



## Stem Rot on Adzuki Bean (*Vigna angularis*) Caused by *Rhizoctonia solani* AG 4 HGI in China

Suli Sun<sup>1†</sup>, Changjian Xia<sup>1†</sup>, Jiqing Zhang<sup>1</sup>, Canxing Duan<sup>1</sup>, Xiaoming Wang<sup>1</sup>, Xiaofei Wu<sup>1</sup>, Suk-Ha Lee<sup>2</sup> and Zhendong Zhu<sup>1\*</sup>

<sup>1</sup>The National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing 100081, People's Republic of China

<sup>2</sup>Department of Plant Science and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea

(Received on July 16, 2014; Revised on October 8, 2014; Accepted on November 2, 2014)

**During late August and early September 2011, stem rot symptoms were observed on adzuki bean plants (*Vigna angularis*) growing in fields located in Beijing and Hebei Province, China, respectively. In this study, four isolates were obtained from infected stems of adzuki bean plants. Based on their morphology, and sequence and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses of the ribosomal DNA internal transcribed spacers (rDNA-ITS) region, the four isolates were identified as *Rhizoctonia solani* in anastomosis group (AG) 4 HGI. Pathogenicity tests showed that all isolates were strongly pathogenic to adzuki bean and resulted in serious wilt symptoms which was similar to observations in the fields. Additionally, the isolates infected several other crops and induced related rot on the roots and basal stems. To our knowledge, this is the first report of *Rhizoctonia solani* AG 4 HGI causing stem rot on adzuki bean.**

**Keywords :** adzuki bean, anastomosis group, PCR-RFLP, *Rhizoctonia solani*, stem rot

Adzuki bean (*Vigna angularis*), an important edible legume crop, has attracted more and more attention from scientists and consumers because of its high nutritional and medicinal value. As of 2011, China had the largest planting area at 250,000 ha and the greatest yield of approximately 300,000 t of adzuki bean (Wang et al., 2013). However, one of the main constraints on adzuki bean production in

China in recent years has been the occurrence of diseases in the fields (Wang et al., 2000).

During late August and early September 2011, many wilting plants were observed in fields of adzuki bean in Beijing and Hebei Province, China, respectively. The wilting plants had girdled stem rot on the lower stems. Initially, the infected stems showed water-soaked lesions at the infection point (Fig. 1a); however, the lesions rapidly expanded up and down. The stem became girdled, dry and discolored as the disease developed (Fig. 1b). Finally, the infection caused withering and death of plants (Fig. 1c, 1d). Similar symptoms had been recorded on adzuki bean in China previously. At that time *Rhizoctonia solani* was thought to be the causal agent of the disease, but it was not confirmed (Wang et al., 2000). Therefore, this study was designed to identify and characterize the causal pathogen of stem rot on adzuki bean plants based on colony morphology combined with molecular techniques, including sequence and PCR-RFLP analyses of the rDNA-ITS region. Additionally, we determined the pathogenicity of the causal agent to several crops, including adzuki bean.

Adzuki bean plants showing typical symptoms of stem rot were collected from fields located in Beijing and Hebei Province, China, during the 2011 growing season. The causal pathogen was obtained from infected stems using the tissue isolation method. Briefly, advancing lesions of infected stems were cut into small sections (3 mm × 3 mm). The small pieces of stem tissue were surface-disinfected in 70% ethanol for 30 s, 1% sodium hypochlorite for 5 min, rinsed three times in sterile distilled water, and then placed on 2% water agar acidified with lactic acid (pH 4.5). The resulting colonies were examined for typical *Rhizoctonia* growth after incubation for 1 to 2 days at 25°C with a 12-h light/dark regime. Hyphal tips excised from branches were transferred to potato-dextrose agar (PDA; AoBoXing, Bio-

<sup>†</sup>These authors contributed equally to this work.

\*Corresponding author.

