

Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily

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Abstract *Botrytis elliptica* is a necrotrophic pathogen that specifically infects *Lilium* species. Previous records show that *B. elliptica* collected in the field can successfully develop apothecia in vitro, however, there are no formal descriptions of apothecia of *B. elliptica*. The aim of this study was to analyse the sequence of the mating type loci of *B. elliptica* and produce apothecia in the laboratory in order to describe their morphology. The sequences of both *MAT* alleles (*MAT1-1* or *MAT1-2*) of *B. elliptica* were determined and compared to the sister taxa, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Two strains of each mating type were used in crosses under controlled conditions to produce apothecia. Primordium

rupture from sclerotial tissue occurred 74 days after fertilization and a mature apothecium formed within 1 month after rupture. The apothecia are 7 to 12 mm in height with a disk of 3 to 4 mm in diameter and 0.5 to 1 mm in thickness. The apothecial disk is usually umbilicate, depressed to funnel and rounded in shape. The number of apothecia growing on a sclerotium was one to nine. Asci are long, cylindrical with a size of $208 \times 14 \mu\text{m}$, thin walled and bearing eight ascospores. Ascospores are hyaline in colour, ellipsoidal with rounded ends, usually 18 to 24 μm in length and 6 to 10 μm in width (mean $19.5 \times 8 \mu\text{m}$). Ascospores were infectious on lily leaves.

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Introduction

There are various modern lily cultivars on the market which originate from the genus *Lilium* of the family Liliaceae. The genus *Lilium* comprises about 100 species and was classified into seven sections, of which three comprise important hybrid cultivars for breeding: Asiatic, Oriental and Longiflorum (MacRae 1998; Shahin et al. 2012). Lilies are the second largest flower bulb crop in the Netherlands, and the largest flower bulb product in the world (Kamenetsky and Okubo 2012). Fire blight, also known as leaf blight is a major threat to lily production worldwide. The disease poses a major economic threat and has been recorded in Argentina,

China, Korea, Italy, Japan, Netherlands, Taiwan, United Kingdom, and the United States (Bastiaansen et al. 1997; Chiou and Wu 2001; Doss et al. 1988; Furukawa et al. 2005; Hwang 2001; Mansfield and Hutson 1980; Migheli et al. 1990). During the growing season, fire blight may affect numerous lily species such as *Lilium candidum*, *Lilium longiflorum*, *Lilium auratum*, and *Lilium speciosum* (Bastiaansen et al. 1997; Doss et al. 1988; Fang Hsieh et al. 2001; Furukawa et al. 2005). The disease is caused by *Botrytis elliptica* (Berk) Cooke, a necrotrophic pathogen that only infects lilies. Host specificity of *B. elliptica* was proposed to be caused by the production of an as yet unidentified, secreted necrosis-inducing protein that causes programmed cell death (Van Baarlen et al. 2004) and therefore presumably serves as a host-specific necrotrophic effector (Vleeshouwers and Oliver 2014). Symptoms such as necrotic spots on leaves and petals are the early signs of infection. The necrotic lesions develop and expand quickly under favourable environmental conditions (Chiou and Wu 2001; Hwang 2001; van Baarlen et al. 2004). Control measures using fungicides for *B. elliptica* are common practice worldwide, however, it has been reported that *B. elliptica* acquires resistance to several fungicides including benzimidazoles, dicarboximides, diethofencarb, and two sterol biosynthesis inhibitors (Chastagner and Riley 1990; Migheli et al. 1990).

Fruiting bodies of species in the genus *Botrytis*, called apothecia, are rarely found in the field. Apothecia are ascomas with an open cup shape, on top of a stipe, and brown in colour (Whetzel 1945). Apothecia develop from sclerotia and act as a platform to discharge ascospores from the asci (Pöggeler et al. 2006). Apothecia of the genus *Botrytis* vary in size depending on the species, ranging from 2 to 25 mm in height and 1 to 6 mm in diameter of the apothecial disk. Several studies have shown that sclerotia must be fertilized with microconidia in order to develop apothecia (Beever and Parkes 1993; Faretra and Antonacci 1987; Fukumori et al. 2004; Groves and Loveland 1953). Mature *Botrytis* apothecia can normally be observed 2 months after fertilization (Faretra and Antonacci 1987; Hennebert 1973; Terhem and van Kan 2014).

