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A New Method for Sclerotial Isolation of Two Species of *Sclerotium* from Infested Soils

Yong-Ki Kim*, Mi-Kyung Kwon, Hong-Sik Shim, Wan-Hae Yeh, Taek-Soo Kim, Weon-Dae Cho and Choong-Hoe Kim

Plant Pathology Division, National Institute of Agricultural Science of Technology, Rural Development Administration, Suwon 441-707, Korea

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White rot on *Allium* crops recently had a high incidence with increased cultivating areas of tropical garlic types in Korea. Two types of *Sclerotium* have known as causal agents that produce different size and shapes of sclerotia in infested fields. Therefore, we developed a new method for isolation of sclerotia from infested field soils that can be used for ecological study of *Sclerotium* spp. and establishment of control strategy. Soil samples collected from heavily infested fields were evenly mixed and placed on a automatic sieve shaker connected with tap water. After 10 min of shaking, residues on 0.5 mm and 0.25 mm sieves were separately collected and suspended with 70% sugar solution, which method floats sclerotia in aqueous layer. Then, floated fraction was carefully separated and mixed with a same volume of 1% sodium hypochlorite solution to differentiate with organic materials. This method provides a direct count of sclerotia under a dissecting microscopy.

Keywords : Inoculum density, isolation, *Sclerotium* spp., White rot of garlic

Garlic white rot occurs on most of *Allium* crop-cultivated areas, especially southern parts in Korea including Jeju island, Muan, Haenam, Goheung, Changyeong, Yeongchon. This disease becomes one of the major limiting factors for continued commercial production of *Allium* crops including garlic, onion, welsh onion, leek, wild garlic. Garlic white rot, one of the most serious diseases of *Allium* crops, has known to be caused by only *Sclerotium cepivorum* (Summer, 1995). However in Korea, it was confirmed that the disease was caused by two species of *Sclerotium, S. cepivorum* and unidentified *Sclerotium* species forming larger sclerotia compared to those of *S. cepivorum* (Cho et al., 2002). Garlic white rot is mainly maintained and disseminated by sclerotia of the fungus in soil. Sclerotia of *S. cepivorum* can lie dormant in soil for many years until roots of host plants grow nearby (Coley-Smith, 1959; Coley-Smith and Javed, 1970; Scott, 1956). It was reported that two major factors affecting the incidence of white rot are the size of the initial population of sclerotia in the field and the composition of the population regarding germination ability of the sclerotia (Gerbrandy, 1992). Therefore, in order to establish effective control strategy of soil-borne diseases, precise evaluation of population dynamics of the plant pathogens in the rhizosphere of garlic is very important. The development of isolation methods for plant pathogens in soil is needed to investigate inoculum density of soil-borne disease. There are various reports on the quantitative isolation of *S. cepivorum* (Adams, 1979; McCain, 1967; Harper and Stewart, 2000; Papavizas, 1972; Ukehede and Rahe, 1979). However among them were no reports for isolating both of two species of *Sclerotium* associated with garlic white rot. In addition, methods described previously were difficult to follow due to its complications and they were developed to isolate only one species, *S. cepivorum.* We thought that the development of new method for simultaneous sclerotial isolation of two species of *Sclerotium* associated with white rot of garlic in Korea is very needed to facilitate enumeration and recovery of sclerotia from naturally infested field soils. In this study we developed a new technique for sclerotial isolation of two species of *Sclerotium* causing white rot of *Allium* crops.

Materials and Methods

Fungal isolates used. Two strains, *Sclerotium cepivorum (Sc-1)* forming small sclerotia (diameter, 340-570 μm; average, 440 μm) and an unidentified *Sclerotium* sp. *(Ss-1)* forming large sclerotia (diameter, 420-750 μm; average, 550 μm), isolated from garlic bulbs in infested fields of Jeonnam province were used in this study to develop method for sclerotial isolation of two kinds of casual agents of white rot.

