Genetic and Phenotypic Diversity of *Sclerotium rolfsii* in Groundnut Fields in Central Vietnam

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Abstract

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Groundnut (*Arachis hypogaea*) is an economically important legume crop in Vietnam and many other countries worldwide. Stem and pod rot, caused by the soilborne fungus *Sclerotium rolfsii*, is a major yield-limiting factor in groundnut cultivation. To develop sustainable measures to control this disease, fundamental knowledge of the epidemiology and diversity of *S. rolfsii* populations is essential. In this study, disease incidence was monitored in eight groundnut areas in central Vietnam with a total of 240 observational field plots. The results showed that 5 to 25% of the field-grown groundnut plants were infected by *S. rolfsii*. Based on internal transcribed spacer (ITS) ribosomal DNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfsii* field isolates, with the majority of the isolates (n = 90) in one ITS group. *S. rolfsii* isolates originating from groundnut, tomato, and taro were all pathogenic on groundnut and relatively sensitive to the fungicide tebuconazole but displayed substantial diversity of various genetic and phenotypic traits, including mycelial compatibility, growth rate, and sclerotial characteristics.

Groundnut (*Arachis hypogaea* L.) is an annual legume crop cultivated in more than 80 countries in the tropics, subtropics, and warm temperate zones (15). It is a major source of edible oil, vitamins, and amino acids and is used extensively for feed and food (36). In terms of economic importance, it ranks 13th among the world food crops and tops the list of oil seed crops both in terms of acreage and production. In 2009, groundnut was grown on 23.5 million ha worldwide, with an estimated total production of 35.5 million tons (13). In Vietnam, groundnut is the most important oil crop, with a total area of 256,000 ha and a production of 534,000 tons in 2008 (13).

Groundnut cultivation is adversely affected by a wide range of pests and diseases, including subterranean pests and foliage feeders (11), leaf spots, rust, stem rot, seedling diseases, limb and pod rot, nematodes, and viral diseases (38). In Vietnam, black collar rot caused by *Aspergillus niger* Tiegh., damping-off caused by *Rhizoctonia solani* Kühn, and stem and pod rot caused by *Sclerotium rolfsii* Sacc. (teleomorph: *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr.) are the most important soilborne fungal diseases of groundnut (20,23). *S. rolfsii* overwinters as mycelium or sclerotia in infected plant tissues and soil. Under favorable conditions, hyphae or germinating sclerotia infect the plant and subsequently colonize and invade the root and stem tissue with typical silky-white mycelium (10). Infected plants become yellow and then wilt, and the collar root turns brown and rots; in addition, *S. rolfsii* infects the groundnut pegs and pods, leading to yield losses.

S. rolfsii is difficult to control by physical and cultural practices due to its wide host range of over 500 plant species (3,28) and persistent sclerotia (19,28). Currently, there are only a few resistant cultivars commercially available (6,7,43). In Vietnam, methods to control *S. rolfsii* include rotation with non-host crops or deep coverage of infected crop debris with soil during land preparation. However, these methods are laborious and not effective due to the broad host range and persistence of *S. rolfsii*. Fungicides frequently used to control *S. rolfsii* include pentachloronitrobenzene, flutolanil (37), and tebuconazole (4,5,9). All three fungicides are effective in many cases, although tolerance to these fungicides was reported for *S. rolfsii* populations from groundnut fields in the United States (14,39,41). In Vietnam, these fungicides are not yet used on a regular basis and large scale due to their relatively high costs for subsistence farmers.

To successfully implement management practices (e.g., chemical and biological) to control *S. rolfsii*, knowledge of the distribution and diversity of the pathogen is essential. The diversity of *S. rolfsii* has been assessed for field populations in Georgia, United States (14) and Ibaraki, Japan (25) but, for most other groundnut-producing countries, including Vietnam, the information on the distribution, severity and diversity is scarce or not available. Here, we monitored the incidence of Sclerotium stem rot of groundnut in fields in central Vietnam and characterized *S. rolfsii* populations genetically and phenotypically. The implications of our findings for developing sustainable and appropriate strategies to control stem and pod rot in Vietnam are discussed.

Materials and Methods

Disease incidence, *S. rolfsii* isolation, and preservation. In 2009, the disease incidence of stem and pod rot was monitored in groundnut fields in four provinces in central Vietnam (i.e., Quang Nam, Thua Thien Hue, Ha Tinh, and Nghe An; Fig. 1). In each province, samples were obtained from groundnut plants grown at two locations at least 20 km apart, one with sandy soil and the other with clay loam soil. The disease incidence was assessed at flowering, the developmental stage at which groundnut is infected by *S. rolfsii* (20,23) For all eight locations, disease incidence was determined in at least 10 farmer fields for a total of 30 randomly

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selected plots of 1 m^2. Therefore, in total, 240 groundnut plots of 1 m^2 (i.e., eight locations × 30 plots) were monitored in central Vietnam to determine the disease incidence of stem and pod rot caused by *S. rolfsii*. From each of the eight locations, 50 samples of diseased tissue or sclerotia were collected, stored in plastic bags, and brought to the laboratory for *S. rolfsii* isolation. Samples were collected mainly from groundnut; however, diseased tissues from two other host crops (i.e., tomato [*Solanum lycopersicum*] and taro [*Colocasia esculenta*]) grown in the same fields were also collected and included for pathogen isolation. Besides sampling the eight locations, groundnut plants from a remote upland location (100 m above sea level) in Nghe An province were also sampled (Fig. 1).

Sclerotium rolfsii was isolated and maintained according to the methods of Punja and Rahe (31). Diseased tissues and sclerotia

