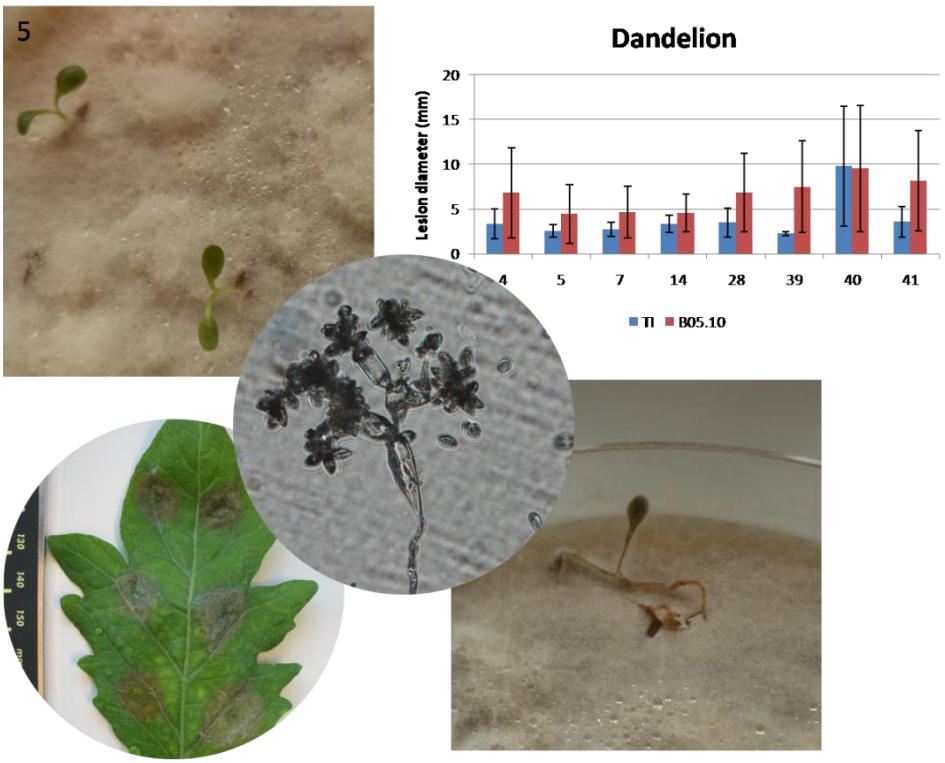
Endophytic *Botrytis* on dandelion (*Taraxacum officinale*):

Truly endophytic or a latent pathogen waiting for a suitable host?



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Summary

The genus *Botrytis* is well known to contain necrotropic pathogens on various plant species. One of the best studied species is *Botrytis cinerea* that can infect over 200 hosts. Several other *Botrytis* species infect a limited number of hosts. However, recent studies found that some *Botrytis* isolates can have endophytic lifestyle. 47 endophytic *Botrytis* isolates were sampled from dandelion plants. The species of those isolates and their phylogenetic positions among known *Botrytis* species remained unknown. It was also important to investigate the mating types of those endophytic isolates. Since several endophytic fungus reported could become pathogenic, therefore the pathogenicity of those isolates also needed to be investigated. The aims of this study were to find out the phylogenetic position, mating type and pathogenicity of endophytic *Botrytis* isolates. For isolates that cannot be identified as one of known *Botrytis* isolates, their morphology characters were also studied. Most of those endophytic isolates belong to clade 1 which 35 isolates appeared as *B. cinerea* species complex, 10 grouped as *B. pseudocinerea*, 1 unknown species (isolate 5) closely related to *B. caroliniana/B. fabiopsis* and the last one remained unknown species (isolate 39) related to *B. mali* based on combined HSP60+G3PDH genes phylogenetic tree. 16 isolates were sequenced for G3890, FG1020 and MRR1 to provide better resolution of *B. cinerea* species complex. Nine of them grouped as *Botrytis* group S, some as *Botrytis* group X and others almost similar to *B. cinerea* B05.10 based on G3890. 12 isolates appeared similar to *B. cinerea* B05.10 and 4 others slightly different to *B. cinerea* B05.10 based on FG1020. However, 13 of those isolates appeared as *Botrytis* group S and distinct from *B. cinerea* B05.10 based on MRR1 sequences while three others cannot be amplified for this gene. Four isolates of *B. pseudocinerea* were amplified for MS547 sequence and all of those isolate appeared identical to *B. pseudocinerea*. Both mating types, MAT1-1 and MAT1-2 were found on endophytic *B. cinerea* and *B. pseudocinerea*. Isolate 5 had mating type MAT1-1 while isolate 39 had MAT1-2. Isolate 4, 7, 41 (*B. cinerea*), 14 (*B. pseudocinerea*) and 39 (related to *B. mali*) except isolate 5 (related to *B. caroliniana/B. fabiopsis*) found pathogenic on dandelion, tomato and tobacco. Isolate 28 and 40 (*B. pseudocinerea*) also found pathogenic on dandelion and tomato but infection assays on tobacco failed for those isolates. Isolates 5 and 39 showed better growth rates on MEA and PDA at 20^{o}C. They produced spores on all five tested media (CDA, MEA, Oatmeal Agar, PDA and V8 Agar) and at both light spores induction treatments (UV light and combination of UV light and fluorescens light). The size of the spores were length 12.6±1.8µm and width 7.2±1.1 µmfor isolate 5 while length 11.8±1.3 µm and width 7.6±1.1µm for isolate 39.They also produced sclerotia on the same tested media while incubated at 15^{o} and 20^{o}C.

Introduction

1.1. Endophytic fungi

Endophytic fungi are fungi which colonize plant tissues and grow inside the plant at particular moment in which the colonized parts of the plant do not show any apparent symptoms. All organisms detected at particular time point inside apparent healthy plant tissues are considered as endophytes including fungi with different life styles. Symptomless microbial colonization is hypothesized as a balanced antagonism between the two partners and the interaction depends on both partner genetic dispositions, their developmental stage, nutritional status and environmental factors (Fig. 1.1) (Schulz and Boyle, 2006). Many endophytes could become latent pathogens. Both of them, endophytes and pathogens, have many virulence factors which are countered by plant defense mechanisms. The interaction remains symptomless if fungal virulence and plant defense are balanced. If plant defense mechanisms completely overcome fungal virulence factors, the fungus will shatter. In contrast, if plant defense mechanisms fail against fungal virulence factors, the association will lead to plant disease (Kusari *et.al.,* 2012).

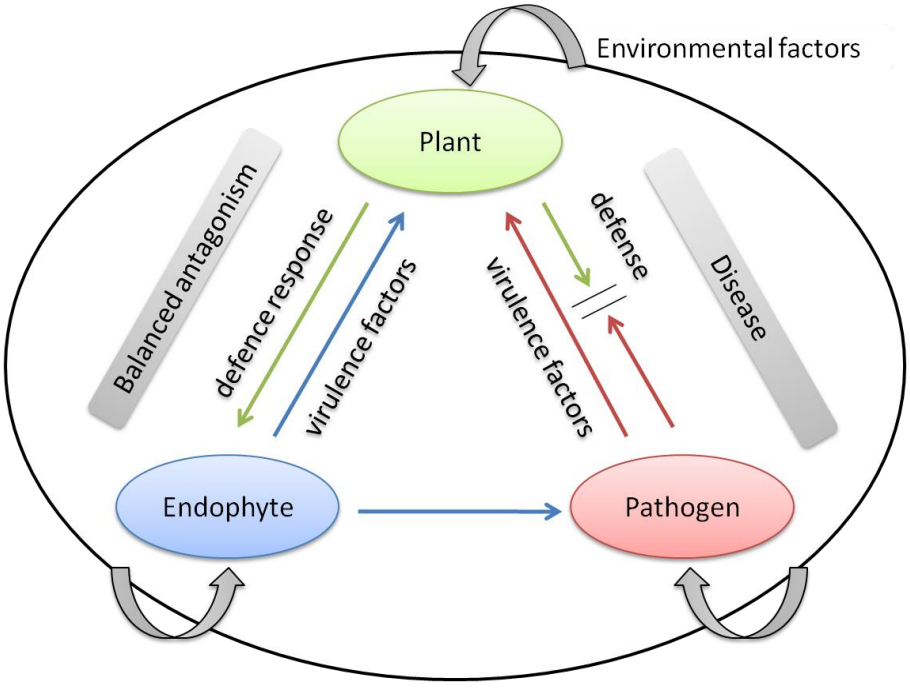


Fig. 1.1. Endophytic concept as balanced antagonism. The microorganism will remain as endophytic when the interaction between plant and microorganism is in balance, however, it can turn into pathogen when the balanced interaction is disturbed (reproduced from Schulz and Boyle, 2006)

Many scientists investigated the importance of endophytic fungi. They were reported to produce biologically active metabolites (Huang *et.al.,* 2007), have biocontrol potential against several plant pathogens (Ownley*et al.,* 2008; Zhang *et al*., 2014) and cover a largely unexplored fungal genetic diversity (Sun and Guo, 2012). On the other hand, some endophytes could also

appear to be latent pathogens (Hyde and Soytong, 2008). Endophytes could become mild pathogenic on their hosts. Certain hosts are known to have resistance against those endophytic fungi virulence factors, therefore they are considered not to be highly virulent. However, they could become serious pathogens for other plant species which can not develop resistance against them. Therefore, this matter should be taken into account when a new endophytic fungus is introduced into a new environment (Sieber, 2007). Some members of Botryosphaeriaceae that found as endophytic fungi become aggressive pathogens especially when their hosts were under stress condition (Slippers and Wingfield, 2007).

The success rate in isolating endophytic fungi was very dependent on methodology used especially when using culture based method with surface sterilization treatment. Weak surface sterilization treatments would result in inconclusive finding because of the high possibility to isolate epiphytes, too harsh sterilization treatments could kill all fungus including endophytes. As alternative to this method, direct analysis of DNA could be a better way to reveal endophyte existences on plant tissues (Hyde and Soytong, 2008). Other obstacle in endophytic fungus study was the problem with fungal identification based on traditional morphology especially for those found as mycelia sterilia. However, this problem has been fixed since molecular based identification was found (Selim *et.al.,* 2012).

Endophytic fungi harbored in one plant species can be very diverse and derived from many orders (Huang *et.al.,* 2008; Huang *et.al.*, 2009; Zhang *et al.,* 2014). Some endophytic fungi exhibit host and tissue specificity (Huang *et.al.,* 2008). Ten *Botrytis* spp. were found from spotted knapweed (*Centaurea stoebe*) (Shipunov *et al.,* 2008) and *Botrytis cinerea* isolates were sampled from lettuce (*Lactuca sativa*) as endophytic fungi that did not cause any disease symptoms (Sowley *et al.,* 2010). However, the genus *Botrytis* is known to consist of species that are pathogenic on many host plants and has a necrotrophic lifestyle.

1.2. Diversity of *Botrytis* species

More than twenty species of *Botrytis* are already known to cause diseases on many plant species. They were grouped into clade 1, containing species that are pathogenic on eudicots and clade 2 that contains species that can be pathogenic either on eudicot or monocot plants (Staats *et.al.,* 2005). More recently published species such as *B. fabiopsis* (Zhang *et.al.,* 2010a)*, B. sinoallii* (Zhang *et.al.,* 2010b)*, B. pseudocinerea* (Walker *et.al.,* 2011), *B. caroliniana* (Li *et.al.,* 2012) and *B. deweyae* (Grant-Downtown *at.al.,* 2014) were also found to have pathogenic lifestyles. Even more species will likely be found along with advanced technology. Surprisingly, however, many isolates of *Botrytis* were found as endophytes on dandelion. Those isolates showed *Botrytis* specific characters

(Navarette and van Kan, unpublished) when cultured on *Botrytis* selective medium. Those isolates need to be identified.

B. cinerea is the most studied *Botrytis* species. This species is reported to be a species complex. *B. cinerea* populations in German and French had multi drug resistance (MDR) that conferred resistance to fungicides. There were three MDR phenotypes in *B. cinerea.* MDR1 which correlated with point mutation in transcription factor MRR1, conferred partial resistance to fludioxonil and cyprodinil (Kretschmer*et.al.,* 2009). Leroch *et.al.*(2013) reported a stronger variant of MDR1 called MDR1h that is distinct from, but closely related to *B. cinerea* and *B. fabae*. This variant was mainly investigated on strawberry and it had about 5% nucleotide divergence compared to *B. cinerea* strain T4 and grouped as a new subclade called *Botrytis* group S.

B. pseudocinerea, previously described as *B. cinerea* group I, was proposed as a new species. Walker *et. al.* (2011) proved that this species was distinct from *B. cinerea* by confirming there was a barrier of sexual reproduction between both species and also by showing their different phylogenetic positions, supported by high bootstrap value. They also proposed several tools to distinguish between *B. cinerea* and *B. pseudocinerea.* However, it was difficult to distinguish them by morphological characters because they had such similar features.

Morphological identification of fungi might not be sufficient to reveal fungal species identity. Molecular based identification techniques have recently shown a rapid progress. Phylogenetic identification is used by many scientists to investigate the position of certain isolates among their relatives, by comparing their gene sequences to sequences of some known species of the group. In this study, this method was used to identify endophytic *Botrytis* isolates of dandelion plants. However, morphological characteristics are also needed to provide complete description of one species.

1.3. Mating types of *Botrytis* species

The genus *Botrytis* is highly diverse. High genetic variation in *B. cinerea* might be caused by sexual reproduction and meiotic recombination. A mating type with two alleles, MAT1-1 and MAT1-2, controls sexual compatibility between isolates among this species. The field isolates are found mostly as heterothallic fungi that had mating type MAT1-1 or MAT1-2 but there are also homothallic isolates that contain mating type MAT1-1/2 (Faretra *et.al.,* 1988). The heterothallism on *Botrytis cinerea* evolved from homothallism in its closely related sister species *Sclerotinia sclerotiorum* as revealed from differences in the organization and structures of mating type loci. *B. cinerea* strain B05.10 had MAT1-1 identity while strain T4 had MAT1-2 identity (Amselem *et. el.,* 2011).

