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Karyotype Analysis and Research of Infection Process of *Rhizoctonia solani* AG-1 IC

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***Rhizoctonia solani* AG-1 IC (teleomorph: *Thanatephorus cucumeris*) is an important soilborne plant pathogenic fungus. This study carried out the morphological feature observation of AG-1 IC, karyotype analyses, and infection process observation of Chinese kale and soybean. We found the sclerotia consisted of dense mycelium using scanning electron microscopy. For the most part, per cell of hyphae contained between 4 and 6 nuclei, and in most cases, each nucleus possessed 12 or 13 chromosomes. Symptom development on Chinese kale and soybean by AG-1 IC was studied. The disease symptoms of Chinese kale were more serious than soybean. AG-1 IC grew over the plant, adhered, and formed infection cushions and appressoria. Multiple invasions from the epidermal cell and hyphal invasion Chinese kale through stoma were observed.**

Key words: *Thanatephorus cucumeris*, Karyological analysis, Chromosome, Appressorium.

Rhizoctonia solani J.G. Kühn (anamorph: *Thanatephorus cucumeris* Frank Donk) is a soilborne plant pathogenic basidiomycetous fungus that causes economically important diseases to great variety of crop plants, vegetables, ornamentals, turf grasses, fruit and forest trees worldwide[1,16,19]. *R. solani* is composed of at least 14 anastomosis groups (AGs) based on the occurrence of hyphal anastomoses[2,5]. Based on geographic origin, host range, cultural morphology, pathogenicity, and molecular techniques, some AGs of *R. solani* have been further separated into subgroups. For instance, AG-1 has been divided into six intraspecific groups: AG-1 IA, AG-1 IB, AG-1 IC, AG-1 ID, AG-1 IE, and AG-1 IF[6,9,13,17].

A number of studies have analyzed subgroups based on the nuclei per cell in somatic hyphae[3,20]. Different fungal species propagate as haploid, dikaryons, multinucleates, polyploids or aneuploids, and the life cycles of some fungus contain not one or two but several of these different states[4,7]. There are different nuclear numbers per cell in somatic hyphae of *R. solani*[3,10]. The classification system for *Rhizoctonia* species is based on the number of nuclei per cell in somatic hyphae. In this study, we analyzed the number of nuclei per cell of range of *R. solani* AG-1 IC.

To obtain genetic information and determine chromosomal number of *R. solani*, Keijer *et al*[11] used pulsed field gel electrophoresis (PFGE). PFGE is an electrophoresis technique that separates large pieces of DNA to can estimate the number of chromosomes. In our study, we utilized

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chromosome tabletting and microscopic observation to determine chromosome number in AG-1 IC.

Among the 14 AGs, the plant-pathogenic isolates of AG-1 have extensive hosts[5]. The subgroups of AG-1 can cause different disease symptoms: rice-sheath blight (AG-1 IA), web blight (AG-1 IB, IE and IF), damping-off (AG-1 IC) and necrosis leaf spot (AG-1 ID)[6,17]. The infection process of *R. solani* includes penetration, adhesion, colonization and host reaction. Different AGs in *R. solani* may have dissimilar infection structures. The pathogenicity studies showed that individual *R. solani* isolates can be highly pathogenic to one or several hosts, yet they cannot infect others[12]. This experiment observed AG-1 IC infected leaves of Chinese kale and soybean, to understand infection structure and invasion pathways.

MATERIALS AND METHODS

Fungus and cultivation

The standard strain of *R. solani* AG-1 IC was provided by the South China Agricultural University. AG-1 IC was cultivated on potato dextrose agar (PDA) medium at 28°C in the dark, 2 d old cultures were used as the inocula throughout this study.

Karyological analysis

The nuclei of AG-1 IC were stained with using 4', 6- diamidino-2- phenylindole (DAPI, Sigma). The speciments were incubated in the staining reagent (0.36mM DAPI, 1.4 M NaCl, 42.9 mM Na_2HpO_4, 14.7mM KH_2pO_4), and kept in the dark for 30 min. Then, a cover slip was placed on the hyphae and pressed with filter paper to remove excess fluid and to exclude bubbles. Nuclei were observed by using fluorescence microscopy. In this study, a total of 1000 nucleate cells were counted.