Phone) +86-10-8210-9609, FAX) +86-10-8210-9608  
E-mail) zhuzhendong@caas.cn

tech, Beijing, China) acidified with lactic acid and supplemented with 50 mg/l streptomycin sulfate to inhibit bacterial growth (Sigma-Aldrich, St. Louis, MO, USA). All the plates were incubated at 25°C for 3 to 4 days. The obtained isolates were stored on PDA slants at 4°C for further use.

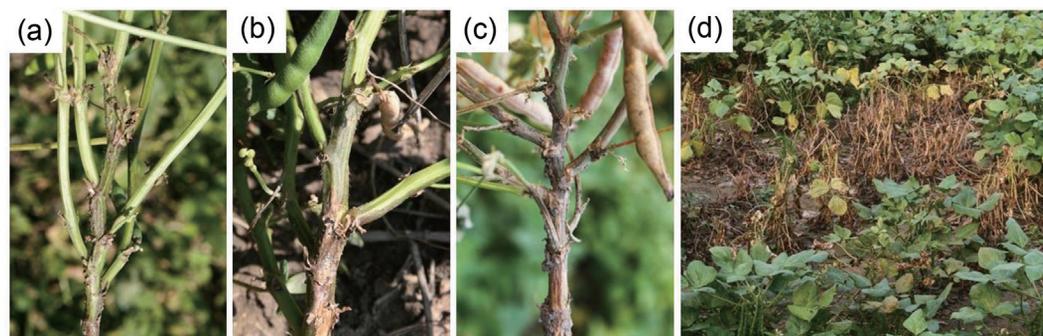
Four isolates, XDR1 and XDR2 from Hebei Province, and XDR3 and XDR4 from Beijing, were selected to determine their identities. Young hyphae of the isolates were stained with 0.03% safranin-O and 3% KOH aqueous solution according to the protocol of Bandoni (1979). The numbers of nuclei in the stained hyphae were examined using an Olympus CX31 microscope at 400× to determine binucleate or multinucleate status. The four isolates were further characterized by sequence and PCR–RFLP analyses of the rDNA-ITS region following the procedures described by Guillemaut et al. (2003) and Pannecouque et al. (2008). Briefly, for the sequence analysis of the rDNA-ITS region, DNA of the four isolates was extracted and amplified with universal primers ITS1/ITS4 (White et al., 1990). The PCR products were further analyzed by sequencing the ITS region. The obtained sequences were blasted against the GenBank (National Center for Biotechnology Information, NCBI) database using Blastn. For the PCR–RFLP analysis of the rDNA-ITS region, genomic DNA from each of the four isolates was amplified with primers RS1/RS4, and the resulting PCR products were further analyzed by RFLP using four restriction enzymes, *MunI*, *MseI*, *AvaII* and *HincII* (Guillemaut et al., 2003).

The pathogenicity of four *R. solani* isolates was tested. Because *R. solani* has a wide range of hosts, the pathogenicity test was performed not only on the original host, adzuki bean (cv. Baihong 1 and line BH06-169-6), but also on other crops, including mung bean (*Vigna radiata*), common bean (*Phaseolus vulgaris*), cowpea (*V. unguiculata*), cotton (*Anemone vitifolia*), towel gourd (*Luffa cylindrica*), maize (*Zea mays*) and wheat (*Triticum aestivum*). All of

the tested crops were sown into 250-ml paper cups (five disinfected seeds/cup) filled with sand that had been sterilized by autoclaving at 121°C for 1 h. The planted cups were randomly distributed on a greenhouse bench under a temperature of 25±2°C. Inoculums of the four isolates were cultured in potato-dextrose broth (PDB) (100 ml in 250-ml flasks) with constant shaking at 150 rpm for 7 days at 26°C. The resulting cultures were homogenized for 30 s in a Waring blender, and the concentration of the mycelial suspension was adjusted to approximately 1.0×10<sup>6</sup> cfu/ml. Then, 10-day-old seedlings were inoculated by pouring the mycelial suspension around the base of the plants (5 ml/plant). A negative control was treated in the same manner using sterile PDB. Eight days after inoculation, the disease severity of the seedlings was scored using a scale range described by Li (1991). The above experiments were performed twice with three replicates.