Isolation of *Sclerotium* spp. from infested soils. 2 kg-soil samples were collected from infested fields, air-dried and screened to pass through 2 mm sieve. Two strains of *Sclerotium* (*Sc-1 and Ss-1*) were isolated as follows. For a soil sample

*Corresponding author.
Phone) +82-31-290-0430, FAX) +82-31-290-0406
E-mail) yongki@rda.go.kr

containing *Sc-1* or *Ss-1*, three 20 g-soil samples were assayed. Each soil sample was weighed 20 g soil, suspended in distilled water, shaken in rotary shaker (150 rpm) for 2 hours, and sieved with sieve shaker (1700/850/500/250 μm) for 10 minutes. The residues remaining on the 500 μm and 250 μm fraction were rinsed in tap water and collected in 500 ml plastic beaker. The collected particles were suspended in 20 ml of 70% sugar solution, mixed evenly, stood for 10-30 sec and collected the floating fraction. The collected fraction were mixed with the same volume of 1% NaOCl evenly. The floating fraction-NaOCl mixtures were filtered with non-woven fabric. Water residues contained in non-woven fabric were discarded with filter paper. Sclerotia were located visually among soil particles filtered with non-woven fabric. Sclerotia were observed under a dissecting microscope at 10-30X.

Determination of sclerotial viability. Sclerotial viability was tested using Coley-Smith and Javed' method (1970). To determine viability, recovered sclerotia were surface-sterilized in 5% sodium hypochlorite for 5 min, washed in distilled water, touched-dried onto filter paper and cut into two pieces with surgical blade to break the rind, to trigger sclerotial germination. They were then plated onto potato dextrose agar media amended with 80 μg ml^{-1} of streptomycin (Sigma)

Recovery of sclerotia from artificially infested soils using a newly developed isolation method. The effectiveness of the newly developed isolation method was tested as follows. Batches of 100 sclerotia of two *Sclerotium* species were mixed with 20 g-soil samples. The number of sclerotia recovered was investigated under a dissecting microscope at 10-30X by the previously described isolation method with three replications.

Recovery of sclerotia from naturally infested soils using a newly developed isolation method. Diseased garlic bulbs from naturally infested fields with two species *of Sclerotium* were collected from two main garlic cultivated areas, Jeonnam and Gyeongnam provinces. Six garlic bulb samples naturally infested with *S. cepivorum* and twelve samples with *Sclerotium* species (large sclerotia-forming species) were assayed with the newly developed isolation method. The number of sclerotia recovered was investigated with three replications. Also sclerotial population of *S. cepivorum* was investigated at three different soil depths in infested garlic fields at garlic-seeding time.

Results and Discussion

Sclerotia contaminated in soil samples can be isolated well by the newly developed isolation method as shown in Figure 1. The problem with this method, as with others, is the difficulty of obtaining a sub-sample that is a representative of the field, field plot. We tried to minimize the effects of sample variation to collect soil samples at same soil depth and same distance from garlic plants, if