Chromosome preparation

The sterile slide was inserted into 9 cm Petri dishes containing 15 ml PDA medium, inoculated with a small piece of AG-1 IC, and then placed in a 28°C incubator until emergent hyphae reached the surface of the slide. The hyphal tips were kept in the slide. Hyphal tips were fixed in Carnoy's solution (a mixture of 3 parts of ethanol and 1 part glacial acetic acid which should be prepared fresh daily) for 24 h at room temperature, and quickly dried with a gentle alcohol flame. The fixed slide was dehydrated with 75% and 95% alcohol two times, for 30 min each. Cell walls of speciments were digested in lyticase solution ($10mg^{-1}$ l lyticase, 0.6M $MgSO_4 \cdot 7H_2O$, pH 5.8) for 1 h at 37°C. The chromosomes of hyphal tips were stained with magenta solution (1.5 mg^{-1}l basic fuchsion, 45% glacial acetic acid (v/v), 1% sorbierite, 15% phenol (m/v)) for 10 min. A cover slip was placed on the hyphal tips and tapped gently with a pencil to scatter tissue. The excess liquid was immediately removed by gently pressing the cover slip without moving it. Chromosomes were observed using an Olympus BX53 microscope and images of selected spreads were captured using a DP21 digital camera.

Infection process observation

The hyphal pieces (each $5\times5\times1mm^3$) cut from the edge of the PDA cultures of the AG-1 IC were placed on leaves of Chinese kale (*Brassica alboglabra Bailey*) and soybean and cultured in a humidity environment at 28°C. After inoculation for 2 h, pieces were observed and sampled every 2 hour for 12 h. To further observe the infection process, samples were also taken at 16, 24, 36, 48, 72, 96 and 144 h. The samples were fixed and decolored in 95% ethanol: acetic acid (9:1, v/v) for 24 h. Half of the samples were stained with lactophenol cotton blue and observed for the infection process by optical microscopy. The other samples were immersed in 2.5% glutaraldehyde for 1 h, rinsed twice with deionized water, dehydrated by passage through a graded ethyl alcohol series, and finally placed in absolute ethyl alcohol. Samples were then dried in a critical-point dryer and coated with a thin layer of gold, and scanned on the scanning electron microscopy screen (JSM-7500F; JEOL Ltd. Japan) to obtain infection structure. Moreover, disease severity index was statistics according to Keijer *et al*[12].

RESULTS

Morphological features of AG-1 IC

In the early stages, *R. solani* AG-1 IC rapidly grew on PDA medium and the average radial growth was 34.01mm d^{-1}. The fungus formed circular colonies with a dense, colorless mycelium (Fig. 1a). As the pathogen grew, a large number of

white groups formed on the colony (Fig. 1b). In the later stages, the mycelium became brown, and the white groups became dark brown sclerotia which were scattered on the plate (Fig. 1c). As observed by scanning electron microscope, the sclerotium was intertwined with the compact hyphae (Figs. 1d and e).

Karyological analysis of somatic hyphae

Karyological analysis of DAPI-stained nuclei was performed on hyphal cells of *R. solani* AG-1 IC (Fig. 2). The mycelium was comprised of multicellular hyphae, which were divided into individual cells by septa (Fig.2a), and hyphal fusion was generally observed (Fig. 2b). The number of nuclei ranged from 1 to 17, but most of the hyphal cells contained between 4 and 6 nuclei (Fig.3).

Number of chromosome analysis

In this study, we investigated the number of chromosomes per nuclei by tabletting and staining with magenta. Chromosomes were small