Sexual reproduction in heterothallic fungi in general is only applicable for crossing between two different mating types from the same fungal species. This phenomenon allows the differentiation between closely related species. Walker *et.al.* (2011) proved the differentiation between *B. cinerea* and *B. pseudocinerea*. Crossings were only possible among the same species but they reported two exceptions. The first exception was a successful crossing between isolate VD184 (*B. pseudocinerea*) and isolate SAS56 (*B. cinerea*). Another exception was a crossing between two *B. pseudocinerea* isolates both carrying a MAT1-2 allele also produced apothecia. They assumed this phenomenon could be caused by contamination or dual mating type strain.

1.4. Necrotrophic and endophytic lifestyle of *Botrytis* species

A well-known *Botrytis* species, *B. cinerea* can cause disease in over 200 host plants (Staats *et.al.,* 2005), while other species are known to have limited hosts. *B. elliptica* causes leaf blight in Lily (van den Ende and Pennock-Vos, 1997), *B. fabiopsis* causes chocolate spot on broad bean (Zhang *et.al.,* 2010a)*B. sinoalli* causes grey mould disease on *Allium* crops (Zhang *et.al.,* 2010b), *B. caroliniana* infects blackberry and broad bean leaves (Li *et.al.,*2012) and a recently identified *Botrytis* species, *B. deweyae,* infects *Hemerocallis* (Grant-Downton *et.al.,* 2014).

The symptomless infection of *B. cinerea* on young primula (*Primula x polyantha*)(Barnes and Shaw, 2003), lettuce (Sowley *et.al.,* 2010) and *C. stoebe* (Shipunov *et.al.*2008) was reported. The fungus was present mostly on outside of the seeds but sometimes within the seeds, grew systemically throughout the plant and caused disease symptoms only at flowering stage (Barnes and Shaw, 2003). Shipunov *et.al.* (2008) found ten *Botrytis* isolates which had endophytic lifestyle on *C. stoebe* and can be placed in both phylogenetic clades, of which eight fell into clade 1. It is important to study the diversity of endophytic *Botrytis* in dandelion based on their position in the phylogenetic tree among *Botrytis* genera and to understand whether those endophytic *Botrytis* spp are fully endophytes or also able to become necrotrophic pathogens on their original host or other plant species.

1.5. Research objectives

This research was conducted (i) to describe the phylogenetic position of recently isolated endophytic *Botrytis* isolates among known *Botrytis* species, (ii) to analyze the mating type of endophytic *Botrytis*, (iii) to investigate pathogenicity of representative isolates from several *Botrytis* species on dandelion (*Taraxacum officinale*), tomato *(Solanum lycopersicum*), and tobacco (*Nicotiana benthamiana*) and (iv) to characterize morphology of endophytic *Botrytis* isolates that do not belong to known *Botrytis* species.

2. Materials and Methods

2.1. Phylogenetic trees construction

Fungal strains

Fungal strains used in this research were 48 isolates of endophytic *Botrytis* isolated from dandelion and obtained from Laboratory of Phytopathology, Wageningen University collections (Table 2.1). All strains were cultured on Malt Extract Agar (MEA, Oxoid) prior to DNA extraction processes.

Table 2.1. Fungal strains used in this research

No	Isolate code	Isolated from	Location	Collectors
1	IB. 2	Leaf	Wageningen (Campus, Binnenveld, Uiterwaarden and Eng)	K.J. Hoevenaars & V. Onland
2	IB. 11	Leaf		K.J. Hoevenaars & V. Onland
3	IB. 19	Leaf		K.J. Hoevenaars & V. Onland
4	2B. 11	Leaf		K.J. Hoevenaars & V. Onland
5	2B. 14	Leaf		K.J. Hoevenaars & V. Onland
6	2B. 20	Leaf		K.J. Hoevenaars & V. Onland
7	2B. 22	Leaf		K.J. Hoevenaars & V. Onland
8	3B. 2	Leaf		K.J. Hoevenaars & V. Onland
9	3B. 8	Leaf		K.J. Hoevenaars & V. Onland
10	3B. 9	Leaf		K.J. Hoevenaars & V. Onland
11	3B. 13	Leaf		K.J. Hoevenaars & V. Onland
12	3B. 24	Leaf		K.J. Hoevenaars & V. Onland
13	4B. 3	Leaf		K.J. Hoevenaars & V. Onland
14	4B. 13	Leaf		K.J. Hoevenaars & V. Onland
15	IH. 11	Flower		K.J. Hoevenaars & V. Onland
16	IH. 25	Flower		K.J. Hoevenaars & V. Onland
17	2H. 4	Flower		K.J. Hoevenaars & V. Onland
18	2H. 11	Flower		K.J. Hoevenaars & V. Onland
19	3H. 15	Flower		K.J. Hoevenaars & V. Onland
20	4H. 8	Flower		K.J. Hoevenaars & V. Onland
21	4H. 10	Flower		K.J. Hoevenaars & V. Onland
22	4H. 13	Flower		K.J. Hoevenaars & V. Onland
23	4H. 15	Flower		K.J. Hoevenaars & V. Onland
24	2S. 21	Stem		K.J. Hoevenaars & V. Onland
25	3S. 13	Stem		K.J. Hoevenaars & V. Onland
26	4S. 15	Stem		K.J. Hoevenaars & V. Onland
27	A7LA	Leaf	Arboretum de Dreijen 6703 BL Wageningen Latitude : 51.967068 Longitude : 5.677716	Z.E.Y. Navarrete
28	A14L	Leaf		Z.E.Y. Navarrete
29	A25SA	Stem		Z.E.Y. Navarrete
30	A25SB	Stem		Z.E.Y. Navarrete
31	A12F	Flower		Z.E.Y. Navarrete
32	A24F	Flower		Z.E.Y. Navarrete
33	B1LA	Leaf	Bornsesteeg 6721 Bennekom Latitude :	Z.E.Y. Navarrete
34	B1FA	Flower		Z.E.Y. Navarrete
35	B13F	Flower		Z.E.Y. Navarrete

36	B14FA	Flower	51.995452 Longitude : 5.653710	Z.E.Y. Navarrete
37	B18F	Flower		Z.E.Y. Navarrete
38	B21F	Flower		Z.E.Y. Navarrete
39	B24F	Flower		Z.E.Y. Navarrete
40	B25F	Flower		Z.E.Y. Navarrete
42	H23SB	Stem	Veerdam 6703 PA, Wageningen Latitude : 51.963223 Longitude : 5.688082	Z.E.Y. Navarrete
43	H24S	Stem		Z.E.Y. Navarrete
44	V8S	Stem		Z.E.Y. Navarrete
45	V9S	Stem		Z.E.Y. Navarrete
46	V17S	Stem		Z.E.Y. Navarrete
47	V5F	Flower		Z.E.Y. Navarrete
48	V18F	Flower		Z.E.Y. Navarrete

DNA extraction and PCR amplification

Genomic DNA of fungal isolates was extracted from three day old mycelia cultured in MEA using Puregene DNA Purification Kit. One cm^2 of mycelium and three metal beads were placed on a 1.5 ml tube. They were grinded by tissue extractor with 30 frequency/second for one minute. A mixture of 600 µl cell lysis solution and 3 µl proteinase K (20 mg/ml) was added to each sample tube. The cell lysate was incubated at temperature of 55^{o}C and speed of 500rpm for at least 1 hour to overnight in order to inactivate DNase. Sample was cooled down to room temperature and then 200 µl protein precipitation solution was added to the cell lysate. High speed vortex was used to mix them thoroughly for 20 seconds. Sample was centrifuged at 13.000-16.000 g for three minutes after it was incubated for 5-15 minutes on ice.

The DNA was available in supernatant and it was pipetted into a new 1.5 ml tube containing 600 µl 100% isopropanol. They were mixed by inverting the tube gently. The tube was centrifuged at 13.000-16.000 g for 1 minute. DNA formed an off white pellet. The supernatant was poured off and the tube was drained briefly on clean absorbent paper. 600 µl of ethanol was added to the pellet and the tube was inverted several times to wash the DNA pellet. The liquid was pipetted out and the tube was inverted in a clean absorbent paper to remove the excess of liquid. The tube was opened for 10-15 minutes to let it air dry. 60 µl milliQ water was added to DNA and the DNA concentration was measured by using NanoDrop (Thermo Scientific). The DNA was stored at -20^{o}C for the next processes.

PCR amplifications were carried out using several primer pairs (Table 2.2) that amplify HSP60, RPB2, G3PDH (Staats *et.al.* 2005), G3890, FG1020 and MRR1. PCR reactions were performed in a 25 µl reaction mixture containing 1 µl genomic DNA, 1 µl 5x GoTaq buffer, 0.5 µl 2.5 mM dNTPs, 0.5 µl of each primer, 0.1 GoTaq polymerase (promega) and 17.4 µl milliQ water. All amplification processes were performed in thermocycler (Biorad).

PCR condition

Thermocycling protocol used to amplify the HSP60, G3PDH, RPB2, G3890, MS547, FG1020 were performed with 1 cycle of 95^{o}C for 5 minutes, 35 cycles of 94^{o}C for 30s, 54^{o}C for 30s and 72^{o}C for 60s and finally 1 cycle of 72^{o}C for 5 minutes.The similar protocol was used to amplify MRR1 fragments with some modifications, the annealing temperature was decreased to 52-55^{o}C and the elongation time was extended to 90 seconds.

Five µl of PCR product was mixed with 2 µl loading dye (Promega), loaded into a well on a 1% agarose gel, stained with ethidium bromide and visualized under UV light camera. The size of the fragment was verified by comparing to 1Kb DNA ladder from Promega.

Table2.2. Primer pair used in this study

Gene	Primers	Primer sequence (5'-3')	Reference
HSP60	HSP60 For	CAACAATTGAGATTTGCCCA	Staats *et. al*, 2005
	HSP60 Rev	GAT GGA TCC AGT GGT ACC GA	
RPB2	RPB2 For	ACG ATG ATC GAG AT	Staats *et. al.*, 2005
	RPB2 Rev	ACC CCA TAG CTT G	
G3PDH	G3PDH For	ATTGACATCGTCGCTGTCAA	Staats *et. al.*, 2005
	G3PDH Rev	ACCCCAGTCGTTGTCGTACC	
G3890	G3890_117_For	GTTCCGGCAAGCCATGAAA	Matthias Hahn (Pers. Comm.)
	G3890_1219_Rev	GATAGCATCGCTACYTTCAG	
RNA helicase	MS547 For	AAGGAGGACGTTGGAAGGAT	Leroch *et al*., 2013
	MS547 Rev	AAG TCCAGAATCTCGATGTATTTGT	
Putative ubiquitin conjugation factor	FG1020 For	GGA GGA TGA TAT GGC AAA GTC	Leroch *et al*., 2013
	FG1020 Rev	GGA TTA AGA GCT TCA CTA CCA	
MRR1 (1)	MRR1-ATGF	ATA CCC GGG TCA ACA TCA TGA ATC CAA CAG TC	Kretschmer*et. al.*, 2009
	MRR1-TF2NR	CTA TCC GAT CGA CCG GTA	Leroch *et al*., 2013
MRR1 (2)	MRR1-TF3NF	TGC TGT GAC GAG CAT GAC	Leroch *et al*., 2013
	MRR1-TF1-4R	GGA TAG GGT ATT GCG TAG ATC G	Kretschmer*et. al.*, 2009
MAT1-1	MAT1-1F	CCAGCAGTAAATGCAGAAGAGCCAA	Grant-Downtown *et.al.,* 2014
	MAT1-1R	CATCATACCAGTGGACCAAGGAGG	
MAT1-2	MAT1-2F	GACTAGGAAAATGGGTACCGCATC	Grant-Downtown *et.al.,* 2014
	MAT1-2R	GAATGTGTAGAGATCCTGTTGTTG	
MAT1-1-1	QMAT111F	AGTATTGGAACTCCAGAACGTC	Terhem, 2015
	QMAT111R	GGAGCAAGGAACATTCTGTTGA	
MAT1-1-5	QMAT115F	GGACGAGGTTCAGCATCATTTA	Terhem, 2015
	QMAT115R	TTTCACCAGTCCCAAATCAGCT	
MAT1-2-1	QMAT121F	AGTATTGGAACTCCAGAACGTC	Terhem, 2015
	QMAT121R	GGAGCAAGGAACATTCTGTTGA	
MAT1-2-4	QMAT124F	GGACGAGGTTCAGCATCATTTA	Terhem, 2015
	QMAT124R	TTTCACCAGTCCCAAATCAGCT	

The amplification products were separated by agarose gel electrophoresis 1% w/v and purified by Nucleospin gel and PCR clean-up (Macherey-Nagel). The purification procedure was performed as explained by the manufacturer. The DNA concentration was measured by NanoDrop (Thermo Scientific).

Sequencing and phylogenetic analysis

The sequencing of PCR product was done by Macrogen (Amsterdam, The Netherlands) in both directions using the universal primers M13UF and M13UR. Phylogenetic position of isolates within the genus *Botrytis* was assessed by combining sequence segments of three nuclear genes and 27 representative isolates of *Botrytis*. *Monilia fructigena* and *Sclerotinia sclerotiorum* were also included as outgroup isolates (Table 2.3).

The nucleotide sequence pattern was checked by BioEdit software. DNA sequences were trimmed at the front and the rear of the sequences to remove unreliable sequence reads. The trimmed DNA sequences were saved infasta format. The alignment was made among all DNA sample sequences by using ClustalW. The phylogeny was made based on sequence data of protein coding genes HSP60, G3PDH and RPB2 that encoding heat-shock protein 60, glyceraldehyde-3-phosphate dehydrogenase and DNA-dependent RNA polymerase subunit II (Staats *et al.,* 2005). The alignment was checked manually to properly fix the alignment by comparing the sequences with the template. The data were saved in mega format and the phylogenetic trees were made based on this data by using maximum likelihood method (Aguileta *et.al.,* 2008) on Mega 6 software (Tamura *et.al.,* 2013) with 1000 bootstrap replications.

Table 2.3. *Botrytis* representative and outgroup isolates used to construct phylogenetic trees.