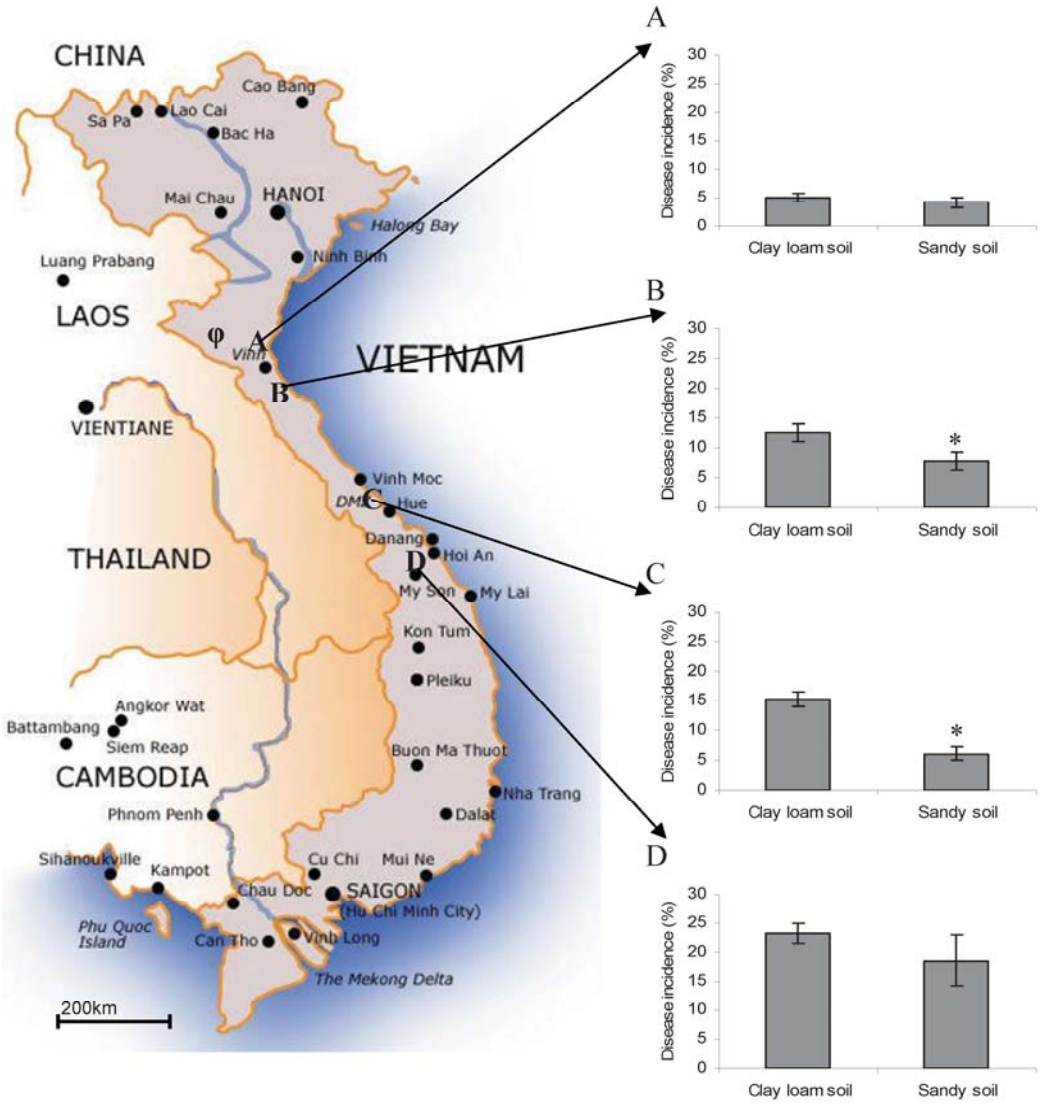

Fig. 1. Map of Vietnam showing the provinces where the incidence of stem rot of groundnut was monitored in 2009 and where *Sclerotium rolfsii* isolates were collected. The four provinces surveyed are **A,** Nghe An (18°46′N 105°38′E); **B,** Ha Tinh (18°21′N 105°51′E); **C,** Thua Thien Hue (16°33′N 107°31′E); and **D,** Quang Nam (15°47′N 108°21′E). Three more isolates of *S. rolfsii* were collected from groundnut grown in the remote upland area of Nghe An province (φ). For each of the four provinces, stem rot incidence was determined for groundnut plants grown in two soil types (clay loam and sandy soils). For each soil type and each province, the disease was recorded in 30 field plots of 1 m^2. In the graphs on the right, the average disease incidence is presented and the standard error of the mean is indicated. An asterisk indicates a statistically significant difference ($P < 0.05$) between the disease incidence in sandy soil and that in clay loam soil.

Table 1. Internal transcribed spacer ribosomal DNA sequences of reference strains of *Sclerotium rolfsii, S. delphinii*, and *S. coffeicola* used in the phylogenetic analyses

Accession number	Host	Origin	Year of isolation	Name	Reference
GQ358518	*Ascocentrum* and *Ascocenda* spp.	South Florida, United States	2008	*S. rolfsii*	12
DQ484060	Unknown	Unknown	Unknown	*S. rolfsii*	21
DQ484061	Unknown	Unknown	Unknown	*S. rolfsii*	21
DQ484062	Unknown	Unknown	Unknown	*S. rolfsii*	21
AB075307	*Arachis hypogaea*	Georgia, United States	1991	*S. rolfsii*	26
GU080230	*Capsicum annuum*	Spain	2009	*S. rolfsii*	34
DQ059578	*Nicotiana tabacum*	North Carolina, United States	2007	*S. rolfsii*	35
AB075318	Unknown	Washington, United States	1991	*S. delphinii*	26
AB075314	Unknown	Japan	1995	*S. delphinii*	26
AB075312	Unknown	Japan	1992	*S. delphinii*	26
AB075319	Unknown	Surinam	1919	*S. coffeicola*	26

were surface sterilized for 2 min in 75% (vol/vol) ethanol, transferred to wet filter paper in petri plates, and incubated at 28°C for 2 to 3 days. Outgrowing mycelium was transferred to water agar medium and incubated for 2 days. From each sample, one or two hyphal tips were transferred to potato dextrose agar (PDA) plates to purify the fungal isolates. *S. rolfsii* isolates were kept on PDA slants in duplicate; one sample was covered with mineral oil for long term storage at 20°C. From a total of more than 400 collected samples, 198 *S. rolfsii* isolates were successfully purified, preserved, and subjected to internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequencing.

ITS-rDNA amplification, sequencing, and phylogeny. Total DNA of *S. rolfsii* isolates was extracted based on the method of Tendulkar et al. (40). Each *S. rolfsii* isolate was cultured on PDA for 48 h at 25°C, and then approximately 5 mg (fresh weight) of mycelium was transferred to a 1.5-ml tube. Tris-EDTA buffer (50 µl of 10 mM Tris/HCl and 1 mM EDTA buffer, pH 8) was added and the sample was kept at room temperature (approximately 20°C) for 10 min, microwaved for 30 s at 650 W, and centrifuged at a speed of 10,000 rpm for 10 min. The supernatant containing genomic DNA was used directly for ITS-rDNA amplification. Genomic DNA template (4 µl) was used in a total reaction mixture volume of 50 µl. The mix contained 2.0 µl of each of the four dNTPs (5 mM stock each), 5.0 µl of 10× polymerase chain reaction (PCR) buffer; 0.2 µl of SuperTaq (5 U/µl; SphaeroQ); 2 µl of primers ITS1 and ITS4 (10 mM stock each) (42), and sterile MQ water. The PCR reaction involved 1 cycle at 94°C for 5 min, and 35 repetitive cycles with 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The PCR product (2 µl) was loaded on a 1.5% (wt/vol) agarose gel to assess the expected size (approximately 700 bp) and quantity of the PCR product. ITS-rDNA fragments were purified and sent for sequencing (Macrogen). The obtained forward and reverse sequences were assembled and edited in Vector NTI and deposited in GenBank with accession numbers HQ895865 to HQ895967. For the phylogenetic analyses, the edited sequences were trimmed (approximately 550 bp) and aligned to reference sequences available in databases (Table 1); the phylogenetic tree was obtained with MEGA4 software (http://megasoftware.net) using the ITS-rDNA sequence of *S. coffeicola* as an outgroup.

