

RESEARCH NOTE

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A New Record and Characterization of Asparagus Purple Spot Caused by *Stemphylium vesicarium* in Korea

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ABSTRACT

In 2017, small, elliptical, brownish purple spots on spears and ferns of asparagus were found in fields of Gangwon-do. The isolated fungal species was identified as an ascomycete *Stemphylium vesicarium* based on morphological characteristics and molecular phylogenetic analyses including nucleotide sequences of the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome b (cytb). A pathogenicity test revealed that *S. vesicarium* was the causal agent of purple spot disease on asparagus. The occurrence of purple spots caused by *S. vesicarium* on asparagus is the first report in Korea.

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Asparagus (*Asparagus officinalis* L.) in the family Asparagaceae is a spring perennial vegetable, which is rich in vitamins and minerals [1]. Asparagus also contains anti-inflammatory nutrients and a variety of antioxidant nutrients, including vitamin C, beta-carotene, vitamin E, mineral zinc, manganese, and selenium [2]. Therefore, asparagus has been used as a botanical drug for thousands of years [3]. In recent years, beneficial effects of asparagus on health have been uncovered [4,5]. Its consumption as medicine and food is continuously increased [6]. In 2016, asparagus has produced about 8.73 million tons per year in an area of 1.53 million ha worldwide. China produced about 7.68 million tons, accounting for about 88% of the total world production of asparagus, followed by Peru with 0.38 million tons and Mexico with 0.22 million tons [7]. Asparagus is mainly growing in Gangwon province in Korea, but domestic consumption relies primarily on imports having a marginal domestic production.

Tender young shoots (spears) of asparagus are commonly eaten as a vegetable, emerging out in spring season from underground root system (crown). Asparagus lives for up to 20 years so that good soil preparation before planting and disease management after planting are unavoidable for sustainable cultivation. Fungal diseases of asparagus are reported in many parts of the world, which include anthracnose caused by *Colletotrichum gloeosporioides* [8], leaf blight by *Cercospora asparagi* [9], crown-root rot and spear spot by *Fusarium oxysporum* [10], *Fusarium proliferatum* [11], gray mold by

Botrytis cinerea [12], spear and crown spot by *Phytophthora megasperma* [13], purple spot by *Stemphylium botryosum* [14] and *Stemphylium vesicarium* [15], rust by *Puccinia asparagi* [16]. Fungal pathogens of asparagus reported in Korea are *Cercospora asparagi*, *Phoma asparagi*, *F. oxysporum*, *F. oxysporum* f. sp. *asparagi*, *Colletotrichum* sp. and *B. cinerea* [17,18]. Although the incidence and prevalence of fungal disease have increased in asparagus plants in Korea, detailed information for fungal disease, frequency, diagnosis, and management is currently absent together with lack of fungicides and biofungicides applicable to fungal diseases in asparagus.

Among fungal diseases of asparagus, purple spot caused by *S. vesicarium* becomes a severe problem. Asparagus purple spot develops on emerging spears in spring and occurs ferns in summer destroying stem, branches, and leaves, which result in reduction of the flow of carbohydrates to the roots and lowering next year yields [19,20]. Symptoms of asparagus purple spot are reported to be small (1–2 mm), elliptical, slightly sunken, and brownish purple spots that blemished the spears, damaging marketability during the harvest season [21]. In the wet season during summer of 2017, severe symptoms of purple spots occurred on asparagus ferns in Chuncheon and Yanggu in Gangwon-do, South Korea. The disease appears as numerous, slightly sunken, purplish spots with brown centers occurring on asparagus ferns (Figure 1(A)). Infected tissues were taken to the laboratory, and isolated 24 strains. Surfaces of