Species	Strain	Species	Strain
B. aclada	MUCL8415	*B. hyacinthi*	MUCL442
B. byssoidea	MUCL94	*B. narcissicola*	MUCL2120
B. calthae	MUCL1089	*B. paeoniae*	MUCL16084
B. caroliniana	CB15	*B. pelargonii*	CBS497.50
B. cinerea	MUCL87	*B. polyblastis*	CBS287.38
B. convolute	MUCL11595	*B. porri*	MUCL3234
B. croci	MUCL436	*B. pseudocinerea*	VD110
B. deweyae	CBS134649	*B. ranunculi*	CBS178.63
B. elliptica	BE9714	*B. sinoalli*	HMAS250008
B. fabae	MUCL98	*B. sphaerosperma*	MUCL21481
B. fabiopsis	BC-2	*B. squamosa*	MUCL1107
B. ficariarum	MUCL376	*B. tulipae*	BT9830
B. galanthina	MUCL435	*B. mali*	B26, B48
B. gladiolorum	MUCL3865	*M. fructigena*	9201
B. globosa	MUCL444	*S. sclerotiorum*	484

2.2. Mating type analysis

The genomic DNA was extracted as previously described. Specific primer pairs were used for each mating type allele (Table 2) to identify MAT alleles of endophytic isolates. Primer pair namely MAT1-1F and MAT1-1R was used to identify MAT1-1 allele while primer pair namely MAT1-2F and MAT1-2R was used to identify MAT1-2 allele. In addition primer pairs namely QMAT111F and QMAT111R, QMAT115F and QMAT115R, QMAT121F and QMAT121R, QMAT124F and QMAT124R were used to identify the MAT1-1 and MAT1-2 alleles for isolates 3, 14, 19, 20, 28, 39 and 48. 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, 58°C for 30 s and 72°C for 1 min, followed with a final extension of 72°C for 5 min.

2.3. Pathogenicity tests

2.3.1. Pathogenicity of endophytic*Botrytis* isolates on dandelion, tomato and tobacco

The pathogenicity of *Botrytis* isolates was tested on dandelion (*T. officinale*), tomato (*S. lycopersicum*) and tobacco (*N. benthamiana*) leaves. Several representative isolates were chosen based on their group positions on phylogenetic tree of combining HSP60 and G3PDH gene sequences (Table 2.4).The test isolates and the reference strain B05.10 were grown for 3 days on MEA plates and exposed to UV light for one night to promote sporulation. The spores were collected 4 days after UV light exposure. Five ml of distilled water was added into the plates and all spores were scratched by sterile spreader. The spore suspension was filtered through glass wool to remove mycelium and collected in a 50 ml tube. The tube was centrifuged at 1000 rpm (215g) for 5 minutes. The liquid was discarded and the pellet was rinsed twice with distilled water to remove the excess medium from spore suspension. Spores were collected and stored in 1 ml distilled water. Spore concentration was adjusted to 10^7 spores/ml.

Table 2.4. The test isolates used for infection assays

Isolate number	Isolate code	Species based on HSP60 + G3PDH sequences
4	2B. 11	*B. cinerea*
5	2B. 14	Related to *B. caroliniana/B. fabiopsis*
7	2B. 22	*B. cinerea*
14	4B. 13	*B. pseudocinerea*
28	A14L	*B. pseudocinerea*
39	B24F	Related to *B. mali*
40	B25F	*B. pseudocinerea*
41	H23SA	*B. cinerea*

Infection assays were performed on three plant species i.e. dandelion (*T. officinale*), tomato (*S. lycopersicum*) and tobacco (*N. benthamiana*). Four to six dandelion plants were used for each isolate. Five leaves/plant were inoculated at 4 inoculation sites on each leaf. Two composite leaves of tomato cv. Money Maker were used for each isolate with three replications with 2-3 inoculation sites/leaf. Three *N. bentamiana* plants were used with one inoculation site/leaf with two replications. All treated leaves were inoculated with tested isolate on the left side and B05.10 on the right side. Disease incidence (%) was calculated on the basis of the number developed lesions and the total number of the inoculation sites. Lesion diameter more than 2 mm was considered as developed lesion. Disease severity was investigated on the basis of lesion diameter. The lesion diameter was measured at 3 to 5 days after inoculation.

Botrytis infestation in dandelion leaves was tested before infection assays were performed. One symptomless leaf of each plant was surface sterilized. The leaves were soaked in 70% ethanol for 1 minute, transferred to 1% sodium hypochlorite for 1 minute and dipped in 70% ethanol for 30 seconds. The leaves were rinsed in sterile water three times with 3 minutes for every rinsing time, then blotted dry on sterile filter paper and placed on BSM plates afterwards. The plates were incubated at 20 °C for 3-4 weeks and evaluated for the outgrowth of *Botrytis*, visible as brown fungal colonies emerging from the leaf tissue.

2.3.2. The effect of endophytic*Botrytis* isolates on dandelion seed germination and disease symptom

Two endophytic *Botrytis* isolates 14 and 39 were chosen to examine their effect on seed germination and disease symptom. *B. cinerea* B05.10 was included as reference. Two µl of 10^6/ml spore suspension were inoculated at four sites in *Botrytis* selective medium (BSM) plates one day prior to seed placement. BSM was prepared following Kerssies (1990) with modifications in antibiotic compounds added. This medium consists of several components (g/l distilled water): $NaNO_3$, 1.0; K_2HPO_4, 1.2; $MgSO_4.7H_2O$, 0.2; KCl, 0.15; glucose, 20.0 and agar, 25.0. The medium was sterilized at 121^oC for 20 minutes and cooled to 65^oC prior to addition of following components (g/l distilled water): Terrachlor (pentachlorobenzene 75%WP, PCNB), 15 x 10^{-3}; Tetracycline, 2 x 10^{-2}; Chloramphenicol, 5 x 10^{-2}; $CuSO_4$, 1.0 and tannic acid, 5.0. The pH of the medium was adjusted to 4.5 with 5.0 N NaOH.

Eight surface sterilized seeds were placed on each plate, in between fungal inoculation sites. Ten biological replications were performed. Non surface sterilized seeds and surface sterilized seeds without fungal inoculation were also included as control. Surface sterilization procedures were performed as described by Hallmann *et al.* (2006). The seeds were soaked in 70% ethanol for 1

minute, in 5% sodium hypochlorite (v/v) for 5 minutes and in 70% ethanol for 30 seconds. The seeds were rinsed 3 times in sterile water with 3 minutes each and blotted dry on sterile filter paper. Seed germination and disease symptom were examined everyday for one month.

2.4. Morphology characterization

Radial growth of *Botrytis* isolates 5 and 39 was measured on Czapek Dox agar (CDA, Difco), malt extract agar (MEA, Oxoid), oat meal agar (OM, Difco), potato dextrose agar (PDA, Difco) and V8 juice agar (V8, Campbell Soup Company) incubated at 0°, 15° and 20^{o}C in darkness. V8 juice agar was made by mixing 800 ml distilled water, 200 ml V8 juice, 2 g CaCO3 and 15 g agar. All media were autoclaved at 121^{o}C for 15 minutes. Mycelium plugs of isolates 5 and 39 were cultured on MEA plates for 5 days. The fungal growth was examined every day until it reached the edge of the petridish.

The spores of isolates 5 and 39 were harvested from cultures on MEA plates. Growing condition used was similar with previously described method to prepare spore suspension for infection assays. Spore length and width were measured under an optical microscope with 50 spores as replication units.

Sclerotia production was investigated by culturing mycelia of isolates 5 and 39 on five media as used in radial growth measurement. Culture plates were incubated at 15^{o} and 20^{o}C in darkness with five replications. The size and total number of sclerotia were investigated after two months of incubation.

3. Results

3.1. Phylogenetic position of endophytic *Botrytis* among *Botrytis* species

The phylogenetic clustering of 47 endophytic *Botrytis* isolates with known *Botrytis* species was determined based on HSP60 and G3PDH gene sequences (Fig. 3.1). 35 out of 47 isolates grouped with the *B. cinerea* species complex and 10 grouped with *B. pseudocinerea,* both of which are members of clade 1 in the genus *Botrytis* (Staats et al., 2005). Two other isolates grouped into clade 2 i.e. isolates 5 and 39. Based on this result, 8 isolates of *B. cinerea* and 3 isolates of *B. pseudocinerea,* together with isolates 5 and 39, were selected for analysis of the RPB2 gene sequence.

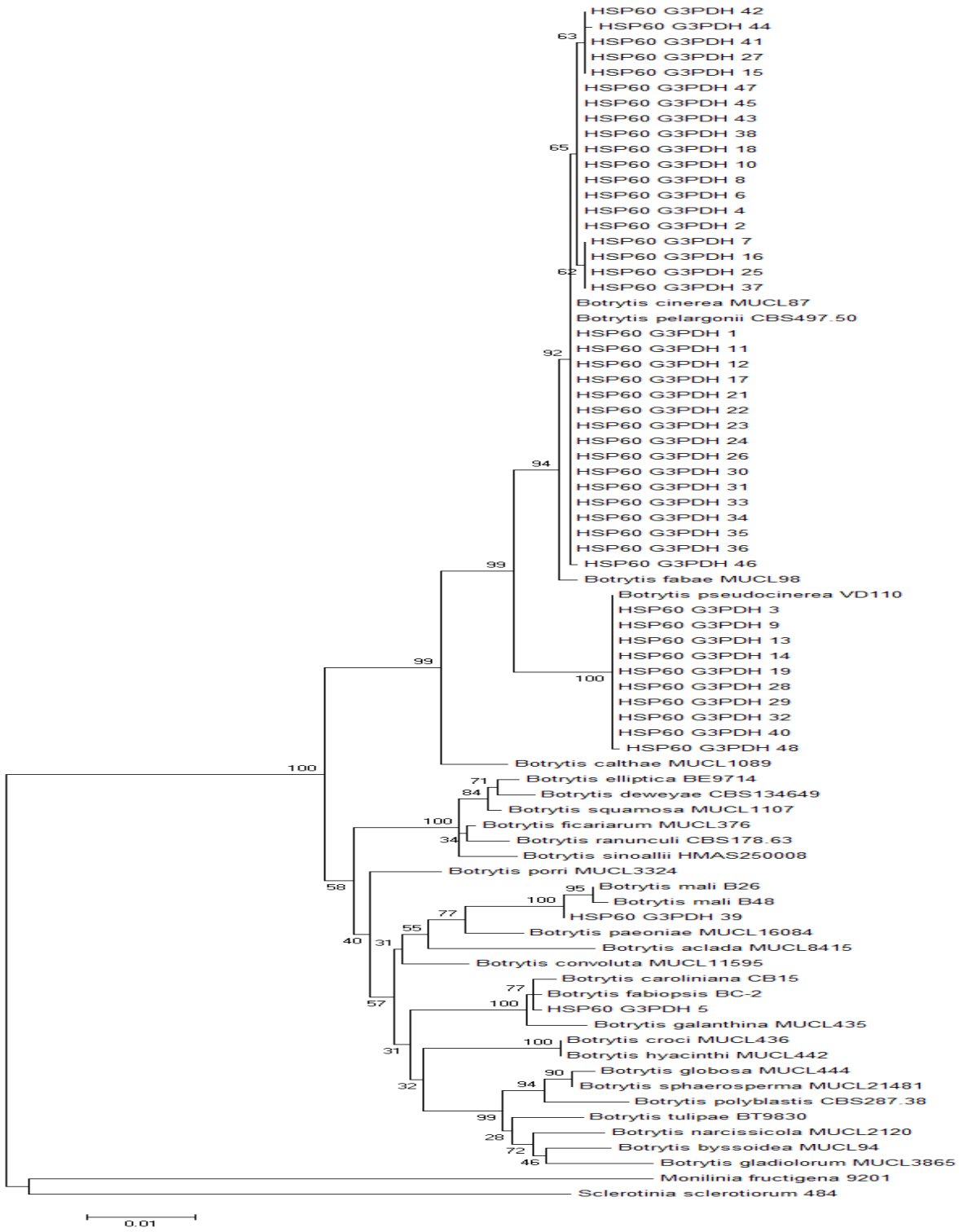


Fig. 3.1. Phylogenetic position of 47 endophytic *Botrytis* isolates in the genus based on combined HSP60 and G3PDH gene sequences. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The number on each node indicated percentage of bootstrap value.

13 isolates sampled from symptomless dandelion were included for phylogenetic tree construction using three genes (HSP60, G3PDH and RPB2). Eight isolates are related, but not identical to *B. cinerea*. Isolates 5 and 39 remained in clade 2 based on this combined phylogenetic

tree (Fig. 3.2) and there was good agreement between the phylogenetic trees for individual gene sequences HSP60 (Fig. S1), G3PDH (Fig. S2), RPB2 (Fig.S3) or combined HSP60 and G3PDH (Fig. 3.1).Isolate 5 was related to *B. caroliniana* and *B. fabiopsis* while isolate 39 related to *B. mali*.