Pathogenicity assays. Pathogenicity of the *S. rolfsii* isolates was assessed on *Arachis hypogaea* L. 'L14', the predominant groundnut cultivar grown in Vietnam. In total, 18 *S. rolfsii* isolates, selected based on origin (i.e., groundnut [n = 8], tomato [n = 5] and taro [n = 5]) and ITS group, were tested for pathogenicity. A single

Fig. 2. Disease scales of groundnut infected by *Sclerotium rolfsii*; 0 = no disease symptoms, 1 = disease symptoms without visible outgrowth of the fungus, 2 = disease symptoms with visible outgrowth, 3 = partial wilting of the plant, and 4 = complete wilting and plant death.

Fig. 3. A, Overview of the sampling location in Quang Nam province; **B,** effects of *Sclerotium rolfsii* on emergence and growth of groundnut seedlings in the field; and symptoms of stem rot of **C,** groundnut; **D,** tomato; and **E,** taro. The sclerotia on the infected plant tissues are indicated by an arrow.

groundnut seed was sown in a square plastic pot (6 cm in width by 8 cm in height) containing 250 g of natural field soil obtained from Hue (Vietnam). Pots were placed in a nethouse and watered regularly. One week after sowing, a mycelial plug (1 cm in diameter) of a 3-day-old *S. rolfsii* PDA plate culture was placed at the base of the stem. Disease incidence and severity were determined 2 weeks after fungal inoculation. Disease incidence refers to the percentage of diseased plants and disease severity was rated on a scale from 0 to 4, with 0 = no disease symptoms, 1 = disease symptoms without visible fungal outgrowth, 2 = disease symptoms with visible fungal outgrowth, 3 = partial wilting of the plant, and 4 = complete wilting and plant death (Fig. 2). For each *S. rolfsii* isolate, three trays with five pots (one plant per pot) each were used.

Phenotypic characterization. *S. rolfsii* isolates were cultured on PDA plates at 25°C. Per isolate, two 25-ml PDA agar plates (9 cm in diameter) were inoculated in the center with a mycelial plug (5 mm in diameter), and radial mycelial growth of the fungal colony was determined 48 h after incubation. For each plate, the number of sclerotia was determined 21 days after incubation and the sclerotial diameter was determined for 30 randomly selected sclerotia.

In total, 26 randomly selected *S. rolfsii* isolates were tested for mycelial compatibility. Multiple combinations of two isolates of *S. rolfsii* were inoculated on PDA plates and incubated at 25°C for 2 weeks. Mycelial compatibility was assessed macroscopically by the method of Punja and Grogan (30). If there was a distinct barrage zone at the contact area between two isolates, these isolates were considered to belong to a different mycelial compatibility group (MCG).

The ergosterol biosynthesis inhibitor fungicide tebuconazole is widely used to control *S. rolfsii*. In our study, *S. rolfsii* isolates were cultured in duplicate on PDA amended with tebuconazole (Sigma-Aldrich Chemie B.V.) to final concentrations of 0, 0.02, and 0.2 mg liter^{-1}. Hyphal growth was assessed 48 h after incubation at 25°C. Sensitivity of *S. rolfsii* to tebuconazole was calculated with the formula used by Franke et al. (14): inhibition (%) = 100 – [(colony diameter on amended medium/colony diameter on control medium) × 100].

Results

Disease incidence. Our survey showed that the incidence of stem rot of groundnut caused by *S. rolfsii* ranged from approximately 5 to 25% (Figs. 1 and 3). Furthermore, a gradient in stem rot disease incidence was observed across central Vietnam with an increase in disease incidence from the northern to the southern field sites (Fig. 1). This might be related to the effect of temperature on disease development (28) because, along with the lower latitude, the average daily temperature increases from the north to the south of Vietnam. For two of the four provinces (i.e., locations B and C), the disease incidence of groundnut plants cultivated in clay loam soil was significantly higher than in sandy soil (Fig. 1).

Genetic diversity of *S. rolfsii*. In total, 198 purified *S. rolfsii* isolates, obtained from more than 400 randomly collected samples from different host plants (groundnut, tomato, and taro) in the four provinces and in the remote upland region were subjected to ITS-rDNA sequence analysis. For 103 *S. rolfsii* isolates, high-quality forward and reverse sequences were obtained whereas, for the