Most members of the genus *Botrytis* are typically designated as having heterothallic mating systems. However, some species in the genus are reported to have homothallic reproductive mating systems, for example *Botrytis porri* and *Botrytis globosa* (Buchwald 1953;

Elliott 1964). *B. elliptica* is believed to have a heterothallic mating system, requiring a cross between different alleles to undergo sexual development and form apothecia (van den Ende and Pennock-Vos 1996). The mating type locus in the genus *Botrytis* is designated as *MAT*, with two alleles defined as *MAT1-1* and *MAT1-2*. As is common in ascomycetes, the *MAT* alleles lack sequence similarity but are flanked by identical genes, i.e., *APN2* and *SLA2* (Pöggeler et al. 2006). The presence of both *MAT* alleles will activate sexual development. Two decades ago, efforts were made to generate apothecia from *B. elliptica*, but complete apothecia failed to form. The apothecia failed to expand the apothecial disk which resulted in empty asci and ascospores (Chastagner et al. 1992). Some years later, van den Ende and Pennock-Vos (1996) managed to collect apothecia in a lily production field in Lisse, the Netherlands and successfully performed crosses with *B. elliptica* strains, but a formal description of the apothecia of *B. elliptica* was not provided. Staats et al. (2007) could not detect apothecia of *B. elliptica* during a field survey; however, they performed AFLP analysis to study the genetic diversity in the population. Over 60 % of haplotypes of *B. elliptica* were unique to a single isolate and only 2 % of all pairwise haplotype comparisons differed by less than four markers. Furthermore, haplotypes that were prominent in one growing season were not found again in the next year. These data collectively provided strong evidence for the occurrence of sexual reproduction in the field (Staats et al. 2007). Also field surveys in North America and Taiwan failed to reveal the presence of apothecia of *B. elliptica* in the field (Huang et al. 2001). Currently, there is no documentation or description of apothecia of *B. elliptica* available to the public. The aim of this study was to analyse the sequence of the *MAT* loci of *B. elliptica* and to produce apothecia in the laboratory in order to describe their morphology.

Materials and methods

Strains

Six *B. elliptica* strains (Be9601, Be9605, Be9610, Be9612, Be9623, Be0004) collected in a lily production field in Lisse, the Netherlands in the years 1996 and 2000, were provided by dr. J. van Doorn (PPO Lisse). Strains were plated on malt extract agar (MEA) and

incubated at 20 °C with continuous exposure to near-UV light and white light until cultures sporulated.

B. elliptica genome sequence

Genomic DNA was isolated from strain Be9601 using a Gentra Puregene DNA purification kit (Qiagen, Venlo, the Netherlands), and used for whole genome sequencing by DNAnvision in Liège, Belgium. The library containing inserts with an average length of 157 bp was sequenced on an Illumina HiSeq2000 platform by paired-end sequencing with a read length of 100 bp. A total of 33.2 million reads (yielding a dataset of >5 Gb) were assembled using Velvet 1.2.05 (Zerbino and Birney 2008) with a k-mer value of 43. The Be9601 assembly consisted of 7,860 contigs (N50=32,840) and had a total length of 47.6 Mbp.

Identification of mating type alleles

Freeze-dried mycelium was used for genomic DNA extraction using a Gentra Puregene DNA purification kit (Qiagen). The entire *MAT* locus could be amplified from strains with either of the *MAT* alleles, using primers annealing in the regions flanking the locus: BOTMAT5 (GTGACTGTAAAACGACGGCCAGTTCCGTATTCACAATCCATCC) and BOTMAT3 (GTGACCAGGAACAGCTATGACCACATACTCGCATTAGTGGAAC). PCR performed on 10–50 ng DNA with Expand polymerase (Roche) and primer pair BOTMAT5/BOTMAT3 in a 25 µl reaction volume yielded a fragment of ~4.3 kb. Amplification conditions were: 95 °C 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min, followed with a final extension of 72 °C for 7 min. The amplicon from *B. elliptica* strain Be9623 was cloned in a TOPO-XL PCR plasmid (Life Technologies) and entirely sequenced by Baseclear (Leiden, The Netherlands) using a primer walking strategy.

To identify the *MAT* alleles of strains or single ascospore progeny cultures by PCR, primer pairs were used that are specific for either of the alleles: MAT1-1 F (TGGTGTAAAAAGATTCCGTATCCG) and MAT1-1R (CACCATATGCATTCTGAGTGAAG); or MAT1-2 F (ATCACGGCACTGTCCTCGAAAC) and MAT1-2R (GAAGAGGTTCCAGACACAGATTG).