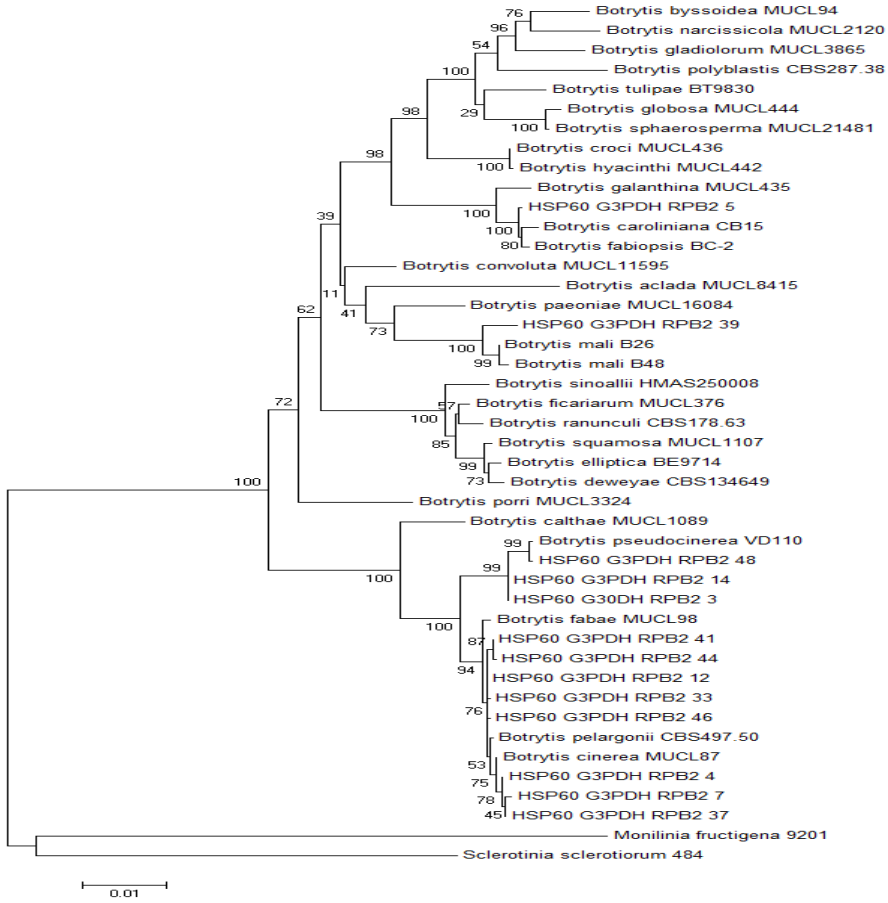


Fig. 3.2. Phylogenetic position of 13 endophytic *Botrytis* isolates in the genus based on concatenated HSP60, G3PDH and RPB2 sequences. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The number stated on each node indicated percentage of bootstrap value.

In order to provide a better resolution, 16 isolates that were most closely related to *B. cinerea* were analyzed in more detail for the sequences of three additional genes (G3890, FG1020, MRR1) and 4 isolates most closely related to *B. pseudocinerea* analyzed in more detail for the sequence of the RNA helicase gene (MS547).

Nine isolates appeared related but not identical to reference strain *B. cinerea* B05.10. The sequence of those 9 isolates for the G3890 gene was more closely related to *Botrytis* type S (Leroch *et.al.* 2013). Seven other isolates were more closely related to *B. cinerea* but distinct from both *B. cinerea* group S and *B. cinerea* group N. They formed a separate group distinct from other types of *B. cinerea* based on G3890 gene sequences (Fig. 3.3). This group was temporarily assigned as *B. cinerea* group X (Matthias Hahn, pers. comm).

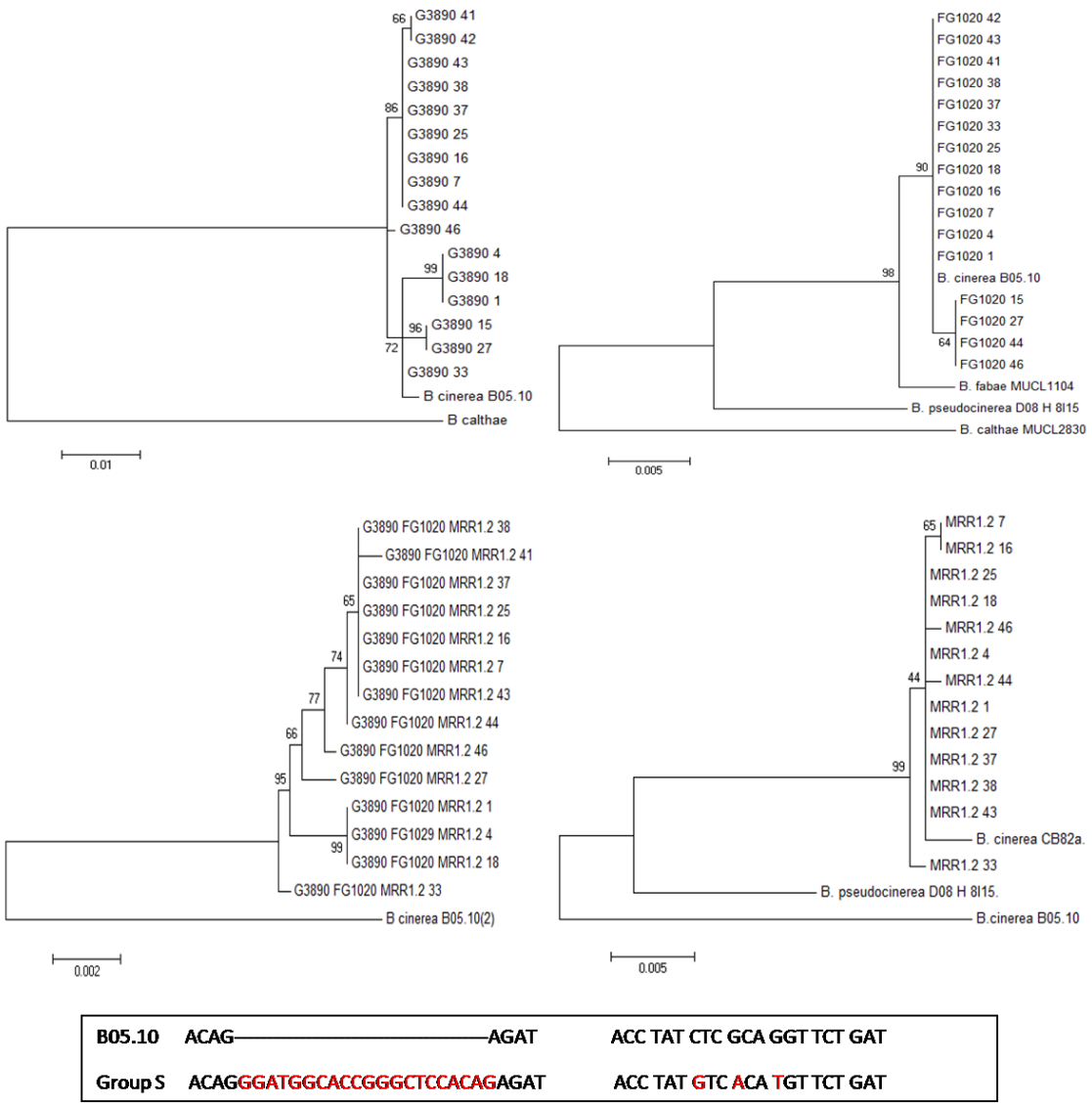


Fig. 3.3. Phylogenetic position of endophytic *Botrytis cinerea* based on G3890, FG1020, MRR1 (part 2) and combined (G3890+FG1020+MRR1) gene sequences. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The number stated on each node indicates percentage of bootstrap value. Sequences in the box indicate important sequence differences that distinguished *B. cinerea* strain B05.10 and the *Botrytis* group S non-MDR1h group (adapted from Leroch *et.al.,* 2013).

FG1020 and MRR1 genes were sequenced for further differentiation of *B. cinerea* isolates. 12 isolates appeared to be similar to reference strain *B. cinerea* B05.10 based on a combined phylogenetic tree using 3 genes (Fig. 3.3). However, all of the 13 isolates were distinct from B05.10 when only the MRR1 gene was used for tree construction (Fig. 3.1.4). This differentiation was strongly supported by a bootstrap value of 99%. All of the endophytic isolates had an insertion of 21 bp in the second part of the MRR1 sequences, while this insertion was not found in B05.10. This insertion was one of characteristics of *Botrytis* group S. Another characteristic was in a short stretch of nucleotides that was specific for *Botrytis* group S and differed from *B. cinerea* B05.10 (Leroch

et.al., 2013). The MRR1 sequence of 13 endophytic *Botrytis* isolates also showed the sequence polymorphisms that are typical for group S (Fig. 3.3)

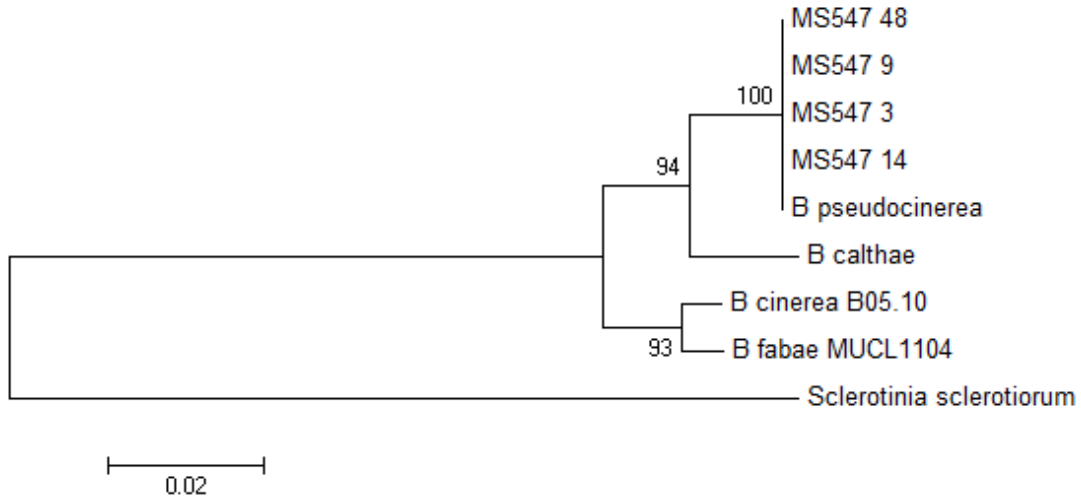


Fig. 3.4. Phylogenetic tree of 4 endophytic *Botrytis* isolates based on MS547 gene sequence. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The numbers stated at each node indicate percentage of bootstrap value.

The RNA helicase gene of four endophytic isolates was identical in sequenceto *B. pseudocinerea*. Those isolates appeared as one group similar to *B. pseudocinerea* group A that is found in Europe and their grouping was highly supported by a 100% bootstrap value (Fig. 3.4).

3.2. Mating type analysis

To investigate the mating type of the 47 endophytic isolates, PCR reactions were carried out that amplified a gene in the MAT locus. Both mating type alleles were found in the isolates from the *B. cinerea* species complex and from *B. pseudocinerea*. Isolate 5 had mating type MAT1-1 and isolate 39 had mating type MAT1-2 (Table 3.1).

Table 3.1 The mating types of endophytic *Botrytis* isolates. Species identification was based on combined HSP60+G3PDH phylogenetic tree. Isolates 20 was amplified with ITS1 and ITS4 primers. The mating types written in bold letters were amplified with primer pairs QMAT115F and QMAT115R while others amplified with primer pairs MAT1-1F and MAT1-1R or MAT1-2F and MAT1-2R. Mating type with question mark (?) showed bright band when amplified with MAT1-2 primer pairs and showed faint band when amplified with MAT1-1 primer pairs.

Isolate code	Species	Mating type
1	*B. cinerea*	MAT1-2
2	*B. cinerea*	MAT1-1
3	*B. pseudocinerea*	**MAT1-2**
4	*B. cinerea*	MAT1-1
5	Related to *B. caroliniana/B. fabiopsis*	MAT1-1
6	*B. cinerea*	MAT1-2
7	*B. cinerea*	MAT1-1

8	*B. cinerea*	MAT1-2
9	*B. pseudocinerea*	MAT1-1
10	*B. cinerea*	MAT1-1
11	*B. cinerea*	MAT1-2
12	*B. cinerea*	MAT1-1
13	*B. pseudocinerea*	MAT1-1
14	*B. pseudocinerea*	**MAT1-2**
15	*B. cinerea*	MAT1-1
16	*B. cinerea*	MAT1-1
17	*B. cinerea*	MAT1-1
18	*B. cinerea*	MAT1-1
19	*B. pseudocinerea*	**MAT1-2**
20	***Fusarium* sp.**	**-**
21	*B. cinerea*	MAT1-2
22	*B. cinerea*	MAT1-1
23	*B. cinerea*	MAT1-1
24	*B. cinerea*	MAT1-2
25	*B. cinerea*	MAT1-2
26	*B. cinerea*	MAT1-1
27	*B. cinerea*	MAT1-2
28	*B. pseudocinerea*	**MAT1-2**
29	*B. pseudocinerea*	MAT1-1
30	*B. cinerea*	MAT1-1
31	*B. cinerea*	MAT1-1
32	*B. pseudocinerea*	MAT1-1
33	*B. cinerea*	MAT1-2(?)
34	*B. cinerea*	MAT1-2
35	*B. cinerea*	MAT1-1
36	*B. cinerea*	MAT1-1
37	*B. cinerea*	MAT1-1
38	*B. cinerea*	MAT1-1
39	Related to *B. mali*	**MAT1-2**
40	*B. pseudocinerea*	MAT1-1
41	*B. cinerea*	MAT1-2
42	*B. cinerea*	MAT1-2
43	*B. cinerea*	MAT1-2
44	*B. cinerea*	MAT1-2
45	*B. cinerea*	MAT1-2
46	*B. cinerea*	MAT1-2
47	*B. cinerea*	MAT1-1
48	*B. pseudocinerea*	**MAT1-2**

3.3. Pathogenicity tests

3.3.1. Pathogenicity of endophytic *Botrytis* isolates on dandelion (*T. officinale*), tomato (*S. lycopersicum*) and tobacco (*N. benthamiana*)

To prepare the dandelion plants for infection assay, a set of experiments was done. The seeds were checked for *Botrytis* infestation before sowing in soil. The outgrowth of the fungus was

examined by growing the seeds on BSM for three days. *Botrytis* infestation was recognized by the color change of medium from light brown into dark brown color near the placement sites of the seeds. All seeds without surface sterilization (NST) contained *Botrytis* on them while only 3% of the seeds showed outgrowth of fungi resembling *Botrytis* after surface sterilization of seeds (SST) (Fig. 3.5).Ten fungal isolates from non surface sterilized seeds and three isolates from surface sterilized seeds were sampled to be checked with an immunoassay. All isolates were positively identified as *Botrytis*.

