed for making sound disease management decisions.

Illinois is a leading producer of soybean in the United States; unfortunately, a statewide investigation of the predominant *Rhizoctonia* spp. causing soybean seedling diseases has not been carried out to date. In the early 1990s, two highly aggressive AG 2-2 isolates, 61D-3 and 65L-2, were recovered from diseased soybean plants collected from a soybean breeding plot at the University of Illinois (Liu and Sinclair 1991) but nothing is known about the variability in the AG associated with *Rhizoctonia* root and hypocotyl rot of soybean in the state. Similarly, the diversity of *Rhizoctonia* spp. associated with soybean

seedling diseases in Arkansas, Michigan, and Kansas is unknown. Therefore, the objectives of this study were to (i) identify and characterize the species of *Rhizoctonia* associated with soybean seedling disease in Illinois, Arkansas, Michigan, and Kansas; (ii) determine whether there has been a change in the AG of *R. solani* that damage soybean seedlings in Minnesota and in Ontario, Canada; and (iii) determine the pathogenicity of the isolates recovered from Illinois on soybean and corn.

Materials and Methods

Isolate collection and storage. Soybean seedlings showing hypocotyl and root rot symptoms typical of *R. solani* infections were collected from 13 Illinois counties in 2012, 2013, and 2014 (Table 1). Plants were dug carefully to minimize root damage, then placed in sealed plastic bags and placed in a cooler during transport back to the lab, where they were stored overnight at 4°C. Roots of plants were washed under running tap water to remove soil particles. Plants were then disinfected in 1% NaOCl solution for 30 s, followed by a thorough rinse in distilled water for 1 min. Cut sections of about 3 mm in length from symptomatic plants were gently pressed onto solidified Ko and Hora medium (Ko and Hora 1971) and incubated in the dark at 25°C for 24 to 48 h. Hyphal tips from colonies with right-angled branches typical of *Rhizoctonia*-like fungi were transferred to potato dextrose agar (PDA; Becton, Dickson and Company, Sparks, MD) amended with rifampicin at 25 mg/liter and were incubated under light at 25°C. Isolates recovered from Arkansas, Kansas, Michigan, Minnesota, and Ontario were collected as part of a multistate research project by the laboratories of Dr. Craig Rothrock (University of Arkansas, Fayetteville), Dr. Christopher Little (Kansas State University, Manhattan), Dr. Martin Chilvers (Michigan State University, East Lansing), Dr. Dean Malvick (University of Minnesota, St. Paul), and Mr. Albert Tenuta (Ontario Ministry of Agriculture and Rural Affairs, Ridgetown, ON, Canada), respectively. These isolates were then sent to Dr. Ahmad Fakhoury's laboratory (Southern Illinois University, Carbondale), where they will continue to be curated as part of the multistate research project. Tester isolates of *R. solani* used for identification of AG and their respective sources are listed in Table 2. All isolates were maintained on sterile table beet seed. These were dried under a sterile flow hood for 48 h, placed in 2.0-ml Nalgene cryogenic vials (Sigma-Aldrich, St. Louis), and stored at 4°C. For long-term storage, three plugs (5 mm in diameter) of isolates on PDA were placed in 1.5-ml microcentrifuge tubes containing 850 µl of 15% glycerol and maintained at -80°C.

Morphological characterization and AG typing of isolates. All isolates were characterized based on cultural morphology, cellular nuclei number in young vegetative hyphae, and anastomosis reactions with tester isolates. Cultural characteristics were determined by growing each isolate on PDA and incubating at 25°C for 14 days. Isolates identified as members of AG-2-2 were further maintained at 35°C to enable rapid delineation of the AG-2-IIIB isolates (Sneh et al. 1991). Cultures were examined for colony color, sclerotia formation and color, aerial mycelia formation, and growth zonation at 5 and 14 days after plating. Binucleate *Rhizoctonia* (BR) were differentiated from multinucleate *Rhizoctonia* by an examination of the nuclear condition using the Safranin O staining technique (Bandoni 1979; Yamamoto and Uchida 1982).

For AG determination, a modified version of the clean slide technique (Kronland and Stanghellini 1988) coupled with Safranin O staining was used for observing hyphal fusion reactions between testers and unknown isolates. Briefly, the unknown isolates and testers initially were grown on PDA for 3 to 5 days. Plugs (5 mm in diameter) of the unknown isolates were cut from the growing edge of the plate and transferred to the opposite ends of a 100-by-15-mm petri dish containing 1.5% water agar. A 5-mm plug of the tester isolate was placed about 4 cm away from the unknown isolate and incubated at 25°C for 24 to 72 h, depending on the growth of the interacting isolates. Cut sections of overlapping hyphae were placed on a plain microscope glass slide, stained with safranin O and 3% KOH, and covered with a coverslip before viewing under a compound microscope. Reaction types observed between interacting hyphae were assigned to one of the four categories (C0, C1, C2, or C3) described by Carling et al. (1988) (Fig. 1). In the C0 reaction type, interaction or contact between hyphae of paired isolates is nonevident. Isolates

exhibiting such reaction types are said to be genetically different and belong to different AG. For the C1 reaction, cell wall contact between hyphae of paired isolates is occasionally observed; however, contact between cell membranes does not occur, and the death of anastomosing cells may or may not be observed. This form of reaction is mostly observed among distantly related isolates belonging to the same or

different AG. For genetically distinct isolates within the same AG, cell wall fusion and the death of anastomosing and adjacent cells, otherwise referred to as the “killing reaction” (Flentje et al. 1967), are usually apparent; a characteristic feature defining the C2 reaction type. The last reaction type, C3, is characterized by a perfect fusion of cell walls and cell membranes of paired isolates and by an absence of

Table 1. Origin, year of isolation, and identification method used for the isolates of *Rhizoctonia* collected from soybean in Arkansas, Illinois, Kansas, Michigan, Minnesota, and Ontario

