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A Binucleate *Rhizoctonia* anastomosis group (AG-W) is the causal agent of sugar beet seedling damping-off disease in China

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Abstract Two binucleate *Rhizoctonia* (BNR) isolates (HLJ-21 and HLJ-57) were recovered from diseased sugar beet seedlings in Heilongjiang Province, Northeastern China in May, 2015. The two isolates were identified as members of the anastomosis group (AG)-W based on morphological characteristics, hyphal anastomosis, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), and phylogenetic analysis of the internal transcribed spacer of the ribosomal DNA (rDNA ITS). The optimum temperature for hyphal growth was 25 °C and the mycelia retained a whitish appearance on potato dextrose agar as cultures aged. No sclerotia, however, were observed during the period of culture. Both of the isolates exhibited anastomosis with AG-W, but failed with other reference strains of BNRs. Restriction fragments resulting from the eight enzymes indicated that HLJ-21 and HLJ-57 isolates had the same restriction pattern as AG-W isolates. Phylogenetic analysis also indicated that the rDNA ITS region of the HLJ-21 and HLJ-57 isolates clustered with other AG-W isolates in the same clade. The AG-W isolates from sugar beet and potato were the causal agent of damping-off disease on sugar beet seedlings as well as root disease on wheat and corn seedlings. The AG-W isolates from sugar beet could also induce stem canker disease on potato plants. Notably, the isolates from sugar beet seedlings had a higher virulence on all of the tested crops than those isolates obtained from potato. This appears to be the first report of a AG-W BNR causing sugar beet seedling damping-off disease in China, and perhaps globally.

Keywords Sugar beet · Seedling damping-off · Binucleate *Rhizoctonia* · AG-W · rDNA ITS · RFLP

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Introduction

Rhizoctonia is one of the most important pathogens impacting seedling health of sugar beet (*Beta vulgaris* L.) all over the world. Infection of sugar beet by *Rhizoctonia* causes pre-emergence death or post-emergence damping-off, the latter of which causes wilting and death of seedlings (Nagendran et al. 2009; Hanson and McGrath 2011). *Rhizoctonia* can also cause serious crown and root rot disease on mature plants, which severely reduces yield and sucrose levels in stored roots (Kiewnick et al. 2001; Strausbaugh et al. 2011b).

Thus far, it is generally recognized that *R. solani* (teleomorph *Thanatephorus cucumeris*) AG-2-2 and AG-4 are the most pathogenic on sugar beet. *R. solani* AG-2-2 (intraspecific groups IIIB and IV) can cause both damping off, and crown and root rot disease of sugar beet (Windels and Nabben 1989; Bolton et al. 2010; Hanson and McGrath 2011; Stojšin et al. 2011;

Zhao and Wu 2014), while *R. solani* AG-4 principally causes damping-off disease in sugar beet seedlings (Soltaninezhad et al. 2008; Zhao et al. 2019).

Isolates of binucleate *Rhizoctonia* (BNR) have been categorized into 19 anastomosis groups (AGs), ranging from AG-A to AG-I, AG-K, AG-L, AG-O to AG-S, AG-U to AG-W; based on the category of their hyphal interaction (C0, C1, C2, and C3) (Ogoshi et al. 1979; Carling 1996; García et al. 2006; Yang et al. 2015a; Dong et al. 2017). AG-B and AG-D are further divided into subgroups, based on differences in their morphology, whole-cell fatty acid composition, the DNA sequence of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA ITS), host range, and virulence, etc. (Toda et al. 1999; Priyatmojo et al. 2001; Hayakawa et al. 2006). Michal et al. (2007) has also suggested that AG-F isolates should be divided into different subgroups based on their ITS sequences.

Some BNR isolates are economically-important phytopathogens causing different diseases on a wide variety of agricultural crops (García et al. 2006; Yang et al. 2014). BNR AG-A, AG-C, AG-K, and *R. cerealis* isolates were reported to cause seedling damping-off on sugar beet (O'Sullivan and Kavanagh 1990; Wang and Wu 2012; Zhao et al. 2019). Furthermore, AG-F has been reported to cause dry rot canker of sugar beet in Nebraska (Harveson and Bolton 2013).

In 2015, two binucleate-like *Rhizoctonia* isolates, designated as HLJ-21 and HLJ-57, were recovered from roots of diseased sugar beet seedlings in Heilongjiang Province, Northeastern China. The objectives of the present study were to characterize the two isolates based on their (i) morphological characteristics, including their appearance when cultured on potato dextrose agar (PDA), nuclear condition, hyphal diameter, and mycelia growth rate; (ii) unique anastomosis reaction with BNR AGs; (iii) rDNA ITS sequence and their polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP); and (iv) pathogenicity on sugar beet seedlings and crops (corn, wheat, and potato) commonly used as part of a rotation in the production of sugar beet in China.

Materials and methods

Sample collection and fungal isolation

Sugar beet seedlings exhibiting symptoms of damping-off disease were collected in 2015 from a nursery bed located in Yi'an County (latitude-47°52′47″N, longitude-125°18′9″E), Heilongjiang Province, northeastern China. Twenty roots with brown lesions of sugar beet seedlings were randomly collected from the nursery bed. After washing under running tap water, roots were cut into fragments (about 5 mm) from the margins of the diseased tissues. Surface-disinfection of the cut root samples was conducted using 70% ethanol for 30 s, followed by soaking in 0.5% sodium hypochlorite for 3 min, and three subsequent rinses of the samples with sterile distilled water. After drying on sterilized filter paper, the fragments were placed on water agar (WA) plates containing 50 $\mu g \cdot mL^{-1}$ streptomycin sulfate, and incubated at 25 °C for 24 to 72 h. Mycelia that subsequently appeared in the cultures possessing right angle branches, a septum near the branch, and a slight constriction at the branch base, were transferred onto PDA plates using a single hyphal method. After purification of the cultures, two *Rhizoctonia*-like isolates were obtained and named HLJ-21 and HLJ-57. The isolates were kept at 4 °C in culture plates for short-term storage. For long-term storage, the isolates were cultured on sterile barley grains and maintained at 4 °C (Webb et al. 2011).

Nuclear staining and colony characterization

A modified clean slide method (Kronland and Stanghellini 1988) was used to determine whether the two *Rhizoctonia* isolates are binucleate or multinucleate. Multiple 7-mm-diameter disks were cut from the margins of different colonies, and placed on sterilized microscope slides in moist 9 cm diameter Petri dishes and incubated at 25 °C in the dark for 24 to 48 h. The slides were then removed from the moist Petri dishes and mycelia were stained with 1 $\mu g\ mL^{-1}$ 4′-6-diamidino-2-phenylindole (DAPI; cat. No. D9542, Sigma) for 10 min in the dark. The slides were subsequently rinsed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Nuclei of at least 30 cells from more than one hypha were counted under a fluorescence microscope at a 400× magnification in order to determine the nuclear status of each isolate.

The mycelia growth rate was determined on PDA plates at 0, 5, 10, 15, 20, 25, 30, 35, and 40 °C. Mycelial plugs, 5 mm in diameter, were cut from the margin of actively-growing colonies of three-day-old cultures, and one plug was placed on the center of 9-cm Petri dishes. The cultures (four replicates for each isolate and

temperature) were incubated in the dark. Two perpendicular measurements of colony diameter were taken at 24-h intervals until the colony reached the edge of the Petri dish. The morphological characteristics of cultures growing on PDA plates at 25 °C in the dark; including color, size, presence and shape of sclerotia, and colony pigmentation; were continuously examined and recorded every 24-h for 3 weeks.