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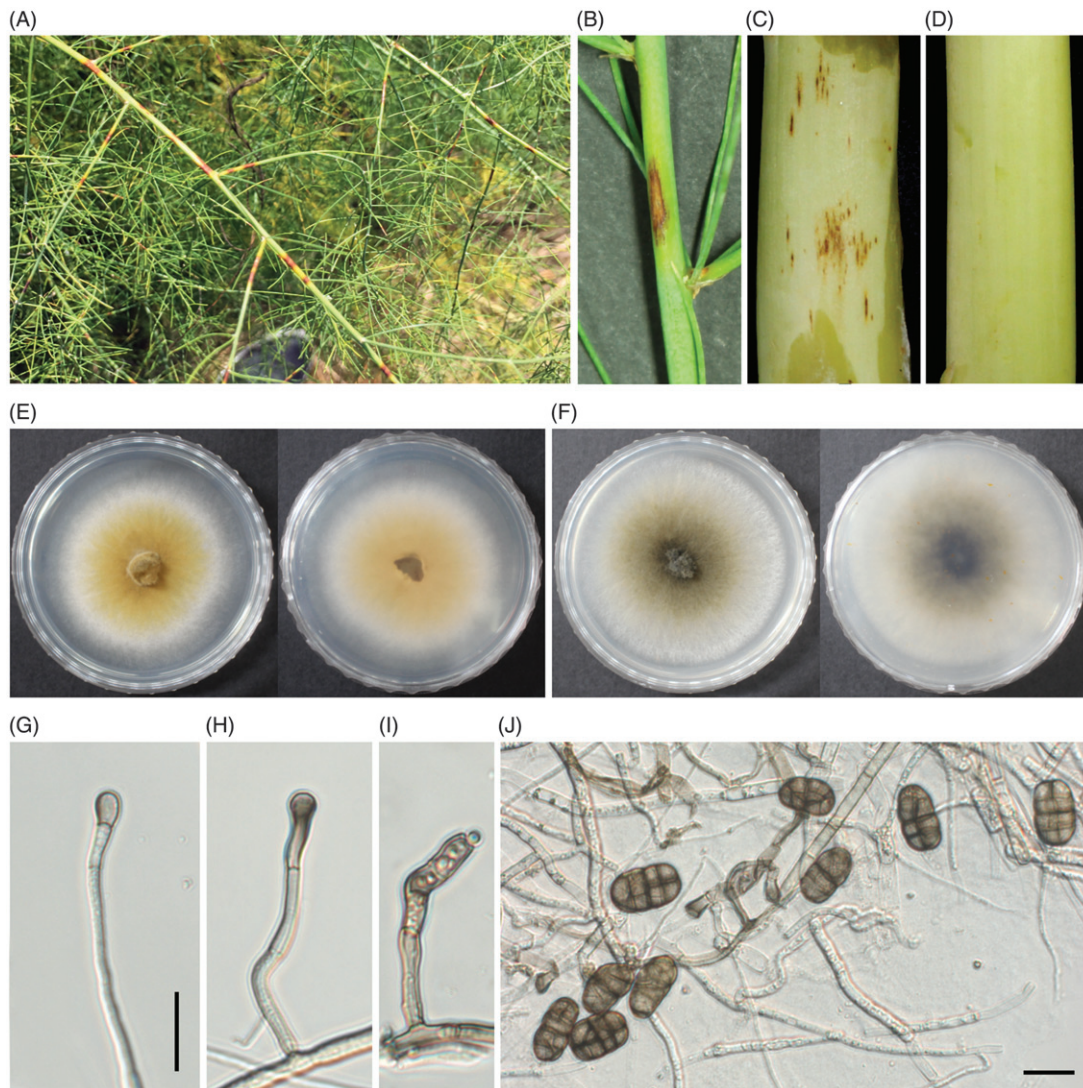


Figure 1. Purple spot caused by *Stemphylium vesicarium* on asparagus. (A) Symptoms of purple spot of asparagus ferns in field; (B,C) Signs of the causal fungus of purple spot on asparagus fern and spear by artificial inoculation; (D) Control of non-inoculation; (E,F) Fungal mycelial colonies on PDA and V8 juice agar plates (left, front view; right, back view); (G–I), Conidiophores; and (J) Morphological characteristics of conidia. Scale bars = 10 μ m.