Isolate ID	GenBank ^a	Year	County, state	<i>Rhizoctonia</i> spp. ^b	Identification method ^c
S_P_19a	KX118381	2013	Pike Co., IL	AG-11	ITS, PCR-RFLP
S_P_19b	KX118382	2013	Pike Co., IL	AG-11	ITS, PCR-RFLP
K_4_18b	KX118367	2013	St. Clair Co., IL	AG-11	ITS, PCR-RFLP
BVT_18	KX118336	2013	St. Clair Co., IL	AG-11	ITS, PCR-RFLP
X12SDSa	KX118394	2013	Illinois	AG-11	ITS, PCR-RFLP
K_ARSO2_1_11	KX118356	2013	Arkansas	AG-11	ITS, PCR-RFLP
K_ARSO2_1_20	KX118358	2013	Arkansas	AG-11	ITS, PCR-RFLP
K_ARSO2_5_1	KX118363	2013	Arkansas	AG-2-1	ITS, PCR-RFLP
DK_11	KX118344	2013	DeKalb Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
DK_8	KX118345	2013	DeKalb Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
X42210_b	KX118397	2013	Illinois	AG-2-2IIIB	ITS, PCR-RFLP
ER_15	KX118346	2013	Whiteside Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
ER_19a	KX118347	2013	Whiteside Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
ER_19b	KX118348	2013	Whiteside Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
ER_2	KX118349	2013	Whiteside Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
ER_4	KX118350	2013	Whiteside Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
X12RS41	KX118392	2012	Williamson Co., IL	AG-2-2IIIB	ITS, PCR-RFLP
ONSO2_18	KX118372	2012	Ontario	AG-2-2IIIB	ITS, PCR-RFLP
WONS 13_8_5	KX118391	2013	Ontario	AG-2-2IIIB	ITS, PCR-RFLP
WONS 13_12_3	KX118390	2013	Ontario	AG-2-2IIIB	ITS, PCR-RFLP
PDONS 13_8_1	KX118373	2013	Ontario	AG-2-2IIIB	ITS, PCR-RFLP
X248_3b KH	KX118396	2012	Jackson Co., IL	AG-3PT	ITS
C_far_500_10a	KX118341	2013	Champaign Co., IL	AG-3PT	ITS
C_far_500_3	KX118342	2013	Champaign Co., IL	AG-3PT	ITS
C_far_500_6	KX118343	2013	Champaign Co., IL	AG-3PT	ITS
C_far_500_1	KX118340	2013	Champaign Co., IL	AG-3PT	ITS
EV_3	KX118352	2013	Jackson Co., IL	AG-4 HGI	ITS, PCR-RFLP
EV_6	KX118353	2013	Jackson Co., IL	AG-4 HGI	ITS, PCR-RFLP
BVT_28	KX118338	2013	St. Clair Co., IL	AG-4 HGIII	ITS, PCR-RFLP
BVT_3	KX118339	2013	St. Clair Co., IL	AG-4 HGIII	ITS, PCR-RFLP
EV_19	KX118351	2013	Jackson Co., IL	AG-7	ITS
EV_7	KX118354	2013	Jackson Co., IL	AG-7	ITS
BVT_16	KX118335	2013	St. Clair Co., IL	AG-7	ITS
BVT_20	KX118337	2013	St. Clair Co., IL	AG-7	ITS
K_ARSO2_1_6	KX118359	2013	Arkansas	AG-7	ITS, PCR-RFLP
K_ARSO2_1_8	KX118360	2013	Arkansas	AG-7	ITS, PCR-RFLP
K_ARSO2_2_5	KX118362	2013	Arkansas	AG-7	ITS, PCR-RFLP
K_ARSO2_1_9	KX118361	2013	Arkansas	AG-7	ITS, PCR-RFLP
K_2_1a	KX118366	2013	Gallatin Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
S_P_18a	KX118378	2013	Pike Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
S_P_18b	KX118379	2013	Pike Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
S_P_18c	KX118380	2013	Pike Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
S_P_2	KX118383	2013	Pike Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
K_1_24c	KX118365	2013	Pope Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
X12SDSb	KX118395	2013	Illinois	<i>Ceratobasidium</i> sp.	ITS, CNN
X4SDS	KX118398	2013	Illinois	<i>Ceratobasidium</i> sp.	ITS, CNN
X9SDS	KX118399	2013	Illinois	<i>Ceratobasidium</i> sp.	ITS, CNN
m_24_b	KX118370	2012	Whiteside Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
m_24_c	KX118371	2012	Whiteside Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN
K_ARSO2_1_19	KX118357	2013	Arkansas	<i>Ceratobasidium</i> sp.	ITS, CNN
K_ARSO2_5_20	KX118364	2013	Arkansas	<i>Ceratobasidium</i> sp.	ITS, CNN
WMIS02_6_19	KX118389	2013	Michigan	<i>Ceratobasidium</i> sp.	ITS, CNN
MNSO1_3_14	KX118369	2012	Minnesota	<i>Ceratobasidium</i> sp.	ITS, CNN
X12RS42	KX118393	2012	Champaign Co., IL	<i>Ceratobasidium</i> sp.	ITS, CNN

(continued on next page)

^a GenBank accession number; NA = not applicable.

^b AG = anastomosis group.

^c ITS = internal transcribed spacer region of the ribosomal DNA, PCR-RFLP = polymerase chain reaction restriction fragment length polymorphism analysis of the amplified ribosomal DNA, CNN = cellular nuclear number, and M = morphological identification on culture media.

dead anastomosing and adjacent cells. This is commonly observed during self-anastomosis as well as during fusion among clones. Pairing for each unknown isolate was replicated twice, and at least four anastomosing points were required for a definitive placement of an unknown isolate in an AG. Pictures of cellular nuclear number and anastomosis reaction types were taken with a Zeiss Axiocam ERc5s and viewed via a Zeiss ZEN imaging software attached to a PC.

DNA isolation, polymerase chain reaction restriction fragment length polymorphism analysis of the ribosomal DNA internal transcribed spacer regions, and polymerase chain reaction analysis with AG-specific primers for AG typing of *R. solani* isolates. For genomic DNA extraction, each isolate was grown on potato dextrose broth (Becton, Dickson and Company) and incubated in continuous light without shaking at 25°C. Mycelia were harvested after

Table 1. (continued from preceding page)

Isolate ID	GenBank ^a	Year	County, state	<i>Rhizoctonia</i> spp. ^b	Identification method ^c
W2_1_12	KX118387	2013	Pope Co., IL	<i>R. zeae</i>	ITS, M
W2_2_12	KX118388	2013	Pope Co., IL	<i>R. zeae</i>	ITS, M
Maxwell 1_1_ KH	KX118368	2012	Sangamon Co., IL	<i>R. zeae</i>	ITS, M
12RS52	NA	2012	Jackson Co., IL	AG-11	PCR-RFLP
IL 2014a	NA	2014	Champaign Co., IL	AG-2-2IIIB	PCR-RFLP
DK_10	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_13	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_14	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_15a	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_15b	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_16	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_19	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_3a	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_3b	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_4a	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_4b	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_6a	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_6b	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
DK_7	NA	2013	DeKalb Co., IL	AG-2-2IIIB	PCR-RFLP
X42210_c	NA	2013	Illinois	AG-2-2IIIB	PCR-RFLP
X42304_b	NA	2013	Illinois	AG-2-2IIIB	PCR-RFLP
X42304_g	NA	2013	Illinois	AG-2-2IIIB	PCR-RFLP
K_IL_SO2_3_25c	NA	2013	Warren Co., IL	AG-2-2IIIB	PCR-RFLP
ONSO2_17	NA	2012	Ontario	AG-2-2IIIB	PCR-RFLP
ONSO2_16	NA	2012	Ontario	AG-2-2IIIB	PCR-RFLP
ONSO2_14	NA	2012	Ontario	AG-2-2IIIB	PCR-RFLP
ONSO2_13	NA	2012	Ontario	AG-2-2IIIB	PCR-RFLP
ONSO2_15	NA	2012	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_4	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_2	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_6	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_4	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_5	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_6	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_3	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_7	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_8_8	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_8	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_3	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_7	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_1	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
PDONS 13_12_3	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
WONS 13_8_4	NA	2013	Ontario	AG-2-2IIIB	PCR-RFLP
BVT_11	NA	2013	St. Clair Co., IL	AG-4 HGI	PCR-RFLP
K_ARSO2_1_7	NA	2013	Arkansas	AG-7	PCR-RFLP
W_2_2_15	NA	2013	Gallatin Co., IL	<i>Ceratobasidium</i> sp.	CNN
W2_2_20	NA	2013	Gallatin Co., IL	<i>Ceratobasidium</i> sp.	CNN
NBK_IL_3	NA	2013	Sangamon Co., IL	<i>Ceratobasidium</i> sp.	CNN
NBK_IL_5	NA	2013	Sangamon Co., IL	<i>Ceratobasidium</i> sp.	CNN
X2SDS	NA	2013	Illinois	<i>Ceratobasidium</i> sp.	CNN
m_20_h	NA	2012	Whiteside Co., IL	<i>Ceratobasidium</i> sp.	CNN
m_20_k	NA	2012	Whiteside Co., IL	<i>Ceratobasidium</i> sp.	CNN
W_KSSO2_2_13	NA	2013	Kansas	<i>Ceratobasidium</i> sp.	CNN
211	NA	2012	Champaign Co., IL	<i>R. zeae</i>	M
12RS39	NA	2012	DeKalb Co., IL	<i>R. zeae</i>	M
12RS40	NA	2012	Jackson Co., IL	<i>R. zeae</i>	M
248_1a KH	NA	2012	Jackson Co., IL	<i>R. zeae</i>	M
248_2KH	NA	2012	Jackson Co., IL	<i>R. zeae</i>	M
12RS36	NA	2012	McLean Co., IL	<i>R. zeae</i>	M
12RS48	NA	2012	Peoria Co., IL	<i>R. zeae</i>	M

