



## Dieback of rose caused by *Acremonium sclerotigenum* as a new causal agent of rose dieback in Iran

Maryam Mirtalebi, Zia Banihashemi, Fatemeh Sabahi and Hamze Mafakheri

Shiraz University, College of Agriculture, Department of Plant Protection, Shiraz, Iran

### Abstract

Severe dieback of rose has been recently observed in several rose greenhouses in Fars province of Iran. During 2014 and 2015, stems of rose plants showing yellow to brown discoloration and dieback were collected from rose greenhouses. *Coniothyrium fuckelii*, *Botrytis cinerea* and *Acremonium* were subsequently isolated from the margin between healthy and symptomatic tissue. *B. cinerea* and *C. fuckelii* isolates were similar to those previously reported for dieback of rose worldwide. Morphological and cultural characters along with molecular analysis based on partial sequences of the internal transcribed spacer (ITS) region of the ribosomal RNA genome allowed confirming the affiliation of the *Acremonium* isolates, corresponding to *A. sclerotigenum* as a new causal agent of rose dieback. To determine its pathogenicity on rose, Koch's postulates were fulfilled by stem inoculation of nine rose cultivars under greenhouse conditions. While *A. sclerotigenum* is considered as a soil-born pathogen, and produces sclerotia that are resistant to adverse conditions enables the fungus to survive extended period in soil, propagule trapping in our study revealed that conidia can become airborne, imply that an aerial phase, forms an important component of the disease cycle.

**Additional key words:** pathogenicity; spore trapping; airborne; growth rate; spore germination.

**Abbreviations used:** ITS (internal transcribed spacer region of the ribosomal RNA genome); MEA (malt extract agar); OA (oatmeal agar); PDA (potato dextrose agar); TMN-PDA (trimethylnonanol-potato dextrose agar); WA (water agar).

**Authors' contributions:** Conceived and designed the experiments, and wrote the paper: MM and ZB. Performed the experiments, and contributed reagents/materials/analysis tools: MM, FS and HM. Analyzed the data: MM. Critical revision of the manuscript for important intellectual content: ZB.

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**Correspondence** should be addressed to Maryam Mirtalebi: [mmirtalebi@shirazu.ac.ir](mailto:mmirtalebi@shirazu.ac.ir).

### Introduction

Stem canker and dieback are very serious diseases of rose (Sweets *et al.*, 1982). This disease is usually more prevalent on plants that are under some type of stress due to poor cultural practices. The stem canker and dieback phase of the disease appears as a yellow to tan or dark brown to black lesion that enlarges until the stem is girdled. Once the stem is girdled, the foliage above the canker wilts and dies that causes brown discoloration and death of the terminal areas of the cane (Waterman, 1982).

Several fungi are capable of causing stem canker and dieback of roses. The most commonly reported fungi worldwide are *Coniothyrium fuckelii* Sacc., *Botryodiplodia theobromae* pat., *Botrytis cinerea* Pers. ex Fr., *Trichothecium roseum* (Pers.) Link ex. S.F. Gray, *Pho-*

*mopsis* sp. and *Pestalotia* spp. (Guba, 1961; Pitta & Teranishi, 1973; Sweets, 1982). *B. cinerea*, *Phomopsis oblonga*, *Pestalotiopsis* sp. and *C. fuckelii* have been reported on roses in Alborz and Esfahan provinces of Iran (Mirabolfathi & Ershad, 2004; Mahdizadehnaraghi & Bakhtiari, 2014). Recently, a relatively high incidence of rose dieback disease has been observed in Shiraz and Noorabad areas rose greenhouses in the Fars province of Iran. There is a lack of information concerning pathogens associated with cane dieback of greenhouse roses in Fars province. During 2014 and 2015, isolations were made from roses with stem dieback obtained from Fars province greenhouses. Fungi isolated from these stems included *C. fuckelii*, *B. cinerea* and *Acremonium* sp. (Domsch *et al.*, 2007). *C. fuckelii* and *B. cinerea* are both well recognized pathogens as causal agents of stem

dieback of roses worldwide (Sweets, 1982). To date there is no available information related to *Acremonium* spp. as causal agent of rose dieback, worldwide. This study was therefore carried out to investigate the identity of *Acremonium* species in terms of morphological and molecular characteristics and pathogenicity test on different rose cultivars.