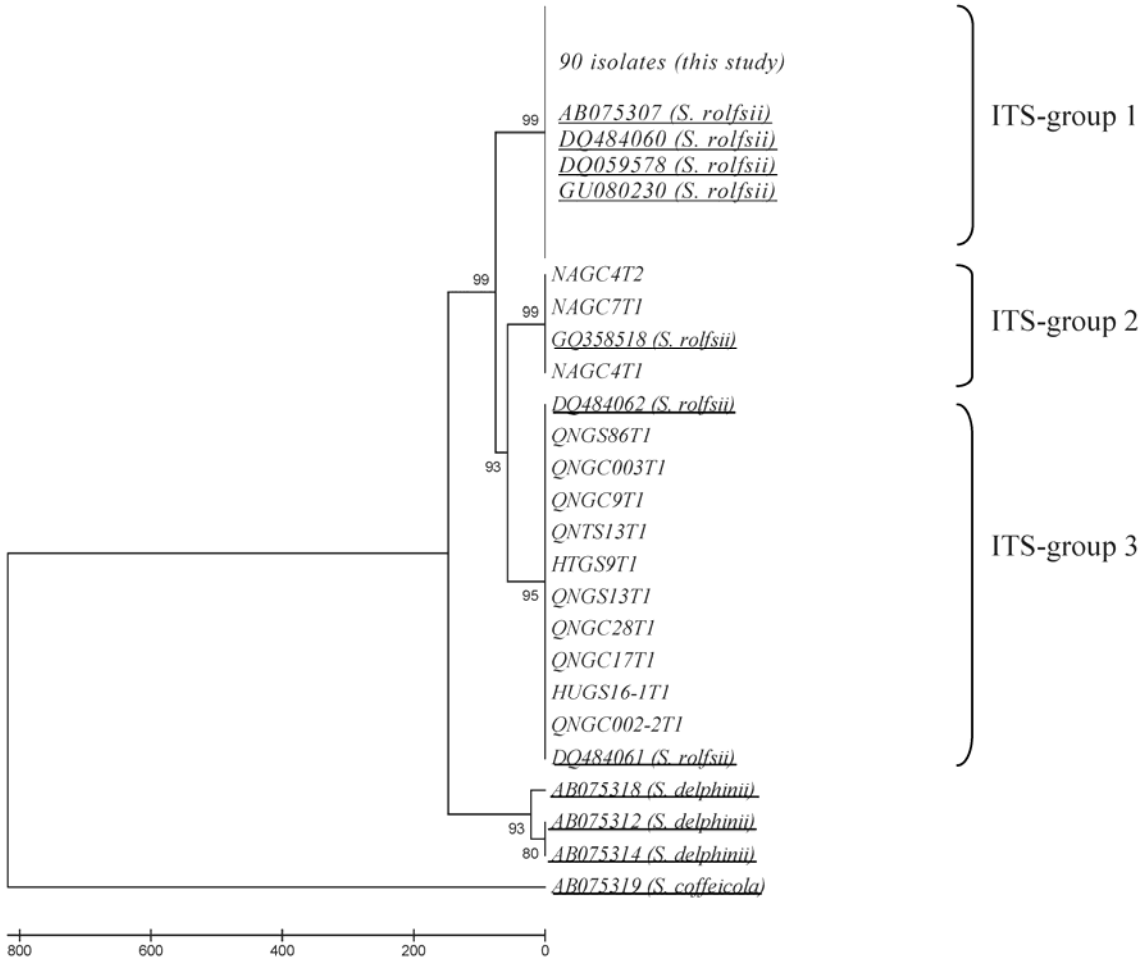

Fig. 4. Phylogeny of internal transcribed spacer (ITS) ribosomal DNA sequences of *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam and of reference strains of *S. rolfsii, S. delphinii*, and *S. coffeicola* (underlined, see Table 1) using the unweighted pair-group method. Bootstrap values with 1,000 replications are indicated at the nodes of the branches. The bar represents the evolutionary distances computed using the maximum composite likelihood method. Sequence codes of the isolates collected in this study refer to the province (first two letters), host crop (third letter), soil type (fourth letter), and the isolate number (digits): NA = Nghe An province, HT = Ha Tinh province, HU = Thua Thien Hue province, QN = Quang Nam province; G = groundnut, T = tomato, M = taro; C = clay loam soil, S = sandy soil.

other 95 isolates, discrepancies were observed in the assembly of the forward and reverse sequences. Resequencing of the ITS fragments of 12 selected isolates did not resolve this inconsistency, which may suggest polymorphisms in different ITS copies in each of these isolates. ITS polymorphisms within a single isolate have been found for several fungal genera and were also reported earlier for *S. rolfsii* by Okabe et al. (24,26).

Phylogenetic analysis of the ITS-rDNA sequences of the reference isolates of *S. rolfsii* (i.e., isolates for which ITS-rDNA sequences are present in the National Center for Biotechnology Information database; Table 1), revealed three main groups designated ITS groups 1 to 3 (Fig. 4). Most isolates (n = 90) collected from central Vietnam belong to ITS group 1 and were identical to reference isolates of *S. rolfsii* that were previously collected from groundnut, tobacco, and sweet pepper (Fig. 4; Table 1). The three isolates in ITS group 2 were collected from the remote upland region of Nghe An province (Fig. 4) and cluster with an *S. rolfsii* isolate collected from Ascocenda orchids in Florida (12). Of the 10 isolates in ITS group 3, 8 were collected in Quang Nam province (Fig. 4). Collectively, these results suggest that the *S. rolfsii* population in groundnut fields in central Vietnam appears relatively uniform. To get more insight into the intraspecific diversity of the collection of *S. rolfsii* isolates from central Vietnam, a range of other traits, including pathogenicity, growth rate, sclerotial characteristics, mycelial compatibility, and tebuconazole sensitivity, were analyzed.

Pathogenicity. Pathogenicity assays showed that *S. rolfsii* isolates obtained from groundnut (n = 8), tomato (n = 5), and taro (n = 5) and representing ITS groups 1 to 3 were all pathogenic on groundnut (Table 2). The time for the first symptoms to appear ranged from 2 to 5 days; however, no statistically significant differences (analysis of variance, $P < 0.05$) in disease incidence and severity were found between the isolates (Table 2). These results confirmed pathogenicity of (i) a subset of *S. rolfsii* isolates collected from groundnut, (ii) all *S. rolfsii* isolates collected from other crops (i.e., tomato and taro), and (iii) the three isolates collected from groundnut cultivated in the remote upland region in Nghe An province (ITS group 2).

Table 2. Pathogenicity of *Sclerotium rolfsii* isolates on groundnut[a]

Host crop	ITS group[b]	Incubation (day)[c]	Incidence (%)[d]	Severity ± SE (%)[e]
Groundnut (n = 5)	1, 3	2–3	100	92.7 ± 2.21
Tomato (n = 5)	1, 3	4–5	100	86.3 ± 4.26
Taro (n = 5)	1	3–4	100	95.3 ± 1.11
Groundnut (n = 3)	2	3–4	100	75.0 ± 2.89

[a] Isolates used for these assays are indicated in Table 4 with an asterisk.
[b] Internal transcribed spacer (ITS) group as indicated in Figure 4.
[c] Incubation period = days to first symptom appearance after fungal inoculation.
[d] Disease incidence (%) = (diseased plants/total number of plants) × 100%.
[e] Disease severity ± standard error (SE): diseased plants were ranked on a 0-to-4 scale based on the symptoms, with 0 = no disease symptoms, 1 = disease symptoms without visible outgrowth of the fungus, 2 = disease symptoms with visible outgrowth, 3 = partial wilting of the plant, and 4 = complete wilting and plant death. Disease severity (%) = [(1 × number of plants classified in scale 1) + (2 × number of plants classified in scale 2) + (3 × number of plants classified in scale 3) + (4 × number of plants classified in scale 4)] × 100%/(4 × total number of plants)].

Growth rate and sclerotial characteristics. *S. rolfsii* is notorious for its high growth rate in vitro (2,28,29). Indeed, the 103 Vietnamese isolates tested here exhibited a high growth rate of 0.28 to 0.79 mm h^{-1} (Tables 3 and 4). On average, isolates from ITS groups 1, 2, and 3 showed no significant differences in growth rate (Table 3). On PDA, the *S. rolfsii* isolates produced typical silky-white mycelium and brown or dark brown sclerotia (Fig. 5; 16). For the 103 isolates tested, considerable variation was observed in the time to form sclerotia, their maturation time, and their number and size (Tables 3 and 4). For example, the number of sclerotia formed per plate after 21 days of incubation ranged from 79 to 1,080 and their size from 0.88 to 2.24 mm (Table 3). When comparing the sclerotial traits, the three isolates in ITS group 2 were different from the isolates in ITS groups 1 and 3 (Table 3). ITS group 2 isolates produced substantially fewer sclerotia per plate; also, the average maturation time and sclerotial diameter were approximately two times greater than for isolates of ITS groups 1 and 3.

Mycelial compatibility. Within each of the three ITS groups identified in our study, a high variation in mycelial compatibility was observed (Table 5). For isolates of ITS group 1, 91 combinations were tested and only 6.6% of these combinations showed a compatible interaction pointing to at least nine MCGs. For ITS groups 2 and 3, we found two and six MCGs, respectively (Table 5).

Tebuconazole sensitivity. At a tebuconazole concentration of 0.02 mg $liter^{-1}$, the inhibition of mycelial growth of the 103 *S. rolfsii* isolates from Vietnam ranged from 18.2 to 58.8%, with an overall average of 36.5% (Tables 4 and 6). The three isolates from ITS group 2, collected from the remote upland region, were significantly more sensitive to tebuconazole than those from ITS group 1 (Table 6). In the study by Franke et al. (14), the variation in tebuconazole sensitivity of *S. rolfsii* isolates from Georgia was substantially higher: at the same tebuconazole concentration of 0.02 mg $liter^{-1}$, inhibition of mycelial growth of 473 field isolates ranged from –54 to 79% with an overall average of 28%; several isolates from their collection showed negative inhibition values, which presumably reflects a positive effect of tebuconazole on hyphal growth.