14 days, placed in 1.5-ml microcentrifuge tubes, and stored at -20°C . DNA extraction was carried out using the FastDNA spin kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's recommendations for fungal DNA isolation. Not all isolates identified as *R. solani* could be classified to AG using the traditional hyphal reaction technique; therefore, a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) was initially conducted on all tester isolates and then on the recovered *R. solani* isolates to allow for a comparison of restriction patterns and final AG typing. For this analysis, genomic DNA was amplified using primer pairs RS1 (5'-CCTGTGCACCTGTGA GACAG-3') and RS4 (5'-TGTCCAAGTCAATGGACTAT-3') (Guillemaut et al. 2003). PCR assays were performed in a 40- μl mixture containing 20 μl of GoTaq Green Master Mix 2X (Promega Corporation, Madison, WI), 4 μl each of RS1 and RS4 primers, 4 μl of nuclease-free water, and 8 μl of template DNA. PCR amplification was done in a 2720 thermal cycler (Applied Biosystems, Foster City, CA) using the following cycle parameters: initial denaturation at

94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Restriction analysis of PCR products was carried out in separate digestion reactions with the enzymes *MseI* (Fisher Scientific, Pittsburgh), *MunI* (Fisher Scientific), *AvaII* (Promega Corporation), and *HincII* (Promega Corporation) following the manufacturers' protocols. *AvaII* and *HincII* were combined in a single reaction for double digestion. Aliquots (5 μl) of restriction fragments were checked on 2.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC) and viewed with GelCam 310 Camera attached to a GelDoc-It2 Imager (UVP, LLC, Upland, CA). Restriction patterns of the unknown isolates and tester isolates were compared with those reported by Guillemaut et al. (2003) for correct placement of isolates into AG and subgroups.

The rDNA-ITS region of the AG-2-IIIB isolates were amplified using AG-2-IIIB-specific primers (Carling et al. 2002), while AG-3 isolates were amplified using both AG-3 (Lees et al. 2002) and AG-3 subgroup-specific primers (Kuninaga et al. 2000). Forward and reverse primers for the AG-2-IIIB isolates were 5'-AGGCAGAG(A/G)CATG GATGGGAG-3' and 5'-ACCTTGGCCA(A/C)CCTTTTATC-3', respectively. For the AG-3 isolates, AG-3-specific primer pairs used were Rs1F2 (5'-TTGGTTGTAGCTGGTCTATTT-3') and Rs2R1 (5'-TAT CACGCTGAGTGGAAACCA-3'), while the AG-3 subgroup-specific primer pair was 5'-GTTTGGTTGTAGCTGGGCC-3' and 5'-CTGA GATCCAGCTAATGT-3' for AG-3TB (tobacco type) and 5'-GTTTGGTTGTAGCTGGTCT-3' and 5'-CTGAGATCCAGCTAA TAC-3' for AG-3PT (potato type).

PCR amplification of rDNA-ITS regions, sequencing, and Bayesian inference of phylogeny. The rDNA-ITS regions of 57 collected (Table 1) and 15 tester (Table 2) isolates were amplified with the ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers (White et al. 1990) in a final volume of 40 μl using the PCR assays and cycle parameters described above. Successful amplification was confirmed by analyzing aliquots (5 μl) of PCR products on 1% agarose gel and viewing with a GelCam 310 Camera attached to a GelDoc-It2 Imager (UVP, LLC). The PCR product for each isolate was purified with the Wizard SV Gel and PCR Clean-Up System (Promega Corporation) and sequenced at the McLab Molecular Cloning Laboratories (South San Francisco, CA) with the ITS-4 and ITS-5 primers. Using the software Sequencher 5.4 (Gene Codes Corporation, Ann Arbor, MI), the ITS sequences for both complimentary strands for each isolate were manually edited and assembled to produce a consensus sequence. Reference sequences representative of the AG of

Table 2. Tester isolates used for anastomosis group (AG) typing of unknown *Rhizoctonia* isolates recovered in this study

Tester isolate ID	<i>Rhizoctonia solani</i> AG or <i>Rhizoctonia</i> spp.	Accession numbers	Source ^a
Rh051307	AG-2-1	KX118375	Tim Paulitz, WSU
Rh051324	AG-2-1	KX118376	Tim Paulitz, WSU
Rh051339	AG-4 HGII	KX118329	Tim Paulitz, WSU
Rh0911029	AG-5	KX118377	Tim Paulitz, WSU
R63_42A	<i>R. zeae</i>	KX118374	Craig Rothrock, UA
HPIN22A	AG-11	KX118355	Craig Rothrock, UA
AG-9_Carling	AG-9	KX118333	Craig Rothrock, UA
AG-6_Carling	AG-6	KX118332	Craig Rothrock, UA
ST81548	AG-7	KX118384	Craig Rothrock, UA
AG-4_Carling	AG-4 HGIII	KX118331	Craig Rothrock, UA
USA_AG-3	AG-3	KX118385	Berlin Nelson, NDSU
USA_AG-5	AG-5	KX118386	Berlin Nelson, NDSU
AG2-2_Nelson	AG-2-2	KX118328	Berlin Nelson, NDSU
AG-4_Nelson	AG-4 HGII	KX118330	Berlin Nelson, NDSU
65L-2 ^b	AG-2-IIIB	KX118334	Sinclair, UIUC

^a WSU = Washington State University, UA = University of Arkansas, NDSU = North Dakota State University, and UIUC = University of Illinois at Urbana-Champaign.

^b 65L-2 was originally collected by Liu and Sinclair (1991).

Fig. 1. Hyphal anastomosis reaction types for anastomosis group designation of *Rhizoctonia solani* isolates. C1 = cell wall contact only. C2 = cell wall fusion and death of anastomosing cells (killing reaction). C3 = perfect fusion of cell walls and cell membranes, and an absence of the killing reaction.