Four isolates (XDR1 and XDR2 from Hebei Province, and XDR3 and XDR4 from Beijing) were obtained from stems of adzuki bean plants exhibiting stem rot symptoms. The four isolates grew rapidly on PDA plates, producing 2.5 cm and 7.0 cm diameter colonies at 1 and 2 days after inoculation, respectively. After 3 days of incubation, the colonies overgrew the 9.0-cm diameter Petri-dishes. The colonies of isolates growing on PDA were whitish initially and turned brown to dark brown after 4 days (Supplementary Fig. 1a). Brown sclerotia were observed on the lids of Petri-dishes at 9 days after inoculation. Hyphae of isolates presented rectangular or acute branches with constricted bases (Supplementary Fig. 1b). Nuclear staining of hyphae using safranin-O revealed that the isolates were multinucleate (Supplementary Fig. 1c). All morphological characteristics and staining reactions indicated that the four isolates belonged to the multinucleate species *R. solani*.

For further confirmation, sequence analyses of the four isolates' rDNA-ITS regions were performed. The obtained

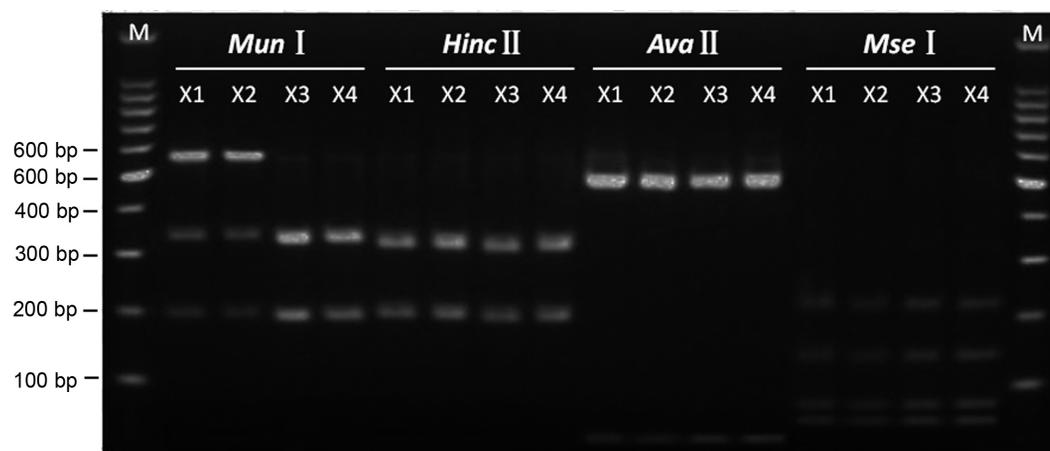


**Fig. 1.** Stem rot symptoms caused by *Rhizoctonia solani* AG 4 HGI observed on adzuki bean plants in the field. (a) Early symptoms on an infected stem; (b) Developing symptoms on an infected stem; (c) Late symptoms on an infected stem with sclerotial formation; (d) Lots of seriously withered plants.