PCRs were performed with GoTaq polymerase (Promega, Leiden, The Netherlands), according to the manufacturer's instructions. Amplification conditions

were: 95 °C 5 min, then 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, followed with a final extension of 72 °C for 5 min.

Sexual crosses

To obtain sclerotia, strains were plated on MEA and incubated in complete darkness at 15 °C for 4 weeks, followed by incubation at 0 °C for 4 weeks. Sexual crosses were set up in a reciprocal way using a standard protocol for crosses in *B. cinerea* (Farettra et al. 1988; van der Vlugt-Bergmans et al. 1993). In total, 24 sclerotia were used in one microtiter plate of six wells. Every well contained four sclerotia (maternal parent) that were fertilized with 3 ml of microconidia (paternal parent) of the mating partner. The microtiter plate was then sealed and incubated at 12 °C under neon light with a 12-h photoperiod. Non-spermatized sclerotia in sterile MilliQ water were used as a negative control. All crosses were set up with two strains of opposite mating types.

Sampling of ascospores and production of single ascospore progeny

To obtain ascospores, an apothecium was gently grinded with a mortar and pestle, followed by the addition of sterile MilliQ water, and mixed. The homogenate containing complete asci, ascospores and hymenium debris was filtered over glasswool fibre in a 5 ml pipette tip. The flowthrough contained predominantly ascospores, with little contamination by asci and hymenium tissue. Ascospores were counted using a haemocytometer. To obtain single ascospore progeny cultures, ascospores were plated on MEA at low density, up to 30 ascospores per plate, and incubated at 20 °C. After 2 days of incubation, germlings emerging from single ascospores were transferred to fresh MEA plates. At least 15 single ascospore cultures were collected from each individual apothecium. Genomic DNA was extracted from single ascospore cultures after 4 days of growth on MEA. PCR was performed as described above to identify their *MAT* allele. A chi-square test for the segregation of *MAT* alleles in the progeny was performed with a confidence level $P=0.05$.

Light microscopy

Transverse sections of apothecia were mounted on a slide with filter-sterile MilliQ water. Observations were made with a Nikon Eclipse 90i (Nikon Instruments,

Badhoevedorp, the Netherlands) compound microscope attached with a Nikon DS-5MC camera. Measurements were performed using N.I.S-Elements AR 2.30 software (Nikon Instruments, Badhoevedorp, the Netherlands). At least 40 ascospores, asci, hymenia, paraphyses, ectal excipulum, and medullary excipulum were measured. The mean, standard deviation, and 95 % confidence intervals were calculated.

Infection assay

Ascospores were collected from a mixture of 10 apothecia using the method described above. The ascospore suspension was adjusted to $1 \times 10^6 \text{ ml}^{-1}$ in potato dextrose broth (12 g/l). 6–12 droplets of 3 μl of ascospore suspension were inoculated on the lower sides of lily leaves freshly cut from mature plants. Inoculated leaves were stored in a box with high relative humidity, > 95 %, and placed at room temperature. The infection assay was performed with 3–5 leaves per treatment and was repeated three times.

Results

The mating type locus of *Botrytis elliptica*

As in most ascomycetes, the mating type (*MAT*) locus in *Sclerotiniaceae* is flanked by two highly conserved genes, *APN2* (a homologue of the *Saccharomyces*

cerevisiae *APN2* gene encoding a DNA lyase) and *SLA2* (a homologue of the *S. cerevisiae* *SLA2* gene encoding a cytoskeleton assembly protein) (Amselem et al. 2011). The draft genome sequence of *B. elliptica* isolate Be9601 was determined (van Kan and Staats, unpublished). Nucleotide alignment was performed to identify the region that is homologous to the *MAT* locus of *B. cinerea* (Amselem et al. 2011). The *MAT* locus of Be9601 (Fig. 1) appeared to contain the *MAT1-1* allele, which includes the *MAT1-1-1* gene (encoding the MAT-alpha domain) and the *MAT1-1-5* gene, in the same configuration as in *B. cinerea* strain B05.10 (Amselem et al. 2011). Using primers BOTMAT5 and BOTMAT3 on DNA of *B. elliptica* Be9623, a fragment was amplified that contains the *MAT1-2* allele, which includes the *MAT1-2-1* gene (encoding a high mobility group domain protein) and the *MAT1-2-4* gene (Fig. 1), in the same configuration as in *B. cinerea* strain T4 (Amselem et al. 2011). The four *MAT* genes in *B. elliptica* were >90 % similar to their orthologs in *B. cinerea* and >75 % similar to their orthologs in *Sclerotinia sclerotiorum* (Table 1; Supplemental Figure 1). Alike in the *MAT* loci in *B. cinerea*, there was a truncated fragment of the *MAT1-2-1* gene in the *MAT1-1* locus and a truncated fragment of the *MAT1-1-1* gene in the *MAT1-2* locus (Fig. 1). Both gene fragments lack start codons as well as their typical DNA-binding domain and thus appear non-functional. The sequences of both *MAT* alleles were deposited in Genbank under accession numbers KM219113 (*MAT1-1*) and KM243314 (*MAT1-2*).