R. solani as well as those of other species of *Rhizoctonia* were retrieved from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>) for identification of AG. To ascertain that all unknown isolates belonged to the genus *Rhizoctonia*, the consensus sequence of each isolate was queried using the BLASTN option (Altschul et al. 1997) in the NCBI database. Consensus sequence alignment for the unknown isolates ($n = 57$), tester isolates ($n = 15$), and those of the reference isolates ($n = 24$) was conducted using the ClustalW algorithm in the Molecular Evolutionary Genetics Analysis (MEGA version 6) Software (Tamura et al. 2013). Best-fit substitution model selection for phylogenetic analyses of the aligned consensus sequences was carried out using the Modeltest 3.7 program (Posada and Crandall 1998) implemented in PAUP* (Swofford 1998), with model selection strictly on the basis of the Akaike Information Criterion (AIC) estimate (Akaike 1974). Based on the AIC, the transversion model plus γ (TVM+G) was selected by Modeltest 3.7, with the following parameters: number of substitution types = 6; rates at variable sites = γ ; state frequencies for A, C, G, and T = 0.33, 0.20, 0.20, and 0.27, respectively; substitution rates for the TVM rate matrix AC, AG, AT, CG, CT, and GT = 0.75, 2.71, 1.07, 0.68, 2.71, and 1.00, respectively; shape parameter of the γ distribution of rate variation = 0.37; and proportion of invariable sites = 0. A phylogenetic tree was then computed from the selected model and parameters using a Bayesian Metropolis-coupled Markov Chain Monte Carlo (MCMC) analysis implemented in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003). The MCMC analysis was run for 2 million generations with chain sampling every 500 generations. From the pool of trees constructed after convergence, 50% majority-rule trees were constructed. Bayesian posterior probability (PP) or clade support values were estimated from the consensus tree, and nodes with PP $\geq 95\%$ were considered strongly supported. Isolates were assigned to a species, AG, or subgroup if they clustered with a reference isolate in a strongly supported clade. Bayesian inference of phylogeny was repeated to replicate the results, and phylogenetic trees were visualized using the FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

Greenhouse pathogenicity assay. For greenhouse pathogenicity tests on soybean and corn, representative isolates from the Illinois collection were randomly selected. In all, 48 (Table 3) and 16 (Table 4) isolates representative of each of the identified AG and the other species of *Rhizoctonia* were evaluated in the greenhouse for their ability to cause hypocotyl and root rot symptoms on soybean and corn, respectively. Because isolates of AG-2-IIIB were the most frequently recovered, the number of isolates within this group that was evaluated for pathogenicity was greater than that of other groups. This permitted an evaluation of the variability in aggressiveness of members of this group.

To prepare inoculum, the procedure described by Paulitz and Schroeder (2005) was adopted but with slight modifications. Briefly, sorghum seed were autoclaved for 30 min at 20 psi and 120°C. A second autoclaving was carried out after 24 h using the same parameters described for the first autoclaving. Plugs (5 mm) from 4-day-old cultures of each isolate were transferred to separate 250-ml flasks containing autoclaved sorghum seed and incubated in the dark at 25°C for 2 weeks. Each flask was shaken at least twice per week to ensure uniform colonization of seed. After 2 weeks of incubation, seed were dried under a laminar flow hood for 3 days, packaged in paper bags, and stored at 4°C before use. A modified version of the inoculum-layer technique (Schmitthenner and Hilty 1962) was adopted for inoculation. Ten nontreated seeds of ‘Williams 82’ soybean were sown into 15-cm-diameter plastic pots that had been previously half-filled with steam-pasteurized 2:1 sand-silt loam soil, covered with 5 g of inoculum, and covered with a 5-cm layer of soil. Control pots received noninfested autoclaved sorghum seed. The experiment was arranged as a completely randomized design with three replicates of each experimental unit (pots). The plants were grown in a greenhouse maintained at $24 \pm 3^\circ\text{C}$ with a 14-h photoperiod and was repeated in a second trial. For corn, 20-cm-diameter pots were filled using the inoculum-layer technique described for soybean, and 10 seeds of nontreated ‘Jubilee’ sweet corn were sown directly over

the uppermost soil layer. At 21 days after planting, plants were collected, roots were washed under a high-pressure flow nozzle, and plants were evaluated for disease severity on hypocotyls and roots. Roots were then dried for 4 days at 50°C and weighed. Disease severity was rated on a 0-to-5 scale modified from Nelson et al. (1996) as follows: 0 = no lesion on root or hypocotyl; 1 = lesions <2.5 mm on hypocotyl and $\leq 20\%$ of roots with lesions or rot symptoms; 2 = lesions 2.5 to 5 mm on hypocotyl and 20 to 40% of roots with lesions

Table 3. Median disease rating (DR), mean rank, and estimated relative effect (RE) for the severity of hypocotyl and root rot symptoms caused by isolates of different *Rhizoctonia* spp. on soybean

Isolate	Anastomosis group ^b	DR ^c	RE ^d	Mean rank	95% CI for RE ^a	
					LL	UL
Control	...	0	0.04	14.4	0.03	0.07
X12SDSa	AG11	1	0.3	99	0.28	0.32
K_4_18b	AG11	1.75	0.45	148.5	0.33	0.58
S_P_19a	AG11	1	0.25	84.4	0.18	0.35
211_1a	AG-2-IIIB	4	0.71	229.3	0.62	0.79
X42210_b	AG-2-IIIB	5	0.85	272	0.83	0.86
X42304_b	AG-2-IIIB	0.75	0.18	58.2	0.09	0.31
65L-2	AG-2-IIIB	5	0.85	272	0.83	0.86
DK_11	AG-2-IIIB	5	0.85	272	0.83	0.86
DK_13	AG-2-IIIB	4.5	0.75	241.3	0.65	0.82
DK_14	AG-2-IIIB	5	0.85	272	0.83	0.86
DK_15a	AG-2-IIIB	4.75	0.72	233.6	0.60	0.82
DK_15b	AG-2-IIIB	5	0.85	272	0.83	0.86
DK_16	AG-2-IIIB	5	0.82	262.6	0.75	0.87
DK_19	AG-2-IIIB	3.25	0.61	199.3	0.47	0.74
DK_3a	AG-2-IIIB	5	0.82	263.9	0.76	0.87
DK_3b	AG-2-IIIB	2.5	0.54	178.4	0.44	0.65
DK_4a	AG-2-IIIB	4.5	0.75	242.5	0.66	0.82
DK_6b	AG-2-IIIB	5	0.85	272	0.83	0.86
DK_8	AG-2-IIIB	5	0.82	262.6	0.75	0.87
ER_15	AG-2-IIIB	5	0.85	272	0.83	0.86
ER_19a	AG-2-IIIB	4	0.67	161.1	0.48	0.81
ER_19b	AG-2-IIIB	5	0.82	263.9	0.76	0.87
ER_2	AG-2-IIIB	5	0.85	272	0.83	0.86
ER_4	AG-2-IIIB	5	0.79	253.2	0.70	0.85
K_IL_S02_3_25a	AG-2-IIIB	1	0.25	84.4	0.18	0.35
K_IL_S02_3_25c	AG-2-IIIB	5	0.82	263.9	0.76	0.87
X248_3bKH	AG-3	1	0.3	99	0.28	0.32
C_far_500_10a	AG-3	1	0.22	72.8	0.14	0.33
C_far_500_3	AG-3	1	0.26	87.3	0.20	0.34
C_far_500_6	AG-3	1	0.3	99	0.28	0.32
USA-AG-3	AG-3	1	0.3	99	0.28	0.32
BVT_11	AG-4 HGI	1	0.34	112.7	0.27	0.43
EV_3	AG-4 HGI	1.75	0.49	162.5	0.42	0.57
EV_6	AG-4 HGI	1.5	0.49	158.4	0.32	0.66
BVT_28	AG-4 HGIII	1.25	0.37	121.1	0.25	0.51
BVT_3	AG-4 HGIII	1	0.34	112.7	0.27	0.43
BVT_16	AG-7	2	0.52	170.6	0.43	0.60
BVT_20	AG-7	1	0.38	126.3	0.29	0.49
EV_19	AG-7	2	0.5	164.9	0.42	0.58
EV_7	AG-7	1	0.34	112.7	0.27	0.43
K_2_1a	BR	0.25	0.13	43.6	0.06	0.27
K_1_24c	BR	1	0.25	84.4	0.18	0.35
m_24_c	BR	1	0.26	87.3	0.20	0.34
SP_18_c	BR	1	0.3	98.6	0.21	0.41
W_2_2_15	BR	0.5	0.17	55.3	0.08	0.31
12RS40	<i>Rhizoctonia zeae</i>	0.5	0.14	46.5	0.07	0.27
248_2KH	<i>R. zeae</i>	1	0.22	72.8	0.14	0.33
W2_1_12	<i>R. zeae</i>	1	0.25	84.4	0.18	0.35

^a Confidence interval (CI) for the RE ($\hat{p}_{ij}$) of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002). LL and UL = lower and upper limit, respectively.