sequences from the four isolates, XDR1, XDR2, XDR3 and XDR4, were submitted to the NCBI database and accession numbers (Nos) KC405625, KC405626, KC405627 and KC405628, respectively, were assigned. A Blastn analysis in GenBank of each obtained sequence showed that the four isolates were highly homologous with many *R. solani* strains and the identified subgroups of *R. solani* AG 4 HGI isolates. Our four isolates, XDR1, XDR2, XDR3 and XDR4, showed the higher similarities (95 to 99%) with reported *R. solani* AG 4 HGI isolates from *Cynanchum paniculatum* (NCBI GenBank accession No. JQ343830) and *Vincetoxicum pycnostelma* (JQ669933) in China; from unidentified host (AB000007) in Japan; from *Phaseolus vulgaris* (HE805679) in Turkey; and from stem rot of tomato (AY154307) in Brazil (Kuramae et al., 2003). Moreover, the homology between the two isolates (XDR1 and XDR2, or XDR3 and XDR4) from the same location was higher than between isolates from different locations. To confirm the subgroups of the four *R. solani* isolates, the approximately 540 bp PCR products from the rDNA-ITS regions were digested with the restriction endonuclease enzymes, *MunI*, *HincII*, *AvaII* and *MseI*. The RFLP patterns were observed on an agarose gel (Fig. 2). The amplification products of the rDNA-ITS regions obtained using RS1/4 had only one accessible *MunI*, *HincII* and *AvaII* restriction sites, but *MseI* had multiple accessible recognition sites. The RFLP analysis of the PCR products from the Beijing isolates XDR3 and XDR4 showed two bands of approximately 340 bp and 200 bp when digested by *MunI*. However, the PCR amplification products of the Hebei Province isolates XDR1 and XDR2 showed additional bands when digested by *MunI* (Fig. 2). This result was consistent with the previous observations of sequence heterogeneity (Pan-

necoucq et al., 2008; Pannecoucq and Höfte, 2009; Strausbaugh et al., 2011). PCR products were digested by *HincII* and *AvaII* into two bands of approximately 330 bp and 210 bp, and 490 bp and 50 bp, respectively (Guillemaut et al., 2003; Pannecoucq et al., 2008). Digestion by *MseI* at several recognition sites resulted in multiple smaller fragments. They appear to be approximately 220 bp, 140 bp, 80 bp and 60 bp bands in agarose gels (Fig. 2). It is possible that smaller fragments were not resolved in the agarose gel based on the total length of the PCR products. Thus, bands of less than 40 bp were not taken into consideration, which is in agreement with a previous study (Guillemaut et al., 2003). Based on the size of the restriction fragments, RFLP types generated by the digestion of four enzymes, *MseI*, *AvaII*, *MunI* and *HincII*, are I, E, A, and A, respectively, and these RFLP types are specific to *R. solani* AG 4 HGI according to the classes of restriction patterns described by Guillemaut et al. (2003). Therefore, the RFLP analysis also classified the four isolates into AG 4 HGI subgroups. The sequence and PCR-RFLP analyses of the rDNA-ITS region provided further support for the isolates belonging to *R. solani* AG 4 HGI.

The pathogenicity tests of the four isolates were performed on 10-day-old seedlings of adzuki bean and 7 other crops by inoculating the basal stems. The four isolates induced rot symptoms on roots and basal stems not only on the original host, adzuki bean, but also on most of the other crops tested (Supplementary Figs. 2 and 3). No significant differences in pathogenicity were observed on the adzuki bean among the four *R. solani* AG 4 HGI isolates. All of the diseased plants showed similar symptoms on the basal stems with observations in the fields 8 days after inoculation. Lastly, the four isolates resulted in serious wilt



**Fig. 2.** PCR-RFLP patterns of the rDNA-ITS region of four fungal isolates, XDR1, XDR2, XDR3 and XDR4, digested with *MunI*, *HincII*, *AvaII* and *MseI* restriction endonucleases and resolved in a 1.5% agarose gel. XDR1 and XDR2 were isolated in Hebei Province, and XDR3 and XDR4 were isolated in Beijing. (M: DNA markers; X1: XDR1; X2: XDR2; X3: XDR3; X4: XDR4).

symptoms due to basal stem rot and root rot on adzuki bean and additional tested crops, including mung bean, common bean, cowpea, towel gourd and cotton (Supplementary Figs. 2 and 3). However, the tested cereal crops, wheat and corn, showed mild symptoms (Supplementary Figs. 2 and 3). The four isolates were also re-isolated from respective symptomatic lesion regions in all tested crops, in accordance with Koch's postulates. Neither symptoms appeared nor pathogens were obtained from the control plants. Our results indicate that the isolates have the potential to cause damage not only to beans but also to other crops.