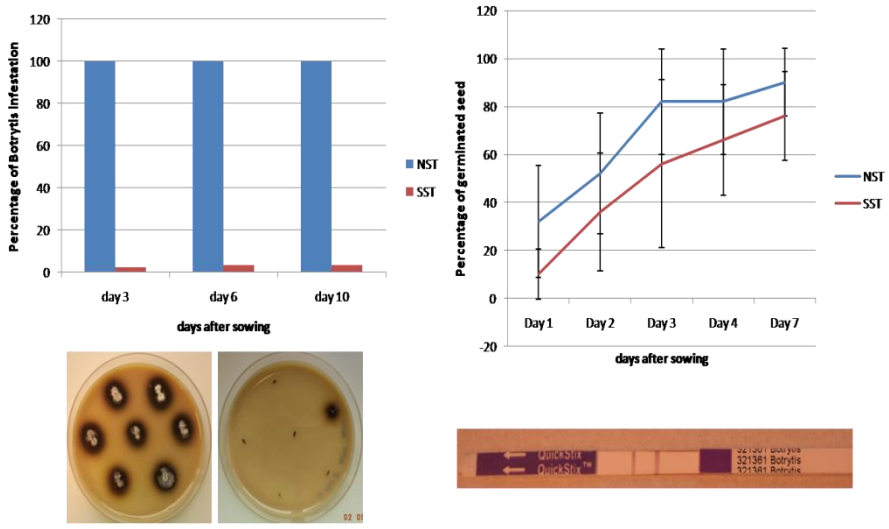
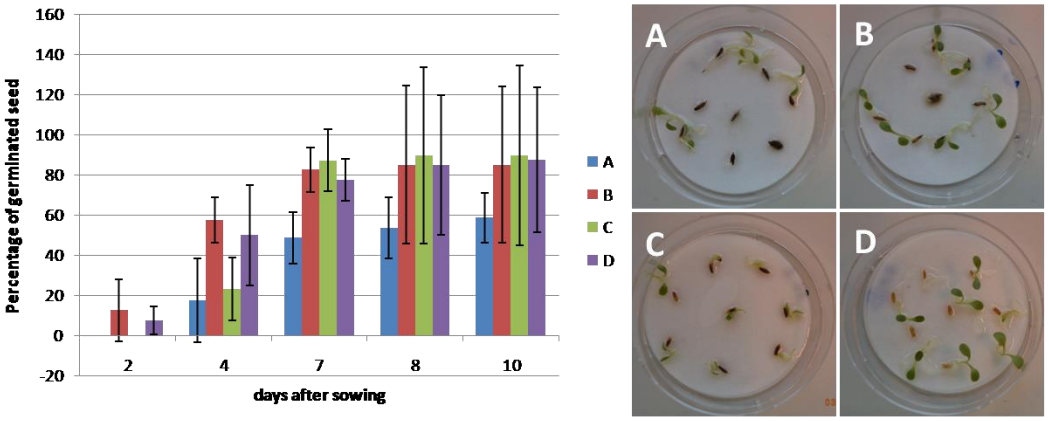


Fig. 3.5. Percentage of *Botrytis* infestation on dandelion seeds treated with surface sterilization (SST, N=91) and without surface sterilization (NST, N=55) on BSM plates at 3, 6 and 10 days after sowing. Percentage of germinated seed on BSM from the seeds treated with surface sterilization (N=50) and non surface sterilized seeds (N=50). Data represent the average mean with standard deviation indicated with the error bar. Positive signs of *Botrytis* infestation on the seeds showed by dark brown color around the outgrowth site of the fungus and two red bands visualization on QuickStix paper stick. N represented number of seeds.

The dandelion seedlings germinated on BSM plates did not look vigorous enough to be sown in the soil although their germination rate on that medium was quite high (Fig. 3.5). To increase the germination rate and to improve the vigor of the seedling a set of treatments was performed. The NST and SST seeds were sown on BSM plates only for three days and transferred into wet sterile filter paper shortly afterwards or directly sown on wet sterile filter paper. The percentage of germinated seed and the vigorous of the seedling were observed visually.



Seedling fresh weight (mg)			
A	B	C	D
5.8 ± 2.0	6.0 ± 0,9	4.1 ± 0.7	6.8 ± 1.4

Fig 3.6. The percentage of seed germination at several days after sowing and visual appearances of dandelion seedlings (A) non surface sterilized seeds incubated three days on BSM and transferred to wet filter paper (N=39), (B) non surface sterilized seeds sowed directly on wet filter paper (N=40), (C) surface sterilized seeds incubated three days on BSM and transferred to wet filter paper (N=46) and (D) surface sterilized seeds sowed directly on wet filter paper (N=40). The data represented the average means of germinated seed and standard deviations indicated with error bars. N represented number of seeds. Seedling fresh weight data represented average seedling means with standard deviation.

The percentage of germinated seeds was higher for NST seeds that were directly sown on wet filter paper than for seeds previously placed on BSM for three days and transferred to wet filter paper afterwards. However, the germination rate was almost similar for SST seeds sown on both treatments. The seedlings seem more vigorous when sown directly on wet filter paper for both NST and SST seeds (Fig. 3.6).

To investigate whether there was a difference in growth performance between NST and SST seeds, twenty one of the best seedlings growing from both NST and SST seeds that were directly sown on wet filter paper were selected to be transferred into soil. Seven plants were randomly selected to measure growth parameters with 10 day intervals. Plants derived from SST seed showed better growth performances at the early stage, but both treatments showed statistically similar vegetative growth at later stage (Table 3.2).

Table 3.2.The vegetative growth of dandelion plants derived from surface sterilized seeds (SST) and non surface sterilized seeds (NST). Data represented average means with standard deviations with N=7 for every ten days interval. N represented number of plants.

Time	Treatment	Number of leaves	Leaf length (mm)	Weight	
				Underground part	Aboveground part
10 days	SST	2.3 ± 0.5^{ns}	19.0 ± 6.8*	64.7 ± 29.8 mg*	
	NST	2.0 ± 0.8	12.8 ± 1.4	35.3 ± 8.4 mg	
20 days	SST	5.9 ± 0.7^{ns}	75.0 ± 16.1^{ns}	0.4 ± 0.4 g^{ns}	0.8 ± 0.3g^{ns}
	NST	5.1 ± 0.9	61.3 ± 19.2	0.3 ± 0.2 g	0.5± 0.3 g
30 days	SST	10.4 ± 1.9^{ns}	206.7 ± 19.3^{ns}	8.9 ± 2.9 g^{ns}	7.8± 0.8g^{ns}
	NST	9.9 ± 1.2	202.4 ± 17.1	7.77 ± 2.96 g	7.5± 1.0 g

*) the means between SST and NST significantly different at P=0.05. ns = the means differences were not significant at P=0.05

Based on the results of this preliminary experiment, one set of treatments was performed to prepare dandelion plants for infection assay. Fourty of both NST and SST seeds were grown on BSM for 3 days. This step was done to confirm that the seeds that we selected were fully infested by *Botrytis* for NST and not infested by *Botrytis* for SST treated seeds. The seeds were transferred to wet filter paper for a week and twenty selected germinated seedlings were transferred into soil immediately afterwards. Those plants were sown in separated plastic pots. Cultural and biological control measures were applied to prevent insect infestation. Plants were grown for two months and then were ready for infection assays. *Botrytis* infestation in dandelion leaves was investigated before the infection assay was performed. One leaf of each plant was selected, surface sterilized and cultured on BSM plate. *Botrytis* infestation was monitored visually by checking the color change in the medium. In the first trial, four out of 20 dandelion leaves derived from NST seeds gave a positive sign for the presence of *Botrytis* while only 1 out 17 leaves gave a positive sign at the second trial (Fig. 3.7).

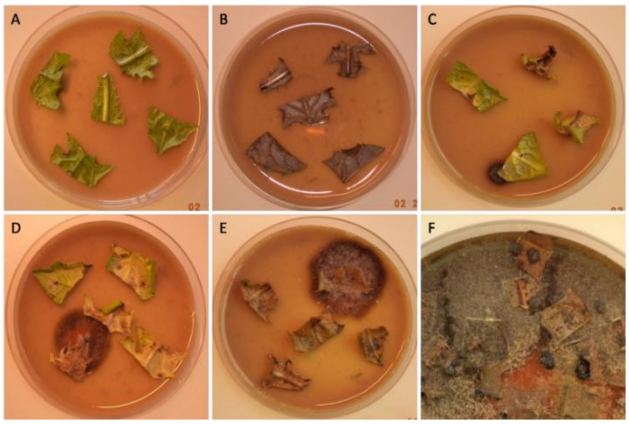


Fig. 3.7. *Botrytis* infestation assays with symptomless dandelion leaves. Leaves were surface sterilized before culturing on BSM plates (A) starting culture, (B) negative for *Botrytis* infestation, (C), (D), (E) and (F) positive signs of *Botrytis* infestation, sclerotia were produced on plates. Samples C,D and E were derived from the first trial and sample F from the second trial.

To investigate whether endophytic *Botrytis* isolates are able to cause disease symptoms, eight selected isolates were prepared for artificial infection assays. Spore suspension with concentration of 10^6spores/ml in PDB solution was used for inoculation. Infection assays were performed on dandelion, tomato and tobacco plants with droplets of spore suspensions inoculated on one leaf half. The reference strain *B. cinerea* B05.10 was inoculated on the opposite leaf half.

Several endophytic *Botrytis* isolates produced disease symptoms on plant leaves as shown at Fig. 3.8. In general, the disease symptoms caused by endophytic *Botrytis* isolates on dandelion leaves were few and mild. The reference strain *B. cinerea* B05.10 also did not show high infection rates on dandelion. *B. cinerea* B05.10 produced only small lesions on dandelion leaves at 5 days after inoculation, whereas on tomato leaves it caused lesions more than 10 mm within 3 days after inoculation. *B. pseudocinerea* isolates sampled as endophytes from symptomless dandelion, differed in disease severity on dandelion, the most virulent strain (isolate 40) caused average lesion diameter as 9.8mm which was similar to lesion size caused by pathogenic strain *B. cinerea* B05.10 whereas the two others (isolate 14 and 28) caused almost similar lesions size as about 3.3-3.5mm. Isolate 5 was not infectious on any of the three tested plant species. It only caused small black dots at the inoculation sites, however the lesions did not develop to expanding lesions at later time points. Isolate 39 caused mild disease symptom on tomato and tobaco but it did not show infectious on dandelion (Fig.3.8 and 3.9).

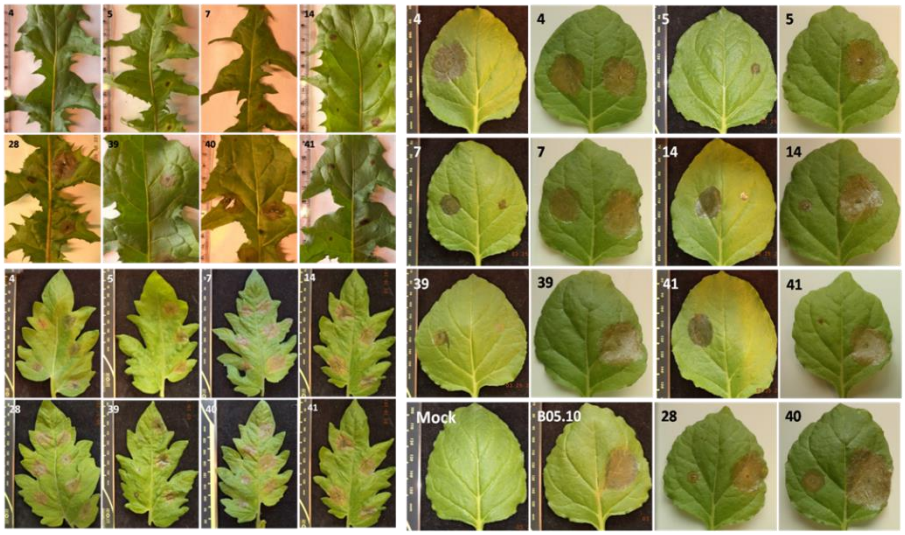


Fig. 3.8. Disease symptoms of *Botrytis* infection on dandelion, tomato and tobacco leaves. The left side of the leaves were inoculated with endophytic *Botrytis* isolates whereas the right side were inoculated with pathogenic reference strain *B. cinerea* B05.10. Lesion diameters were observed at 5 dpi on dandelion, 3 dpi on tomato and 4 dpi on tobacco leaves. N= 6 for dandelion, tomato and for tobacco, except isolate 28 and 40 N= 4. (dpi = days post inoculation).

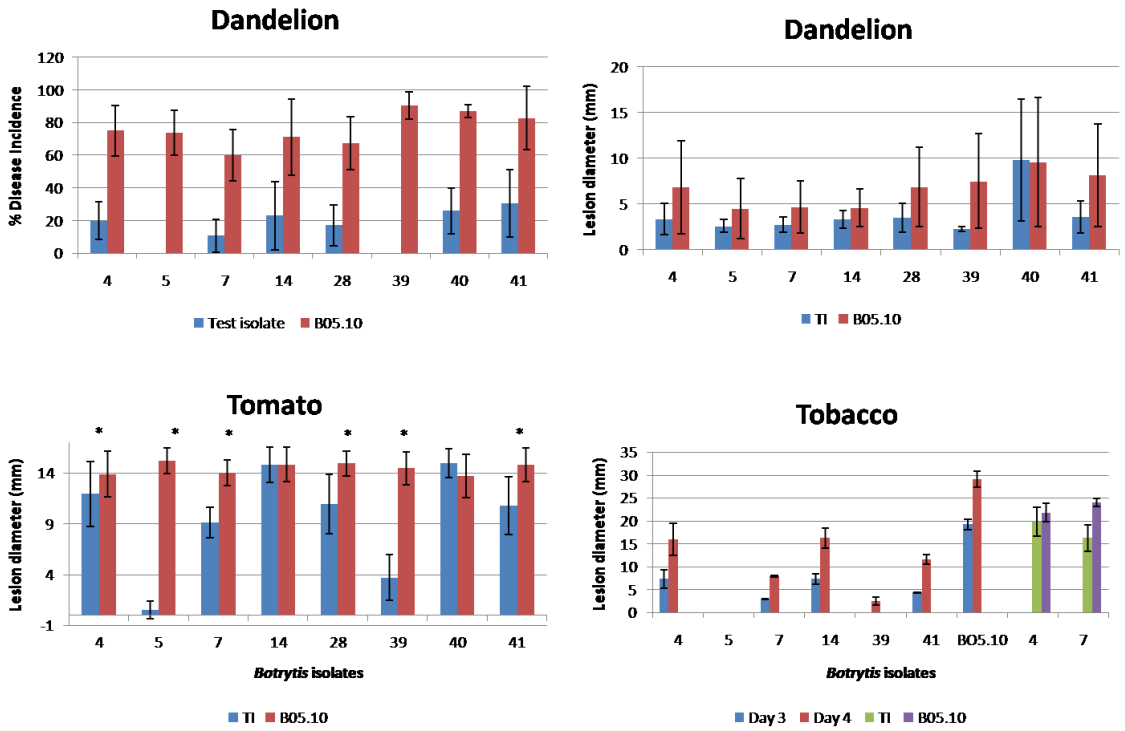


Fig. 3.9.Disease incidence on dandelion and lesion diameter on dandelion at 5 days after inoculation (N=6), tomato at three days after inoculation (N=8) and tobacco leaves at three and four days after inoculation (N=3) caused by endophytic *Botrytis* isolates and reference pathogenic strain *B. cinerea* B05.10 inoculation. Data represented average means with standard deviations indicated with the error bars. Average means of lesion sizes were significantly different with reference strain B05.10 based on T-test at P=0.01 indicated with asterisk sign. For infection assay on tobacco, blue (3 dpi) and red bars (4 dpi) showed the result from the first replicate while the green and purple bars (4 dpi) showed the result of the second replicate. dpi : days post inoculation.