Fig. 1 Structure of the *MAT* locus in *B. elliptica*. Top panel: *MAT1-1* allele of strain Be9601; bottom panel: *MAT1-2* allele of strain Be9623. *MAT* alleles are non-homologous in DNA sequence, but are flanked by identical genes, *APN2* and *SLA2*. Introns in the *MAT* genes are indicated by lines connecting the exons (indicated by boxes). The boxes marked with dotted circles represent truncated fragments of the *MAT1-2-1* gene (in the *MAT1-1* locus in the top panel) and the *MAT1-1-1* gene (in the *MAT1-2* locus in the bottom panel), respectively. The direction of transcription is indicated by arrows

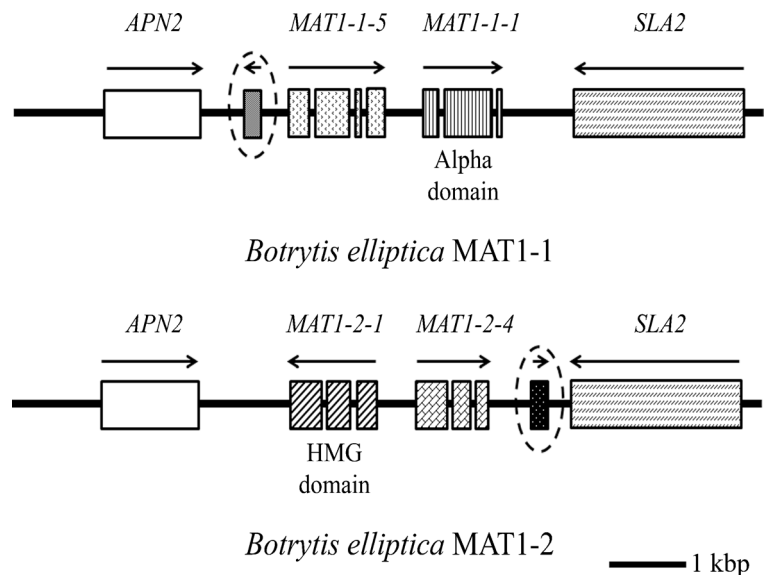


Table 1 Homology of *B. elliptica* MAT genes to orthologs in other Sclerotiniaceae

<i>B. elliptica</i> gene	Protein length (amino acid residues)	Homology to <i>B.cinerea</i> ortholog		Homology to <i>S.sclerotiorum</i> ortholog	
		Identity	Similarity	Identity	Similarity
<i>MAT1-1-1</i>	353	96	97	81	84
<i>MAT1-1-5</i>	376	86	92	75	87
<i>MAT1-2-1</i>	376	94	96	78	86
<i>MAT1-2-4</i>	279	82	90	63	76

Characterization of strains

Four different *B. elliptica* strains were analyzed by PCR for their *MAT* allele. Two strains carried the *MAT1-1* allele and another two strains carried the *MAT1-2* allele. To analyse the production of sclerotia, these strains were plated on MEA plates and incubated in a 15 °C incubator in darkness. All strains formed sclerotia, except for Be9610 (*MAT1-1*). Approximately 30 sclerotia were produced in one plate. Fully developed sclerotia were observed in the MEA plate after 30 days. Table 2 shows the characterization of strains.