AG determination

The determination of the AGs was performed using reference isolates of known AGs. A total of 18 reference strains, including FuK-600 (AG-A), C484 (AG-Ba), C-350 (AG-Bb), Ao-1-2 (AG-C), Ayu-WP-1 (AG-DI), TAK-14KT (AG-DII), KOU04-12FW (AG-DIII), RH-155 (AG-E), AH-6 (AG-Fb), AH-9 (AG-G), STC-11 (AG-H), AV-2 (AG-I), 55D45 (AG-K), FKO-2-16 (AG-L), FKO-6-2 (AG-O), C-578 (AG-P), C-620 (AG-Q), and MWR-20 (AG-U), were kindly provided by N. Kondo (Graduate School of Agriculture, Hokkaido University, Sapporo, Japan). The AG-W isolates (HL-CZ, HL-ZA) were stored in our lab in the Department of Plant Pathology, China Agricultural University, Beijing, China. Anastomosis reactions were observed using the clean-slide method (Kronland and Stanghellini 1988). A 7-mm-diameter disk of each of the two isolates (HLJ-21 and HLJ-57) was placed on a microscope slide with a known AG reference strain placed 2 to 3 cm away. The slides were placed in moist 9 cm diameter Petri dishes and incubated at 25 °C in the dark for 24 to 48 h. When hyphae from the two disks came into contact with each other, the overlapping hyphae were observed under bright-field optics at 40× magnification to identify potential fusion events; which were then confirmed at a higher magnification (100× and 400×). All pairings were repeated twice and at least 10 fusion events were analyzed. The categories of hyphal anastomosis reaction were scored as: C0, no interaction; C1, hyphal contact without fusion; C2, cell wall fused while anastomosing and adjacent cells die; C3, fusion of cell wall and membrane, with no cell death (Carling 1996).

DNA extraction and polymerase chain reaction (PCR) amplification

The two isolates were grown on PDA plates at 25 °C for 3–5 days. Mycelia were collected directly from plates and freeze-dried, and ground to a fine powder in liquid nitrogen. DNA was extracted using a CTAB (cetyltrimethylammonium bromide) method as described in the previous study (Zhao et al. 2019).

The internal transcribed spacer (ITS) region (partial 18 s rDNA, internal transcribed spacer 1, 5.8 s rDNA gene, internal transcribed spacer 2, and partial 28 s rDNA sequence) of ribosomal DNA (rDNA), was amplified from the genomic DNA with ITS1 and ITS4 primers (White et al. 1990). Each 25 μL PCR mixture consisted of 11 μL ddH_2O, 12.5 μL 2x *Pfu* Master Mix [PC1102, Aidlab Biotechnology (Beijing, China), containing 0.05 U μL^{-1} *Pfu* DNA polymerase, 400 μM dNTP and 4 mM Mg^{2+}], 0.5 μL each of primer (10 μM), and 0.5 μL DNA (100 μg mL^{-1}). Negative controls were sample tubes containing the same reagents but without DNA. The amplification reaction was performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf AG, Hamburg, Germany) using the following program: initial denaturation at 94 °C for 3 min; followed by 30 cycles of denaturing at 94 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min; one cycle of extension at 72 °C for 10 min and final incubation at 4 °C. The PCR products of rDNA ITS region were purified with an Aidlab DNA Gel Extraction Kit (Aidlab Biotechnology, Beijing, China).

RFLP of rDNA ITS region

DNA restriction sites for the utilized enzymes in different BNR isolates were predicted using Primer premier 5.0 (Premier Biosoft International, Palo Alto, CA) (Lalitha 2000). Eight enzymes, *Eco*RI, *Hae*III, *Hap*II, *Hha*I, *Hin*fI, *Mbo*I, *Mse*I, and *Mun*I (TaKaRa Biotechnology, Dalian, China), were selected to digest the purified PCR product of each isolate according to the enzyme manufacturer's instructions. The restriction fragments were separated by electrophoresis on a 2% agarose gel and the resulting bands were visualized under UV light after staining with ethidium bromide (EB). A mix of molecular markers with known sizes (100-bp DNA ladder; TaKaRa) was used as a size reference in each gel. The experiment was conducted three times.

DNA sequencing and phylogenetic analysis

Purified PCR products were placed in a cloning vector using a pTOPO-Blunt cloning kit (Aidlab

Biotechnology) according to the manufacturer's instructions. The cloned products were then transformed into competent cells (*Escherichia coli* MC 1022) by heat shock at 42 °C for 90 s, which were then cultured on Luria-Bertani broth medium containing 50 μg mL^{-1} ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-thiogalactopyranoside. White colonies verified by PCR were then sent to Beijing Sunbiotech Co., Ltd. (Beijing, China) for sequencing and the obtained sequences were subsequently edited using DNAMAN Version 7 (Lynnon LLC., San Ramon, CA) and deposited in GenBank (GenBank accessions: KX290846 & KX290847).

The rDNA ITS sequences of the two *Rhizoctonia* isolates from this study were first subjected to BLASTn on the National Center for Biotechnology Information (NCBI) website to determine sequence identity and to find the closest match based on maximal percent identity. Phylogenetic analysis was performed using MEGA 5 Version 5.2.2 (Tamura et al. 2011) with reference sequences (AG-A to AG-I, AG-K, AG-L, AG-O to AG-S, AG-U to AG-W) retrieved from GenBank. The rDNA ITS sequence of *Athelia rolfsii* FSR-052 (GenBank accession: AY684917) was used as outgroup for rooting the phylogenetic trees. Maximum Likelihood (ML) analysis was used to construct a phylogenetic tree based on the distance matrix produced by the Tamura-Nei model. Bootstrap support was estimated based on 1000 pseudoreplicates. The percent sequences identities of the ITS1–5.8S rDNA-ITS2 region were calculated by direct pairwise comparisons within BioEdit software Version 7.2.5 (Hall 1999).

Pathogenicity of AG-W isolates on sugar beet seedlings

Pathogenicity test of the AG-W (HLJ-21, HLJ-57, HL-CZ, and HL-ZA) isolates on sugar beet (cv. HI0305) seedlings was conducted as the method described in our previous study (Zhao et al. 2019). The wheat seeds used for inoculum were moistened with sterile distilled water (60% *v*/*w*) in 250-mL flasks and sterilized by autoclaving twice at 121 °C for 60 min, with a 24 h interval between autoclaving runs. The seeds were then cooled at room temperature and spread on the 4-day-old actively growing PDA cultures of the *Rhizoctonia* isolates. The fungus was allowed to colonize the wheat seeds for 3 days at room temperature in the dark.

A single surface-sterilized sugar beet seed was placed in each plastic pot (120 mL) filled with a mixture soil of sand and sawdust (1:2 *v*/v) that had been subjected to dry heat sterilization at 161 °C for 4 h before use. The pots were arranged in a randomized block design in a greenhouse maintained at 25 to 27 °C, with a 12 h photoperiod, and watered daily to maintain growth. Twenty healthy, two-leaf-stage seedlings were selected for pathogenicity tests as a replicate and three replicates were used for each isolate tested. One infested wheat seed was placed at a depth of 10 mm into the soil next to the root of each seedling. Negative controls were inoculated with un-infested autoclaved wheat seeds. The sugar beet seedlings were incubated in a greenhouse maintained at 25 to 27 °C with a 12 h photoperiod and watered whenever the surface soil appeared dry. After 7 days, all of the seedlings were harvested and assessed for disease incidence and disease index. The experiment was conducted twice.