Discussion

S. rolfsii Sacc. is a major soilborne pathogen of groundnut. Fundamental knowledge of the diversity and epidemiology of *S. rolfsii*

Table 3. Growth rate and sclerotial characteristics of *Sclerotium rolfsii* isolates obtained from groundnut fields in central Vietnam

Characteristics	Growth (mm h^{-1})[a]	Sclerotia production and size			
		Days to form	Days to mature	Number per plate	Diameter (mm)
Values[b]					
Average	0.40	4.8	8.0	589	1.1
Minimum	0.28	4.0	7.0	79	0.9
Maximum	0.79	11.0	16.0	1080	2.2
Standard deviation	0.06	1.1	1.4	204	0.2
ITS group[c]					
1 (n = 90)	0.41 ± 0.06	4.7 ± 0.6	7.8 ± 0.7	595 ± 181	1.1 ± 0.1
2 (n = 3)	0.36 ± 0.04	10.0 ± 1.7	14.3 ± 2.9	98 ± 16	2.0 ± 0.2
3 (n = 10)	0.41 ± 0.04	4.2 ± 0.4	7.8 ± 1.2	689 ± 236	1.1 ± 0.2
Average	0.40 ± 0.06	4.8 ± 1.1	8.0 ± 1.4	589 ± 204	1.1 ± 0.2

[a] Hyphal growth rate during 48 h.
[b] Averages, minimum, and maximum values of each of these phenotypic characteristics are given for 103 isolates.
[c] Averages ± the standard deviation of these phenotypic characteristics are given for each of the three identified internal transcribed spacer (ITS) ribosomal DNA groups of *S. rolfsii.*

Table 4. Genetic and phenotypic traits of 103 randomly selected *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam[a]

		Origin					Time (days)		Diameter of sclerotia (mm)[b]					
Order	Code	Province	Soil	Host	ITS[c]	Rate (mm/h)[d]	Produce[e]	Mature[f]	Avg	Min	Max	SD	Sclerotia[g]	Inhib. (%)[h]
1	HTGS12T1	Ha Tinh	Sandy	Groundnut	1	0.43	5	8	1.06	0.71	1.32	0.16	1,080	19.80
2	HTGS13-2T1	Ha Tinh	Sandy	Groundnut	1	0.36	5	8	0.89	0.58	1.35	0.19	618	26.26
3	HTGS17T1	Ha Tinh	Sandy	Groundnut	1	0.32	6	9	1.29	1.02	1.57	0.16	278	22.77
4	HTGS18T1	Ha Tinh	Sandy	Groundnut	1	0.42	4	7	0.99	0.72	1.26	0.13	616	37.58
5	HTGS20T1	Ha Tinh	Sandy	Groundnut	1	0.37	5	8	1.14	0.80	1.65	0.20	508	28.19
6	HTGS23T1	Ha Tinh	Sandy	Groundnut	1	0.36	4	8	0.93	0.67	1.17	0.12	658	47.70
7	HTGS24T1	Ha Tinh	Sandy	Groundnut	1	0.39	5	8	1.22	0.78	1.43	0.16	326	38.04
8	HTGS6T1	Ha Tinh	Sandy	Groundnut	1	0.34	5	8	1.00	0.68	1.37	0.14	724	50.07
9	HTGS9T1	Ha Tinh	Sandy	Groundnut	3	0.35	5	8	1.02	0.72	1.37	0.19	584	45.40
10	HTGC10-2T1	Ha Tinh	Clay loam	Groundnut	1	0.41	4	7	1.20	0.86	1.53	0.18	568	36.29
11	HTGC10-1T1	Ha Tinh	Clay loam	Groundnut	1	0.36	5	9	1.07	0.77	1.40	0.19	516	24.25
12	HTGC13-1T1	Ha Tinh	Clay loam	Groundnut	1	0.41	5	8	1.10	0.85	1.47	0.16	452	42.31
13	HTGC13T1	Ha Tinh	Clay loam	Groundnut	1	0.33	5	8	1.09	0.67	1.43	0.19	472	28.09
14	HTGC3T1	Ha Tinh	Clay loam	Groundnut	1	0.40	4	7	1.06	0.81	1.29	0.12	704	38.34
15	HTGC5T1	Ha Tinh	Clay loam	Groundnut	1	0.38	5	8	1.29	0.90	1.57	0.17	346	19.85
16	HTGC9T1	Ha Tinh	Clay loam	Groundnut	1	0.45	4	7	1.24	0.90	1.72	0.18	356	53.90
17	HTGC9T2	Ha Tinh	Clay loam	Groundnut	1	0.44	4	7	1.21	0.99	1.55	0.12	422	42.70
18	NAGS2-1T1	Nghe An	Sandy	Groundnut	1	0.42	5	8	1.09	0.64	1.50	0.25	576	30.06
19	NAGS1T1 *	Nghe An	Sandy	Groundnut	1	0.40	4	8	1.04	0.84	1.29	0.13	472	31.97
20	NAGS5T1	Nghe An	Sandy	Groundnut	1	0.42	4	7	1.09	0.84	1.77	0.18	688	41.64
21	NAGC10T1	Nghe An	Clay loam	Groundnut	1	0.42	5	8	0.98	0.67	1.35	0.19	574	51.92
22	NAGC12T1	Nghe An	Clay loam	Groundnut	1	0.35	6	10	1.47	1.00	2.05	0.27	184	25.61
23	NAGC15T1	Nghe An	Clay loam	Groundnut	1	0.41	5	8	1.14	0.75	1.69	0.22	464	35.90