The correct identification of the causal pathogen is critical to the appropriate selection of isolates for resistance-screening programs and disease control, as well as for intercropping or crop rotation strategies (Fenille et al., 2002). In the present study, the causal pathogen of stem rot of adzuki bean was confirmed as *R. solani* AG 4 HGI by combining colony morphology and molecular identification techniques. The latter includes sequence and PCR-RFLP analyses of the rDNA-ITS region, which have been considered the criteria to identify *R. solani* at the AG and subgroup levels (Boysen et al., 1996; Guillemaut et al., 2003; Pannecouque et al., 2008).

In the PCR-RFLP analysis, it is interesting that variation in PCR-RFLP patterns was observed using the restriction enzymes *MunI* among the four isolates of *R. solani* AG 4 HGI. Two different digestion patterns appeared in the agarose gel (Fig. 2). This result revealed that sequence heterogeneity exists within the isolates. This phenomenon of heterogeneous digestion patterns in rDNA-ITS region of *R. solani* isolates has been observed in recent studies by other researchers (Pannecouque et al., 2008; Pannecouque and Höfte, 2009; Strausbaugh et al., 2011). In addition, the results of direct sequencing revealed that homology within isolates from the same geographical region, XDR1 and XDR2 or XDR3 and XDR4, is higher than between two isolates from different regions. This corroborates results on the genetic structure of populations of *R. solani* AG 4 (Haratian et al., 2013).

In previous studies, *R. solani* AG 4 HGI was reported as a main pathogen causing major damping-off, and root and hypocotyl rot in beans (Nerey et al., 2010). In China, the pathogen *R. solani* AG 4 HGI was observed to cause the head rot of cabbage (*Brassica oleracea* var. *capitata*) and the web blight of the snap bean (*Phaseolus vulgaris*) (Yang et al., 2007). Additionally, *R. solani* AG 4 HGI and the other two subgroups, HGII and HGIII, can cause stem rot or stem canker on tomato, castor bean, green amaranth and Chinese amaranth (Basseto et al., 2008; Kuramae et al., 2003; Yang et al., 2005). Our results and previous stud-

ies show that *R. solani* AG 4 HGI can cause various types of diseases in a wide range of hosts. Thus, to reduce *Rhizoctonia* stem rot on adzuki bean, crop rotational strategies should be given further consideration.

Recently, hypocotyl rot of adzuki bean caused by species *R. solani* AG-1 IB was observed in Japanese fields (Misawa and Komatsu, 2011). To the best of our knowledge, there are no reports to date describing *R. solani* AG 4 HGI occurring on adzuki bean. Thus, this is the first report of *R. solani* AG 4 HGI infecting adzuki bean and causing stem rot. Our results indicated that stem rot caused by *R. solani* AG 4 HGI is a new threat to adzuki bean production and adzuki bean might be acting as a reservoir and spreading this pathogen to other economically important crops in China.

### Acknowledgment

This research was supported by a grant from the China Agriculture Research System (CARS-09).