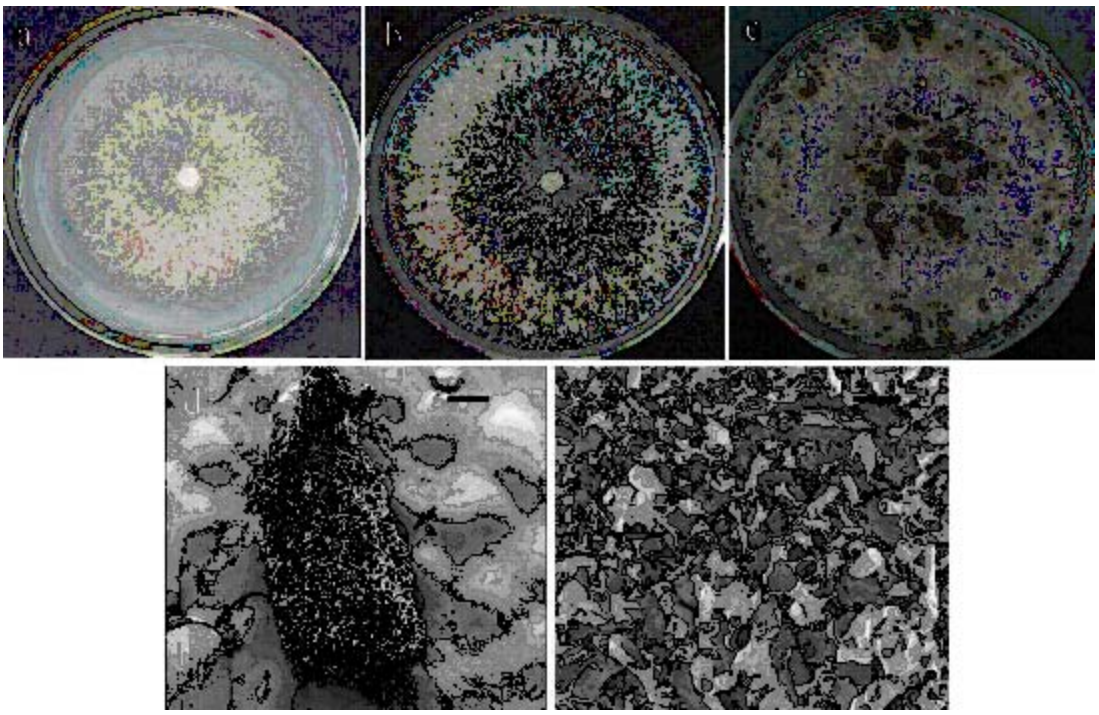

Fig. 1. Colonies of *R. solani* AG-1-IC on PDA solid medium. a The growth of hyphae at 2 days after inoculation. b White groups on hyphae at 4 days after inoculation (arrow). c Mycelia became brown and white groups became dark brown sclerotia (arrow) at 18 days after inoculation. d Scanning electron microscopy of the surface shape of sclerotium (arrow). e Internal structure of a sclerotium comprising dense mycelium. Bars: d=5 µm. e= 10 µm.

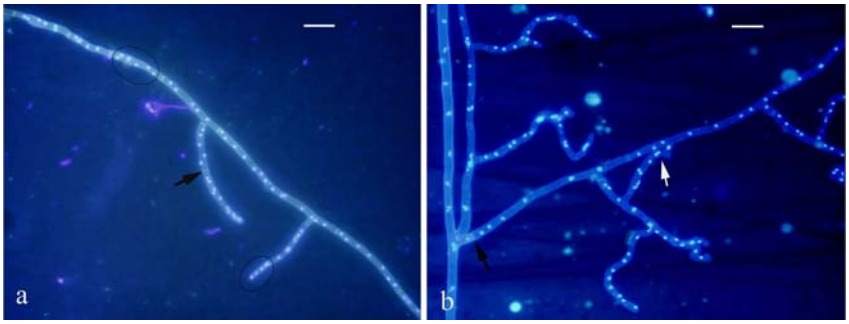

Fig. 2. Karyological observation of *R. solani* AG-1-IC with DAPI. a The hyphae were separated into multicellular mycelia by septum (black arrows), and nuclear number in per cell (black circle).
b Hyphal fusion (white arrow). Bars: a, b=5µm.

and linear shape (Fig. 4). The nuclei ranged between 10 to 18 chromosomes per nucleus, with 12 to 13 nuclei per chromosomes most frequently observed (Fig. 5).