## Material and methods

### Sample collection and fungal isolation

During 2014 and 2015, rose plants with yellow to brown discoloration and dieback of the tips of shoots, were collected randomly from the major rose-producing greenhouses of the Fars province. Wood segments were cut from the affected stems, washed under running tap water, surface disinfested in a 1% sodium hypochlorite solution for 5 min, and washed twice with sterile distilled water. Small pieces from the margin between healthy and discolored stem tissue were plated on acidified potato dextrose agar (PDA; potato extract 300 g/L, dextrose 20 g/L, agar 15 g/L). Plates were incubated at 25 °C for 5 to 10 days, and growing colonies were transferred to PDA. Single spore colonies were derived prior to morphological and molecular identification using the serial dilution method (Dhingra & Sinclair, 1995). Among isolated fungi, *Acremonium* species was found to be the most abundant one. Using compound microscope, morphological characters were used to distinguish the species of isolated fungi included conidiophore morphology, phialides type and shape, and conidial size and shape. The length and width of 50 conidia per isolate were measured. Colony characters and pigment production on PDA, 2% malt extract agar (MEA; 20 g/L malt extract, 16 g/L agar) and oatmeal agar (OA; 60 g/L oatmeal, 16 g/L agar) incubated at 25 °C were evaluated after 10 days (Domsch *et al.*, 2007). Presence/absence of chlamydo spores and sclerotia was evaluated using OA and 2% water agar (WA; agar 20 g/L) cultures with sterilized nettle (*Urtica* sp.) stems respectively and incubating them at 25 °C (Domsch *et al.*, 2007). Isolates were examined weekly for formation of chlamydo spores and sclerotia.

Representative isolates were deposited in the culture collection of Plant Protection Department, College of Agriculture, Shiraz University.

### Growth rates and percentage of spore germination

Plugs of agar, 2 mm in diameter, were cut from the edge of 7 day-old colonies and placed in the center of

PDA plates which were then incubated at temperatures ranging from 15±2 to 35±2 °C in 5 °C increments using three replicate plates per isolate. Growth rates of colonies were recorded after 10 days.

Concentration of about 10<sup>4</sup> spore/mL of each isolate was made and dispersed to 2% WA plates using a sterilized glass rod. Percent germination of spores was determined under a compound microscope Zeiss at × 100 magnification following 16 h at temperatures ranging from 15 to 35 °C in 5 °C increments.

### Spore trapping in rose greenhouses

The spore trapping method described by Eskalen & Gubler (2001) was used for this study with the following modification. Spore traps including TMN-PDA (PDA amended with 0.5 mL/L trimethylnonanol and 25 µg/mL pentachloronitrobenzene) (Banihashemi & de Zeeuw, 1969) plates placed in selected greenhouses where rose dieback were known to occur. The plate traps were collected after 24 h, incubated at 25 °C and observed for pathogen presence after 7 days. The colonies of each fungus were recorded and a representative of each isolate of fungus was subculture onto PDA.

### Pathogenicity tests

Nine rose cultivars ('Red One', 'Otopia', 'Tinto', 'Avalanche', 'Attracta', 'Fiesta', 'Samurai', 'Dolce Vita', 'Shiraz') were used for pathogenicity tests in the greenhouse test. Six shoots of each cultivar were used for each isolate. Wounds were made on the internode of the shoots with a disinfested pruning shear. Agar plug from 10-day-old cultures were placed in the wounds and parafilm was wrapped over the wounds to prevent desiccation. Fresh PDA plugs instead of mycelium plugs were used as control. Four weeks after inoculation, the canes were inspected for lesion development and disease symptoms. Small pieces of necrotic tissue from the edge of each lesion were cut and placed on PDA and the pathogen was re-isolated from the inoculated plants to confirm Koch's postulates.