### References

- Bandoni, R. J. 1979. Safranin O as a rapid nuclear stain for fungi. *Mycologia* 71:873–874.
- Basseto, M. A. 2008. First report of *Rhizoctonia solani* AG 4 HG-II attacking castor bean plants (*Ricinus communis*) in Brazil and evaluation of two castor bean cultivars for resistance to damping-off. *Austral. Plant Dis. Notes* 3:121–123.
- Boysen, M., Borja, M., Del Salazar, M. C. and Rubio, V. O. 1996. Identification at strain level of *Rhizoctonia solani* AG 4 isolates by direct sequence of asymmetric of the ITS regions. *Curr. Genet.* 29:174–181.
- Fenille, R. C., de Souza, N. L. and Kuramae, E. E. 2002. Characterization of *Rhizoctonia solani* associated with soybean in Brazil. *Eur. J. Plant Pathol.* 108:783–792.
- Guillemaut, C., Edel-Herman, V., Comporota, P., Alabouvette, C., Richard-Molard, M. and Steinberg, C. 2003. Typing of anastomosis groups of *Rhizoctonia solani* by restriction analysis of ribosomal DNA. *Can. J. Microbiol.* 49:556–568.
- Haratiana, M., Safaiea, N., Sharifnabib, B., Mahmudic, S. B. and Arianab, A. 2013. Genetic structure of populations of *Rhizoctonia solani* AG-4 from five provinces in Iran. *Plant Pathol.* 62:649–656.
- Kuramae, E. E., Buzeto, A. L., Ciampi, M. B. and Souza, N. L. 2003. Identification of *Rhizoctonia solani* AG 1-IB in lettuce, AG 4 HG-I in tomato and melon, and AG 4 HG-III in broccoli and spinach, in Brazil. *Eur. J. Plant Pathol.* 109:391–395.
- Li, Y. L. 1991. *Rhizoctonia* root rot of mungbean. In: Methods used in the evaluation of pest resistant potentialities in food crop germplasm resources eds. by Q. A. Wu, K. G. Liang and J. Cao, pp. 58–59. Beijing: China Agriculture Press, Led. (*in*

- Chinese).
- Misawa, T. and Komatsu, T. 2011. Occurrence of *Rhizoctonia* root rot of soybean caused by *Rhizoctonia solani* AG-2-2IV and adzuki bean caused by *R. solani* AG-1 IB in commercial fields of Hokkaido. *Annu. Rep. Soc. Plant Prot. North Jpn.* 62:50–54.
- Nerey, Y., Beneden, S. V., França, S. C., Jimenez, A., Cupull, R., Herrera, L. and Höfte, M. 2010. Influence of soil type and indigenous pathogenic fungi on bean hypocotyls rot caused by *Rhizoctonia solani* AG 4 HGI in Cuba. *Soil Biol. Biochem.* 42:797–803.
- Pannecoucq, J. and Höfte, M. 2009. Detection of rDNA ITS polymorphism in *Rhizoctonia solani* AG 2-1 isolates. *Mycologia* 101:26–33.
- Pannecoucq, J., Van Beneden, S. and Höfte, M. 2008. Characterization and pathogenicity of *Rhizoctonia* isolates associated with cauliflower in Belgium. *Plant Pathol.* 57:737–746.
- Strausbaugh, C. A., Eujayl, I. A., Panella, L. W. and Hanson, L. E. 2011. Virulence, distribution and diversity of *Rhizoctonia solani* from sugar beet in Idaho and Oregon. *Can. J. Plant Pathol.* 33:210–226.
- Wang, L. X., Cheng, X. Z. and Wang, S. H. 2013. Review on genetic study and application of adzuki bean (*Vigna angularis*) germplasm. *J. Plant Genet. Resour.* 14:440–447.
- Wang, X. M., Jin, D. S., Redden, R., Wang, S. M., Lawrence, P., Zong, X. X., Hu, J. P. and Cao, Y. S. 2000. Adzuki bean diseases and insect pests. Beijing: China Agricultural Science and Technique Press, Led. (in Chinese).
- White, T. J., Bruns, T., Lee, S. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications eds. by M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, pp. 315–322. New York: Academic Press, Inc.
- Yang, G. H., Chen, J. Y. and Pu, W. Q. 2007. First report of head rot of cabbage and web blight of snap bean caused by *Rhizoctonia solani* AG-4 HGI. *Plant Pathol.* 56:351.
- Yang, G. H., Chen, H. R., Naito, S. and Ogoshi, A. 2005. Characterization of *Rhizoctonia solani* AG 4 HG-III causing stem canker and wirestem on green amaranth and Chinese amaranth. *J. Phytopathol.* 153:185–187.
- Zhu, Z. D. and Wang, X. M. 2003. Identification of pathogen causing *Phytophthora* stem rot of adzuki bean and screening for resistant germplasm. *ACTA Phytophylacica Sinica* 30:289–294.