Symptom development observation

Hyphae of *R. solani* AG-1 IC began to grow on leaves of Chinese kale and soybean at 8-12 h after inoculation (Figs. 6a and b). Usually,

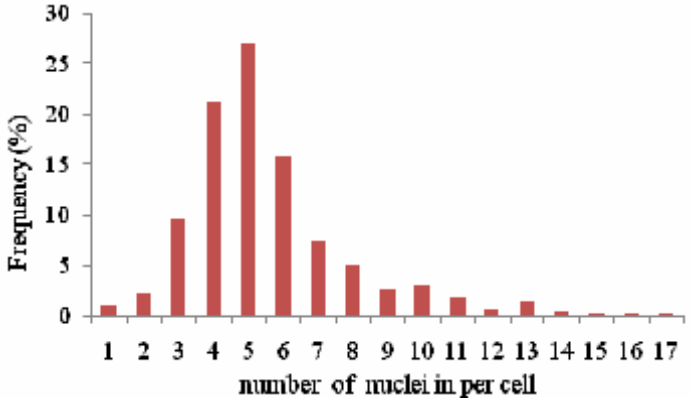

Fig. 3. The number of nuclei in per cell of somatic hyphae of *R. solani* AG-1-IC. The number of nuclei ranged from 1 to 17, with most hyphal cells containing between 4 and 6 nuclei

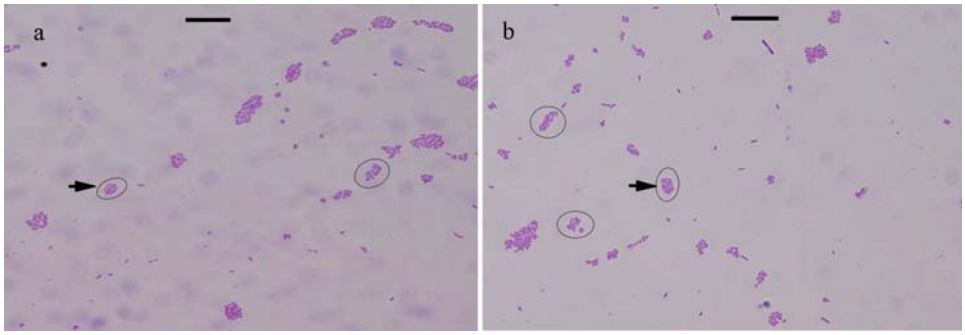

Fig. 4. Chromosomal observation with optical microscopy. a-b Chromosomal number in per nuclear (black circle). Bars: a, b=10μm

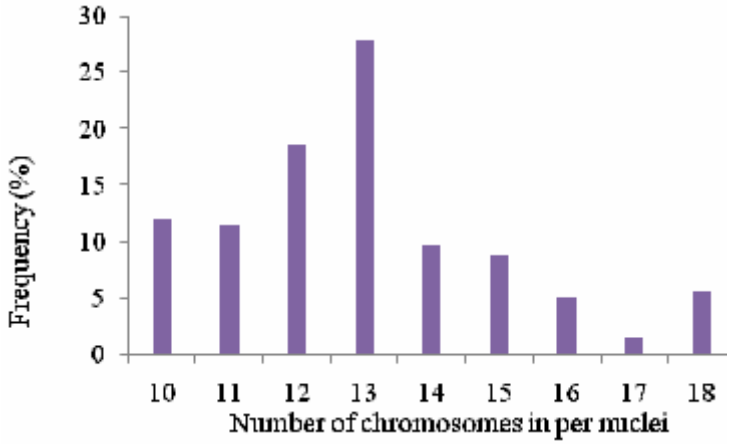

Fig. 5. The number of chromosomes per nuclei of *R. solani* AG-1-IC. Chromosomal number range is 10 to 18, and 12 to13 chromosomes were most frequently observed.

mycelial growth was radial, and in most cases, growth on Chinese kale was faster than that on soybean (28 h after inoculation) (Figs. 6c and d). The fungal mycelium completely covered the leaves surface of Chinese kale leaves in 30-48 h after inoculation. Disease severity index analysis revealed that the disease symptom of Chinese kale severity higher than soybean at the same time after inoculation (Fig. 7). When disease development on leaves of Chinese kale and soybean was compared (96 h inoculated), the whole leaves of Chinese kale appeared tan and had water immersion- shaped symptoms (Fig. 6e), while soybean leaves only presented oval and irregular brown disease spots (Fig. 6f).