Isolates 4, 7, 14, 39 and 41 except isolate 5 were pathogenic on tobacco based on the result of the first replicate. All of those endophytic isolates showed lower disease severity on tobacco leaves compared to *B. cinerea* B05.10 (Fig. 3.9). However, the infection assay at second replicate was failed to confirm the result from the first one. Only endophytic *Botrytis* isolates 4 and 7 produced lesions at second replicate.

3.3.2. The effect of endophytic *Botrytis* isolates on dandelion seed germination and disease symptom

To investigate whether endophytic *Botrytis* isolates had effects on dandelion seed germination and disease symptoms on dandelion seedlings, 2 species of endophytic *Botrytis* isolates namely isolate 14 and 39 were chosen as representative isolates. Surface sterilized dandelion seeds were sown on BSM plates that were inoculated one day before with a spore suspension of those isolates and reference strain B05.10 at four inoculation sites. SST seeds and NST seeds without fungal inoculation were also included.

More than 60% of dandelion seeds germinated on medium that was previously inoculated with endophytic *Botrytis* isolate 14 whereas for other treatments the percentage of germination was about 50% after 4 weeks incubation at room temperature. Several of the germinated seedlings became diseased by *Botrytis* afterwards. High rates of diseased seedlings were observed on plates that were previously inoculated with isolate 39 and on plates with non surface sterilized seeds without fungal inoculation. Lower percentage of *Botrytis* infections were observed on plates inoculated with isolate 14 and with strain *B. cinerea* B05.10. High infection rates on plates inoculated with isolate 39 and NST seeds seemed to be associated with rapid spread of those isolates over petridishes, whereas on plates inoculated with isolate 14 and B05.10, the fungi only grew at limited areas near the inoculation sites. Therefore, many seedlings probably escaped from fungal infection (Fig. 3.10). Based on visual observation, dandelion seedlings growing on BSM with fungal inoculation showed better growth performance compared to SST seedlings without fungal inoculation when the seedlings did not contact with fungal mycelia. This phenomenon seemingly related to the ability of *Botrytis* isolates to degrade toxic compounds on BSM medium which might hamper plant growth.

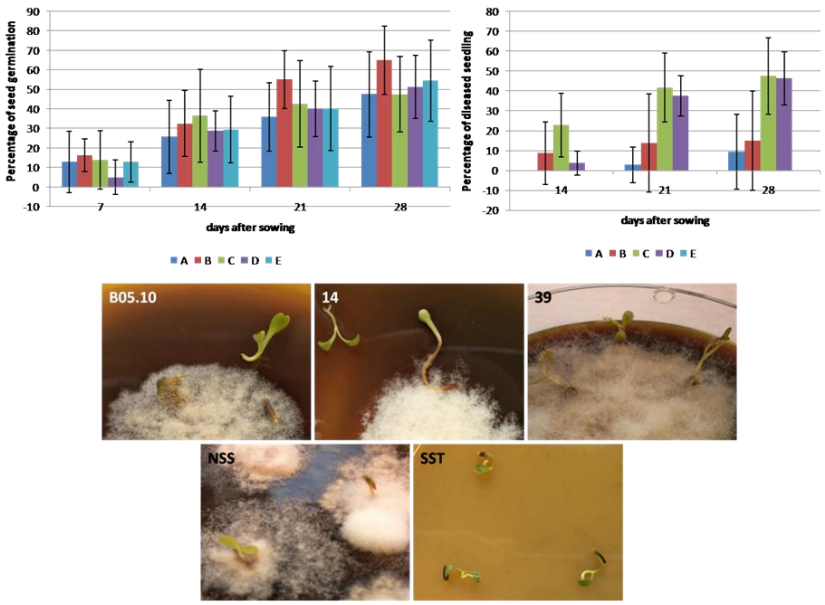


Fig.3.10.Percentage of germinated seeds and percentage of diseased seedling of dandelion seeds sown on BSM plates (A) SST seeds sown on plates inoculated with 4 µl 10^6 spores/ml of reference strain *B. cinerea* B05.10, (B) SST seeds on plates inoculated with endophytic *Botrytis* isolate no 14, (C) SST seeds on plates inoculated with endophytic *Botrytis* isolate no 39, (D) NST seeds without fungal inoculation and (E) SST seeds without fungal inoculation. SST = surface sterilization treated seeds and NST = non surface sterilization treated seeds. Data represented the average of means with standard deviations indicated with error bars. N=10. N represented number of plates with 8 seeds/plate.

To promote the spread of *Botrytis* isolates across the plate, MEA was selected for growing cultures not only because this medium is favorable for *Botrytis* growth but also because it probably

will not hamper plant growth. Spore solutions of isolate 5, 7, 39 and B05.10 were inoculated at 4 sites on MEA plates three days before seeds were sown. Better seed germination was observed in the presence of the endophytic *Botrytis* isolates, as compared to seeds sown on B05.10 inoculated plates. 53% of seeds cultured on plates inoculated with isolate 7 were germinated and appeared healthy, while 30% of seeds sown on plates inoculated with *B. cinerea* B05.10 were also germinated but became diseased soon so they did not continue to grow (Fig. 3.11). However, the germinated seedlings sown on plates with those endophytic *Botrytis* isolates started to become infected by those fungi a week afterwards.

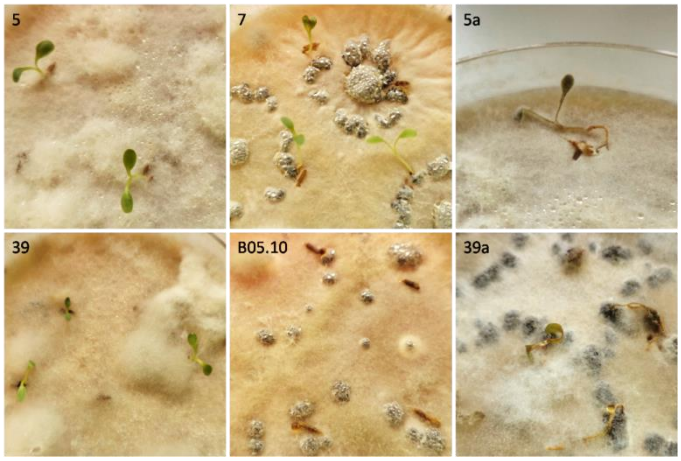


Fig. 3.11. Dandelion seed germination on MEA plates inoculated with endophytic *Botrytis* isolates 5, 7, 39 and pathogenic *B. cinerea* B05.10 (N=3 with 10 seeds/plate) at 10 days after sown. The infected seedlings by isolates 5 and 39 at 3 weeks after sown (5a and 39a).

3.4. Morphological characterization

Isolate 5 and 39 were not assigned to a known *Botrytis* species yet, because their phylogenetic relationship did not permit a species identification. Therefore, it was important to describe the characteristics of those isolates, especially the characters that are commonly used as species description features, such as growth rate on specific media, sporulation conditions, spore shape, spore size, sclerotium production and sclerotium size.

Radial Growth

To find the optimum temperature for growth of isolates 5 and 39, those isolates were cultured on five media such as CDA, MEA, OMA, PDA and V8A and three incubation temperatures i.e. 0^{o}, 15^{o} and 20^{o}C. The plates were incubated in darkness and the radial growth was measured every day until the mycelium reached the edge of petridishes. Both isolates showed the best growth rate on PDA and MEA incubated at 20^{o}C where they reached the edge of the plates within four days of incubation. They reached the edge of the plates after five days at 15^{o}C on PDA (Fig. 3.12). Both

isolates also grew on all media at 0°C but the growth rates were really slower compared to higher temperatures (data not shown).

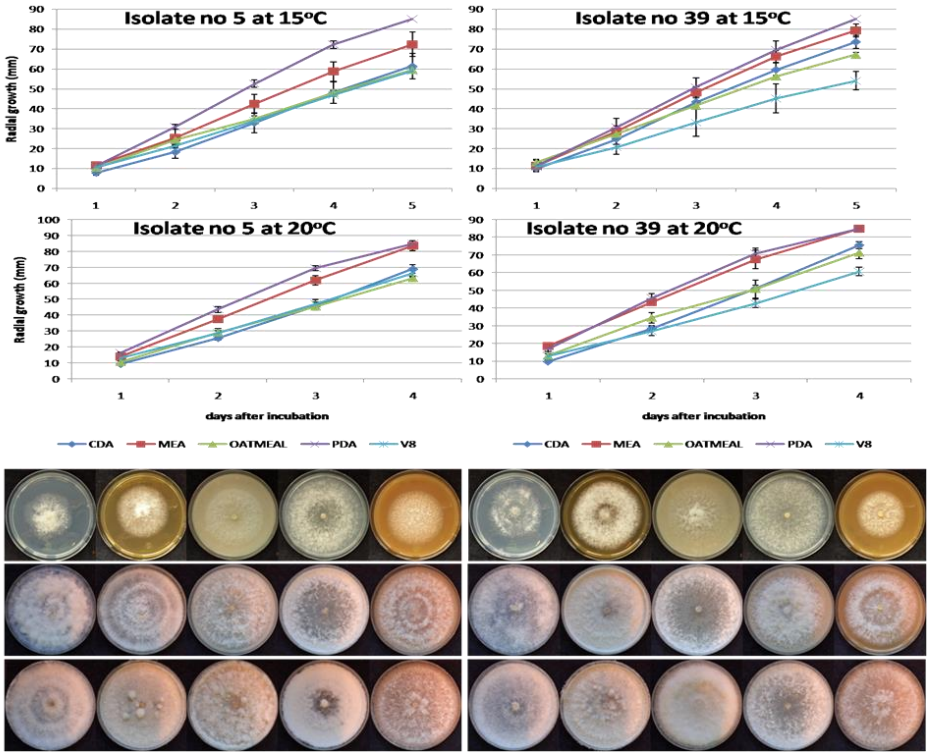


Fig. 3.12. Radial growth of endophytic *Botrytis* isolates no 5 and 39 on five media and at two incubation temperatures 15° and 20°C in the dark. Data represented average means of radial growth (N=5) every day with standard deviation indicated with error bars (above). Cultures of isolate no 5 (left) and 39 (right) after incubation for 10 days at 0°C (upper), 5 days at 15°c (middle) and 5 days at 20°C (lower) cultured in CDA, MEA, OMA, PDA and V8A media respectively from left to right (bottom)

Induction of spore production

Species identification based on morphology according to the morphological species concept (MSC) is one of the methods to identify species. It is not easy for microorganisms such as fungi to use morphological characters for species identification because many species that look similar but actually belong to different species, as is the case for *B. cinerea* and *B. pseudocinerea* (Walker *et.al.,* 2011). However, spore shape and size are still needed to formally describe a species.

To investigate the optimum medium and light condition for spore formation of isolates 5 and 39, the mycelia were cultured on five media in the dark for four days and then exposed to two light conditions i.e. UV light and combining UV and fluorescence light for one night. The best medium for spore induction of isolate 5 was V8 agar exposed to UV light whereas the best medium for isolate 39 was oatmeal agar exposed to UV light. Both isolates can produce spores on all five media and both light conditions (Table 3.3 and Fig. 3.13) but produce a lower number of spores than

B. cinerea strain B05.10 (data not shown). Both isolates produced conidiophores with septa. Conidiophores of isolate 5 formed opposite-branches with ovoid to ellipsoid and sometimes oblong spores with obtuse apex and base flattened abscission scar. Isolate 39 formed alternate branches with ovoid to ellipsoid spores with narrowing area to the point of attachment and obtuse apex. Both primary branches of those isolates then formed secondary branches.

Table. 3.3. Total number of spores of endophytic *Botrytis* isolates 5 and 39 on five media with two light conditions. Data represented average mean of total spores (spores/ml) from two 90 mm plates.

