

Full Length Research Paper

The research of infection process and biological characteristics of *Rhizoctonia solani* AG-1 IB on soybean

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The isolate *Rhizoctonia solani* AG-1 IB collected from the diseased leaves of soybean were identified by the method of internal transcribed spacer sequence analysis. In this study, we focus on the biological characteristics and infection process of AG-1IB. Morphological, nucleus, chromosome, and infection process were observed. Typical infectious structures as infection cushion and appressorium were observed during the infection process.

Key words: *Rhizoctonia solani* AG-1 IB, soybean, biological characteristics, infection process.

INTRODUCTION

Rhizoctonia solani, a basidiomycete fungus, was divided into 12 anastomosis groups (AG1-11 and BI) according to hyphal anastomosis behavior, cultural morphology, host range, pathogenicity and so on (Ogoshi, 1987). Among these groups, isolates of AG-1 have been recovered from many hosts (Huang et al., 2003). Furthermore, AG-1 has been subdivided into three subgroups designated as AG-1IA, AG-1IB and AG-1IC (Ogoshi, 1987; Sneh et al., 1991). The common symptoms of *Rhizoctonia* disease are referred to as damping-off, sheath blight, sheath spot, leaf blight and rot (Duan et al., 2008; Grosch et al., 2003; Takeshi et al., 1998; Yang et al., 2005). *R. solani* AG-1 IB is a widely existing fungus with great harm to many plants.

In Xishuangbanna District of China, *R. solani* isolates collected from the diseased leaves of Chinese cabbage, mint and lettuce were identified to belong to anastomosis group AG-1 IB (Sneh et al., 1991). Web-blight disease of European pear in Okayama prefecture is caused by *R. solani* AG-1, especially IB (Kuramae et al., 2003). However, these studies are only in the level of identified pathogens were *R. solani* AG-1 IB. *R. solani* can cause damping off, root rot, and hypocotyl lesions on soybeans in the United States as well as web blight in the southern United States (Yang, 1999) and *R. solani* rot is a major

disease of soybean (Sinclair and Dhingra, 1975; Yang, 1999). To facilitate breeding of resistant cultivars, it is important to understand the infection process. So we focused on biological characteristics and infection process.

MATERIALS AND METHODS

Pathogen isolation

R. solani isolates were collected from the diseased leaves of soybean. Specimens were rinsed gently in tap water, then cut into small pieces (2 to 5 mm), washed 3 times in sterile distilled water and blotted dry on sterile paper towels. Pieces were placed on 5.0% water agar (WA) and incubated at 28°C for 1 or 2 days. Emerging hyphal tips were transferred to plates of potato dextrose agar (PDA, 200 g of potato, 20 g of dextrose and 20 g of agar) and pure cultures were transferred to PDA slants for storage at 4°C until use (Zhou and Yang, 1998).

Pathogen identification

Mycelium for DNA extraction was grown by inoculating 50 ml PDB in 250-ml conical flasks with mycelia fragments. Cultures were incubated on an orbital shaker (28°C) for 2 to 3 days, depending on the growth rate of the isolate. The culture products were washed twice with sterile distilled water, then dried with filter paper and stored at -20°C for use. The culture products were then ground in liquid nitrogen and total genomic DNA extracted from isolates followed the method of CTAB (Wu et al., 2009). PCR were

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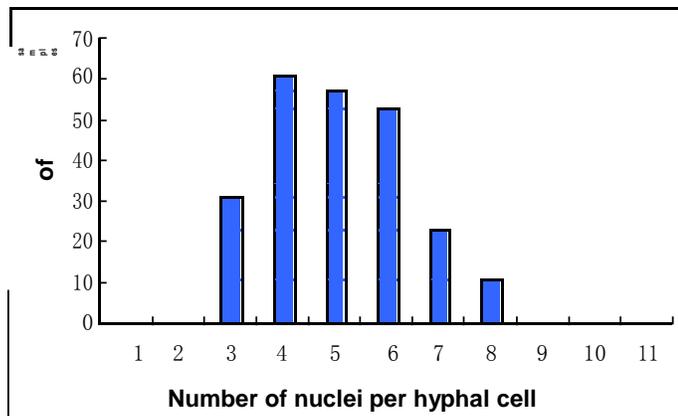


Figure 1. Statistics of nuclei number.

performed in a final mixture of 50 μ l containing 100 ng template DNA, 5 μ l 10 \times reaction buffer (with 1.5 mmol/L Mg^{2+}), 0.2 mmol/L dNTP mixture, 0.2 μ mol/L of each primer (ITS1-F : 5'-CTTGGTCATTTAGAGGAAGTAA-3'; ITS4: 5'-TCCTCCGCTTATTGATAGC-3') (Chen et al., 2010; Takeshi et al., 1998), and 1 Unit of Taq DNA polymerase with the thermal profile: 2 min at 95°C, initial denaturation cycle; 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, 30 cycles; 5 min at 72°C, 1 cycle. PCR products were sent to Premier Scientific Partner for sequence analysis, and sequences were compared on NCBI (<http://www.ncbi.nlm.nih.gov/>).