Infection process observation

Mycelia grew from the inoculum on the leaf's surface of Chinese kale and soybean. After growing for some distance, hyphae produced primary branches (Figs. 8a and 9a). Repeated branching and the development of resultant short hyphal cells in a localized area resulted in infection

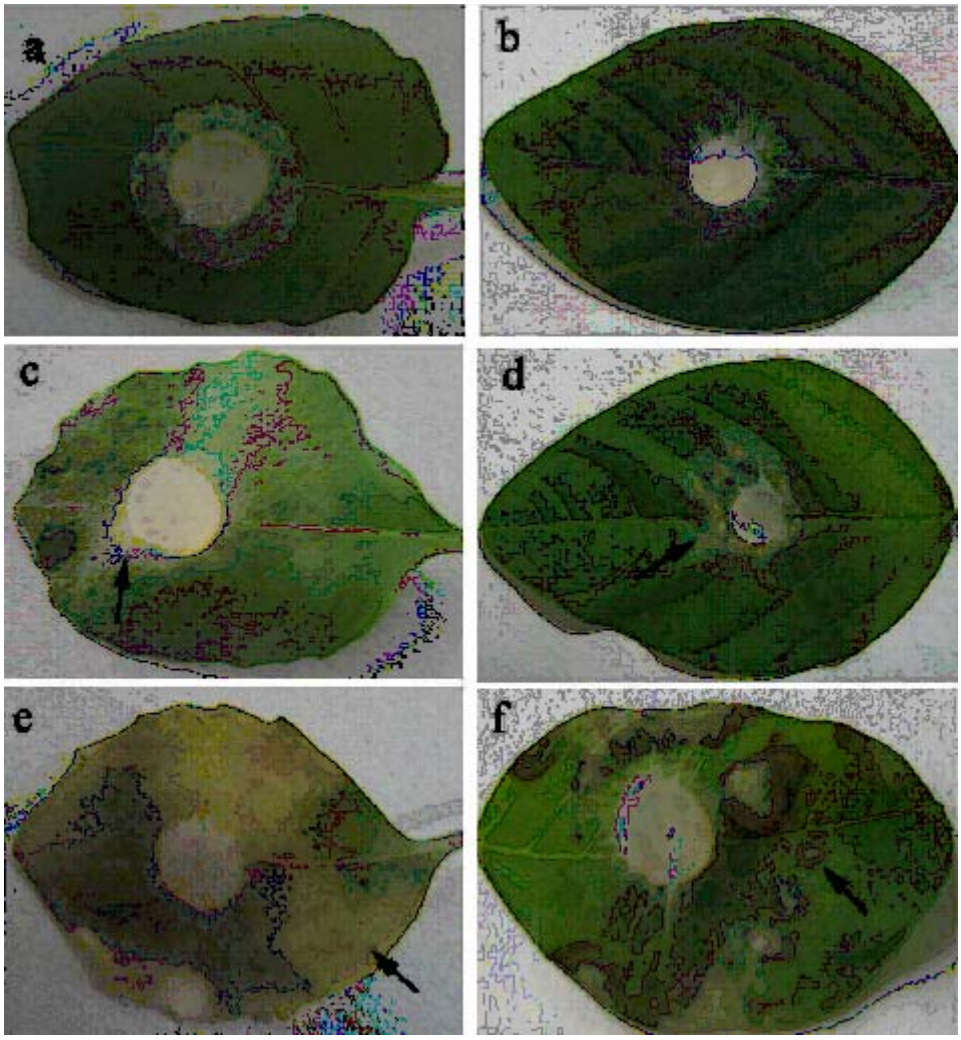

Fig. 6. Symptom development of Chinese kale and soybean during *R. solani* AG-1-IC infection. (a)Hyphae grew on chinese kale at 16 h after inoculation. (b) Hyphae grew on soybean at 16 h after inoculation. (c) Disease symptoms with water immersion appeared on Chinese kale at 30 h after inoculation (arrow). (d) Brown spots on soybeans presented at 30 h after inoculation (arrow). (e) Whole leaf of Chinese kale became brown with water immersion at 96 h after inoculation (arrow). (f) Soybean leaves had oval and irregular brown disease spots at 96 h after inoculation (arrow)

cushions (Figs. 8band 9b). In addition, the hyphal tips produced lobate appressoria, which were swollen hyphal tips (Figs. 8c, d and 9c). In the next step, several of the swollen tips simultaneously formed infection pegs (Fig. 9d). These infection structures directly penetrated into the epidermal cell wall of host tissue (Figs. 8f, 9e and f) or invaded host tissue through stomata (Fig.8e).