Sugar beet seedlings were scored for disease severity using a 0–7 scale: 0 = no disease, 1 = necrosis of root, with a ≥ 1 cm longitudinal crack, and tissue discoloration at the edge of crack, 3 = <75% necrosis of the root, 5 = 75%–100% necrosis of the root, and 7 = seedling death. The disease incidence and the disease index were calculated as follows: disease incidence =100% × $(n_1 + n_3 + n_5 + n_7)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 3n_3 + 5n_5 + 7n_7)$ / (7 N), where n_0 to n_7 were the number of plants in each degree and N was the total number of inoculated plants.

Pathogenicity of AG-W isolates on wheat and corn seedlings

The pathogenicity of the AG-W isolates (HL-CZ, HL-ZA, HLJ-21, and HLJ-57) on two-leaf-stage seedlings of wheat (cv. Lunxuan 266) and corn (cv. Zhengdan 958) was conducted under greenhouse conditions using sugar beet seedling method described above. The disease rating scale for the wheat and corn seedlings was as described by Rush et al. (1994): 0 = no disease, 1 = <25% necrosis of the roots, 2 = 25% -75% necrosis of the roots, 3 = 75–100% necrosis of the roots, and 4 = seedling death. The disease incidence and the disease index of wheat and corn were calculated as follows: disease incidence =100% × $(n_1 + n_2 + n_3 + n_4)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)$ / (4 N), where n_0 to n_4 were the number of plants in each degree and N was the total number of inoculated plants.

Pathogenicity of AG-W isolates from sugar beet on potato

The pathogenicity of the AG-W isolates (HLJ-21, and HLJ-57) isolates from sugar beet on potato (cv. Favorita) was carried out in the greenhouse conditions with the method of Yang et al. (2015a). The surface-sterilized potato seed tubers were allowed to sprout at room temperature in the dark until the sprouts were about 3 mm long. One visually asymptomatic sprouted tuber was placed in a sterile plastic pot (caliber×bottom diameter×height: 90 mm × 70 mm × 90 mm) filled with 30 mm depth of sterilized soil mixture of sand and sawdust mentioned above, then one infested wheat seed was placed 10 mm above the uppermost sprout tip and then the pot was filled with the sterilized soil mixture. Control plants were inoculated with un-infested autoclaved wheat seeds. The experiment was conducted with three replicates per isolate and 20 plants per replicate. The pots were arranged in randomized block design. All potato plants were harvested after incubation in the greenhouse for 3 weeks, and the disease incidence and disease index were calculated. Based on the relative size of necrotic area on subterraneous stems, the disease rating scale was as follows: 0 = no disease, 1 = less than 10%, 2 = 10–50%, 3 = 50–100%, and 4 = plants dead. The disease incidence and disease index were calculated as follows: disease incidence = $100\% \times (n_1 + n_2 + n_3 + n_4)$ / N and disease index = $100 \times (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)$ / (4 N), where n_0 to n_4 were the number of plants in each degree and N was the total number of inoculated plants.

All the sugar beet, wheat, and corn seedlings, and potato stems were used to re-isolate *Rhizoctonia* fungus and the resulting *Rhizoctonia* isolates were identified by morphological and molecular methods described above to fulfill Koch's postulates.

Statistical analysis

All data for disease incidence and disease index of sugar beet, wheat, corn, and potato plants from the pathogenicity tests were analyzed by one-way analysis of variance (ANOVA) using Statistical Product and Service Solutions (SPSS) software version 20.0 (SPSS Inc., Chicago, USA). Homogeneity of variance was assessed using Levene's test. As variances and sample size were unequal, differences between groups were tested via the non-parametric tests of Kruskal-Wallis H. The results were further examined by one-way ANOVA with Dunnett's T3 tests ($P = 0.05$).

Results

Characterization of the two isolates and AG determination

Isolates of HLJ-21 and HLJ-57 growing on PDA plates for three weeks exhibited white mycelia. They did not form sclerotia during the culturing period of 21 days at 25 °C on PDA plates, but did produce white monilioid cells in their fluffy aerial hyphae (Fig. 1). The nuclear stain revealed that both of the isolates possess two nuclei per cell (Fig. 2). The average hyphae width was 4.67 ± 0.12 μm (ranging from 4.06 to 5.80 μm) and 4.98 ± 0.13 μm (ranging from 3.76 to 5.54 μm) in the HLJ-21 and HLJ-57 strains, respectively. Both of the isolates were able to grow on PDA plates at temperatures ranging from 5 to 35 °C, with an optimum growth temperature of 25 °C. The isolates stopped growing at both 0 and 40 °C (Fig. 3).

When paired with the reference strains, both of the tested isolates exhibited a C3 anastomosis with the two AG-W isolates from potato (Fig. 4), but failed to exhibit anastomosis with the other BNR reference strains (AG-A to AG-I, AG-K, AG-L, AG-O to AG-Q, AG-U). The two isolates also exhibited anastomosis with each other.

rDNA ITS PCR-RFLP and sequence analysis

The restriction enzyme cutting sites of the rDNA ITS and the resulting sizes of the restriction fragments could successfully differentiate the BNR strains (Table 1). The tester isolates, HLJ-21 and HLJ-57 from sugar beet, share the same RFLP patterns as AG-W isolates (HL-CZ, HL-ZA) from potato, which were distinct from all of the other anastomosis groups of BNR reference strains. Among the eight restriction enzymes, *Mbo*I, *Mse*I, *Hin*fI, and *Hae*III resulted in producing variation in the pattern of restriction fragment sizes. The PCR products of each isolate had one *Eco*RI cut site, resulting in two different sized fragments. Isolates could not be digested by *Hap*II, except for AG-C, AG-DI, AG-DIII, AG-I, AG-K, and AG-P. The rDNA ITS PCR products were not cleaved by *Mun*I, except for isolates associated with AG-A, AG-Ba, AG-C, AG-E, AG-H, AG-O, AG-