### Molecular identification

Morphological identification of *Acremonium* species as dominant fungi in this study was confirmed by sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA genome. DNA extraction

was carried out following the procedures described by Mirtalebi *et al.* (2013). The ITS region was amplified by polymerase chain reaction (PCR) using the universal primers ITS1 and ITS4 (White *et al.*, 1990). Each 20  $\mu$ L amplification reaction mixture contained 5-10 ng of total DNA, 2  $\mu$ L of 10 $\times$  reaction buffer, 0.2 mM dNTP, 2.5 mM MgCl<sub>2</sub>, 1 U of Taq polymerase (CinnaGen) and 0.8  $\mu$ M of each primer. Amplification conditions consisted of 34 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 90 s, and extension at 72 °C for 2 min. Each PCR reaction included an initial denaturation step at 95 °C for 2 min and final extension step at 72 °C for 5 min. The amplification products were purified with GeneJet PCR Purification Kit (Fermentas) to remove excess of primers and nucleotides. Subsequently, purified amplification products were sequenced in both directions with an ABI PRISM-BigDye Terminator v3.1 sequencing kit (Applied Biosystems) on an ABI-3100 automated sequencer. Phylogenetic analyses were performed using DNA sequences of the ITS region that were either retrieved from published ITS sequences in the GenBank or determined in this study (Table 1). DNA sequences were edited with DNASTAR (Seq Man II) and aligned with ClustalX 1.8 (Larkin *et al.*, 2000). Manual adjustment of sequence alignments was performed to accommodate insertions/deletions. Phylogenetic analyses were conducted in MEGA 5 (Tamura *et al.*, 2011) using the Neighbor-Joining method (Saitou & Nei, 1987). The sequence of *Bionectria ochroleuca* (KF055399) was used as outgroup.

## Results and discussion

### Isolation and identity of causal agent of rose dieback

Disease symptoms on roses in greenhouses including yellow, brown to black areas on a cane or stem, canker and dieback were observed (Fig. 1). In this study, 10 isolates of *Botrytis cinerea* and 34 isolates of *Coniothyrium fuckelii* were isolated. These fungi have been previously reported for dieback of rose worldwide (Sweets, 1982; Mirabolfathi & Ershad, 2004). Based on morphological characteristics (Domsch *et al.*, 2007), 51 isolated fungi were preliminarily identified as *Acremonium* spp. The species of these isolates obtained from single conidia were characterized as follows: Colonies deeply floccose-cottony to tomentosus, whitish to pale pinkish; conidiation abundant, mainly plectonematogenous; phialides very numerous on hyphal strands, mostly simple and long (25-55  $\mu$ m, mean 43  $\pm$  1.6), distinctly chromophilic near the base; conidia were aggregated in heads, cylindrical or tapering towards the tips and slightly fusiform, mostly homopolar, smooth-walled, hyaline 4.2-5.5 (mean 4.9  $\pm$  0.3)  $\times$  1.2-1.7 (mean 1.5  $\pm$  0.2)  $\mu$ m; sclerotia formed on nettle stems, scattered in the mycelium, firm, globose, smooth walled, hyaline, 15-50 (mean 34  $\pm$  1.8)  $\mu$ m, consisting of isodiametrical cells of equal size; chlamydospores were absent (Fig. 1). These characteristics were typical of *A. sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams (Domsch *et al.*, 2007).