24	NAGC18T1	Nghe An	Clay loam	Groundnut	1	0.35	6	10	1.23	0.99	1.61	0.15	282	25.13
25	NAGC1T1	Nghe An	Clay loam	Groundnut	1	0.39	5	8	1.12	0.79	1.40	0.16	538	34.52
26	NAGC21T1	Nghe An	Clay loam	Groundnut	1	0.38	4	7	1.09	0.87	1.44	0.14	564	39.42
27	NAGC22T1	Nghe An	Clay loam	Groundnut	1	0.35	5	8	1.08	0.87	1.40	0.12	542	43.80
28	NAGC2-2T1	Nghe An	Clay loam	Groundnut	1	0.35	5	7	1.02	0.76	1.45	0.13	605	29.17
29	NAGC27T1	Nghe An	Clay loam	Groundnut	1	0.41	5	8	1.00	0.75	1.20	0.13	880	38.23
30	NAGC9T1	Nghe An	Clay loam	Groundnut	1	0.37	6	9	1.23	0.75	1.59	0.20	337	27.75
31	NAGC4T1 *	Nghe An	Clay loam	Groundnut	2	0.32	11	16	1.91	0.85	3.42	0.58	105	45.65
32	NAGC4T2 *	Nghe An	Clay loam	Groundnut	2	0.28	11	16	2.24	1.08	3.39	0.73	79	49.02
33	NAGC13T1	Nghe An	Clay loam	Groundnut	1	0.35	5	8	1.05	0.80	1.37	0.14	532	26.76
34	NAGC7T1 *	Nghe An	Clay loam	Groundnut	2	0.35	8	11	1.97	1.20	3.19	0.53	110	42.89
35	NATC1T1 *	Nghe An	Clay loam	Tomato	1	0.42	4	7	1.15	0.74	1.41	0.16	626	53.66
36	QNGS38-2T1	Quang Nam	Sandy	Groundnut	1	0.46	5	8	1.17	0.79	1.50	0.18	470	29.48
37	QNGS52T1	Quang Nam	Sandy	Groundnut	1	0.45	5	8	1.20	0.93	1.56	0.16	428	43.90
38	QNGS1T1	Quang Nam	Sandy	Groundnut	1	0.40	5	8	1.14	0.81	1.41	0.16	664	23.75
39	QNGC86T1	Quang Nam	Sandy	Groundnut	3	0.45	4	8	0.90	0.53	1.20	0.13	696	41.76
40	QNGS17T1	Quang Nam	Sandy	Groundnut	1	0.38	5	8	1.11	0.70	1.53	0.19	546	34.69
41	QNGS23T1	Quang Nam	Sandy	Groundnut	1	0.40	4	8	1.08	0.76	1.28	0.13	656	49.99
42	QNGS2T1	Quang Nam	Sandy	Groundnut	1	0.42	5	8	1.12	0.82	1.50	0.16	548	31.94
43	QNGS7T1	Quang Nam	Sandy	Groundnut	1	0.40	5	8	1.12	0.79	1.52	0.20	474	35.19
44	QNGS38T1	Quang Nam	Sandy	Groundnut	1	0.39	5	8	1.07	0.83	1.39	0.16	604	38.85
45	QNGS40T1	Quang Nam	Sandy	Groundnut	1	0.38	5	8	1.07	0.72	1.31	0.16	672	34.61
46	QNGS13T1	Quang Nam	Sandy	Groundnut	3	0.43	4	7	1.09	0.83	1.31	0.14	822	28.64
47	QNGS3T1	Quang Nam	Sandy	Groundnut	1	0.40	5	8	1.11	0.85	1.49	0.18	646	41.15
48	QNGS8T1	Quang Nam	Sandy	Groundnut	1	0.44	4	7	1.09	0.69	1.52	0.22	430	31.86
49	QNMS25T1 *	Quang Nam	Sandy	Taro	1	0.48	4	7	1.14	0.76	1.48	0.16	492	38.19
50	QNTS13T1 *	Quang Nam	Sandy	Tomato	3	0.42	4	7	0.95	0.78	1.15	0.08	978	22.77
51	QNGC002-2T1	Quang Nam	Clay loam	Groundnut	3	0.43	4	7	1.00	0.78	1.24	0.12	808	41.87
52	QNGC17T1	Quang Nam	Clay loam	Groundnut	3	0.37	4	8	1.10	0.95	1.40	0.12	844	56.00
53	QNGC61T1	Quang Nam	Clay loam	Groundnut	1	0.39	4	8	1.19	0.86	1.42	0.15	614	32.98
54	QNGC73T1	Quang Nam	Clay loam	Groundnut	1	0.35	5	8	1.21	0.89	1.48	0.15	544	35.96
55	QNGC19-2T1	Quang Nam	Clay loam	Groundnut	1	0.40	4	7	1.11	0.83	1.29	0.12	578	37.99
56	QNGC49T1	Quang Nam	Clay loam	Groundnut	1	0.43	4	7	1.09	0.71	1.59	0.19	592	46.54
57	QNGC003T1	Quang Nam	Clay loam	Groundnut	3	0.33	5	11	1.72	1.04	2.54	0.41	136	58.82
58	QNGC16T1	Quang Nam	Clay loam	Groundnut	1	0.79	5	8	1.09	0.84	1.39	0.14	360	31.84
59	QNGC26T1	Quang Nam	Clay loam	Groundnut	1	0.34	5	7	0.96	0.77	1.33	0.13	682	42.38
60	QNGC28T1	Quang Nam	Clay loam	Groundnut	3	0.46	4	8	1.04	0.59	1.25	0.16	522	33.37
61	QNGC3T1	Quang Nam	Clay loam	Groundnut	1	0.43	4	7	0.96	0.81	1.21	0.09	716	27.20
62	QNGC5T1 *	Quang Nam	Clay loam	Groundnut	1	0.38	4	8	1.05	0.88	1.35	0.11	754	34.00
63	QNGC6T1	Quang Nam	Clay loam	Groundnut	1	0.39	5	8	1.10	0.77	1.34	0.13	732	37.55