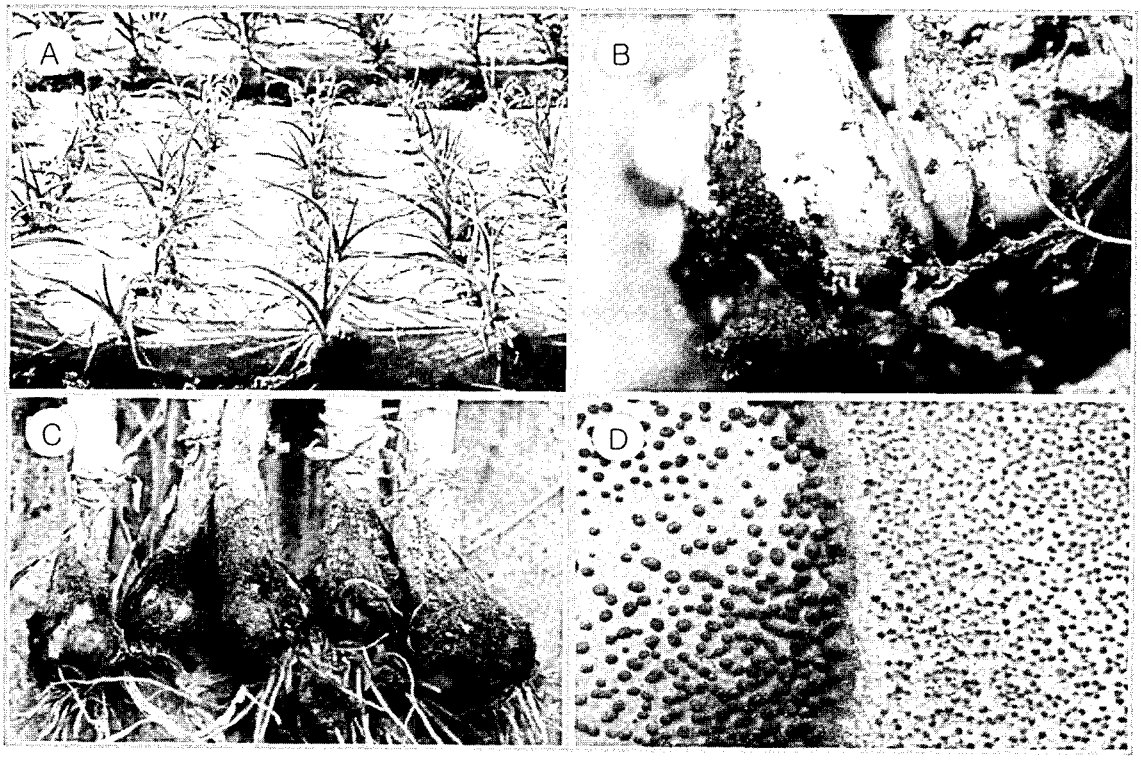

Fig. 1. Symptoms of garlic white rot and its pathogen(sclerotia). **(A)** Symptoms on foliage with premature yellowing and drying older leaves; **(B)** Sclerotia of *Sclerotium cepivorum* and **(C)** Sclerotia of a new *Sclerotium* species forming larger sclerotia compared to *Sclerotium cepivorum*, on the diseased tissues; **(D)** Border line formed by dual culturing *Sclerotium cepivorum* (right) and a new *Sclerotium* species (left).

possible. When viability of sclerotia isolated were tested by the determination method described previously, it was confirmed that most of isolated sclerotia were germinated 7 days after seeding them on potato dextrose agar medium amended with with 80 μg ml^{-1} of streptomycin (data not shown). Therefore, we think that this newly developed isolation method can be used to determine inoculum density of the pathogen, to obtain sclerotia from infested field for various experiments, to determine the percent of viable sclerotia in soil and to monitor population dynamics of the pathogen. Once soil samples were suspended evenly, recovery of sclerotia using newly developed isolation method took less than 30 min per 20 g soil sample, compared with 50-60 min for the methods previously reported. Thus, we think this method allows quick assessment of sclerotial numbers in the infested soil. Utkhede and Rahe (1979) founded that surface sterilization of recovered sclerotia in sodium hypochlorite followed by rinsing in distilled water, splitting, and plating of the halves on potato dextrose agar to be superior to the use of selective media. As the results of Utkhede and Rahe (1979), in this study survival rate of sclerotia obtained by the procedure described above could be evaluated effectively by monitoring sclerotial germination. It was confirmed that splitting sclerotia were germinated and grown faster than intact sclerotia (data no shown).

Infested soil samples were prepared according to previously mentioned method (100 sclerotia per 20 g-soil) and the effectiveness of the newly developed isolation method for recovery of sclerotia was tested. By using the method, recoveries of sclerotia varied markedly according to species of *Sclerotium*. The recoveries of Sc-1 and Ss-1 averaged 82.3 ± 4.9 and 72.0 ± 5.5(Table 1). It suggests that difference in recovery between Sc-1 and Ss-1 is closely bound up with external shape of sclerotia. While external structure of *S. cepivorum* (Sc-1) is round and smooth, that of *Sclerotium* species (Ss-1) is amorphous and indented (figure not shown). Due to this morphological property, it is difficult for sclerotia of *Sclerotium* sp. to pass through a series of sieves for sclerotial isolation. McCain (1967) and Adams (1979) reported wet-sieving techniques to recover sclerotia of *S. cepivorum* from soil. Papavizas (1972) also developed a method for sclerotial isolation of *S. cepivorum* from soil by a modified soil-dilution-plate technique. Uthede and Rahe (1979) reported wet-sieving floatation technique for sclerotial isolation of *S. cepivorum* from muck soil. Harper and Stewart (2000) reported

Table 1. Recovery of sclerotia of two species of *Sclerotium* by a newly developed isolation method

Pathogen	Replication	No. of sclerotia per 20 g soil		Recovery (%)
		Added	Isolated	
Sclerotium cepivorum (Sc-1)	I	100	88	88.0
	II	100	84	84.0
	III	100	75	75.0
			Average	82.3 ± 4.9
New *Sclerotium* sp. (Ss-1)	I	100	77	77.0
	II	100	61	61.0
	III	100	78	78.0
			Average	72.0 ± 5.5