Sexual crosses

To analyse the ability of *B. elliptica* to develop apothecia, the *MAT1-1* strains were crossed with the *MAT1-2* strains in all possible combinations. Because of the failure of Be9610 to produce sclerotia, this strain could only be used as paternal parent (microconidia). Of the nine crosses that were set up, four crosses were fertile and resulted in the formation of apothecia. The crosses that produced apothecia were all set up from *MAT1-2* strains as the maternal parent (sclerotia). No apothecia formed from crosses that used *MAT1-1* strain Be9612 as the maternal parent. As a control, sclerotia from the *MAT1-1* strain or the *MAT1-2* strain that were incubated in sterile MilliQ water did not produce apothecia. Table 3 displays the results of the sexual crosses. The experiment was performed three times with identical results.

Apothecia were sampled from two different fertile crosses, and at least 15 single ascospore progeny were cultured from three independent apothecia of each. To identify the *MAT* alleles in the progeny, amplification of genomic DNA was performed by PCR with *MAT*-allele-specific primers. Table 4 shows that the *MAT* alleles segregated almost 1:1 in progeny. In one of the three

experiments, the segregation was skewed, with the *MAT1-1* allele being dominant for reasons that remain unexplained. A chi-square test indicated that, over the entire set of three biological repeats, the segregation ratio between the *MAT* alleles did not significantly deviate from 1:1 ($P < 0.05$, Table 4).

Morphological description of sclerotia and apothecia

Morphological structures of apothecia of *B. elliptica* were described based on the general descriptions by Whetzel (1945). Sclerotia are plano-convex, black, with irregular shape, and sometimes firmly attached to each other. Sclerotia range in size from 3 to 14 mm, and are frequently concave or hollow on the inward. Optimal conditions for development of sclerotia are in malt extract agar in darkness, in a temperature range between 15 °C and 20 °C.

From 74 days after fertilization onwards, primordia ruptured from the surface of sclerotia and sometimes from the bottom (Fig. 2a). The primordia develop into a stipe in 7 to 10 days after emergence. The stipe is cylindrical and erected (Fig. 2b, c). The stipe surface is pubescent, scabrous, light brown in colour, 7 to 12 mm in height, and 1.5 to 2 mm in width. One to nine (mean = four) apothecia emerged on a sclerotium. The apothecial

Table 2 Morphological and molecular characterization of *B. elliptica* strains

Strain	Microconidia	Macroconidia	Sclerotia	Mating type allele
Be9610	Yes	Yes	No	<i>MAT1-1</i>
Be9612	Yes	Yes	Yes	<i>MAT1-1</i>
Be9605	Yes	Yes	Yes	<i>MAT1-2</i>
Be0004	Yes	Yes	Yes	<i>MAT1-2</i>

Table 3 Results of sexual crosses

	Cross	Sclerotia	Microconidia	Replicates ^a	# Repeats ^b	Apothecia
	1	Be9612	Be0004	3	3	–
	2	Be0004	Be9612	3	3	+
	3	Be9612	Be9605	3	3	–
	4	Be9605	Be9612	3	3	+
	5	Be0004	Be9610	3	3	+
	6	Be9605	Be9610	3	3	+
	7	Be9612	Sterile MilliQ water	3	3	–
^a number of biological replicates within an experiment	8	Be0004	Sterile MilliQ Water	3	3	–
^b number of repeated experiments	9	Be9605	Sterile MilliQ Water	3	3	–

disk is umbilicate, depressed to funnel and round in shape, usually 3 to 4 mm in diameter and 0.5 to 1 mm thick. It is slightly shallow, cup-shaped and becomes flat when mature; light brown in colour, becoming dark brown at maturity (Fig. 2d, e). Apothecia are mature 94 to 104 days after fertilization. At first the margin of the disk is entire, becoming serrate to irregular (not shown). Asci formed in the apothecial disk were long and cylindrical in shape with an average dimension of $200 \times 14 \mu\text{m}$ ($n=40$) (Fig. 2f). An apical pore was not clearly observed in the ascus tip. The ascus has a thin wall and bears eight ascospores (Fig. 2f). The ascospores are hyaline, ellipsoidal with rounded ends, usually 18.5 to $24 \mu\text{m}$ in length (mean $19.5 \pm 1.3 \mu\text{m}$, $n=40$) and 6 to $10 \mu\text{m}$ in width ($8.1 \pm 0.8 \mu\text{m}$, $n=40$) (Fig. 2g). The medullary excipulum ranges in shape between *textura globosa* and *textura angularis*, with a thickness 240 to $344 \mu\text{m}$ ($n=40$) (Fig. 2h). The underside of the

apothecial disk is pubescent. Ectal excipulum is in the shape of *textura globosa* with a thickness of 59 to $87 \mu\text{m}$ ($n=40$) (Fig. 2i). The hymenium ranges from 87 to $106 \mu\text{m}$ in height ($n=40$) and the subhymenium from 100 to $124 \mu\text{m}$ ($n=40$). The size of paraphyses ranges from $197 \mu\text{m}$ to $291 \mu\text{m}$ ($n=40$).