(*continued on next page*)

[a] Isolates used in the pathogenicity assays are indicated with an asterisk; isolates from the remote upland area of Nghe An province are underlined.
[b] Average (Avg), minimum (Min), maximum (Max), and standard deviation (SD).
[c] Internal transcribed spacer (ITS) group.
[d] Mycelial growth rate during 48 h (mm/h).
[e] Time to produce sclerotia.
[f] Time for maturation of the sclerotia.
[g] Number of sclerotia per plate.
[h] Sensitivity to tebuconazole at 0.02 mg/liter (percent inhibition).

Table 4. (*continued from preceding page*)

Order	Code	Origin			ITS[c]	Rate (mm/h)[d]	Time (days)		Diameter of sclerotia (mm)[b]				Sclerotia[g]	Inhib. (%)[h]
		Province	Soil	Host			Produce[e]	Mature[f]	Avg	Min	Max	SD		
64	QNGC7T1	Quang Nam	Clay loam	Groundnut	1	0.39	5	8	1.18	0.88	1.49	0.14	546	37.03
65	QNGC9T1	Quang Nam	Clay loam	Groundnut	3	0.41	4	7	0.96	0.74	1.56	0.18	844	48.56
66	QNGC22T1	Quang Nam	Clay loam	Groundnut	1	0.45	5	8	1.15	0.81	1.67	0.23	530	51.76
67	HUGS12T1	Thua Thien Hue	Sandy	Groundnut	1	0.46	7	11	1.07	0.86	1.41	0.15	520	38.59
68	HUGS11T2	Thua Thien Hue	Sandy	Groundnut	1	0.42	5	8	1.04	0.73	1.36	0.14	582	27.29
69	HUGS13T1	Thua Thien Hue	Sandy	Groundnut	1	0.37	5	8	1.09	0.83	1.28	0.11	638	45.72
70	HUGS14T1	Thua Thien Hue	Sandy	Groundnut	1	0.38	4	8	1.16	0.82	1.36	0.12	616	39.74
71	HUGS16-1T1	Thua Thien Hue	Sandy	Groundnut	3	0.46	4	7	1.05	0.66	1.44	0.18	652	49.36
72	HUGS1T1	Thua Thien Hue	Sandy	Groundnut	1	0.41	4	8	1.33	0.87	1.60	0.17	368	19.63
73	HUGS6T1	Thua Thien Hue	Sandy	Groundnut	1	0.40	4	7	1.03	0.76	1.23	0.12	726	43.08
74	HUGS4T1	Thua Thien Hue	Sandy	Groundnut	1	0.42	4	7	1.09	0.59	1.33	0.19	488	40.58
75	HUGS5T1	Thua Thien Hue	Sandy	Groundnut	1	0.39	5	8	1.05	0.64	1.44	0.20	578	37.77
76	HUGS16-2T1	Thua Thien Hue	Sandy	Groundnut	1	0.45	4	7	1.14	0.80	1.36	0.13	476	27.17
77	HUGS7T1	Thua Thien Hue	Sandy	Groundnut	1	0.40	4	8	1.08	0.71	1.41	0.17	930	27.78
78	HUGS18T1	Thua Thien Hue	Sandy	Groundnut	1	0.41	4	7	0.90	0.70	1.25	0.15	932	36.81
79	HUGS18T2	Thua Thien Hue	Sandy	Groundnut	1	0.42	4	7	1.03	0.83	1.35	0.14	954	55.54
80	RH001	Thua Thien Hue	Sandy	Groundnut	1	0.42	4	7	1.14	0.89	1.57	0.18	508	38.96
81	H001	Thua Thien Hue	Sandy	Groundnut	1	0.45	5	8	1.23	0.76	1.55	0.17	338	23.42
82	HUMC22T1 *	Thua Thien Hue	Sandy	Taro	1	0.40	4	7	1.16	0.85	1.70	0.18	495	22.27
83	HUTS1T2 *	Thua Thien Hue	Sandy	Tomato	1	0.41	4	7	1.05	0.68	1.41	0.12	562	49.78
84	HUGC10-1T1	Thua Thien Hue	Clay loam	Groundnut	1	0.42	5	8	1.17	0.74	1.46	0.20	314	38.90
85	HUGC10-2T1	Thua Thien Hue	Clay loam	Groundnut	1	0.33	5	8	1.09	0.79	1.32	0.12	516	35.37
86	HUGC15T1 *	Thua Thien Hue	Clay loam	Groundnut	1	0.43	4	7	0.98	0.67	1.28	0.15	752	29.98
87	HUGC16T2	Thua Thien Hue	Clay loam	Groundnut	1	0.48	4	7	1.02	0.69	1.22	0.11	734	22.73
88	HUGC17T1	Thua Thien Hue	Clay loam	Groundnut	1	0.44	4	8	0.99	0.69	1.26	0.13	880	23.88
89	HUGC19T1 *	Thua Thien Hue	Clay loam	Groundnut	1	0.44	4	7	1.02	0.85	1.20	0.10	876	31.16
90	HUGC21T1	Thua Thien Hue	Clay loam	Groundnut	1	0.37	5	8	1.06	0.72	1.40	0.19	830	25.59
91	HUGC24T1	Thua Thien Hue	Clay loam	Groundnut	1	0.35	5	8	1.16	0.86	1.48	0.15	342	34.11
92	HUGC26T1	Thua Thien Hue	Clay loam	Groundnut	1	0.36	5	8	1.06	0.76	1.38	0.16	632	43.57
93	HUGC27T1	Thua Thien Hue	Clay loam	Groundnut	1	0.40	5	8	1.15	0.77	1.37	0.15	516	18.22
94	HUGC27T2	Thua Thien Hue	Clay loam	Groundnut	1	0.45	5	8	1.20	0.82	1.46	0.17	576	39.80
95	HUGC29T1	Thua Thien Hue	Clay loam	Groundnut	1	0.43	4	7	0.88	0.74	1.17	0.10	1042	38.41
96	HUGC30T1	Thua Thien Hue	Clay loam	Groundnut	1	0.42	5	8	0.95	0.70	1.15	0.10	926	37.67
97	HUGC4T1	Thua Thien Hue	Clay loam	Groundnut	1	0.47	4	7	0.96	0.80	1.20	0.11	1,020	31.73
98	HUGC7T1	Thua Thien Hue	Clay loam	Groundnut	1	0.33	5	8	0.97	0.63	1.31	0.20	868	30.59
99	HUGC7T2	Thua Thien Hue	Clay loam	Groundnut	1	0.41	5	8	0.93	0.66	1.39	0.15	724	29.21
100	HUGC7T3	Thua Thien Hue	Clay loam	Groundnut	1	0.43	5	8	1.01	0.65	1.21	0.15	702	43.07
101	HUGC9T1	Thua Thien Hue	Clay loam	Groundnut	1	0.41	5	8	1.12	0.78	1.53	0.15	642	41.21
102	HUMC20T1 *	Thua Thien Hue	Clay loam	Taro	1	0.42	5	8	1.02	0.62	1.46	0.20	635	37.35
103	HUTC1T1 *	Thua Thien Hue	Clay loam	Tomato	1	0.45	4	7	1.23	0.88	1.60	0.19	492	46.75

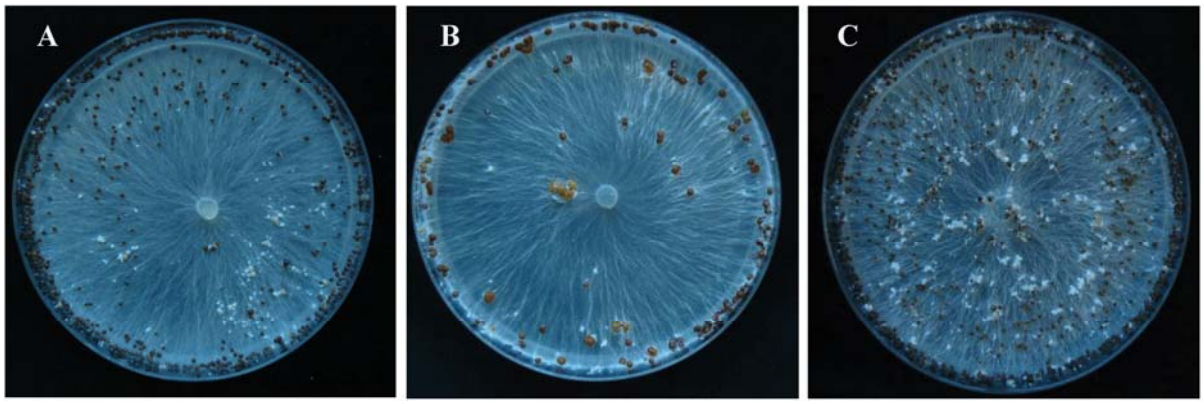

Fig. 5. Phenotypic characteristics of mycelium and sclerotia of three *Sclerotium rolfsii* isolates belonging to internal transcribed spacer ribosomal DNA groups 1 **(A)**, 2 **(B)**, and 3 **(C)**. A 5-mm-diameter mycelial agar plug was inoculated in the center of a 9-cm-diameter potato dextrose agar plate and incubated at 25°C. All isolates have the typical white-silky mycelium with round or irregular, light- to dark-brown sclerotia.

populations in groundnut fields may help to adopt and develop effective and sustainable control measures. Our field survey in central Vietnam in 2009 showed that the incidence of stem rot disease of groundnut caused by *S. rolfsii* was 5 to 25%. This is similar to that reported in Georgia from 1983 to 1985 (i.e., approximately 8.5%) (8) but much higher than the disease incidence reported in Texas in 1992 and 1993 (less than 1%) (39). On the other hand, it was lower than in Ibaraki (Japan) where disease incidence was 10 to 40% (25). The observed gradient in stem rot disease incidence across central Vietnam, with an increase in disease incidence from the northern to the southern field sites, might be related to the effect of temperature on disease development (28). Important to note is that the field sites surveyed in the two southern provinces were sites where tomato and taro are also cultivated. Both crops are hosts of *S. rolfsii* and may have contributed to the build-up of pathogen inoculum. For two of the four provinces (i.e., locations B and C), the

disease incidence of groundnut plants cultivated in clay loam soil was significantly higher than in sandy soil. This difference may be related, in part, to the higher organic content of the clay loam soil, a characteristic that is known to support germination of sclerotia and subsequent hyphal growth toward the host plant (28).