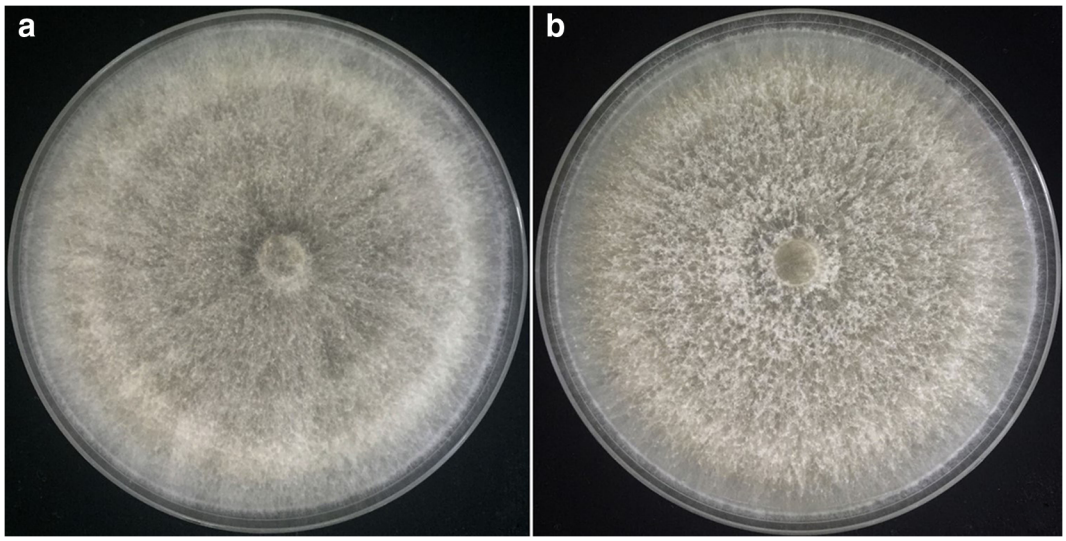

Fig. 1 Morphology of the two binucleate *Rhizoctonia* isolates after 21 days of incubation on potato dextrose agar (PDA) in the dark. A: HLJ-21. B: HLJ-57

P, AG-W, HLJ-21, and HLJ-57. *Hha*II cut the PCR products of all of tested isolates into two fragments, except for AG-Bb and AG-Q, which had two cut sites resulting in three restriction fragments. The bands generated by restriction digestion were consistent with the fragments predicted by Primer premier 5.0. A few exceptions occurred for some short fragments of <100 bp which were not visualized due to the limited resolution that can be obtained using 2.0% agarose gels.

The sequences of the rDNA ITS region of the reference strains retrieved from GenBank and the HLJ-21 and HLJ-57 isolates clustered into different clades in a

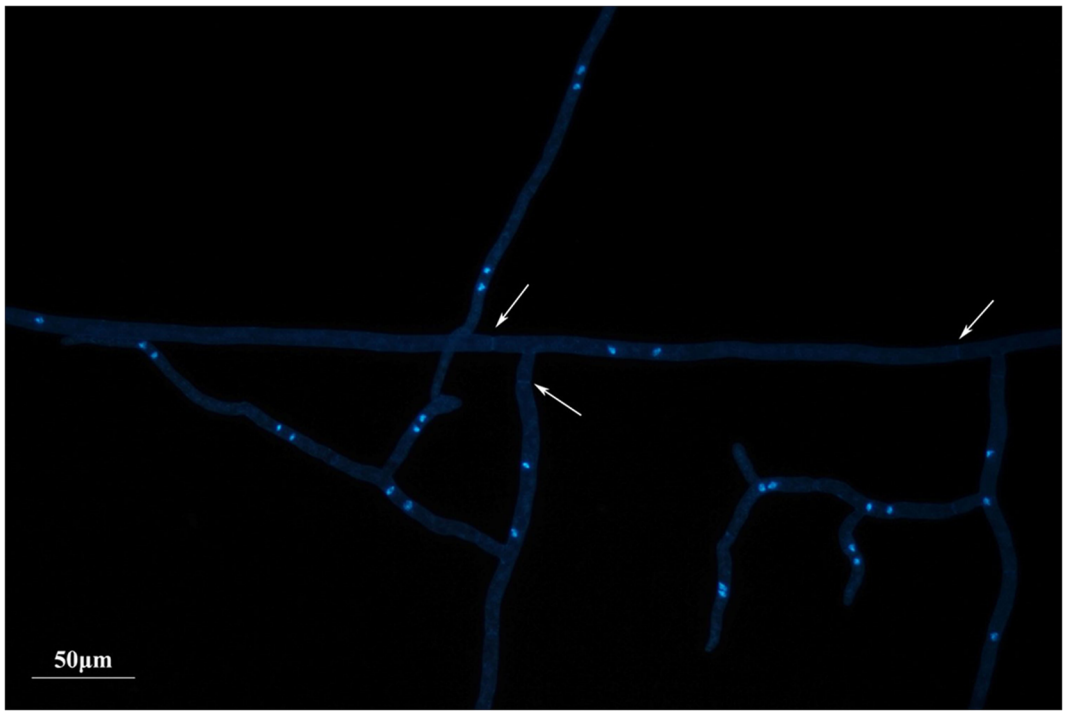

Fig. 2 DAPI-staining of nuclei (scale bar = 50 μm) observed in *Rhizoctonia* hyphae after 1–2 days of incubation in water agar on a microscope slide in a Petri dish kept at 25 °C. Arrows indicate *Rhizoctonia* hyphal septa

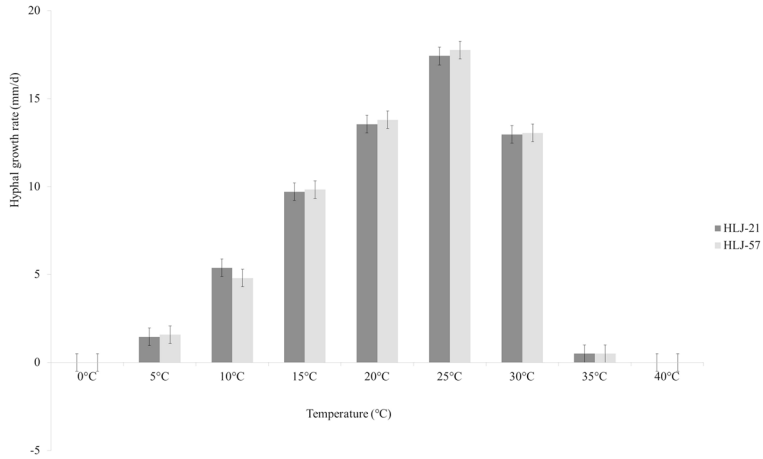

Fig. 3 Effect of temperature on mycelia growth rate of *Rhizoctonia* isolates HLJ-21 and HLJ-57 grown on potato dextrose agar (PDA) in the dark. The bars indicate the standard error

phylogenetic tree according to their AGs (Fig. 5). HLJ-21 and HLJ-57 clustered in the same clade with the AG-W isolates from potato; which were separated from the other reference strains. Collectively, the data indicate that HLJ-21 and HLJ-57 isolates belong to AG-W group of BNR isolates, based on their morphology and their rDNA ITS sequence.

Greenhouse pathogenicity assay

All the control plants remained asymptomatic. For pathogenicity on sugar beet seedlings, the AG-W isolates could be capable of inducing a brown necrosis of the primary shoots in sugar beet seedlings. The disease incidence and disease index resulting from HLJ-21 were 81.2% and 28.9, while those resulting from HLJ-57 were 82.2% and 23.2, respectively (Table 2). The disease incidence and disease index resulting from HL-CZ were 37.1% and 9.7, while those resulting from HL-ZA were 26.3% and 7.5, respectively (Table 2).