Morphological characterization

A 6-mm mycelial disc from a 3-day-old PDA culture of IB was placed in the middle of PDA Petri dishes and the Petri dishes were incubated at 28°C. The diameter of colony was measured and colony morphology was observed every 24 h. Sterile slides were inserted into the plates of PDA which were inoculated. The Petri dishes were incubated at 28°C until mycelium covered half of the slides. A drop of lactophenol cotton blue was added on slide, mycelial were examined microscopically 5 min later.

Nucleus and chromosome observations

Slides which were covered with mycelium were fixed in 1:3 glacial acetic acid: ethyl alcohol for 3 to 5 h, and transferred to 75 and 95% ethyl alcohol 30 min respectively. Then slides were kept in 4 mg/ml lysozyme 1 to 3 h (28°C). Phenol magenta was added on slides after slides were rinsed with distilled water. DAPI was used to count the number of nucleus, by adding stain on slides which were covered with mycelium (Coleman et al., 1981).

Infection process observations

A 6-mm mycelial disc from a 2-day-old PDA culture of IB was placed on leaves of soybean, cultured in a high humidity environment at 28°C. Culturing started at 4 h, and was observed and sampled each hour until 12 h. Then it was further observed and sampled at 16, 20, 24, 36, 48, 72, 96, and 130 h. Half of the samples were fixed and discolored in 1:3 glacial acetic acid: ethyl alcohol for 24 h. Trypan blue was added on samples to observed infectious structures by optical microscope (Xie, 2008). The other samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.2, overnight.

Subsequently, the samples were rinsed with phosphate buffer and gradually dehydrated in a series of ascending concentrations of ethyl alcohol. This sample was replaced with isoamyl acetate and critical point drying, then coated with gold particles and observed by scanning electron microscope (Takuya et al., 2008).

RESULTS

Pathogen identification

The internal transcribed spacer sequence was amplified using PCR. Agarose gel showing internal transcribed spacer sequence length is about 750 bp, which is in accord with except length (Figure 1). Sequences were compared with standard on NCBI, the coincidence rate is 100%. So pathogen was identified to belong to anastomosis group AG-1 IB.

Morphological characteristics

The vegetative mycelium of *R. solani* AG-1 IB is colorless when young, but become brown as they grow (Figure 2A and B). Aerial mycelium is undeveloped. Mycelium forms a white group, and gradually becomes a brown sclerotium, which are scattered on the plate (Figure 2C and D). The surface of sclerotia will appear with some drops in a high humidity environment (Figure 2E). The hyphae often branch less than a 90° angles (Figure 3). The mycelium consists of hyphae partitioned into individual cells by a septum, and hyphal fusion can be seen normally (Figure 4). The growth rate of mycelium is 1.11 mm/h.

Nucleus and chromosome characteristics

We census the number of nuclei per cell by the use of DAPI (Figure 5). Mycelia possess more than 2 and less than 11 nuclei, and usually possess between 4 and 6 nuclei per hyphal cell.

Infection process observations

The infection process of *R. solani* AG-1 IB on soybean includes pre-infection, penetration of epidermal cell, spreading in the codex, colonization and showing of symptoms. Hyphae begin to grow to leaves at 4 h after inoculation. 6 h later, tips of hyphae begin to swell (Figure 6). Infection cushion and appressorium are formed at 8 to 10 h after inoculation (Figure 7A and B), and then penetrate host. A layer of mycelium tile in the leaves can be observed at 12 h (Figure 8). Either the hyphae at the base of infection or the infection hyphae developing from the appressorium penetrated host cuticle directly or through stomata (Figure 9A, B and C) hyphae, spread