^b BR = binucleate *Rhizoctonia*.

^c Median DR was on a scale of 0 to 5.

^d RE were determined as described by Shah and Madden (2004).

or rot symptoms; 3 = lesions >5 mm on hypocotyl and 40 to 60% of roots with lesions or rot symptoms; 4 = lesions girdling entire hypocotyl and 60 to 80% of roots with lesions or rot symptoms; and 5 = plant dead, or no roots, or >80% of roots with lesions or rot symptoms. Statistical analyses were carried out in SAS (version 9.4; SAS institute Inc., Cary, NC). Disease severity data were subjected to nonparametric analysis using the rank-based method described by Shah and Madden (2004). Briefly, median ratings of the 10 plants constituting an experimental unit were obtained before further analysis. Ordinal ratings were converted to midranks using PROC RANK, and midranks obtained were then used by PROC MIXED to calculate significance levels and nonparametric test statistics. Relative treatment effect ($\hat{p}_{ij}$) of each individual isolate and confidence intervals (CI) of relative treatment effects were obtained using the LD_CI macro written by Brunner et al. (2002) and available at <http://www.ams.med.uni-goettingen.de/sasmakr-de.shtml>.

Results

Morphological characterization. Of the 114 isolates recovered from Illinois, Arkansas, Kansas, Michigan, Minnesota, and Ontario, 80 were identified as *R. solani*, 10 as *R. zeae*, and 24 as BR (*Ceratobasidium* spp.). The 80 *R. solani* isolates were characterized as belonging to five AG as follows: AG-2 (with two subgroups; AG-2-1 [$n = 1$] and AG-2-IIIB [$n = 52$]), AG-3 ($n = 5$), AG-4 ($n = 5$), AG-7 ($n = 9$), and AG-11 ($n = 8$). All *R. solani* isolates exhibited macroscopic and microscopic characteristics typical of members of the species (Figs. 2A–D and 3). On PDA, young vegetative hyphae were hyaline when young but became brown with age, and hyphal cells were multinucleate. Cellular nuclear number of young vegetative hyphae ranged from 4 to 12 nuclei, with considerable variation among AG (Table 5). After 2 weeks of incubation, the AG-2-1 isolate produced thick-walled, brown sclerotia that darkened with prolonged incubation. The AG-2-1 isolate produced a C2 reaction with the AG-2-1 tester isolate Rh051307 (KX118375), indicating genetic distinctiveness. Isolates of AG-2-IIIB exhibited marked similarity in cultural appearance; colonies appeared brown with age with zonation, and the thick-walled, brown sclerotia produced darkened with prolonged incubation. All AG-2-IIIB isolates grew at 35°C, and the 10 AG-2-IIIB selected for anastomosis reaction assays produced

a C2 reaction with the AG-2-IIIB tester isolate 65L-2 (KX118334). Cultures of AG-3 isolates were buff in color, with no zonation or apparent pigmentation. Also, sclerotia, which were seldom produced, appeared embedded in PDA around the edges of the plate. Anastomosis reactions between the recovered AG-3 isolates and the AG-3 tester isolate, USA_AG-3 (KX118385), produced a C2 reaction type. The five AG-4 isolates produced brown colonies on PDA with no zonation or apparent pigmentation. Occasionally, a few olive-brown sclerotia developed around the inoculation point on PDA. There were no differences in growth among the four isolates at 25°C, and all four isolates produced a C3 reaction type when anastomosed with AG-4 tester isolate Rh051339 (KX118329). Traditional anastomosis analysis using AG-4 subgroup-specific tester isolates did not permit classification of the AG-4 isolates into subgroups. The AG-7 isolates exhibited similarity in cultural morphology to the AG-4 isolates; the colony appeared brown at 2 weeks after incubation, with clusters of sclerotia occurring mostly at the point of inoculation in PDA. However, none of the AG-7 isolates anastomosed with any of the AG-4 or AG-7 tester isolates. The AG-11 isolates produced light-brown mycelia on PDA at 5 days after incubation at 25°C but colony color became dark-brown with age. Two of the AG-11 isolates produced yellow pigmentation on PDA. Abundant sclerotia, ranging from light tan to brown, were densely distributed on the surface of the PDA. All AG-11 isolates produced a C2 reaction type when anastomosed with AG-11 tester isolate HPIN22A (KX118355). For all BR, two nuclei per cell were observed in young vegetative hyphae (Fig. 2E), and no sclerotia were observed in culture after 2 weeks of incubation. Isolates identified as *R. zeae* had multinucleate cell compartments with 4 to 7 nuclei and produced salmon-orange colonies on PDA. In addition, white spherical sclerotia formed, which turned orange to red with age and appeared submerged in PDA.

Rhizoctonia spp. by location. Of the total number of isolates characterized in this study, 77 were recovered from Illinois, 10 from Arkansas, 1 from Kansas, 1 from Michigan, 1 from Minnesota, and 24 from Ontario, Canada (Fig. 4). The identity of the Illinois isolates are as follows: AG-2-IIIB ($n = 28$), AG-3PT ($n = 5$), AG-4 HGI ($n = 3$), AG-4 HGIII ($n = 2$), AG-7 ($n = 4$), AG-11 ($n = 6$), *Ceratobasidium* spp. ($n = 19$), and *R. zeae* ($n = 10$). Five of the Arkansas isolates were AG-7, two were AG-11, one was AG-2-1, and two were *Ceratobasidium* spp. The Kansas, Michigan, and Minnesota isolates were *Ceratobasidium* spp., whereas all 24 isolates from Ontario were AG-2-IIIB.

PCR-RFLP analysis of the rDNA-ITS regions and PCR analysis with AG-specific primers for AG typing of *R. solani* isolates. Restriction patterns obtained from the digestion of 71 *R. solani* isolates with the restriction enzymes *Mse*I, *Mun*I, *Ava*II, and *Hinc*II corresponded well with those obtained by Guillemaut et al. (2003). Of the different RFLP types they reported for AG-2-1, only “DANN” was observed for both the AG-2-1 isolate from Arkansas and the AG-2-1 tester isolates Rh051307 (KX118375) and Rh051324 (KX118376). All of the AG-2-IIIB isolates characterized by restriction analysis produced a “CAAN” RFLP type. None of the four AG-3 isolates could be typed to either AG or subgroup by restriction analysis. Restriction analysis permitted the classification of all AG-4 isolates into subgroups, producing “IEAA”, and “LENA” RFLP types for the AG-4 HGI and AG-4 HGIII isolates, respectively. Two RFLP types were observed for the AG-7 isolates; AG-7 tester isolate ST81548 (KX118384) produced “DNNN”, while the AG-7 isolates recovered from Arkansas produced an “ENNA”. The AG-7 isolates from Illinois could not be resolved by restriction analysis. All AG-11 isolates produced a “BNAA” RFLP type. All isolates identified as AG-2-IIIB were successfully amplified with the AG-2-IIIB-specific primers, producing a single amplicon of approximately 0.50 kb. The AG-3PT primer pair gave a single product of approximately 0.5 kb for the five AG-3 isolates recovered from Illinois, while the AG-3TB primer produced a single amplicon of about 0.5 kb for the AG-3 tester isolate USA_AG3.