infected tissues were sterilized by 1% sodium hypochlorite (NaOCl) for 1 min, washed twice with sterilized distilled water, dried on sterilized filter paper, and placed into plates containing potato dextrose agar (PDA; BD Difco, Franklin Lakes, NJ) supplemented with 100 ppm ampicillin. The plates were then incubated at 25 °C for 5 days in a 16 h light of cool white and 8 h dark chamber, emerged fungal hyphae were transferred to new PDA plates. Single spore isolation was conducted for identification and storage of isolates at –70 °C in 20% glycerol for further study. A representative isolate, KNU1709YG was deposited (CFGR 2018-120-00001) at the Center for Fungal Genetic Resources (CFGR) at Seoul National University, Korea.

To determine pathogenicity of a representative isolate, KNU1709YG, we performed artificial inoculation on spears and ferns of asparagus. In brief, Conidia of KNU1709YG were harvested from PDA culture plates grown for 7 days. Spears and ferns of

asparagus grown in Yanggu were sterilized with 1% NaOCl for 2 min and washed twice with sterilized distilled water. Conidia suspension (1×10^5 conidia/mL) was sprayed on asparagus spears and placed in a humid box at 25 °C. The control was sprayed the distilled water. As a result, tan-to-brown sunken and elliptical early common lesions appeared in the ferns and spears after 7 days (Figure 1(B,C)). The symptom was identical to that caused by *S. vesicarium*, and the fungus was subsequently re-isolated from the lesions. However, no symptom was observed in the control (Figure 1(D)). This experiment was repeated three times and the same results were obtained.

When the fungus was cultured at room temperature under white fluorescent light with 16 h photoperiod, colors of mycelial colonies appeared light brown and dark green after 7 days on PDA and V8 medium, respectively (Figure 1(E,F)). The mycelial growth rate was 6 cm on both PDA and V8 medium

Table 1. Morphological characteristics of *Stemphylium vesicarium* isolated in this study.

Characteristic		KNU1709YG	<i>Stemphylium vesicarium</i> (Wallr.) E.G. Simmons ^a
Colony	Color	Light brown on PDA Dark green on V8	Light brown on PCA
	Size	6.0 cm after 7 days	6.0 cm after 7 days on PCA
Hyphae	Color	Pale brown	Pale brown
	wide	4–7 μ m	5–7 μ m
Conidiophore	Shape	Swollen	Swollen
	Color	Dark brown	Dark brown
	Wide	6–7 μ m	6–8 μ m
Conidia	Color	Dark brown	Dark brown
	Shape	Oblong or broadly oval, sometimes inequilateral	Oblong or broadly oval, sometimes inequilateral
	Septate	Transverse 1–6 longitudinal 1–3	Transverse 1–6 longitudinal 1–3
	Size	22–38 \times 13–18 μ m	25–42 \times 12–22 μ m

PCA: potato carrot agar; PDA: potato dextrose agar; V8: V8 juice agar

^aSources of description [15].

for a week. Hyphae were pale brown in color, and 4–7 μ m in width. Conidiophore was swelling and developed dark-brown conidia (Figure 1(G–I)). Conidia were oblong or broadly oval, 22–38 \times 13–18 μ m in size, having inequilaterally, 1–6 transverse septa and 1–3 longitudinal septa per transverse sector (Figure 1(J)). The length of the fungal tissues was measured using a Carl Zeiss Axio Imager A2 microscope (Carl Zeiss Microscope Division, Oberkochen, Germany). Morphological characteristics were summarized in Table 1. As a result, it was found that KNU1709YG was morphologically identical to the fungus *S. vesicarium* [22]. The genus *Stemphylium* was first reported in 1833 [23] with *Stemphylium botryosum* (Teleomorph: *Pleospora tarda*) as the type species. *Stemphylium* is a dematiaceous hyphomycete, which can be distinguished from other hyphomycetes forming phaeodictyo spores based on the percurrent rejuvenation of its conidiophores, and apically swollen conidiogenous cells [24]. Identification of *Stemphylium* species has relied on morphological characters such as variation in conidium, conidiophore, and ascospore morphology. However, accurate identification of species in *Stemphylium* is difficult due to overlapped morphological characters. Furthermore, the sexual morph *Pleospora* of *Stemphylium* is known to be polyphyletic [25]. Therefore, species identification based exclusively on morphological data was not feasible. Combined morphological and molecular data are necessary for unambiguous identification of species in *Stemphylium*. Recently, a multi-gene phylogenetic analysis of *Stemphylium* species reveals new species [24].