Phylogenetic analysis of the ITS-rDNA sequences revealed three main groups among the 103 isolates from central Vietnam. These results suggest that the *S. rolfsii* population in groundnut fields in central Vietnam is relatively uniform. However, ITS-rDNA sequencing does not give detailed insight into the intraspecific diversity. For *S. rolfsii*, several other techniques and molecular markers have been used to assess intraspecific diversity, especially random amplified polymorphic DNA (RAPD) analysis (32). Multiples of these tests, aimed at characterizing the intraspecific diversity of the isolates, were performed in this study with an initial seven *S. rolfsii* isolates from the collection (i.e., three isolates from Vietnam and four reference strains from culture collections). However, RAPD analysis, as well as repetitive element PCR and enterobacterial repetitive intergenic consensus PCR (22), gave inconsistent or irreproducible results and were not considered useful. Therefore, a range of other traits, including pathogenicity, growth rate, sclerotial characteristics, mycelial compatibility, and tebuconazole sensitivity, were analyzed to get more insight into the intraspecific diversity of the collection of *S. rolfsii* isolates from central Vietnam.

On average, isolates from ITS groups 1, 2, and 3 showed no significant differences in growth rate but exhibited considerable variation in the time to form sclerotia, their maturation time, and their number and size. These results confirm and extend results obtained in previous studies on *S. rolfsii* isolates from other crops and geographic locations (18,27,29,45). For example, the number of sclerotia produced by *S. rolfsii* isolates from chickpea ranged from 150 to 1,210 and their diameter from 0.5 to 2.0 mm (18). Similar degrees of variation in sclerotial characteristics were found by Xu et al. (45) for *S. rolfsii* isolates from different hosts and geographic locations. The ITS group 2 isolates from this study produced substantially fewer sclerotia per plate; also, the average maturation time and sclerotial diameter were approximately two times greater than for isolates of ITS groups 1 and 3. These characteristics combined with the light brown color of the sclerotia of the three ITS group 2 isolates were also reported for isolates classified morphologically or by LSU sequencing as *S. rolfsii* var. *delphinii* (3,29,45).

For several fungi, mycelial compatibility is used to get insight into the genetic relatedness of isolates or to identify intraspecific variation within field populations of plant pathogens (17). When confronted with one another, a demarcation or barrage zone between the fungal colonies places these isolates in different MCGs. Isolates in the same MCG can be clonal (17) or generally exhibit a higher degree of genetic relatedness than isolates from different MCGs (33,44). In a study by Punja and Sun (32) on *S. rolfsii* isolates from 13 countries and 36 different host species, 71 MCGs were found and there was no clear relationship between the original host plant and the MCG, except for *S. rolfsii* isolates from turfgrass. Within each of the three ITS groups identified in our study, a high variation in mycelial compatibility was observed. For isolates of ITS group 1, 91 combinations were tested and only 6.6% of these combinations showed a compatible interaction, pointing to at least nine MCGs. In contrast, studies by Adandonon et al. (1) on *S. rolfsii* from cowpea fields in Benin revealed four MCGs among a total of 66 isolates. Similarly, Okabe and Matsumoto (25) found four MCGs among a total of 132 isolates from groundnut fields in Ibaraki (Japan) and concluded, based on RAPD analysis, that many isolates were clonal. Based on the MCG analysis, our results suggest that *S. rolfsii* populations from groundnut fields in central Vietnam are more diverse. This may be partly due to the fact that the fields sampled in our study are far apart (i.e., approximately 400 km from the northern fields sites to the southern field sites compared with only 2.5 km in the study by Okabe and Matsumoto; 25). It should be emphasized, however, that mycelial compatibility refers to macroscopic observations in which hyphae of different isolates readily intermingle without forming a barrage or inhibition zone. Therefore, the MCGs found here cannot automatically be considered as vegetative compatibility groups or anastomosis groups, because anastomosis and heterokaryon formation was not confirmed microscopically.

The results further showed small variations in tebuconazole sensitivity of *S. rolfsii* isolates from groundnut fields in central Vietnam. This is substantially less than reported earlier by Franke et al. (14) for the *S. rolfsii* population from Georgia. The three isolates from ITS group 2, collected from the remote upland region, were significantly more sensitive to tebuconazole than those from ITS group 1. Although this may be due, in part, to a sample size difference, it may also be linked to the fact that farmers in this remote area do not use tebuconazole. In the other farmer fields surveyed, however, no records are kept on fungicide use; therefore, for the fields included in this study, no correlation can be established between sensitivity and fungicide use. Franke et al. (14) found a correlation between reduced sensitivity to tebuconazole among isolates from fields with a history of repeated applications of this fungicide. In Vietnam, tebuconazole is used to control *S. rolfsii* but not on a regular basis or a large scale due to its relatively high costs for subsistence farmers.

In conclusion, the work presented here is the first study on the distribution, incidence, and diversity of *S. rolfsii* populations in groundnut fields in central Vietnam. *S. rolfsii* isolates originating from groundnut, tomato, and taro were all pathogenic on groundnut and displayed substantial diversity of various genetic and phenotypic traits, including mycelial compatibility, growth rate, and sclerotial characteristics. The observation that the *S. rolfsii* isolates tested were all relatively sensitive to tebuconazole provides opportunities to use this fungicide to control stem rot disease of groundnut in central Vietnam. Combination with other control measures, including biological control, is recommended to prevent resistance development, as was observed previously for other *S. rolfsii* populations exposed repeatedly to this fungicide.

Table 6. Sensitivity to tebuconazole of *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam[a]

ITS[b]	Growth inhibition (%) (mean ± SD)[c]
1	35.5 ± 8.7
2	45.9 ± 3.1
3	42.7 ± 11.6

[a] The mean inhibition of radial mycelial growth was determined at the tebuconazole concentration of 0.02 mg liter^{-1} of potato dextrose agar. The relative inhibition of mycelial growth was calculated based on the formula inhibition (%) = 100 – [(colony diameter on amended medium/colony diameter on control) × 100%].
[b] Internal transcribed spacer (ITS) group.
[c] SD = standard deviation.

Table 5. Mycelial compatibility between *Sclerotium rolfsii* isolates for each of the three identified internal transcribed spacer (ITS) groups

ITS group	Number of isolates	Number of combinations	Compatible combinations	Compatibility (%)	Number of MCGs[a]
1	14	91	6	6.6	9
2	3	3	1	33.3	2
3	9	36	4	11.1	6

[a] Mycelial compatibility groups.

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