Phylogenetic analysis of rDNA-ITS region. Phylogenetic analysis of the rDNA-ITS sequence data permitted the classification of the recovered isolates into well-established AG or subgroups of different

Table 4. Median disease rating (DR), mean rank, and estimated relative effect (RE) for the severity of hypocotyl and root rot symptoms caused by isolates of different *Rhizoctonia* spp. on corn

Isolate	Anastomosis group ^b	DR ^c	RE ^d	Mean rank	95% CI for RE ^a	
					LL	UL
Control	Control	0	0.25	26.0	0.18	0.34
X12SDSa	AG-11	2	0.68	69.5	0.64	0.71
K_4_18b	AG-11	1	0.50	51.6	0.39	0.61
S_P_19a	AG-11	2.5	0.73	75.2	0.66	0.79
65L-2	AG-2-IIIB	4.5	0.92	94.3	0.89	0.94
DK_3a	AG-2-IIIB	3.5	0.82	84.0	0.79	0.85
ER_2	AG-2-IIIB	3	0.81	82.8	0.77	0.84
C_far_500_6	AG-3	0	0.25	26.0	0.18	0.34
USA_AG3	AG-3	0.25	0.38	39.2	0.25	0.53
X248_3bKH	AG-3	0	0.25	26.0	0.18	0.34
EV_6	AG-4 HGI	5	0.96	98.0	0.94	0.96
BVT_20	AG-7	1	0.54	55.3	0.41	0.66
EV_19	AG-7	0	0.25	26.0	0.18	0.34
K_2_1a	BR	0	0.27	28.3	0.18	0.40
W_2_2_15	BR	0	0.26	26.7	0.18	0.35
12RS40	<i>Rhizoctonia zeae</i>	0	0.25	26.0	0.18	0.34
248_2KH	<i>R. zeae</i>	0.5	0.39	40.8	0.26	0.55

^a Confidence interval (CI) for the RE ($\hat{p}_{ij}$) of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002). LL and UL = lower and upper limit, respectively.

^b BR = binucleate *Rhizoctonia*.

^c Median DR was on a scale of 0 to 5.

^d RE were determined as described by Shah and Madden (2004).

Rhizoctonia spp. Of the 57 isolates analyzed for phylogenetic relationship with reference isolates from the GenBank and tester isolates, 56 were identified based on the PP obtained from the Bayesian analysis (Fig. 5). Three isolates clustered and formed a strongly supported clade (PP = 100%) with tester isolate R63_42A (KX118374) and with an *R. zeae* accession from Japan (AB213594). Sixteen isolates were identified as *Ceratobasidium* spp. and were located in two distinct clades. In the first clade, which consisted mainly of BR isolates, 1 isolate clustered with the *Ceratobasidium* sp. AG-L reference isolates from Japan (AB286933; PP = 100%), while 12 isolates clustered with a *Ceratobasidium* sp. AG-A reference isolate from Japan (AF354092; PP = 100%). The remaining three *Ceratobasidium* spp. were found to group in a clade consisting of *R. solani* isolates. Although two of these isolates grouped closely with two *Ceratobasidium* sp. AG-F reference isolates (PP = 100%) in a clade comprising AG-4 and AG-7 isolates, one clustered with an AG-6 tester isolate (KX118332) and an AG-6 reference isolate from Japan (AF354102). Within the *R. solani*

clade, two *R. solani* AG-7 clades were observed. The first clade (PP = 100%) comprised the AG-7 tester isolate ST81548 (KX118384) and a reference AG-7 isolate AF354098 from Japan, while the second clade (PP = 100%) included nine recovered isolates: four from Illinois, four from Arkansas, and an *R. solani* AG-7 reference isolate from Arkansas (AF153793). Two isolates grouped with *R. solani* AG-4 HGI reference isolate AB000007 from Japan (PP = 100%) and two others clustered with AG-4 tester isolate KX118331 and *R. solani* AG-4 HGIII reference isolate AY154659 from Brazil (PP = 100%). One recovered isolate (KX118363) clustered with an *R. solani* AG-2-1 reference isolate AY154317 from Brazil with a PP of 98%. Thirteen recovered isolates and two tester isolates, 65L-2 (KX118334) and AG-2-2_Nelson (KX118328), clustered together and formed strongly supported clades with two *R. solani* AG-2-2IIIB reference isolates, AF354116 and GU811670, from Japan and the United States, respectively. Seven isolates were identified as *R. solani* AG-11 based on a PP value of 100% from a clade formed with tester isolate HPIN22A

Fig. 2. Microscopic and macroscopic characteristics of *Rhizoctonia solani*. **A**, Right-angled branching of septate hyphae. **B**, Dark-brown sclerotia of a growing colony on potato dextrose agar. **C**, Monilioid cells of sclerotia. **D**, Multinucleate *Rhizoctonia*. **E**, Binucleate *Rhizoctonia*.

Fig. 3. Colony morphology of the binucleate *Rhizoctonia*, *Rhizoctonia zeae*, and some select anastomosis groups (AG) of *Rhizoctonia solani*.

(KX118355) and an AG-11 reference isolate AF354114 from the United States. Five isolates formed a strongly supported clade (PP = 100%) with USA_AG-3 tester isolate (KX118385) and an AG-3 reference isolate (GQ885147) from the United States.

Pathogenicity on soybean. Forty-eight isolates were evaluated for their ability to incite disease on the roots and hypocotyls of soybean in the greenhouse. Of these, 23 were AG-2-2IIIB, 5 were AG-3, 5 were AG-4, 4 were AG-7, 3 were AG-11, 5 were BR, and 3 were *R. zeae*. Based on median disease ratings, estimated relative effect ($\hat{p}_{ij}$), and mean ranks, isolates of AG-2-2IIIB were the most aggressive on the roots and hypocotyls of soybean, followed by those of AG-7, AG-4, AG-11, AG-3, BR, and *R. zeae* (Table 3). The median disease severity ratings of the 48 isolates evaluated ranged from 0.25 to 5, with relative treatment effects of 0.13 to 0.85. Isolates of AG-2-2IIIB had the greatest median disease severity rating (ranging from 2.5 to 5), $\hat{p}_{ij}$ (ranging from 0.50 to 0.85), and mean ranks (ranging from 178.4 to 272), with the exception of two isolates that had median disease severity ratings of 0.75 and 1 and significantly lower $\hat{p}_{ij}$ (0.18 and 0.25, respectively) and mean ranks (58.2 and 84.4, respectively). Isolates of other AG of *R. solani*, BR, and *R. zeae* gave median disease ratings less than 2.0 and $\hat{p}_{ij}$ less than 0.50.