DISCUSSION

These experiments demonstrate the morphology of colonies of *R. solani* AG-1 IC. A few characteristics differed from other subgroups of AG-1. This study reported the sclerotium structure of AG-1 IC observed using scanning electron microscopy. The interior composition of the sclerotia was made of hyphae; therefore, the sclerotia can germinate and act as a preliminary infection source.

The microscopic observations of AG-1 IC nuclei number per cell in somatic hyphae were performed after staining the nuclei with the fluorochrome DAPI. The number of nuclei per cell is a novel result in AG-1 IC. Our observation of the mitosis process in *R. solani* was consistent with

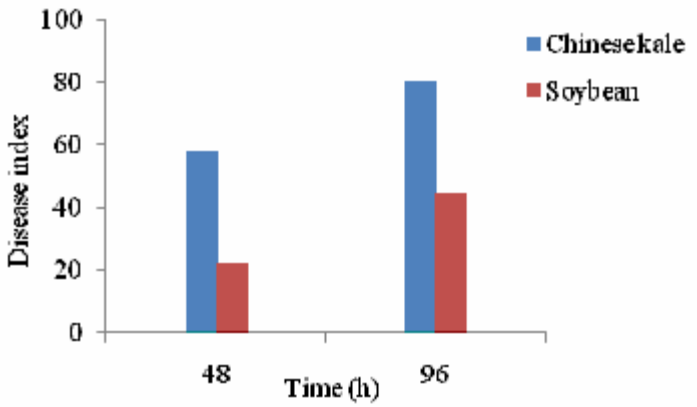

Fig. 7. The disease severity index on whole leaves of Chinese kale and soybean. Disease symptom of Chinese kale severity higher than soybean at 48 and 96 h after inoculation.

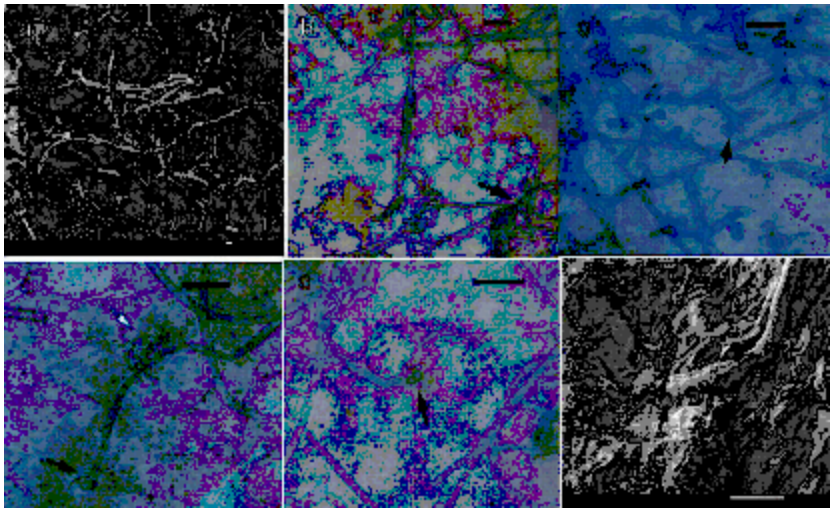

Fig. 8. Observation of the infection process of *R. solani* AG-1-IC in Chinese kale. a Hyphae produced primary branches. b Infection cushions (arrow). c Lobate appressoria (arrow). d Hyphae penetrated through stomata (white arrow) and top swollen (black arrow). e Hyphal invasion through stomata (arrow). f Hyphal invasion through epidermal cell (arrow). Bars: a, c, d, e, f=10ìm. b= 5µm
Notes: Optical microscope- b, c, d, e; Scanning electron microscope- a, f.