Infectivity of ascospores

Droplets of ascospore suspensions were inoculated on the lower side of lily leaf surfaces. As a control, suspensions of asexual conidia were inoculated at the same density. Symptom development became visible at 20–24 h post inoculation (hpi) and was indistinguishable between the ascospores and conidia (not shown). The infected area turned brown in colour and necrotic spots developed at the infection site. At 24 hpi, the lesion was approximately 1 mm in diameter, equivalent to the size

Table 4 Segregation of *MAT* alleles in the progeny

Cross	Sclerotia	Microconidia	Apothecium (biological replicates)	Mating types		Total no. of ascospores	Chi square value
				MAT1-1	MAT1-2		
1	Be0004	Be9612	1	11	13	24	0.09*
			2	7	8	15	0.02*
			3	18	2	20	5.28
			Total	36	23	59	2.86*
2	Be9605	Be9612	1	11	10	21	0.02*
			2	7	9	16	0.09*
			3	16	2	18	4.45
			Total	34	31	55	3.07*

* no significant deviation from 1:1 segregation in the progeny ($P < 0.05$)

of the inoculation droplet. Hyphae were growing on the infected area and could be examined under the light microscope. The lesions expanded to 2–4 mm in diameter at 36 hpi and lesions were circular to oval in shape (Fig. 3). At 48 hpi, the first botryose clusters of spores could be observed in some infected areas. At 4 weeks post inoculation, sclerotia formed on lily leaves and had a typical appearance of *B. elliptica* sclerotia (not shown)

Discussion

In this study we confirmed that *B. elliptica* has a heterothallic mating system with two alleles: *MAT1-1* and *MAT1-2*. Apothecia of *B. elliptica* can be produced in the laboratory after sexual crosses of two strains of opposite mating types, using a standard protocol developed for *B. cinerea* (Faretra et al. 1988; van der Vlugt-Bergmans et al. 1993) although *B. elliptica* apothecia

Fig. 2 Morphology of sexual structures of *B. elliptica*. **a,b** primordia, **c** stipe, **d,e** mature apothecia, **f** ascospores in asci, **g** ascospores, **h** medullary excipulum, **i** ectal excipulum