All the tested AG-W isolates (HL-CZ, HL-ZA, HLJ-21, and HLJ-57) could cause dry and brown lesions, and dry and brown sunken lesions on the roots of wheat and corn seedlings, respectively. The disease incidence of AG-W isolates on wheat seedlings ranged from 25.0 to 54.8%, and disease index from 7.5 to 15.3, respectively (Table 3). The disease incidence of these isolates on corn seedlings

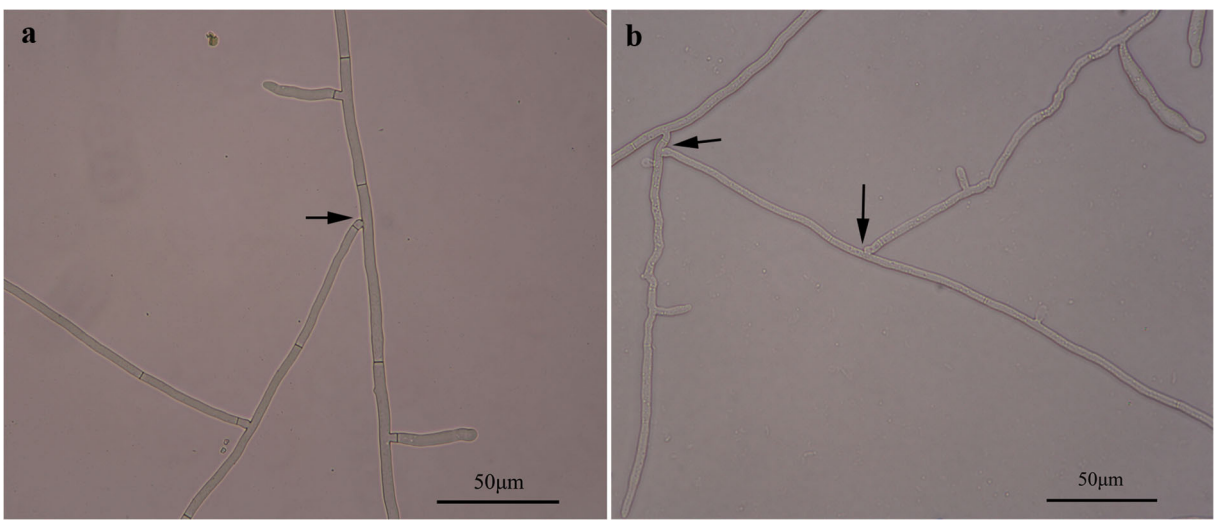

Fig. 4 Hyphal anastomosis reaction of HLJ-21 and HLJ-57 with AG-W *Rhizoctonia* isolates obtained from potato as a C3 reaction (scale bar = 50 μm), A: HLJ-21, B: HLJ-57. Arrows indicate the fused hyphae cell

Table 1 Restriction enzyme cut sites and restriction fragment size (bp) of rDNA ITS PCR products from the reference strains and HLJ-21 and HLJ-57 isolates of binucleate *Rhizoctonia* spp. digested with *Eco*RI, *Hae*III, *Hap*II, *Hha*I, *Hin*fI, *Mbo*I, *Mse*I, and *Mun*I

Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Eco*RI		*Hae*III	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
A(FuK-600)	658	293	293, 365	131, 408, 540	118, 131, 132, 277
Ba(C484)	676	311	311, 365	131, 558, 563	5, 113, 131, 427
Bb(C-350)	718	334	334, 384	61, 599	61, 119, 538
C(Ao-1-2)	653	296	296, 357	61, 538	61, 115, 477
DI(Ayu-WP-1)	686	320	320, 366	77, 164, 568	77, 87, 118, 404
DII(TAK-14KT)	684	313	313, 371	62, 562	62, 122, 500
DIII(KOU04-12FW)	672	315	315, 357	80, 158, 563	78, 80, 109, 405
E(RH-155)	688	315	315, 373	563	125, 563
Fb(AH-6)	677	311	311, 366	62, 557	62, 120, 515
G(AH-9)	656	290	290, 366	61, 537	61, 119, 476
H(STC-11)	655	297	297, 358	61, 539	61, 116, 478
I(AV-2)	655	398	257, 398	62, 540	62, 115, 478
K(55D45)	674	302	302, 372	438, 553	115, 121, 438
L(FKO-2-16)	650	287	287, 363	61, 533	61, 117, 472
O(FKO-6-2)	658	293	293, 365	131, 408, 540	118, 131, 132, 277
P(C-578)	707	335	335, 372	584	123, 584
Q(C-620)	695	331	331, 364	183, 577	118, 183, 394
U(MWR-20)	692	321	321, 371	63, 571	63, 121, 508
W(HL-ZA)	671	304	304, 367	553	118, 553
W(HL-CZ)	671	304	304, 367	553	118, 553
W(HLJ-21)	672	305	305, 367	554	118, 554
W(HLJ-57)	674	307	307, 367	556	118, 556
Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Hap*II		*Hha*I	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
A(FuK-600)	658	NONE	658	331	331, 327
Ba(C484)	676	NONE	676	349	327, 349
Bb(C-350)	718	NONE	718	372, 470	98, 248, 372
C(Ao-1-2)	653	539	114, 539	334	319, 334
DI(Ayu-WP-1)	686	165, 511, 569	58, 117, 165, 346	358	328, 358
DII(TAK-14KT)	684	NONE	684	351	333, 351
DIII(KOU04-12FW)	672	159, 564	108, 159, 405	353	319, 353
E(RH-155)	688	NONE	688	353	335, 353

Table 1 (continued)

Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Eco*RI		*Hae*III	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
Fb(AH-6)	677	NONE	677	349	328, 349
G(AH-9)	656	NONE	656	328	328
H(STC-11)	655	NONE	655	335	320, 335
I(AV-2)	655	541	114, 541	336	319, 336
K(55D45)	674	137	137, 157	340	334, 340
L(FKO-2-16)	650	NONE	650	325	325
O(FKO-6-2)	658	NONE	658	331	327, 331
P(C-578)	707	91	91, 616	373	334, 373
Q(C-620)	695	NONE	695	369, 451	82, 244, 369
U(MWR-20)	692	NONE	692	359	333, 359
W(HL-ZA)	671	NONE	671	342	329, 342
W(HL-CZ)	671	NONE	671	342	329, 342
W(HLJ-21)	672	NONE	672	343	329, 343
W(HLJ-57)	674	NONE	674	345	329, 345
Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Hin*fI		*Mbo*I	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
A(FuK-600)	658	302, 310	8, 302, 348	23, 230, 471, 598, 603	5, 23, 55, 127, 207, 241
Ba(C484)	676	154, 320, 328	8, 154, 166, 348	23, 248, 452, 487, 616, 621	5, 23, 35, 55, 129, 204, 225
Bb(C-350)	718	45, 343, 351	8, 45, 298, 367	23, 271, 528, 658, 663	5, 23, 55, 130, 248, 257
C(Ao-1-2)	653	45, 305, 313	8, 45, 260, 340	23, 233, 471, 593, 598	5, 23, 55, 122, 210, 238
DI(Ayu-WP-1)	686	46, 329, 337, 464, 572	8, 46, 108, 114, 127, 283	23, 257, 497, 626, 631	5, 23, 55, 129, 234, 240
DII(TAK-14KT)	684	142, 322, 330, 458	8, 128, 142, 180, 226	23, 250, 422, 491, 624, 629	5, 23, 55, 69, 133, 172, 227
DIII(KOU04-12FW)	672	46, 324, 332, 459, 503, 567	8, 44, 46, 64, 105, 127, 278	23, 252, 492, 612, 617	5, 23, 55, 120, 229, 240
E(RH-155)	688	324, 332	8, 324, 356	23, 252, 492, 628, 633	5, 23, 55, 136, 229, 240
Fb(AH-6)	677	320, 328	8, 320, 349	23, 248, 487, 617, 622	5, 23, 55, 130, 225, 239
G(AH-9)	656	299, 307	8, 299, 349	23, 227, 468, 596, 601	5, 23, 55, 128, 204, 241