Media	Light condition	Isolate 5	Isolate 39
CDA	UV	$9{,}6 \times 10^4$	$1{,}5 \times 10^7$
MEA	UV	$1{,}3 \times 10^5$	$3{,}4 \times 10^7$
OMA	UV	$1{,}6 \times 10^5$	$6{,}2 \times 10^7$
PDA	UV	$6{,}4 \times 10^4$	$1{,}1 \times 10^7$
V8A	UV	$5{,}7 \times 10^6$	$1{,}5 \times 10^7$
CDA	UV + FL	$9{,}6 \times 10^4$	$4{,}6 \times 10^7$
MEA	UV + FL	$1{,}3 \times 10^5$	$1{,}6 \times 10^7$
OMA	UV + FL	$4{,}2 \times 10^5$	$1{,}7 \times 10^7$
PDA	UV + FL	$6{,}4 \times 10^4$	7×10^6
V8A	UV + FL	$1{,}2 \times 10^6$	$3{,}5 \times 10^7$

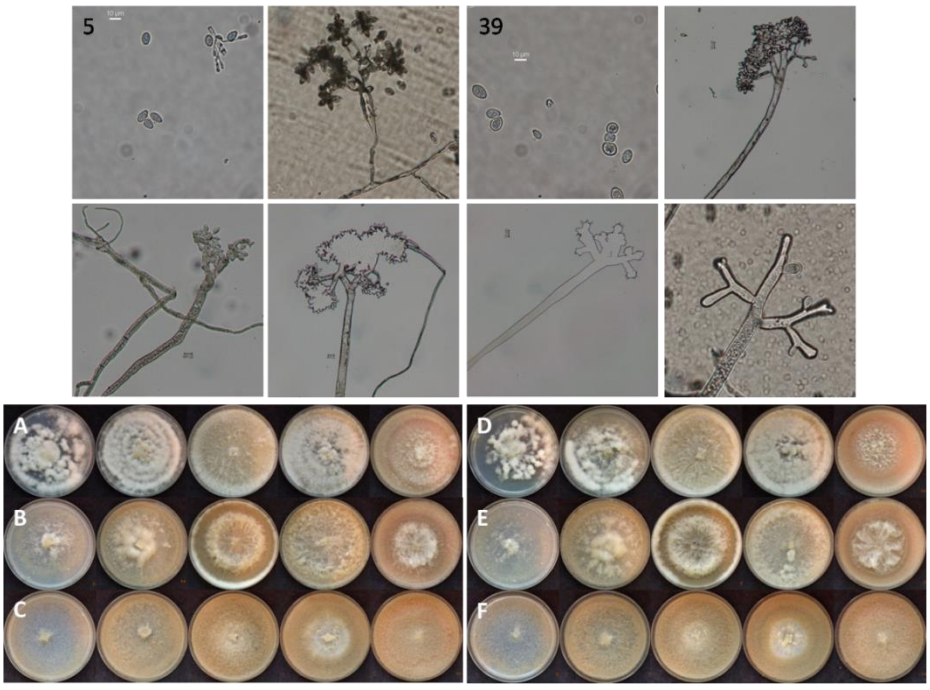


Fig. 3.13. Conidiophores of isolate 5 and 39 (above). Sporulating cultures at 4 days after exposure to UV or UV+fluorescens light (N=3 plates). (A) isolate no 5, (B) isolate no 39, (C) reference strain *B. cinerea* B05.10 exposed to UV light for one night, (D) isolate no 5, (E) isolate no 39, and (F) B05.10 exposed to UV and fluorescence light for one night to induce spore formation (bottom figure).

Spore measurements

Spore sizes of isolate 5 and 39 were measured under an optical microscope. Average spore length and width for both isolates were similar (Table 3.4), and they were similar in size to *B. cinerea*(11.86 ± 1.45 µm) and *B. pseudocinerea* (12.04 ± 1.55 µm) (Walker *et.al.,* 2011). Isolate 5 produced light brown spores while isolate 39 produced dark brown to black spores.

Table.3.4. Spore length and width (µm) of isolates 5 and 39 (N=50). Measurement was performed under optical microscope.

Measurement	5	39
Length	12.6 ± 1.8	11.8 ± 1.3
Min	9.5	9.2
Max	16.8	14.2
Width	7.2 ± 1.1	7.6 ± 1.1
Min	5.3	4.9
Max	10.8	9.8

Sclerotia formation

To induce sclerotia formation, cultures of isolate 5 and 39 were incubated in darkness for about 2 months at 15^{o} and 20^{o}C. Five media were used similar to those used for spore induction. Both isolates produced black sclerotia on all media and both incubation temperatures. However, they produced different numbers and shapes of sclerotia in those media (Table 3.5 and Fig. 3.14).

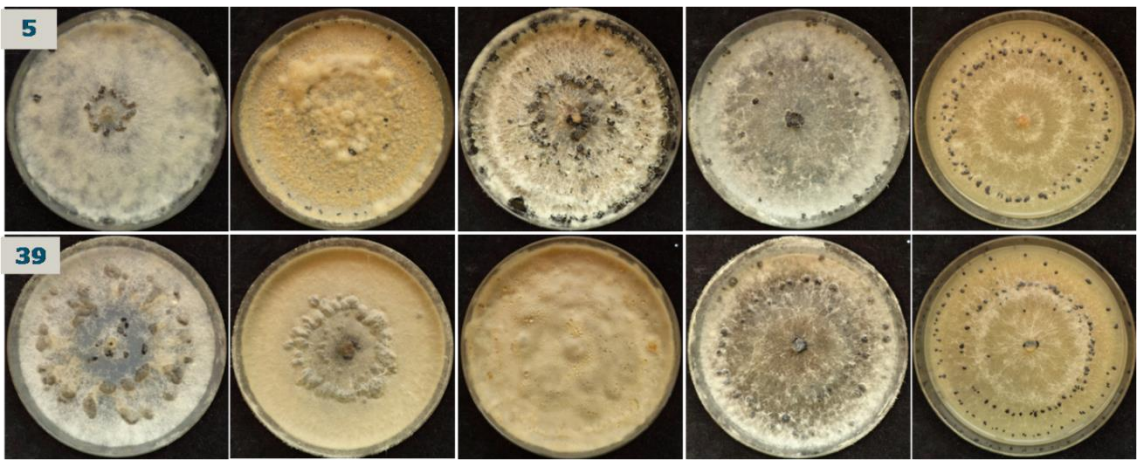


Fig. 3.14. The sclerotia formation of isolate 5 and 39 on five media, CDA, MEA, Oatmeal Agar, PDA and V8A, left to right respectively after about two months incubated at 15^{o}C in darkness.

Table. 3.5. Sclerotia produced by isolate 5 and 39 (N=5 plates). +/- indicated isolate produced sclerotia on some plates but did not on other replicates. + indicated on average < 20 sclerotia formed on each plates, ++ indicated 20-50 sclerotia formed and +++ >50 sclerotia formed. Number between the brackets was the average length of sclerotia (mm) with a standard deviation (N=20)

Media	Isolate 5		Isolate 39	
	T 15°C	T 20°C	T 15°C	T 20°C
CDA	+++(3.5±1.5)	++(4.2±1.6)	++(4.4±1.4)	++(5.1±1.5)
MEA	++(2.4±0.9)	++(6.4±3.1)	++(3.9±1.3)	++(6.5±2.7)
Oatmeal Agar	++(3.0±0.6)	++(6.2±3.2)	+(3.1±0.9)	+/-(5.9±1.5)
PDA	+++(3.3±1.3)	+(1.5±0.3)	+++(3.5±1.2)	++(4.3±1.6)
V8A	+++(2.2±0.8)	+++(2.2±0.4)	+++(2.2±0.3)	+++(2.3±0.5)

4. Discussion

Based on phylogenetic analysis of 47 *Botrytis* isolates, sampled as endophytes from seemingly healthy dandelion plants, the isolates were divided into 4 species i.e. *B. cinerea, B. pseudocinerea,* 1 unknown species related to *B. caroliniana/B. fabiopsis* and 1 unknown species related to *B. mali.* Most (35) of the isolates belong to the *B. cinerea* and several others grouped with *B. pseudocinerea*. Several studies reported that *B. cinerea* forms a species complex. Giraud *et. al.* (1997) reported that *B. cinerea* can be divided into two groups based on the absence (*vacuma* strains) and presence (*transposa* strains) of two transposable elements (TEs) *boty* and *flipper*. Fournier *et.al.* (2003) also discovered that *B. cinerea* consists of two groups, however, they described group I as isolates that did not have TE (*vacuma* strains) whereas group II contained isolates that comprised both *vacuma* and *transpose* strains. However, later on many isolates that previously belong to *B. cinerea* based on two housekeeping gene sequences (HSP60 and G3PDH) appeared to be different species. Some of them grouped with *Botrytis* group S that was proposed as a novel clade (Leroch *et.al.* 2013). Our finding is in line with this report because we also found some isolates that were previously assigned as *B. cinerea* showed MRR1 sequences similar to *Botrytis* group S. Leroch *et.al.* (2013) identified there were sequence polymorphisms on MRR1 gene sequence that can distinguish between *B. cinerea, Botrytis* group S with MDR1h and *Botrytis* group S without MDR1h. Analysis of MRR1 sequences showed that 13 endophytic *Botrytis* isolates belong to *Botrytis* group S without MDR1h (Fig. 3.4).

Endophytic *Botrytis* isolates that were assigned to *B. pseudocinerea* seem phylogenetically more homogenous than the endophytic isolates assigned to *B. cinerea*. Several differences that were found between the housekeeping gene sequences of *B. pseudocinerea* isolates, suggested that there might be further subgroups within the *B. pseudocinerea.* However further analysis using the MS547 gene sequence confirmed that all isolates belong to one species, i.e. *B. pseudocinerea* (Fig. 3.5). The

endophytic *B. pseudocinerea* were assigned as group A while another group of *B. pseudocinerea* isolates from New Zealand has been assigned as group B and for a while were still indicated as variation within one species (Johnston *et.al.,* 2013).

Shipunov *et.al.* (2008) reported 2 isolates that are related to *B. peoniae* as endophytic fungi on spotted knapweed. Isolate 39 was closely related to one of those isolates based on HSP60 sequence (Fig. S1). Later on, those isolates and isolate 39 appeared more related to *B. mali* than *B. peoniae* when the sequences of *B. mali* (DNA samples were kindly provided by Agriculture and Agri-Food Canada) were included to construct phylogenetic tree. HSP60 sequence of isolate 39 was identical to sequence of *B. mali* B26 while one nucleotide miss match was found on G3PDH sequence between isolates 39 and *B. mali* B26. However, more than 10 miss matches were investigated on RPB2 sequences between isolate 39 and *B. mali*. Some miss matches were detected as problems in sequences pattern. It might be important to repeat sequencing process of isolate 39 RPB2 gene to get more reliable data.

Both mating types MAT1-1 and MAT1-2 were found in endophytic isolates that belong to *B. cinerea* and *B. pseudocinerea*. Since many isolates were identified as *B. cinerea* and others as *B. pseudocinerea* with both mating types, it could be one of the reasons why those species are highly diverse as the result of sexual crosses and recombination.

As we found that most of endophytic isolates belong to *Botrytis* species clade 1, which is known to have isolates with a broad hosts range, it was not surprising that those endophytic isolates might be pathogenic. This assumption was confirmed by infection assay results. In fact, several endophytic isolates showed infectious on dandelion, tomato and tobacco, and some of them caused similar lesion diameters as the pathogenic strain *B. cinerea* that is well known as pathogen for over 200 host plants (Williamson *et.al.,* 2007).

The infection rate was lower on dandelion plants. It seems that dandelion has some level of resistance against *Botrytis* infection because even the reference pathogenic strain also showed low disease severity (Fig. 3.9). Hanawa *et.al.* (1995) investigated a phytoalexin of dandelion, lettucenin A, inhibited the growth of pathogen when dandelion was challenged with *Cladosporium herbarum.* Richter *et.al.* (2012) reported that dandelion showed high polyphenol oxidase (PPO) activity which contributed to disease resistance against *B. cinerea* and *Pseudomonas syringae* pv. *tomato.* It could be those compounds manage a level of inhibition against *Botrytis* isolates invasion therefore they cause mild disease symptom on dandelion. However, endophytic *Botrytis* isolates were able to infect dandelion seedlings during *in vitro* inoculation tests. Even isolate 39 that showed lower infection

rates for all tested leaves, infected almost 50% of dandelion seedlings on BSM plates (Fig. 3.10). Isolate 5 that showed non infectious on all tested leaves also caused disease symptoms on dandelion seedlings. It might be these endophytic isolates produce sufficient toxin concentrations that overcome dandelion seedling defense mechanisms, therefore, cause disease symptoms.

The next experiment with dandelion plants grown from NST and SST seeds also supported this assumption. Outgrowth of *Botrytis* fungi almost covered all seeds when NST seeds grew on BSM. If this condition continued, the germinated seeds started to become infected. However, if they were transferred into wet filter paper after three days in BSM plates, only a small portion of mycelia attached to the seeds/seedlings that were brought to new medium. Seedlings managed to escape from fungal infection, and none of the germinated seedlings developed disease symptoms based on visual observation. Dandelion seedlings probably produce sufficient phytoalexin or pathogenesis-related (PR) proteins that protect the seedlings from damaging effect of *Botrytis* infection. However, there seemed to be a trade-off effect between defense and growth at the early stage of dandelion plant development. Seedlings that developed from NST seeds showed slower growth compared to seedlings derived from SST seeds. Once the resistance against fungal infection was established, plants from NST seeds recovered from their delay in growth. Their growth parameters were similar to those developed from SST seeds at 4 weeks after sowing on the soil (Table 3.2).

Three representative isolates of endophytic *B. pseudocinerea* (isolates 14, 28 and 40) caused different disease severity on tomato leaves although they belong to the same species. Two of them, isolates 14 and 40, produced similar lesion sizes as pathogenic strains *B. cinerea* B05.10, whereas 3 representative isolates of endophytic *B. cinerea* (isolates 4, 7 and 41) showed less virulent than pathogenic reference strain. The classes of toxins and toxin concentrations produced by each endophytic isolate could differ and affect its virulence. Reino *et.al.,* (2004) investigated more aggressive strains of *B. cinerea* produced higher toxins concentrations derived from two classes of toxins with botryane skeleton and botcinolide derivatives. In contrast, less virulent strain produced only one class of toxin corresponding to botrydial or its derivatives with low concentration.

Isolate 5 that seems to be related to *B. caroliniana* and *B. fabiopsis* was not infectious to any of the tested plants. *B. caroliniana* is infectious on broad bean and blackberry (Li *et.al,.*2012b) and *B. fabiopsis* can infect broad bean (Zhang *et. al.,* 2010a). It might be important to perform infection assay of isolate 5 on broad bean leaves and blackberry to confirm its pathogenicity. In case, this isolate also cannot infect those hosts, the test should be continued with other related plant hosts that are commonly associated with *Botrytis* infection before we can assume this isolate is truly

endophytic and non-infectious. It might be needed to check pathogenicity of isolate 39 on apple because it was related to *B. mali* that reported as a post harvest pathogen of apple.