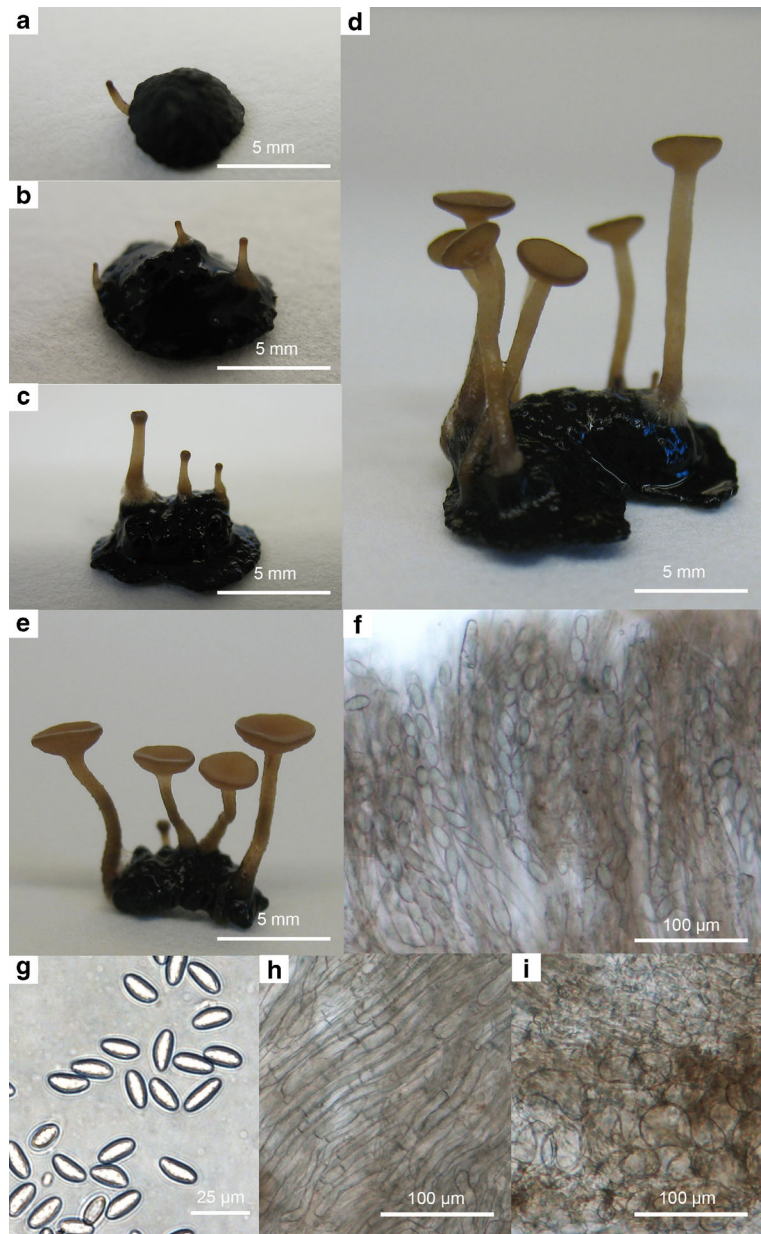
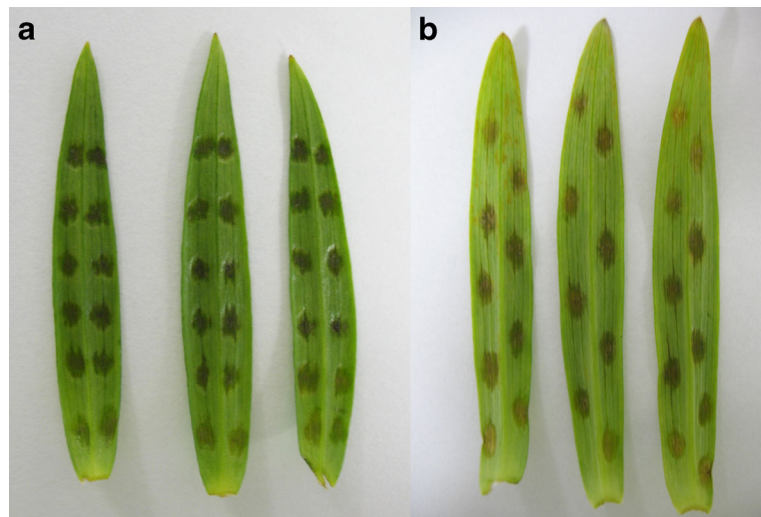


Fig. 3 Disease symptoms of *B. elliptica* on lily leaves, 36 hpi. **a** inoculation with ascospores, **b** inoculation with conidia



took significantly longer to develop than those of *B. cinerea*.

The *MAT1-1-1* and *MAT1-2-1* genes in *B. elliptica* are orthologous to the alpha-domain protein and the HMG-domain protein, respectively, which are conserved in all ascomycetes studied thus far. These two proteins jointly act as master regulators of the initiation of sexual fruiting body development, when expressed together in a dikaryotic cell (Pöggeler et al. 2006). The *MAT1-1-5* and *MAT1-2-4* genes in *B. elliptica* are orthologous to the *MAT1-1-5* and *MAT1-2-4* genes in *B. cinerea* and have no homologues outside the family Sclerotiniaceae (Amselem et al. 2011). Deletion of either the *B. cinerea* *MAT1-1-5* gene or the *MAT1-2-4* gene results in a defect in sexual reproduction, specifically in the developmental transition from stipe to apothecial disk (Terhem and van Kan, unpublished). Whether the *B. elliptica* *MAT1-1-5* and *MAT1-2-4* genes have a similar function in apothecial disk development remains to be established.