For a precise species identification, morphological observation of KNU1709YG was combined with molecular analysis. First, genomic DNA of KNU1709YG was extracted using the quick DNA methods [26,27]. Sequence 18s rRNA of internal transcribed spacer (ITS) was amplified using primers ITS1 and ITS4 [28]. Sequence of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was

amplified using primers GPD1 and GPD2 [29]. Conditions for PCR amplification were described by Graf et al. [29]. The PCR products were purified according to the MEGA quick-spin total fragment DNA purification kit (iNtRON Bio Technology, Daejeon, South Korea) and sequenced with the same primers. The resulting sequences (GenBank accession numbers MK073013 and MK105974) were compared to all available fungal sequence data in the NCBI-GenBank database using the BLAST search tool [30]. Compared with the sequences of *S. vesicarium* of GenBank, the analyzed sequence showed 100% homology with the *GAPDH* sequence (GenBank accession No. KU850710, KU850723, and KU850735), 99% with the ITS sequences (GenBank accession No. KU850563, KU850576, and KU850588). The multi-locus sequences were aligned with closely related strains by ClustalW [31]. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7 [32] (Figure 2). The multi-gene phylogenetic tree using two genes (*ITS* and *GAPDH*) was very similar to that previously done by Woudenberg et al. [24], suggesting that the isolate KNU1709YG may be *S. vesicarium* (Table 2).

Cytochrome b gene in mitochondrial DNA is commonly used to determine phylogenetic relationships between species due to difference in structure and sequence [33–35]. Two causal agents of the asparagus purple spots, *S. vesicarium* and *S. botryosum*, are differentiated with the intron-exon structure of the *cytb* gene, in which many isolates initially classified as *S. botryosum* are also identified as *S. vesicarium* in Germany [29]. In Korea, there is a short report that *S. botryosum* caused the purple spot of asparagus, which suggests that examination of exact identification and abundance of the two species *S. botryosum* and *S. vesicarium* in asparagus would be necessary with collections of more isolates in a future study. *Stemphylium lycopersici*, also known for causing leaf spot in crops [36,37] was revealed to be differentiated from *S. vesicarium* and

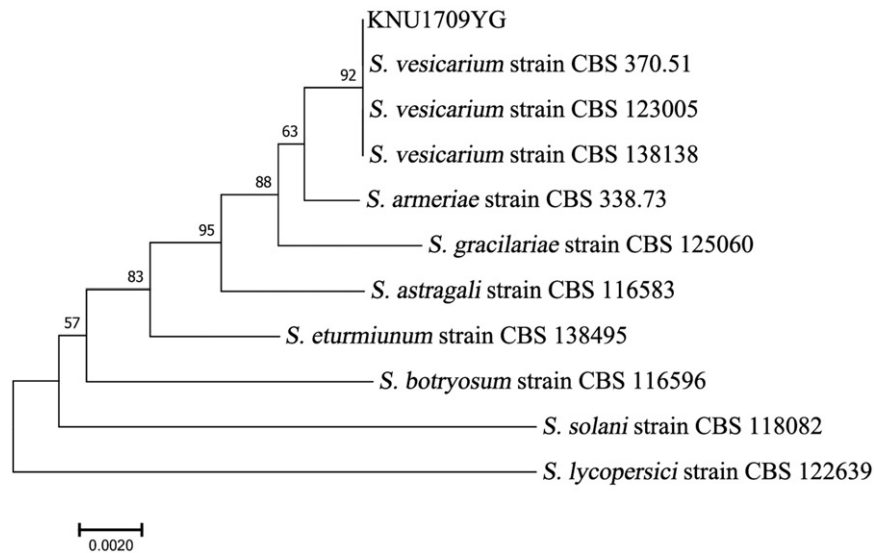


Figure 2. The phylogenetic tree based on combined of ITS and GAPDH gene sequences of *Stemphylium vesicarium* and other *Stemphylium* spp. DNA sequences from the GenBank were aligned using the ClustalW program in MEGA 7.0 and constructed using the neighbor-joining method with 1000 bootstrap replicates. The scale bar indicates the number of differences in nucleotide substitutions per sequences.