Table 1 (continued)

Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Eco*RI		*Hae*III	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
H(STC-11)	655	306, 314, 439	8, 125, 216, 306	23, 234, 472, 595, 600	5, 23, 55, 123, 211, 238
I(AV-2)	655	307, 315, 440	8, 125, 215, 307	23, 235, 407, 473, 595, 600	5, 23, 55, 66, 122, 172, 212
K(55D45)	674	45, 311, 319	8, 45, 266, 355	23, 239, 482, 614, 619	5, 23, 55, 132, 216, 243
L(FKO-2-16)	650	296, 304	8, 296, 346	23, 224, 464, 590, 595	5, 23, 55, 126, 201, 240
O(FKO-6-2)	658	302, 310	8, 302, 348	23, 230, 471, 598, 603	5, 23, 55, 127, 207, 241
P(C-578)	707	344, 352	8, 344, 355	23, 272, 513, 647, 652	5, 23, 55, 134, 241, 249
Q(C-620)	695	146, 340, 348, 473	8, 125, 146, 194, 222	23, 268, 440, 506, 635, 640	5, 23, 55, 66, 129, 172, 245
U(MWR-20)	692	330, 338	8, 330, 354	23, 258, 499, 632, 637	5, 23, 55, 133, 235, 241
W(HL-ZA)	671	313, 321	8, 313, 350	23, 65, 241, 484, 611, 616	5, 23, 42, 55, 127, 176, 243
W(HL-CZ)	671	313, 321	8, 313, 350	23, 65, 241, 484, 611, 616	5, 23, 42, 55, 127, 176, 243
W(HLJ-21)	672	314, 322	8, 314, 350	23, 66, 242, 485, 612, 617	5, 23, 43, 55, 127, 176, 243
W(HLJ-57)	674	316, 324	8, 316, 350	23, 66, 244, 487, 614, 619	5, 23, 43, 55, 127, 178, 243
Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Mse*I		*Mun*I	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
A(FuK-600)	658	175, 210, 396, 456, 636	22, 35, 60, 175, 180, 186	402	256, 402
Ba(C484)	676	29, 146, 192, 196, 414, 472, 607, 654	4, 22, 29, 46, 47, 58, 117, 135, 218	420	256, 420
Bb(C-350)	718	29, 146, 157, 216, 249, 449, 459, 496, 513, 696	10, 11, 17, 22, 29, 33, 37, 59, 117, 183, 200	NONE	718
C(Ao-1-2)	653	180, 399, 440, 456, 631	16, 22, 41, 175, 180, 219	405	248, 405
DI(Ayu-WP-1)	686	29, 68, 205, 423, 482, 664	22, 29, 39, 59, 137, 182, 218	NONE	686
DII(TAK-14KT)	684	67, 197, 476, 499, 550, 662	22, 23, 51, 67, 112, 130, 279	NONE	684
DIII(KOU04-12FW)	672	29, 71, 200, 477, 650	22, 29, 42, 129, 173, 277	NONE	672
E(RH-155)	688	38, 73, 154, 177, 197, 202, 418, 618, 666	5, 20, 22, 23, 35, 38, 48, 81, 200, 216	424	264, 424
Fb(AH-6)	677	38, 68, 142, 147, 152, 173, 414, 655	5, 21, 22, 30, 38, 74, 241	NONE	677
G(AH-9)	656	132, 175, 393, 453, 573, 587, 593, 634	6, 14, 22, 41, 43, 60, 120, 132, 218	NONE	656

Table 1 (continued)

Anastomosis group	Amplicon size of the rDNA ITS region (bp) [a]	Restriction enzyme			
		*Eco*RI		*Hae*III	
		Cutting site [b]	RFLP patterns [c]	Cutting site	RFLP patterns
H(STC-11)	655	457, 633	22, 176, 457	406	249, 406
I(AV-2)	655	458, 633	22, 175, 458	NONE	655
K(55D45)	674	183, 219, 405, 467, 542, 603, 652	22, 36, 49, 61, 62, 75, 183, 186	NONE	674
L(FKO-2-16)	650	130, 150, 168, 172, 390, 449, 523, 569, 582, 587, 628	4, 5, 13, 18, 20, 22, 41, 46, 59, 74, 130, 218	NONE	650
O(FKO-6-2)	658	175, 210, 396, 456, 636	22, 35, 60, 175, 180, 186	402	256, 402
P(C-578)	707	38, 78, 82, 167, 197, 217, 222, 438, 636, 685	4, 5, 20, 22, 30, 38, 40, 49, 85, 198, 216	444	263, 444
Q(C-620)	695	29, 157, 217, 434, 491, 673	22, 29, 57, 60, 128, 182, 217	NONE	695
U(MWR-20)	692	39, 77, 81, 183, 203, 208, 424, 670	4, 5, 20, 22, 38, 39, 102, 216, 246	430	262, 430
W(HL-ZA)	671	73, 167, 185, 189, 409, 469, 649	4, 18, 22, 60, 73, 94, 180, 220	415	256, 415
W(HL-CZ)	671	73, 167, 185, 189, 409, 469, 649	4, 18, 22, 60, 73, 94, 180, 220	415	256, 415
W(HLJ-21)	672	74, 168, 186, 190, 410, 470, 650	4, 18, 22, 60, 74, 94, 180, 220	416	256, 416
W(HLJ-57)	674	74, 170, 188, 192, 412, 472, 652	4, 15, 22, 60, 74, 96, 180, 220	418	256, 418

[a] Length (bp) of the sequences of rDNA ITS region from the reference and testing strains amplified using ITS1/ITS4 primers

[b] Analysis of restriction enzyme cutting site was performed using Primer premier 5.0

[c] Restriction digestion was carried out overnight using 17 μl of PCR product in a 20 μl reaction mixture according to the enzyme manufacturer's recommendation

Table 2 Pathogenicity of the *Rhizoctonia* AG-W (HLJ-21, HLJ-57, HL-CZ, and HL-ZA) isolates on sugar beet seedlings in the greenhouse conditions

Isolates name	Anastomosis group (AG)	Host for isolating *Rhizoctonia*	Disease incidence (%) [a]	Disease index [a]
CK	–	–	0 d	0 d
HLJ-21	AG-W	Sugar beet	81.2 a	28.9 a
HLJ-57	AG-W	Sugar beet	82.2 a	23.2 b
HL-CZ	AG-W	Potato	37.1 b	9.7 c
HL-ZA	AG-W	Potato	26.3 c	7.5 c

[a] Sugar beet seedlings were scored for disease severity using a 0–7 scale: 0 = no disease, 1 = necrosis of root, with a ≥ 1 cm longitudinal crack, and tissue discoloration at the edge of crack, 3 = <75% necrosis of the root, 5 = 75%–100% necrosis of the root, and 7 = seedling death. The disease incidence and the disease index were calculated as follows: disease incidence =100% × $(n_1 + n_3 + n_5 + n_7)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 3n_3 + 5n_5 + 7n_7)$ / (7 N), where n_0 to n_7 were the number of plants in each degree and N was the total number of inoculated plants

ranged from 9.5 to 56.3%, and disease index from 2.4 to 14.8, respectively (Table 4). The virulence of the AG-W isolates from sugar beet (HLJ-21 and HLJ-57) was significantly higher than those collected from potato (HL-CZ and HL-ZA) on sugar beet, wheat, and corn seedlings (Table 2, Table 3, and Table 4).