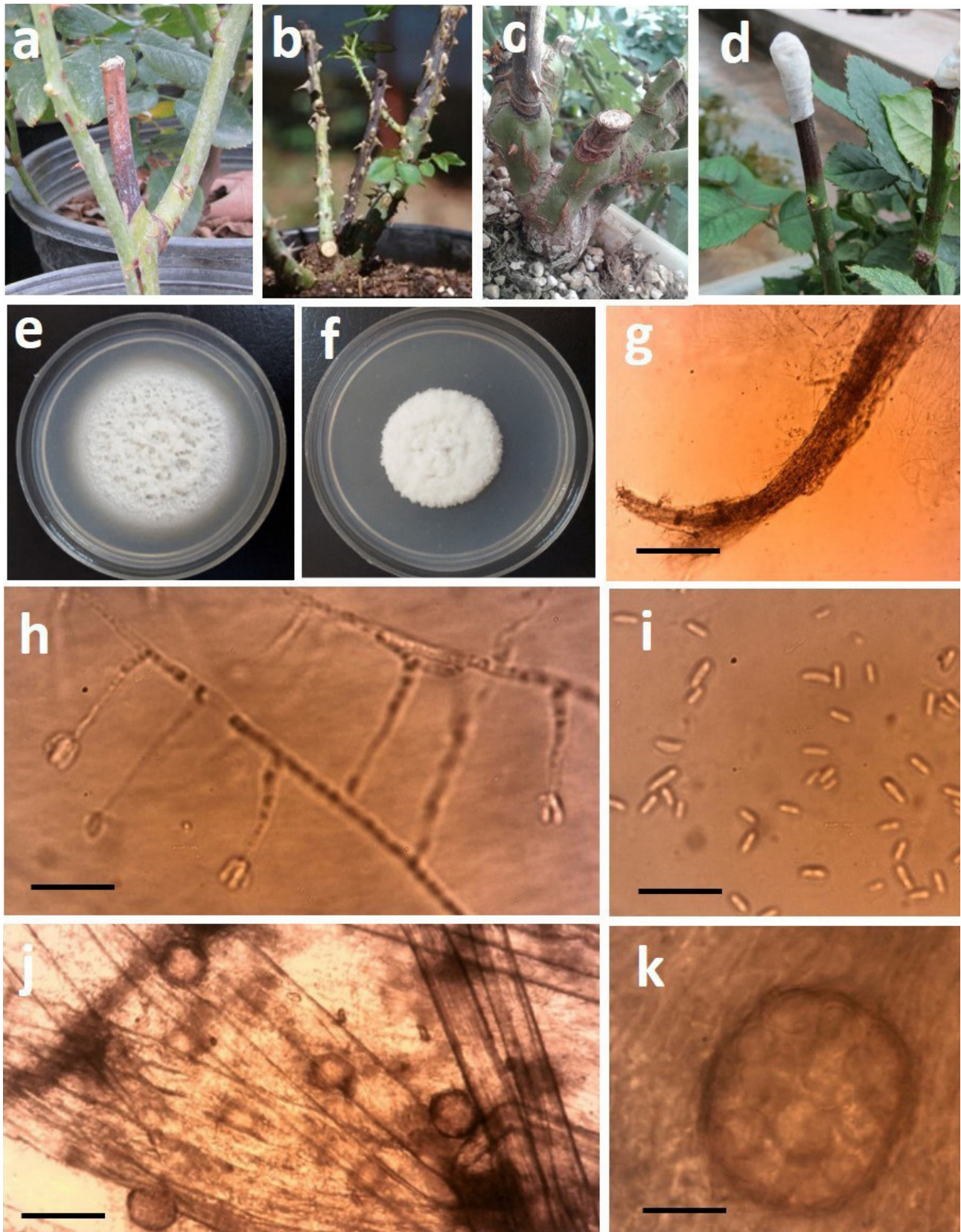
**Table 1.** GenBank accession numbers of internal transcribed spacer sequences of rDNA (ITS) of isolates used in this study. Isolate's name is indicated where known.