Alike in *B. cinerea*, the *MAT* alleles of *B. elliptica* both contain a truncated *MAT* gene fragment derived from the opposite allele (i.e., a non-functional fragment of the *MAT1-2-1* gene in the *MAT1-1* locus and a non-functional fragment of the *MAT1-1-1* gene in the *MAT1-2* locus). The lengths and positions of the non-functional *MAT* gene fragments are nearly identical between *B. cinerea* and *B. elliptica* (J. van Kan, unpublished observations). This configuration suggests that contemporary *MAT* loci of *B. cinerea* and *B. elliptica* have both evolved from an ancestral, homothallic locus, by two

separate deletion events. Amselem et al. (2011) proposed an evolutionary scenario for the *B. cinerea* *MAT* locus, in which deletion of the *MAT1-1-5* gene as well as a large part of the *MAT1-1-1* gene resulted in the emergence of a heterothallic *MAT1-2* allele, whereas deletion of the *MAT1-2-4* gene and the major part of the *MAT1-2-1* gene yielded the heterothallic *MAT1-1* allele. The configuration of the *B. elliptica* *MAT* alleles is in full agreement with the occurrence of such a scenario in a common ancestor of *B. cinerea* and *B. elliptica*.

The morphology of *B. elliptica* apothecia is in agreement with the general description of apothecia in the genus *Botrytis* (Whetzel 1945). The apothecia of *B. elliptica* range between 7 and 12 mm in height and the apothecial disks range from 3 to 4 mm in diameter and 0.5 to 1 mm in thickness. *B. elliptica* apothecia are similar in size to those of *Botrytis ranunculi*, but smaller than apothecia of *B. cinerea*, which can reach up to 25 mm in height (Bergquist and Lorbeer 1972; Faretra and Antonacci 1987). The ascospores of *B. elliptica* ($23 \times 10 \mu\text{m}$) are substantially larger than those of *B. cinerea* ($13 \times 8 \mu\text{m}$). The earliest primordia ruptured from the sclerotium in *B. elliptica* 74 days after fertilization; which is more than 7 weeks later than in *B. cinerea* (Faretra and Antonaci, 1987; Terhem and van Kan 2014). Whether the slow development of *B. elliptica* apothecia is due to conditions employed here, or is intrinsically slower than in *B. cinerea* remains unknown. *B. elliptica* apothecia are brown in colour and become darker when mature, alike typical characteristics of apothecia of the genus *Botrytis* (Whetzel 1945).

When comparing *B. elliptica* apothecia with *B. cinerea* side by side, we noticed that *B. elliptica* apothecia in a young stage appear slightly more yellow-greyish as compared to the yellow-brown appearance of *B. cinerea*. The apothecia of *B. elliptica* are thicker and more deeply cup-shaped than those of *B. cinerea*. Furthermore, the stipes of *B. elliptica* are uniform in width, whereas stipes of *B. cinerea* are tapered to a narrow base (not shown).

Apothecia only developed in crosses with *MAT1-2* strains acting as the maternal parent, but not in crosses of *MAT1-1* strain Be9612 as the maternal parent, in spite of several attempts in multiple replicates. The reasons for the unsuccessful development of apothecia in the crosses with Be9612 as the maternal parent is unclear, but a similar feature was reported for certain *B. cinerea* strains (van der Vlugt-Bergmans et al. 1993). The observation that apothecia were never produced when sclerotia were incubated in sterile water indicates that the *B. elliptica* strains tested are not homothallic.

Individual ascospores collected from *B. elliptica* apothecia were analysed for the segregation of mating type alleles. The analysis of segregation showed that the MAT loci segregate nearly 1:1 in two independent experiments (Table 4). In a third experiment, a skewed segregation was observed, the reason of which remains unexplained. Nevertheless, in the total dataset, the segregation ratio did not significantly deviate from 1:1.

The four strains of *B. elliptica* used in these crosses were obtained from a field in which there was apparently a potential for sexual reproduction, as indicated by the presence of opposite mating types at the same location. Van den Ende and Pennock-Vos (1996) collected apothecia of *B. elliptica* on plant debris in the same fields as the strains used in this study, and they were the first to succeed in inducing production of apothecia of *B. elliptica* in vitro. They also tested the ascospores produced from apothecia by inoculating them on lily leaves. In the present study, inoculation of ascospores of *B. elliptica* on lily leaves caused similar symptoms as described by van den Ende and Pennock-Vos (1996). Disease development and lesion outgrowth was indistinguishable between inoculation with ascospores or conidia of *B. elliptica*. In view of the detection of *B. elliptica* apothecia in plant debris at the end of the winter (van den Ende and Pennock-Vos 1996), a high genetic diversity in the *B. elliptica* population that is indicative of extensive sexual reproduction (Staats et al. 2007) and our observation that ascospores are equally infectious as conidia, we propose that ascospores make

an important contribution in the epidemiology of *B. elliptica*, by infecting young lily shoots emerging in early spring.

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Conflict of interest The authors declare that they have no conflict of interest.

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