Isolate 5 could produce spores on all tested media although it produced lower number of spores as compared to *B. cinerea* B05.10. The spore sizes of isolates 5 and 39 were similar to spores of *B. cinerea* and *B. pseudocinerea*, therefore they could not recognize by looking at spore characters. Li *et.al.* (2012a) described that isolate *B. caroliniana* did not produce spores on PDA and Kings Medium B (KMB) while Zhang *et.al.* (2010) described quite large spore sizes for *B. fabiopsis* 19.7 x 15.0 µm. In line with our finding about radial growth of isolates 5 and 39, Zhang *et.al.* (2010a) observed optimum growth rate of *B. fabiopsis* on PDA also at temperature 20^{o}C.

Isolate 39 grouped with *B. mali* based on phylogenetic analysis using HSP60 and G3PDH gene sequences. This species was reported since 1931 as post harvest pathogen on apple, however the morphology characterization of this species was not formally described. Later on, O'Gorman *et. al.,* (2008) described morphological characters of this species by using herbarium specimen deposited by Ruele (1931). The shape and size of isolate 39 were similar to *B. mali* characters described by O'Gorman *et. al.* (2008) .

By growing non surface sterilized dandelion seeds on sterile soil and later collecting the leaves, surface sterilizing them and culturing in *Botrytis* selective medium, we observed that *Botrytis* grew from some of the dandelion leaves. However, it can be argued that the fungi could have landed on the leaves and the surface sterilization treatments were not sufficient to kill all epiphytes. It is difficult to validate the effectiveness of a surface sterilization treatment, so it is difficult to prove that an organism considered an endophyte is not an epiphyte. To fully confirm endophytic colonization on dandelion plants, it would be possible to grow the surface sterilized dandelion seeds on sterile media infested with endophytic isolate that is tagged with a specific marker (such as GFP) which allows detection of the infested isolate after re-isolation.

Some isolates were found to be pathogenic on tomato and also might be pathogenic on other crops. The endophytic fungal virulence factors and plant defense mechanisms are assumed in balance condition, therefore the association between them remains asymptomatic. However, endophytic fungus can infect a host while plant experiences stress conditions or it may invade senescent tissues of the host (Sieber, 2007). It is also important to check the risk that endophytic isolates are transferred into new hosts and to study their ability to persist in the soil or in plant debris without hosts.

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Supplemental data

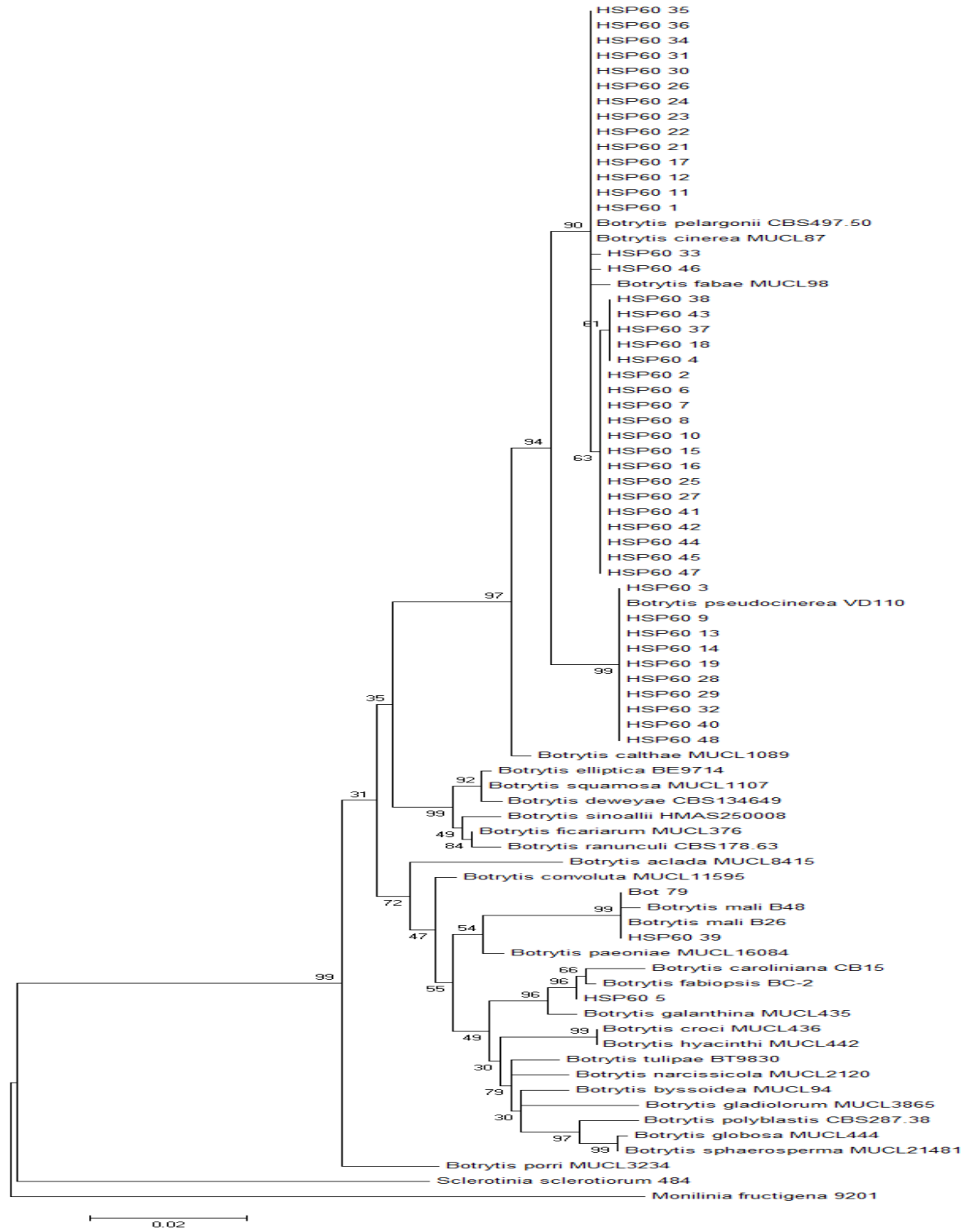

Fig. S1. Phylogenetic position of 47 endophytic *Botrytis* isolates in the genus based on HSP60 sequence. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The numbers stated at each node indicate percentage of bootstrap value. Isolate Bot 79 (Shipunov *et.al.,* 2008) were included to show its HSP60 sequence related to isolate 39.

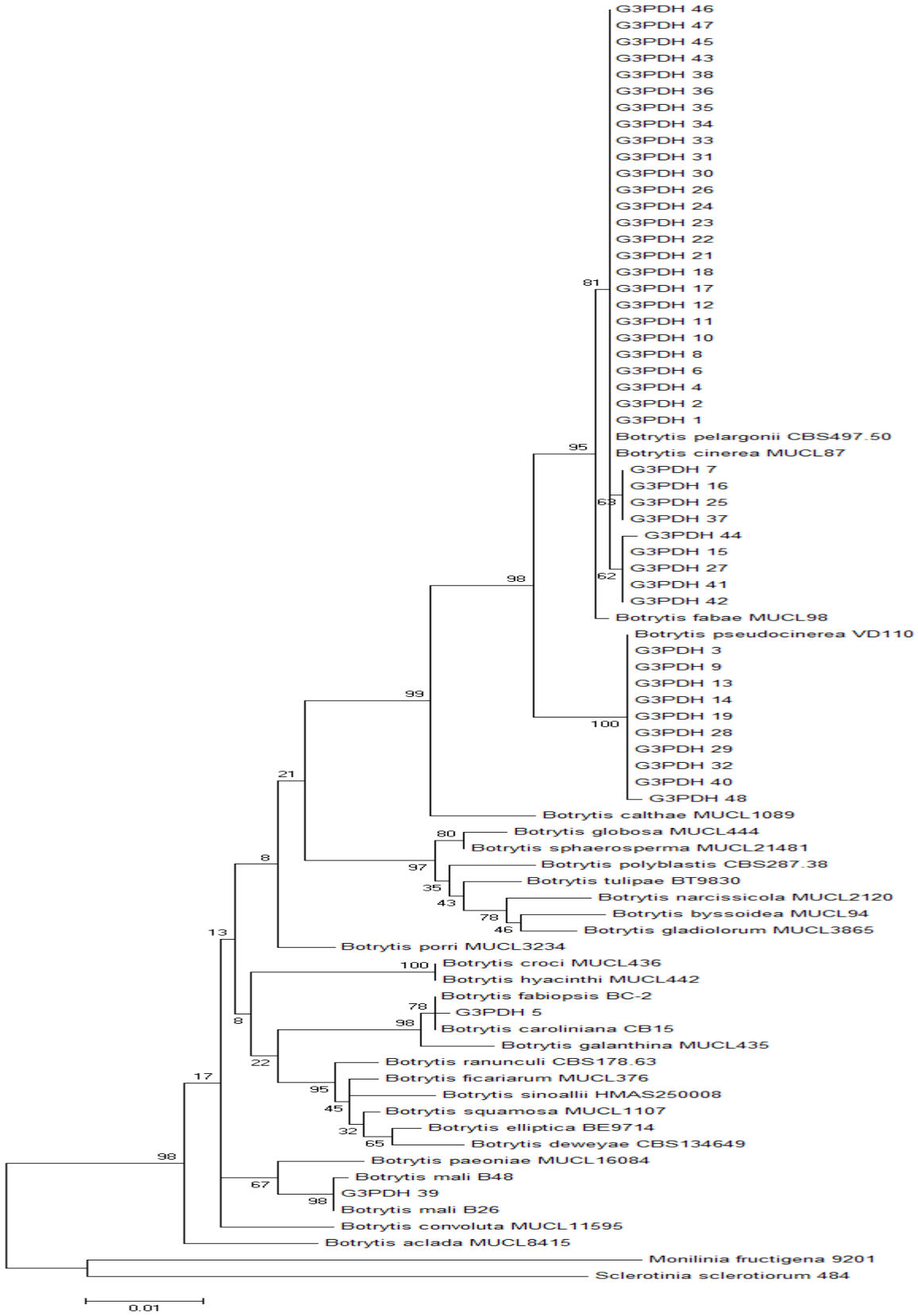

Fig. S2. Phylogenetic position of 47 endophytic *Botrytis* isolates in the genus based on G3PDH sequence. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The numbers stated at each node indicate percentage of bootstrap value.

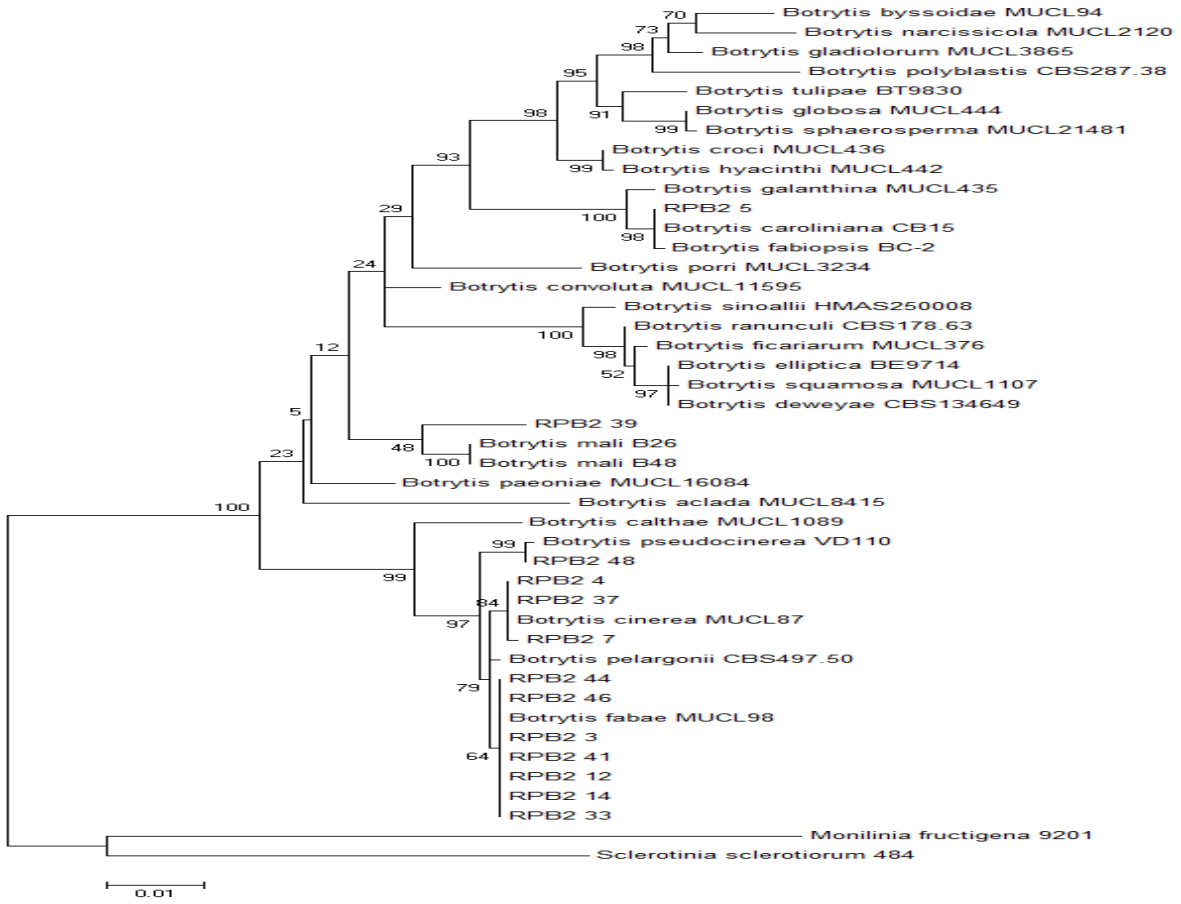

Fig. S3.Phylogenetic position of endophytic *Botrytis* isolates in the genus based on RPB2 gene sequences. Phylogenetic tree construction used maximum likelihood method with 1000 bootstraps. The number on each node indicated percentage of bootstrap value.