Isolate	Species	GenBank accession
Acr1	<i>Acremonium sclerotigenum</i>	KU532330 <sup>1</sup>
Acr2	<i>Acremonium sclerotigenum</i>	KU532331 <sup>1</sup>
07739	<i>Acremonium sclerotigenum</i>	KT878352 <sup>2</sup>
07495	<i>Acremonium sclerotigenum</i>	KT878350 <sup>2</sup>
BAFSCH	<i>Acremonium sclerotigenum</i>	KJ194115 <sup>3</sup>
BAFSCH1	<i>Acremonium alternatum</i>	KF225143 <sup>3</sup>
CBS 223.70	<i>Acremonium alternatum</i>	U57674 <sup>4</sup>
UOA/HCPF 1384.2	<i>Acremonium strictum</i>	KC254091 <sup>5</sup>
OUCMBI 101011	<i>Acremonium strictum</i>	HQ914912 <sup>6</sup>
TX-1054	<i>Acremonium cucurbitacearum</i>	AJ621763 <sup>7</sup>
A-544	<i>Acremonium cucurbitacearum</i>	AJ621755 <sup>7</sup>
-	<i>Acremonium chrysogenum</i>	U57672 <sup>4</sup>
-	<i>Plectosphaerella cucumerina</i>	L36640 <sup>8</sup>
P8	<i>Plectosphaerella cucumerina</i>	AF132805 <sup>9</sup>
Sin80	<i>Bionectria ochroleuca</i>	KF055399 <sup>10</sup>

<sup>1</sup> Submitted in this study. <sup>2</sup> Yao L, Wang H, Wan Z, Li R & Yu J (Unpubl. data). <sup>3</sup> Li *et al.* (2014).

<sup>4</sup> Glenn *et al.* (1996). <sup>5</sup> Arabatzis M & Velegraki A (Unpubl. data). <sup>6</sup> Sun K, Li W & Li C (Unpubl. data).

<sup>7</sup> Martinez-Culebras *et al.* (2004). <sup>8</sup> O'Donnell & Gray (1995). <sup>9</sup> Harrington *et al.* (2000). <sup>10</sup> Schumpp O (Unpubl. data).



**Figure 1.** Rose dieback symptoms observed in greenhouses (a-c). Necrotic lesion development in pathogenicity trials (d); Morphological characteristics of *A. sclerotigenum*; a whitish tomentose (e) and floccose-cottony (f) colony of the fungus on potato dextrose agar; mycelial strand (g); simple, long, stiff phialids with conidial heads (h); hyaline, cylindrical conidia (i); firm, globose, hyaline sclerotia consisting of isodiametrical cells of equal size formed on the nettle stem (j-k). Bars= 20  $\mu\text{m}$  (h, i, k); 100  $\mu\text{m}$  (g, j).

Optimum temperatures for growth and spore germination of *A. sclerotigenum* were 25-30°C (Fig. 2).