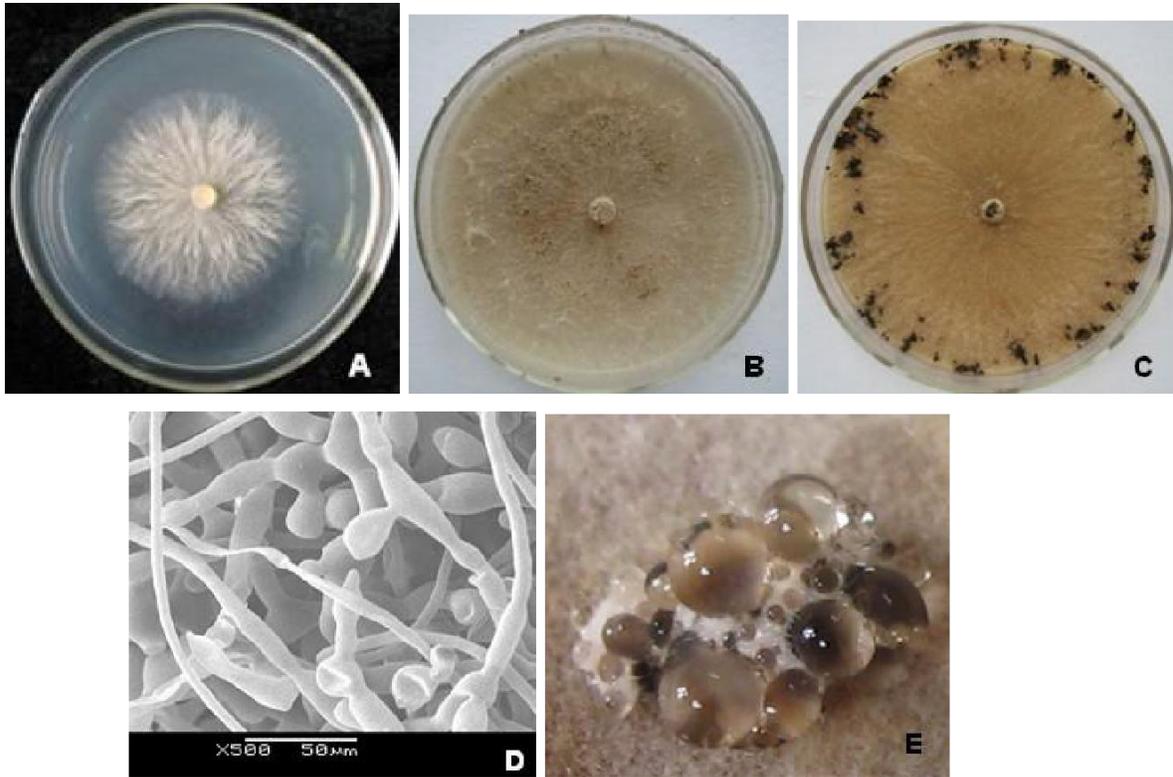


Figure 2. A) Young vegetative mycelium (48 h); B) Matured vegetative mycelium(96 h); C) Sclerotium scattered on the plate (7 days); D) The surface of sclerotia; E) Appearance of much drops on the surface of sclerotia.



Figure 3. Branch less than a 90° angles and septums.

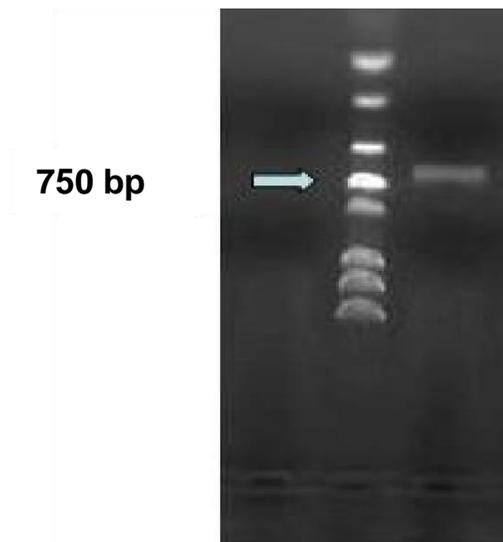


Figure 4. Agarose gel showing PCR amplified product.

thought plant cells (Figure 10), and leaves show symptoms 24 h after inoculation. As the disease progresses, the fungus causes lesions on leaves. Small oval or circular, greenish-gray spots appear on leaves. The spots soon enlarge, with irregular dark brown margins and bleached to grayish white centers or light green

centers (Figure 11). On high humidity and high temperature conditions, diseased organization will rot. Four days later, white sclerotia appear and become brown after maturity (Figure 12).



Figure 5. Hyphal fusion and nuclei.



Figure 6. Tips of hyphae begin to swell.