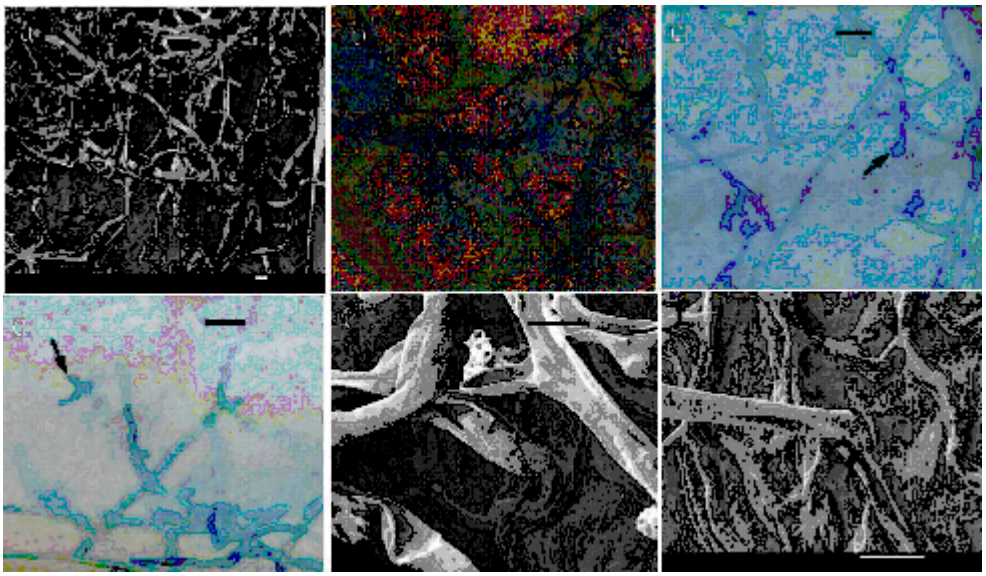

Fig. 9. Observation of the infection process of *R. solani* AG-1-IC in soybean. a Hyphae produced primary branches. b Infection cushions (arrow). c Appressoria (arrow). d Infection pegs (arrow). e-f Hyphal invasion through the epidermal cell (arrow). Bars: a, c, d, e, f=10μm. b= 5μm
Notes: Optical microscope- b, c, d; Scanning electron microscope- a, e, f.

what has been previously reported[3]. Uninucleate cells are not common in *R. solani*, but we found the existence of mononucleate cells in AG-1 IC. Thus, a technique for preparing homokaryotic strains from single protoplast cultures, prepared from a heterokaryotic mycelium is possible for use in AG-1 IC[18].

In this study, the application of the tabletting chromosome method separated nuclear chromosomes in hyphal tips. There are many nuclei per cell, and chromosomes are small; therefore, there is variation in the number of nuclei in hyphal tips. This study is the first to report the morphology and number of chromosomes observed in AG-1 IC using optical microscopy. The results showed that AG-1 IC had at least 10 chromosomes and was not consistent with previous reports[11]. These results will be helpful in future molecular and genetic studies, for example, hybridization *in situ*[8], construction of genomic maps or location of pathogenic genes on the chromosome.

The observation of symptom development in AG-1 IC infected Chinese kale and soybean found different symptoms and severities at the same time after inoculation. These results showed that AG-1 IC possessed different pathogenicity based on the host plant. The plant may have different levels of protection against a pathogen's invasion mechanism[12].

The infection process of *R. solani* includes adhesion, penetration, colonization and host reaction and it forms infection structures[5]. Of these steps, penetration is the most important and conclusive factor in determining whether the plant disease will occur or not. The infection cushion and appressorium are infection structures of *R. solani*. Although these two structures are well studied in the process of *R. solani* infecting rice[14], this study is the first to report that AG-1 IC formed infection cushions and appressoria in invaded Chinese kale and soybean. These structures were commonly observed by using optical microscopy. It was previously reported *R. solani* directly invades host tissue though the epidermal cell, and stomatal penetration is not frequent[15]. However, we observed AG-1 IC invaded Chinese kale through stoma. The infection process is an interaction between pathogens and hosts. The study of the infection process of pathogens is a vital process and it will be helpful in molecular resistance breeding.

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