### Molecular identification

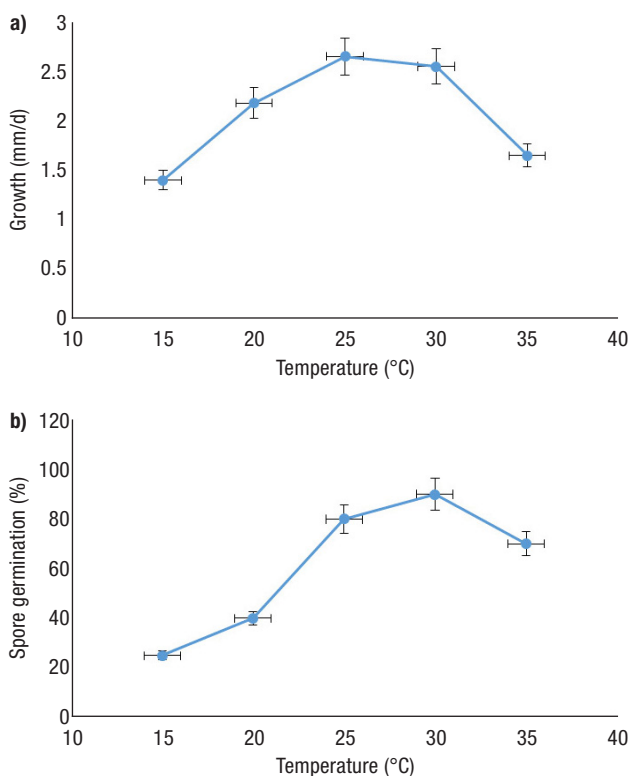
To confirm the identification based on morphology, two isolates of *Acremonium* (Acr1 and Acr2) previously identified based on morphological and culture characters, were amplified using the primers ITS1 and ITS4. An amplicon of about 600 bp was obtained for both isolates. BLASTn searches in GenBank showed that ITS sequences of *Acremonium* isolates had 99-100% identity with isolates of *A. sclerotigenum* (GenBank KT878352, KT878350 and KJ194115). ITS sequences of the two isolates of *Acremonium* have been submitted to the GenBank (Table 1). According to DNA sequence analyses and morphological characteristics, *Acremonium* isolates recovered from stem of roses showing dieback symptoms could be assigned to *A. sclerotigenum* (Fig. 3). This study revealed *Acremonium sclerotigenum* as a new causal agent of rose dieback. Results of inoculation of healthy rose plants with *A. sclerotigenum* isolates confirmed its pathogenic feature, a fact which is particularly important because there is no information in the literature about the capacity of *A. sclerotigenum* to affect roses. Fungi of the genus *Acremonium* have been found

as plant endophytes (Morgan-Jones & Gams, 1982), mycoparasites of plant pathogenic fungi (Choi *et al.*, 2008) and pathogens (Garcia-Jimenez *et al.*, 1994). *Acremonium cucurbitacearum* is the causal agent of *Acremonium* collapse, a soil-borne disease affecting muskmelon and watermelon worldwide (Armengol *et al.*, 1998; Martinez-Culebras *et al.*, 2004). *Acremonium strictum* has proved to be pathogenic to sorghum, cotton, okra, shasta daisy, maize, pea and strawberry (El-Shafey *et al.*, 1979; Chase & Munnecke, 1980; King, 1981; Kamlesh-Mathur *et al.*, 1987; Racedo *et al.*, 2013). *A. sclerotigenum* is obviously a widely distributed saprophyte (Domsch *et al.*, 2007). The species has also been reported as epiphytic fungus on barley and causal agent of bagged apple brown spot (Asgari *et al.*, 2004; Li *et al.*, 2014).

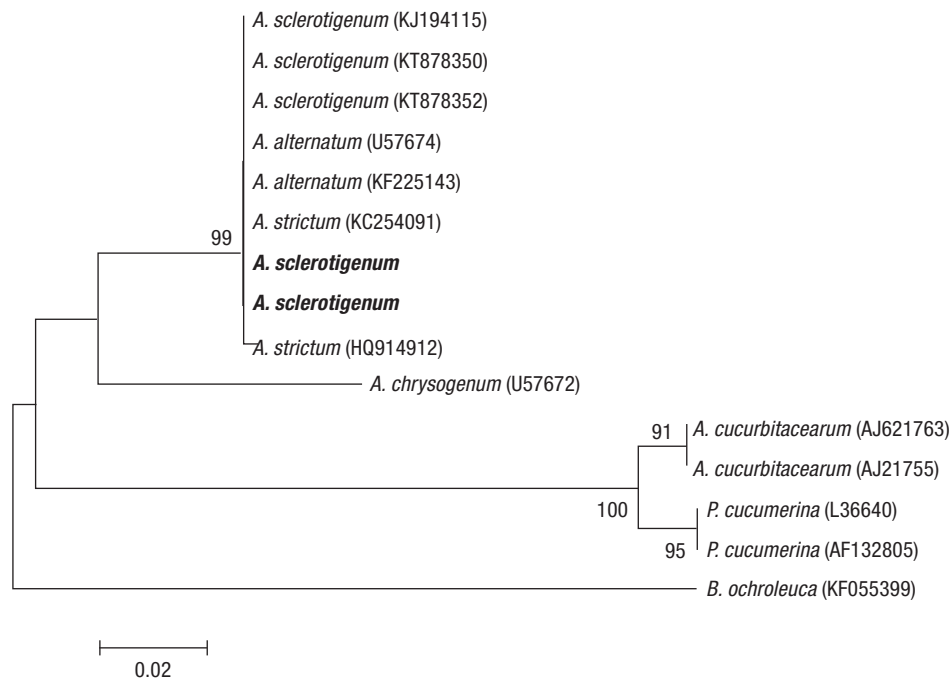
### Spore trapping and pathogenicity tests