Table 5. Variation in cellular nuclear number among anastomosis groups (AG) of *Rhizoctonia solani* identified in this study

Group	Cellular nuclear number	
	Range	Mode
AG-2-2IIIB	4 to 12	8
AG-3	5 to 10	7
AG-4	4 to 6	5
AG-7	4 to 6	10
AG-11	5 to 10	8
Binucleate <i>Rhizoctonia</i>	2	2
<i>R. zeae</i>	4 to 7	5

Pathogenicity on corn. Three isolates each of AG-2-2IIIB, AG-3 and AG-11; two isolates each of AG-7, BR, and *R. zeae*; and one isolate of AG-4 were evaluated for their ability to incite disease on roots of corn. The median disease ratings ranged from 0 to 5, with $\hat{p}_{ij}$ of 0.25 to 0.92 and mean ranks of 24.5 to 94.3 (Table 4). AG-2-2IIIB isolates produced the greatest median disease severity ratings (ranging from 2.5 to 4.5) while isolates of BR gave 0 as the median disease severity rating. Based on $\hat{p}_{ij}$ and their 95% CI, AG-2-2IIIB isolates were the most aggressive, although the single AG-4 isolate tested expressed similar levels of aggressiveness. Isolates of AG-3, AG-7, BR, and *R. zeae* were not significantly different from the noninoculated control.

Discussion

In this study, isolates of *Rhizoctonia* recovered from symptomatic soybean plants between 2012 and 2014 from five states in the United States and from the Canadian province of Ontario were characterized and identified to AG, subgroups, and species. The pathogenicity of isolates representative of the identified groups was also determined to provide information on which of the groups present the greatest risk to soybean health in any soybean production field. Three taxonomic groups—*R. solani* (70%), *R. zeae* (9%), and *Ceratobasidium* spp. (21%)—were identified. Isolates of *R. solani* were further identified to AG and subgroups as follows: AG-2-1, AG-2-2IIIB, AG-3PT, AG-4 HGI, AG-4 HGIII, AG-7, and AG-11. Of the identified AG and *Rhizoctonia* spp., *R. solani* AG-2-2IIIB posed the greatest risk to soybean health at the seedling stage.

The diversity of *R. solani* AG recovered in this study is consistent with reports from other authors (Carling et al. 1994; Liu and Sinclair 1991; Muyolo et al. 1993a; Nelson et al. 1996; Rizvi and Yang 1996; Zhao et al. 2005). Combining all locations in which *R. solani* was recovered, isolates of AG-2-2IIIB represented the most frequently isolated population while AG-2-1 represented the least recovered group; however, considering each location, the number and diversity of AG recovered varied. Muyolo et al. (1993a) identified AG-2-2IIIB as the

Fig. 4. Distribution by location of isolates of *Rhizoctonia* collected from soybean in Arkansas, Illinois, Kansas, Michigan, Minnesota, and the Canadian province of Ontario. BR = binucleate *Rhizoctonia* and AG = anastomosis group.

AG-4, and AG-3, in addition to other species of *Rhizoctonia*, were recovered from diseased soybean plants obtained from 200 fields, with AG-4 isolates being recovered more frequently (Nelson et al. 1996). Similarly, in Iowa, AG-4 was the predominant group isolated from diseased soybean seedlings (Rizvi and Yang 1996). In our study, no AG-5 isolate was recovered and very few AG-4 isolates were recovered from Illinois. The reasons for the disparity in the predominant AG across different soybean-growing regions is uncertain

but may be attributed to differences in cropping systems (Ogoshi 1987) or soil type associated with the different states. The diversity of AG recovered from Arkansas is consistent with previous reports. In the United States, isolates of AG-7 (Rothrock et al. 1993) and AG-11 (Carling et al. 1994) were first reported on soybean seedlings in Arkansas. Also, AG-11 isolates have been associated with fields under repeated rice–soybean rotations in Arkansas (Spurlock et al. 2016) and Texas (Jones and Carling 1999). However, this is the first report of AG-2-1 in this region and the first time AG-2-1 was isolated from soybean seedlings. AG-2-1 is known to be pathogenic on a number of host crops (Sneh et al. 1991), including pea (Hwang et al. 2007; Sharma-Poudyal et al. 2015), canola (Paulitz et al. 2006), tulip (Nakatomi and Kaneko 1971), and crucifers (Ogoshi 1987; Watanabe and Matsuda 1966), and no researchers have reported this AG as a major pathogen of soybean or corn. The role of this AG in the ecology of *R. solani* on soybean deserves further investigation. The diversity of AG observed in Illinois is unexpected given the less diverse cropping pattern typical of this region. With the corn–soybean rotation being the primary rotation regime in Illinois, the detection of AG known to be pathogenic on other crops not cultivated in this region is rather surprising. Isolates of AG-3 are well established pathogens of members of the Solanaceae family (Anderson 1982; Kuninaga et al. 2000; Meyer et al. 1990; Ogoshi 1987), AG-7 are pathogens of cotton and watermelon (Baird and Carling 1995, 1997; Baird et al. 1996), and AG-11 are pathogens of lupine, cotton, and radish (Carling et al. 1994; Sweetingham 1989). The identification of these AG on diseased soybean seedlings may imply an expansion of host range to include soybean. The recovery of only AG-2-IIIB isolates from Ontario suggests that there has not been a change in the AG of *R. solani* causing seedling diseases of soybean in that region.

The single isolate recovered from Kansas, Minnesota, and Michigan was identified as a *Ceratobasidium* sp. *Ceratobasidium* spp. are *Rhizoctonia*-like fungi with binucleate cells (Sneh et al. 1991) and, similar to *R. solani*, members of the genus *Ceratobasidium* have been classified into seven *Ceratobasidium* AG (CAG-1 to CAG-7) (Burpee et al. 1980a), although these were later expanded to 17 AG (AG-A to AG-Q) (Ogoshi et al. 1983). The low frequency of *R. solani* recovery from these states was rather surprising. At least four AG of *R. solani* have been associated with soybean seedlings in Minnesota (Nelson et al. 1996) and, in Michigan, *R. solani* AG-2, AG-3, and AG-4 are widespread (Wharton et al. 2006). Therefore, the reason for their low recovery rate in this study was probably due to sampling or isolation protocols. The isolation of *R. zeae* from soybean seedlings was infrequent. Moreover, all *R. zeae* isolates identified in this study were recovered from Illinois, and this is the first report of this *Rhizoctonia* sp. on soybean seedlings in this region. *R. zeae* are multinucleate *Rhizoctonia* that are differentiated from *R. solani* by their distinctive salmon-colored mycelia in culture (Burpee and Martin 1992). They are major pathogens of corn and turfgrass species (Burpee and Martin 1992; Martin and Lucas 1984a; Sumner and Bell

1982; Voorhees 1934), and their frequent isolation from organic debris and soils associated with symptomatic host plants (Martin and Lucas 1984a) suggests an excellent saprophytic potential. Given that corn is a major rotational crop in Illinois, *R. zeae* recovered on symptomatic soybean seedlings might have originated from the inoculum on corn residue.