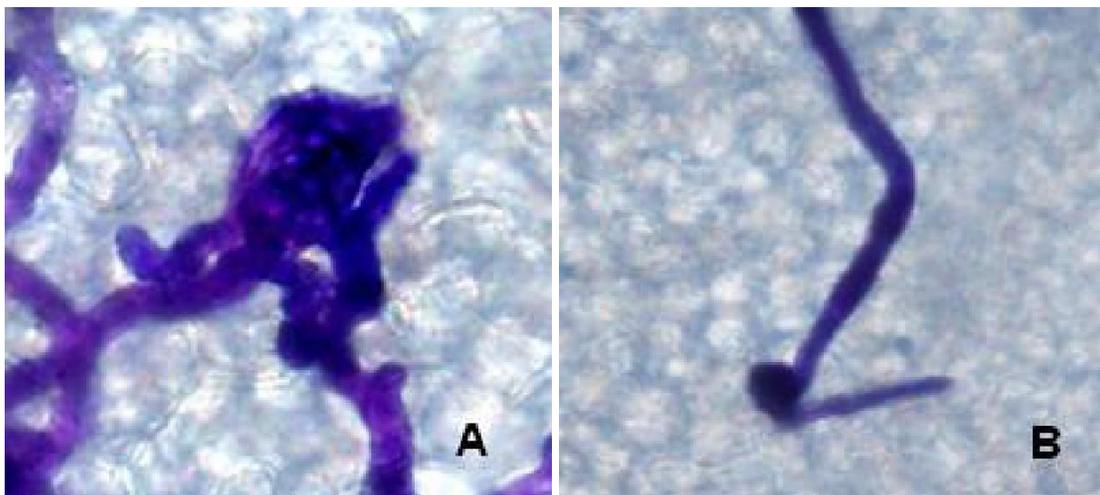


Figure 7. A) Infection cushion; B) Appressorium.



Figure 8. A layer of mycelium tile in the leaves.

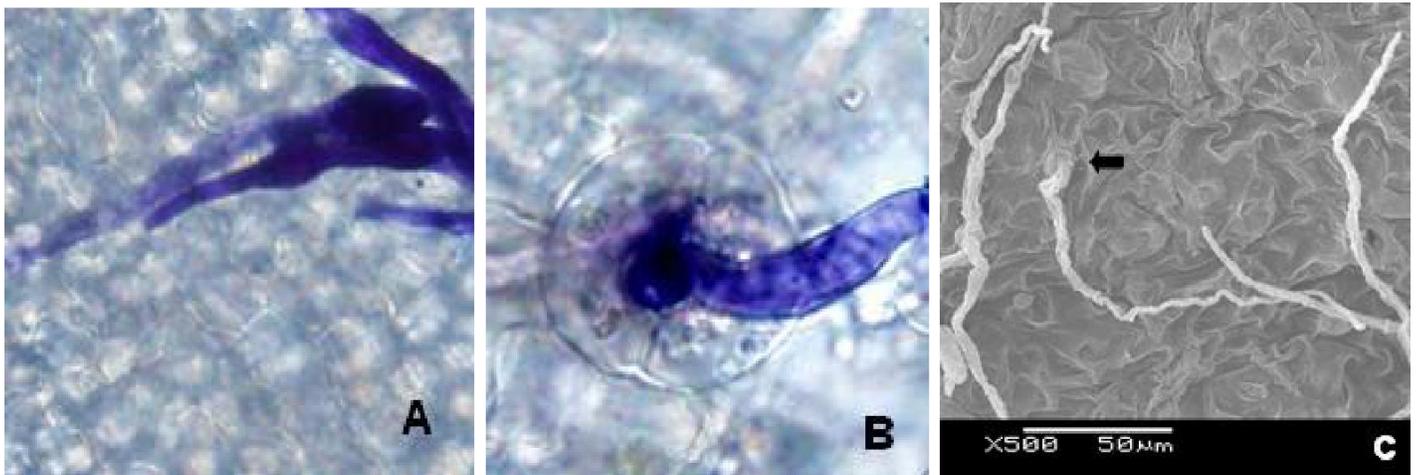


Figure 9. A) Hyphae penetrated directly; B) Hyphae penetrated through stomata (optical microscope); C) Hyphae penetrated through stomata (scanning electron microscope).



Figure 10. Hyphae spread through plant cells.

DISCUSSION

The process of infection includes mycelial growth on plant surface before infection, the formation of infectious structures, penetration, expansion of pathogen within the plant tissue and the appearance of symptom. Among these steps, penetration is the most important cause whether the plant disease takes place or not is depended on the penetration's chances of success. So, the formation and progress of infectious structures play a vital role in the occurrence of plant disease. Infection cushion and appressorium are *R. solani*'s infectious structures. Although these two structures have been deeply considered in the process of *R. solani* AG-1 IA infection of rice; we first observed infection cushion and appressorium in the process of *R. solani* AG-1 IB infection of soybean. Comparing these processes, we can find that infection



Figure 11. Symptom on leaf.



Figure 12. Sclerotia on leaf.

cushion on soybean leaf is sparser than that on rice leaf (Chen et al., 2000; Yang et al., 2008; Liu and Xiao, 1999; Tao and Tan, 1995; Zheng and Wu, 2007). The invasion of *R. solani* has diverse ways. The hyphae at the base of the appressorium or the infection hyphae developing from the appressorium can penetrate host cuticle directly. Also, some hyphae penetrate host cuticle through stomata or intercellular space indirectly. We observed both of these ways in our study. The infection process is a progress of interaction between plants and pathogens. In this study, we focused on the side of pathogens, and we hope to concentrate on plants in future to have a comprehensive understanding of *R. solani* AG-1 IB's infection process.

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