According to records obtained from spore trapping in rose greenhouses, several fungi including *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp., *Alternaria* spp., *Botrytis cinerea* and *Acremonium sclerotigenum* were detected. Trapping frequency was 29-30%, 25-27%, 12-13%, 20-22% and 14-16%, respectively.

Three isolates of *Acremonium sclerotigenum* obtained from roses with dieback symptoms and two isolates obtained from spore trapping were used for pathogenicity tests. Four weeks after inoculation, disease symptoms including brown discoloration lesions, necrosis and dieback of stems were observed for each five isolates on each nine rose cultivars. Brown necrotic lesions extended downwards from the inoculation site (Fig. 1d). *A. sclerotigenum* was re-isolated from all inoculated stems on nine cultivars. Control plants remained symptomless and no fungi were re-isolated. Based on spore trapping results, the possibility of airborne inoculum of *A. sclerotigenum* infecting rose wounds such as pruning wounds is not negligible in our study. Different *formae speciales* of *Fusarium oxysporum* have been shown to enter into greenhouses by airborne inoculum (Scarlett *et al.*, 2015). Infection through leaf wounds by airborne propagules of *F. oxysporum* f. sp. *radicis-lycopersici* in tomatoes (Rekah *et al.*, 2000) and *F. oxysporum* f. sp. *basilici* in basil (Uchida *et al.*, 1996) have also been reported. *A. sclerotigenum* probably survive on disease canes or plant debris in greenhouses and spores of the causal fungus are dispersed by air currents and splashing water. The disease may also be spread by fungus-contaminated pruning tools. Rowe *et al.* (1977) demonstrated that while organic waste can provide a source for the survival of *F. oxysporum* f. sp. *lycopersici*, entry of the



**Figure 2.** Effect of incubation temperature on radial growth rate (a) and percentage of spore germination (b) of *Acremonium sclerotigenum* isolates on potato dextrose agar.



**Figure 3.** Phylogram of neighbor-joining analysis of two *Acremonium sclerotigenum* isolates from this study (indicated in bold), together with 10 isolates of *Acremonium* taxa and two isolates of *Plectosphaerella cucumerina* (indicated using accession number retrieved from GenBank within parentheses) based on the internal transcribed spacer region of rDNA (ITS). *Bionectria ochroleuca* isolate KF055399 is included as outgroup. Bootstrap values (> 70%) are shown as percentages of 1,000 replicates.

pathogen into tomato greenhouse that utilize soilless growth substrates can be due to the aerial inoculum of the pathogen. Understanding the threat associated with airborne conidial germination on wound sites will establish a basis for practices in relation to management the risk of disease associated with aerial inoculum of the fungus (Scarlett *et al.*, 2015).

Although the occurrence of the causal agents of rose dieback such as *Coniothyrium fuckelli*, *Botryodiplodia theobromae*, *Botrytis cinerea*, etc. is much more frequent worldwide (Sweets, 1982), the disease caused by *Acremonium sclerotigenum* is only at the early stage of development and measures should be taken to prevent or reduce its spread into new areas. Currently we are investigating the use of fungicidal pruning wound protectants to reduce the incidence of rose dieback disease development.

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