Table 2. Species and sequences used for phylogenetic analyses.

Species	Isolate/strain	GenBank accession number (identity %)	
		ITS(%)	GAPDH(%)
<i>Stemphylium vesicarium</i>	CBS 370.51 ^a	KU850563 (99)	KU850710 (100)
<i>Stemphylium vesicarium</i>	CBS 123005	KU850576 (99)	KU850723 (100)
<i>Stemphylium vesicarium</i>	CBS 138138	KU850588 (99)	KU850735 (100)
<i>Stemphylium armeriae</i>	CBS 338.73	KU850511 (99)	KU850658 (99)
<i>Stemphylium gracilariae</i>	CBS 125060	KU850552 (99)	KU850699 (99)
<i>Stemphylium astragali</i>	CBS 116583	KU850512 (99)	KU850659 (98)
<i>Stemphylium eturmiunum</i>	CBS 138495	KU850546 (99)	KU850694 (98)
<i>Stemphylium botryosum</i>	CBS 116596	KU850538 (99)	KU850685 (97)
<i>Stemphylium solani</i>	CBS 118082	KU850628 (99)	KU850769 (96)
<i>Stemphylium lycopersici</i>	CBS 122639	KU850611 (98)	KU850756 (96)

^aCBS: culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands.

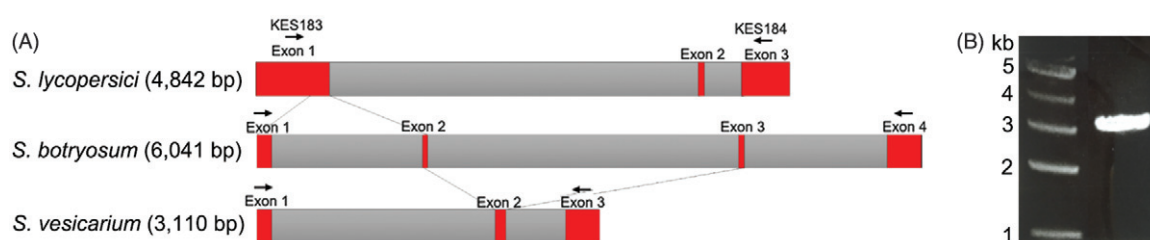


Figure 3. *Stemphylium* cytochrome *b* gene. (A) Structural organization of the three *Stemphylium* cytochrome *b* genes and (B) *cytb* amplicon of 3 kb from KNU1709YG using primer set KES183 and KES184.

S. botryosum, based on the structure of the *cytb* gene [38] (Figure 3(A)). By analyzing the sequence and structure of the *cytb* gene (3 kb) of the isolate KNU1709YG that was amplified with a primer set of KES183 and KES184, the isolate KNU1709YG was revealed to form a clade with *S. vesicarium* (Figure 3(B)). Taken together with symptoms, pathogenicity by artificial inoculation, morphology and molecular analysis, we were able to confirm

that the isolate KNU1709YG was *S. vesicarium* and causal agent of purple spot of asparagus. Although purple spot on asparagus caused by *S. vesicarium* was reported in other countries including Germany and Australia [29,39], the occurrence of asparagus purple spots caused by *S. vesicarium* in Korea is the first report as we know. Further analyses including pathogenicity, control measures, and genetics of *S. vesicarium* population will provide an integrated

strategy for the management of asparagus purple spot.

Disclosure statement

No potential conflict of interest was reported by the authors.

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