Molecular characterization using the ITS sequence of the ribosomal genes has been identified as the most reliable approach for the phylogenetic studies of AG and subgroups of different *Rhizoctonia* spp. (Gonzalez et al. 2001; Sharon et al. 2006). Using the ITS sequence data of the recovered isolates and those from the NCBI database, we were able to corroborate the results obtained using other characterization techniques and make inferences regarding the phylogenetic relationships among the isolates. Phylogenetic analysis also allowed for the identification of certain *R. solani* isolates that remained unidentified using other AG-typing techniques. For example, the four AG-7 isolates from Illinois grouped strongly with AG-7 isolates collected from Arkansas and the AG-7 reference isolate, which also was recovered from Arkansas (AF153793). A second AG-7 clade comprised an AG-7 tester isolate, ST81548, and an AG-7 reference isolate from Japan (AF354098) also was observed. The origin of ST81548 is unknown but it does appear that the two AG-7 clusters observed in this study correspond to different AG-7 subgroups. Additional work involving representative isolates from different hosts and origins needs to be done to test this hypothesis. For the first time in the United States, AG-4 HGI was identified on soybean. In the United States, AG-4 isolates from soybean have been characterized as HGII and HGIII, while those associated with hypocotyl rot in Brazil have been identified as HGI. Phylogenetic analysis also revealed different clades that corresponded to well-established AG of different *Rhizoctonia* spp. Based on PP values, there was strong support (>95%) for AG-2-1, AG-2-IIIB, AG-3, AG-4, AG-7, and AG-11 clusters. The AG-2-1 and AG-2-IIIB isolates did not cluster together on the phylogenetic tree despite being members of the same AG. This observation is not surprising given that polyphyly among members of this AG has been reported by several authors (Budge et al. 2009; González et al. 2006; Kuninaga et al. 1997; Ohkura et al. 2009; Salazar et al. 1999, 2000; Sharon et al. 2008; Stodart et al. 2007).

Despite the strong support (100%) for the separation of the *Ceratobasidium* spp. and the *R. solani* isolates, certain *Ceratobasidium* isolates clustered with isolates of *R. solani*. This suggests that certain *Ceratobasidium* spp. are more closely related to some *R. solani* isolates than they are to other members of genus *Ceratobasidium*. Similar observations indicating genetic relatedness between *Ceratobasidium* spp. and some *R. solani* isolates have been reported by several authors. For example, Gonzalez et al. (2001) found that certain BR belonging to CAG-4 and CAG-6 clustered closely with *R. solani* AG-4 isolates. Similarly, cluster analysis of rDNA-ITS region sequences of *Rhizoctonia* isolates recovered from strawberry in Israel grouped *Ceratobasidium*

Fig. 6. Hypocotyl and root rot symptoms caused by *Rhizoctonia solani* anastomosis group (AG)-2-IIIB and AG-4 on Williams 82 soybean.

sp. AG-F isolates more closely with *R. solani* AG-4 isolates than with other *Ceratobasidium* spp. (Sharon et al. 2007). Also, the genetic relatedness between AG-F and *R. solani* AG-6 has been suggested based on hyphal anastomosis reactions (Yokoyama and Ogoshi 1986). Together, these observations indicate that BR represents a polyphyletic group and that further studies are required to clarify their phylogenetic relationship with *R. solani*.

From the greenhouse pathogenicity experiments on soybean, considerable variation in the aggressiveness among the different *R. solani* AG and other species of *Rhizoctonia* was observed. The most frequently recovered group, AG-2-IIIB, was also the most aggressive on soybean roots and hypocotyls. The AG-2-IIIB isolates also exhibited significant variability in their aggressiveness on soybean, and virulence was not correlated with isolate origin. Variability in aggressiveness of AG-2-IIIB isolates has been reported previously (Dorrance et al. 2003; Muyolo et al. 1993a; Nelson et al. 1996). These observations highlight the importance of determining the aggressiveness of an isolate if the goal of the breeding program is to select for resistant soybean genotypes. The AG-4 isolates were not as aggressive as the AG-2-IIIB isolates. Similar to what has been observed by other researchers (Fenille et al. 2002, 2003; Muyolo et al. 1993a), AG-4 isolates caused more severe symptoms on the hypocotyls while AG-2-IIIB isolates caused more severe symptoms on the roots (Fig. 6). Although the five AG-4 isolates tested represented two homogenous groups (AG-4 HGI and AG-4 HGIII), there were no differences in aggressiveness among the five AG-4 isolates evaluated in the greenhouse. Disease symptoms produced by AG-7 and AG-11 isolates were comparable with those caused by the AG-4 isolates. This contradicts previous observations by other researchers, where these two AG were found to be less aggressive than AG-4 on soybean seedlings (Carling et al. 1994; Rothrock et al. 1993). The long-term coexistence with soybean in the absence of other major host plants may have played a role in expanding the host range of these two AG; however, because we did not evaluate the pathogenicity of isolates of AG-7 and AG-11 recovered outside of Illinois, it is impossible to validate our hypothesis. Pathogenicity tests did not implicate the recovered *Ceratobasidium* spp. and *R. zeae* as pathogens of soybean. BR and *R. zeae* generally are not considered pathogens of soybean, even though they have been recovered from soybean seedlings at different locations (Naito et al. 1993; Nelson et al. 1996). Isolates of certain CAG of BR have been shown to be pathogenic on a number of host crops (Burpee et al. 1980b; Kataria and Hoffmann 1988; Sneh et al. 1991) while those of *R. zeae* mostly attack corn and turfgrass species (Burpee and Martin 1992; Martin and Lucas 1984a; Sumner and Bell 1982; Voorhees 1934). Naito et al. (1993) identified isolates of AG-E from Indonesia as potential pathogens of soybean. However, the *Ceratobasidium* isolates evaluated in our greenhouse assays are members of CAG-2 (AG-A), suggesting that soybean is not a potential host to this AG. Although isolates of *Ceratobasidium* spp. and *R. zeae* produced little to no necrotic lesions on corn roots, we observed a significant reduction in dry root weight of plants inoculated with these groups compared with the noninoculated control. Our observations with these two species of *Rhizoctonia* suggest that root weight reduction may be due to the production of root growth inhibitory compounds.

Results of pathogenicity tests revealed that corn is a host to the AG that are pathogenic on soybean, especially AG-2-IIIB, AG-4, and AG-11. The AG-4 and AG-2-IIIB isolates were highly aggressive on corn, severely rotting both the crown and seminal roots. Isolates of AG-2-IIIB are known to cause root rot of corn in the United States (Ohkura et al. 2009; Sumner and Bell 1982) and Europe (Buddemeyer et al. 2004; Ithurrart et al. 2004), and AG-4 isolates have been reported to produce root rot symptoms that are not as severe as those produced by AG-2-IIIB isolates (Fenille et al. 2002; Muyolo et al. 1993a; Zhao et al. 2005). Because we tested only one isolate of AG-4, which belongs to HGI, we were unable to correlate HG types to virulence on corn or make conclusions regarding variation in aggressiveness of HG types of AG-4 on corn. Root damage caused by AG-11 isolates was mild and mostly restricted to the mesocotyl. The identification of corn as a susceptible host to the

most aggressive AG on soybean indicates a high potential for inoculum build-up in the corn–soybean rotations in Illinois. Therefore, management practices that reduce inoculum build-up of these AG on farmers' fields deserve consideration in this region.

In summary, our work provides evidence for the association of a diverse group of *Rhizoctonia* spp. with soybean, with AG-2-IIIB as the most predominant and aggressive group. Two groups (AG-2-1 and AG-4 HGI) previously unidentified as part of the soybean seedling disease complex and two divergent groups of AG-7 were identified in this study. The susceptibility of corn to AG-2-IIIB and other AG pathogenic on corn highlights the potential risks of the corn–soybean rotation, especially in areas where such AG are prevalent.

Acknowledgments

We thank the United Soybean Board and the North Central Soybean Research Program for financial support of this project; and K. Ames, K. Krausz, D. Pedersen, and U. Reuter-Carlson for technical assistance.

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