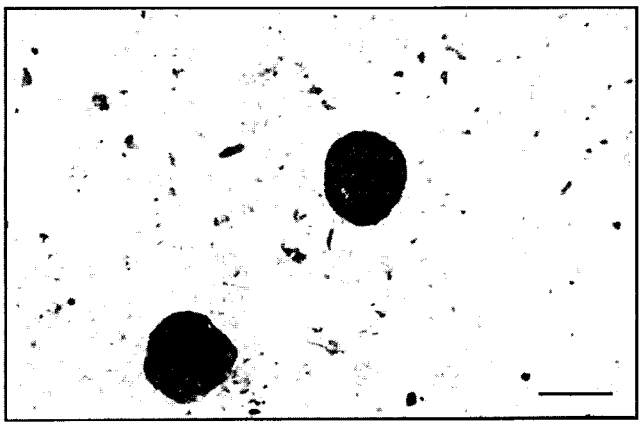

Fig. 2. A micrograph of sclerotia of *Sclerotium cepivorum* isolated using the newly developed isolation method from infested soil. Scale bar represents 300 μm.

Table 2. Natural inocumum densities of *Sclerotium* spp. investigated by this isolation method on diseased garlic bulbs collected in main garlic cultivation area

Pathogen	Soil origin	Number of collected soil samples[a]	Range of inoculum densities (sclerotia/garlic bulbs)
S. cepivorum	Jeonnam, Muan	4	646~14,723
S. cepivorum	Jeonnam, Haenam	2	40~86
Sclerotium sp.	Gyeongnam, Changyeong	5	1~3,402
Sclerotium sp.	Gyeongnam, Namhae	3	66~94
Sclerotium sp.	Gyeongnam, Haenam	4	26~95

[a]Soil samples were collected from garlic fields infested more than 10% based on percentage of diseased field area in 2003.

Table 3. Sclerotial population of *Sclerotium cepivorum* isolated from three different soil depths of infested garlic fields at garlic-seeding time

Soil depth (cm)	Inoculum density (sclerotia/20 g soil) at different garlic field soil samples[a]			
	Field A	Field B	Field C	Average
0~3.0	3	1	13	5.7
3.1~5.0	2	2	1	1.7
5.1~7.0	1	1	0	0.7

[a] Soil samples tested were collected from infested garlic fields at Taean county, Chungnam province in 2002.

magnetic separation technique for the isolation of sclerotia of *S. cepivorum* from iron-rich soil particles. Isolation of *S. cepivorum* from infested soils has been known to be precluded by competitive saprophyte (Papavizas, 1972) and the presence of large number of organic particles (McCain, 1967). We think both of these difficulties are overcome to a large extent by the method reported here. Although the previously reported methods for sclerotial isolation provide excellent results, they require more sophisticated facilities or comparatively much time to obtain the results. In addition the previously reported methods were developed to isolate quantitatively only sclerotia of *S. cepivorum*. To conclude, major advantages of newly developed method is to allow rapid and accurate assessment of sclerotial number in the infested soil.

Using this method, natural inoculum densities of two species of *Sclerotium* were investigated on garlic bulbs collected in main garlic cultivation area, Jeonnam and Kyungnam provinces. Numbers of sclerotia of two white rot pathogens, *S. cepivorum* and *Sclerotium* sp. (large sclerotia forming species) were at the range of 40-15,000 and 1-3,400, respectively (Table 2). This study showed that numbers of sclerotia of white rot pathogens were variable according sampling soil origins and *S. cepivorum* produced more sclerotia than *Sclerotium* species (large sclerotia forming species). Although white rot pathogens produce in a large number of sclerotia shown as Table 2, only several hundreds of sclerotia or less than 50 sclerotia per 100 g soil are known to be actually isolated from infested field soils (Papavizas, 1972; Adams, 1979). This result suggests that most of sclerotia of white rot pathogen be inactivated by competitive saprophytes or decomposed by soil-environmental factors. We think that this newly developed isolation method can be used to monitor inoculum density of pathogen and to isolate viable sclerotia. Also it can be used to confirm how sclerotia of white rot pathogen are disseminated from infested field to the other fields. In addition, the range of inoculum density of *S. cepivorum* were investigated at three different soil depths of infested garlic fields at garlic-seeding time (Table 3). Sclerotial population was variable according to soil samples and soil depth. Generally the deeper soil depth became, the more sclerotial density decreased. The range of inoculum densities of *S. cepivorum* found in three soil samples in main garlic cultivation areas ranged 1.0-13.0 per 20 g soil within 0-3.0 cm of soil depth and less than 1.0 per 20 g-soil between 5.1-7.0 cm of soil depth. It was confirmed that most of sclerotia of garlic white rot pathogen were distributed in soil surface. These results showed that inoculum densities of *S. cepivorum* isolated from infested fields in Korea were similar with those reported in foreign countries (Adams, 1979; Papavizas, 1972).

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