Both of the AG-W isolates (HLJ-21 and HLJ-57) collected from sugar beet seedlings could induce stem canker-like symptoms on potato plants. The disease incidence of the AG-W isolates from sugar beet on potato plants was 54.6 and 44.4%, and the disease index was 8.3 and 13.3, respectively (Table 5).

The same AG-W isolates could be correspondingly re-isolated from all the diseased sugar beet, wheat, corn, and potato plants, while no *Rhizoctonia* isolates were re-isolated from the healthy plants of these crops. Their identities were confirmed by both morphological and molecular characteristics as described above; thus fulfilling Koch's postulates.

Discussion

Based on the morphological characteristics, anastomosis reactions, a PCR-RFLP analysis, and a sequence analysis of rDNA ITS, the HLJ-21 and HLJ-57 isolates from sugar beet were identified as belonging to the AG-W group of *Rhizoctonia*. AG-W isolates were first identified in our lab from stem canker samples of potato (Yang et al. 2015a). In the present study, AG-W isolates were obtained from sugar beet seedlings with symptoms of damping off disease, and could cause seedling damping-off of sugar beet, necrotic lesions on roots of wheat and corn, and stem canker of potato. Wheat, corn, and potato are commonly planted in rotation with sugar beets in northeastern China. In addition, other closely related isolates from Australia have been shown to cause

Table 3 Pathogenicity of the *Rhizoctonia* AG-W (HLJ-21, HLJ-57, HL-CZ, and HL-ZA) isolates on wheat seedlings in the greenhouse conditions

Isolates name	Anastomosis group (AG)	Host for isolating *Rhizoctonia*	Disease incidence (%) [a]	Disease index [a]
CK	–	–	0 d	0 c
HLJ-21	AG-W	Sugar beet	54.8 a	15.3 a
HLJ-57	AG-W	Sugar beet	45.2 b	12.9 a
HL-CZ	AG-W	Potato	26.3 c	7.5 b
HL-ZA	AG-W	Potato	25.0 c	8.3 b

[a] The disease rating scale for the wheat seedlings was: 0 = no disease, 1 = <25% necrosis of the roots, 2 = 25%–75% necrosis of the roots, 3 = 75–100% necrosis of the roots, and 4 = seedling death. The disease incidence and the disease index of wheat were calculated as follows: disease incidence =100% × $(n_1 + n_2 + n_3 + n_4)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)$ / (4 N), where n_0 to n_4 were the number of plants in each degree and N was the total number of inoculated plants

Table 4 Pathogenicity of the *Rhizoctonia* AG-W (HLJ-21, HLJ-57, HL-CZ, and HL-ZA) isolates on corn seedlings in the greenhouse conditions

Isolates name	Anastomosis group (AG)	Host for isolating *Rhizoctonia*	Disease incidence (%) [a]	Disease index [a]
CK	–	–	0 e	0 c
HLJ-21	AG-W	Sugar beet	56.3 a	14.8 a
HLJ-57	AG-W	Sugar beet	40.6 b	10.2 a
HL-CZ	AG-W	Potato	9.5 d	2.4 b
HL-ZA	AG-W	Potato	17.6 c	4.4 b

[a] The disease rating scale for corn seedlings was: 0 = no disease, 1 = <25% necrosis of the roots, 2 = 25%–75% necrosis of the roots, 3 = 75–100% necrosis of the roots, and 4 = seedling death. The disease incidence and the disease index of corn were calculated as follows: disease incidence =100% × $(n_1 + n_2 + n_3 + n_4)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)$ / (4 N), where n_0 to n_4 were the number of plants in each degree and N was the total number of inoculated plants

root rot on strawberries (Fang et al. 2013). Therefore, an appropriate crop rotation needs to be used to control diseases caused by the AG-W isolates.

A sequence analysis of the rDNA ITS region has been reported to be the most reliable approach in conducting phylogenetic studies of AGs and subgroups within *Rhizoctonia* spp. (Gonzalez et al. 2001; Sharon et al. 2006). RFLP analysis of PCR products of the rDNA ITS region has also shown that the AGs in *R. solani* are genetically divergent (Vilgalys and Hester 1990). RFLP analysis of the rDNA ITS region can provide quick, reliable, and repeatable results (Liu and Sinclair 1993). Cubeta et al. (1991) used four different restriction enzymes to separate 13 of 21 AGs of binucleate *Rhizoctonia* into distinct groups that were consistent with prior groupings based on hyphal anastomosis. Further RFLP studies by Elbakali et al. (2003) demonstrated that AG-3 isolates from potato could be clearly separated from isolates belonging to other AGs. Hyakumachi et al. (2005) designated two new AGs (AG-T and AG-U) of binucleate *Rhizoctonia* based on a RFLP analysis of rDNA ITS region and hyphal anastomosis. In the present study, PCR-RFLP analysis of the rDNA ITS region of the HLJ-21 and HLJ-57 isolates from sugar beet revealed that they shared the same pattern of restriction fragments as AG-W isolates from potato, which differed from the pattern obtained from other BNR reference isolates. Based on these results, we propose the HLJ-21 and HLJ-57 isolates belong to the AG-W anastomosis group.

All of the tested BNR isolates clustered in different groups in the ML phylogenetic tree and the groupings were consistent with their AGs. The HLJ-21 and HLJ-57 isolates from sugar beet clustered in the same clade as AG-W isolates from potato. Furthermore, eight AG-G isolates from Australia and three unknown *Rhizoctonia* isolates from France, Canada, and the United States were clustered with the AG-W isolates. These isolates may also belong to this new BNR AG. Fang et al. (2013) found that these eight AG-G isolates may indeed be new groups or subgroups for a deep split was observed between the new clade and AG-G isolates. The clade of the AG-W isolates was located within a larger phylogenetic group of AGs that included AG-A and AG-K, it seems that the AG-W isolates has a close relation with AG-A and AG-K. Though AG-A and AG-K isolates have shown to be non-pathogenic on mature sugar beet roots, they could cause damping-off disease of sugar beet seedlings (Strausbaugh et al. 2011a; Wang and Wu 2012; Zhao et al. 2019).

Table 5 Pathogenicity of the *Rhizoctonia* AG-W (HLJ-21, and HLJ-57) isolates on potato in the greenhouse conditions

Isolates name	Anastomosis group (AG)	Host for isolating *Rhizoctonia*	Disease incidence (%) [a]	Disease index [a]
CK	–	–	0 c	0 c
HLJ-21	AG-W	Sugar beet	54.6 a	13.3 a
HLJ-57	AG-W	Sugar beet	44.4 b	8.3 b

[a] The disease rating scale was as follows: 0 = no disease, 1 = less than 10%, 2 = 10–50%, 3 = 50–100%, and 4 = plants dead. The disease incidence and disease index were calculated as follows: disease incidence = 100% × $(n_1 + n_2 + n_3 + n_4)$ / N and disease index = 100 × $(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)$ / (4 N), where n_0 to n_4 were the number of plants in each degree and N was the total number of inoculated plants

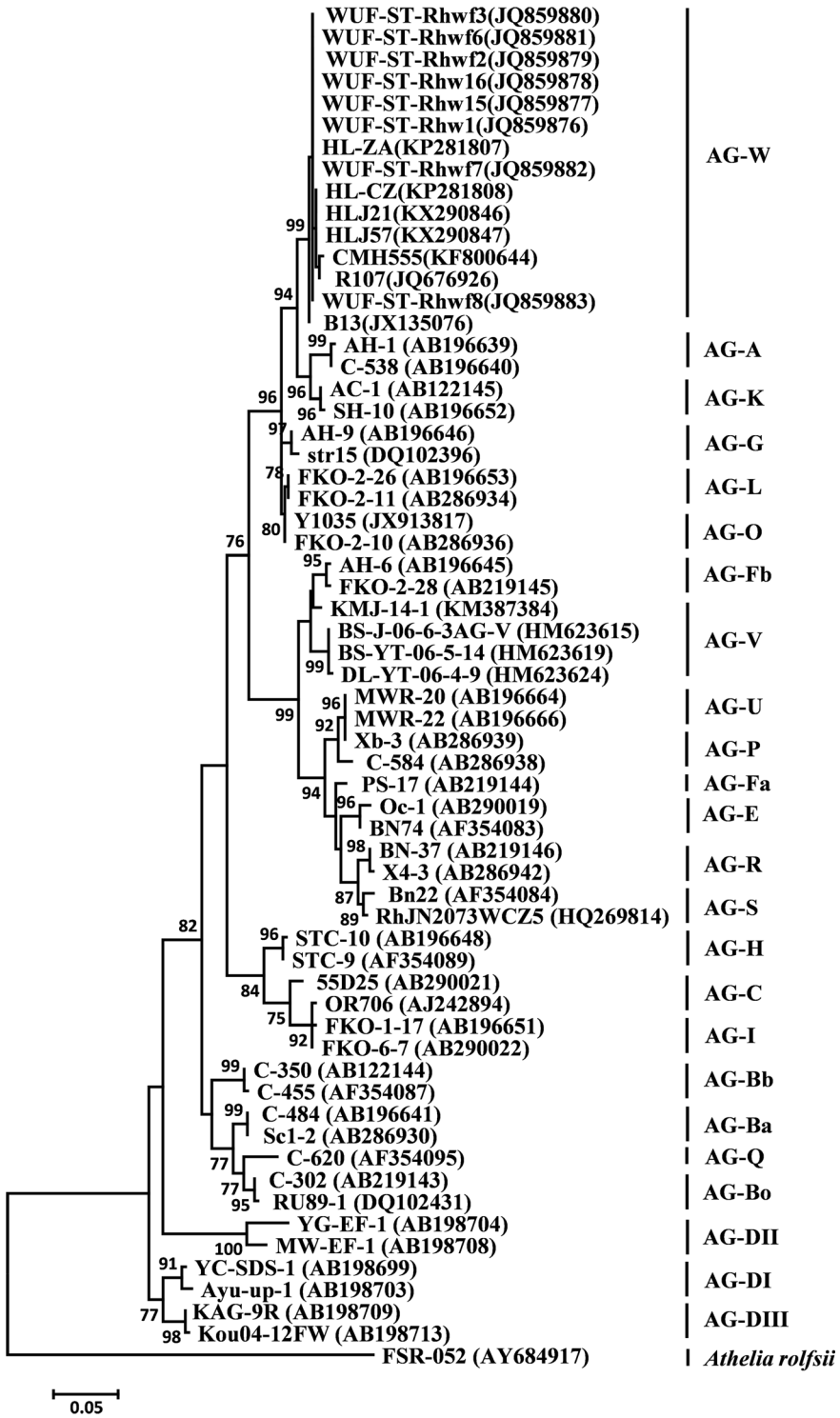

Fig. 5 Phylogenetic tree was constructed using Maximum Likelihood (ML) analysis generated from rDNA ITS sequences of *Rhizoctonia* isolates HLJ-21 and HLJ-57 from this study and those of binucleate *Rhizoctonia* isolates from GenBank. Bootstrap values (1000 replicates) greater than 70 are above the branches. Scale bar represents a genetic distance of 0.05 for horizontal branch lengths. The GenBank accession number for each strain has been provided in the parentheses

Some nonpathogenic and weakly pathogenic BNR isolates have been used as biocontrol agents (Herr 1988; Poromarto et al. 1998; Burns and Benson 2000). More and more BNR isolates, however, have been reported to be pathogenic on a wide variety of crops; including sugar beet (Wang and Wu 2012; Harveson and Bolton 2013; Zhao et al. 2019), potato (Yang et al. 2014; Muzhinji et al. 2015; Yang et al. 2015b), soybean (Ajayi-Oyetunde and Bradley 2017), strawberries (Fang et al. 2013), taro and ginger (Dong et al. 2017). Several new BNR AGs, such as AG-U from Japan, and AG-V and AG-W from China have also been identified in recent years (Hyakumachi et al. 2005; Yang et al. 2015a; Dong et al. 2017).

Observations of increased virulence of BNR have been suggested to be due to several factors, including global environmental change, long-term use of fungicides, and the wider use of susceptible cultivars (Hamada et al. 2011). This raises the question of whether current environmental conditions support the prevalence of specific AGs (Virgen-Calleros et al. 2000). Though AG-W isolates from potato only caused minor disease symptoms on sugar beet, wheat, and corn in the present study, the isolates from sugar beet had a higher virulence on the tested crops than that from potato. Furthermore, a previous study had demonstrated that the Australian isolates presented a moderate to high virulence on strawberries (Fang et al. 2013). Thus, AG-W isolates might have the potential to be more than a minor disease problem on other crops given the right environmental conditions.

The AG-W isolates associated with sugar beet seedling damping-off disease and potato stem canker were obtained in Yi'an County and Kedong County, respectively; which were both located in Qiqihar City of the Heilongjiang Province, Northeastern China. The distance between the two counties is only 60 km and the climate is similar. The growth stage of sugar beet and potato would also be nearly similar when grown in Qiqihar City region, as the temperature ranges from 9 to 28 °C. Our results indicate that the HLJ-21 and HLJ-57 isolates obtained from sugar beet grew well from 10 to 30 °C, with an optimum temperature at 25 °C. These are the same temperature parameters as the AG-W isolates from potato (Yang et al. 2015a). Thus, it appears that the climate in Qiqihar city may be especially well-suited for AG-W isolates. However, factors other than climate such as soil characteristics and rotation crops could also impact the dominance of AG-W isolates.

Potato and sugar beet are the main crops in Heilongjiang Province, and a rotation of potato with sugar beet is common. *R. solani* AG-2, AG-3, AG-4, and AG-5 have been demonstrated to be pathogens of potato in Heilongjiang Province, Northeastern China (Yang et al. 2015c; Yang et al. 2017). These four AGs also contribute to damping-off disease in sugar beet seedlings (Windels and Nabben 1989; O'sullivan and Kavanagh 1991; Soltaninezhad et al. 2008; Bolton et al. 2010; Hanson and McGrath 2010; Hanson and McGrath 2011; Stojšin et al. 2011; Zhao et al. 2019). The pathogenicity test results also suggest that the potato-sugar beet rotation may also be contributing to the presence of AG-W in Heilongjiang Province.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal studies This article does not contain any studies with human or animal subjects.

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