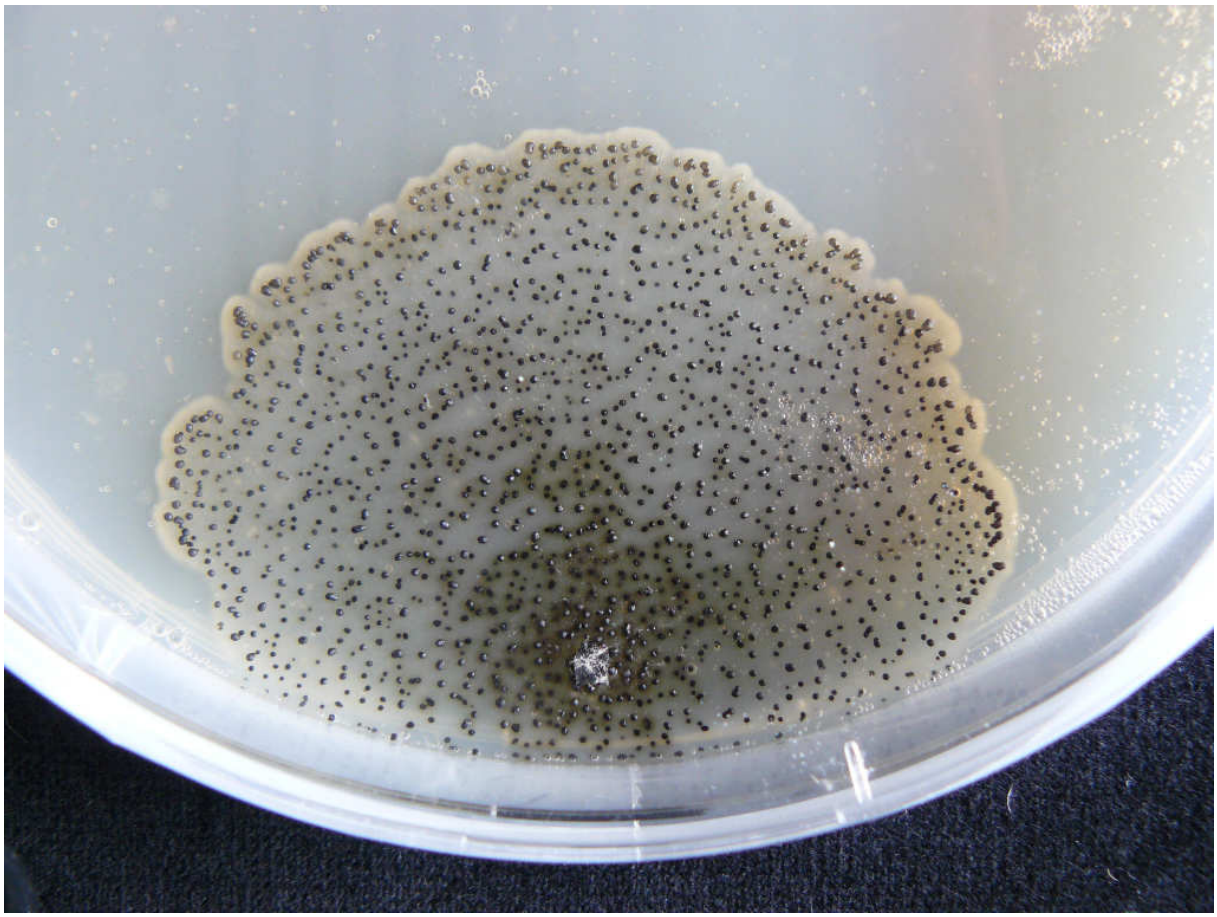
Ecology and Evolution of Senescence in Species of the Plant Pathogen *Botrytis*



Picture showing a plate containing 2%PGA GB5 medium with *B. tulipae* Bt9830 showing senescence

Frank Segers

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Preface

When writing this preface I am almost finished with my thesis on senescence in *Botrytis*. Looking back I am very happy I chose to do my master thesis at the department of genetics. I could not have found better or more exciting supervisors as Fons Debets and Anne van Diepeningen. The work environment was always very stimulating and had a friendly atmosphere. Furthermore I would like to thank Jan van Kan for his help and expertise on *Botrytis*. Marijke Slakhorst was always in for a chat and was more than a great help on the lab, much appreciation for that. On the molecular lab I found that Bertha Koopmanschap was a great help and I could not have done it without her. Next to learning a lot about senescence in fungi and about *Botrytis* I learned to plan my work better and to develop experiments. Furthermore I found out that using several computers with different versions in computer programs can lead to difficulties.

Frank Segers
April 21, 2009

Abstract

Senescence in fungi has been studied for more than 50 years now, and still only a few have been properly described. *Podospora anserina* is used as a model organism for fungal senescence. For *P. anserina*, mitochondrial DNA instability leads to senescence. Reactive oxygen species (ROS) are thought to be the cause of these instabilities. Because the molecular phylogeny of *Botrytis* is complete senescence in *Botrytis* species will be studied. *Botrytis* species are necrotrophic plant pathogens, which live on ephemeral substrate and live in a ROS rich environment. Senescence was found erratically in *B. aclada*, *B. cinerea* and *B. porri*, while found regularly in *B. paeoniae* and *B. tulipae*. Survival structures as sclerotia are produced just before death. The *B. cinerea* mutant Δbcg3 stopped growing after 2 weeks and showed senescence. Senescence has been seen in several *Botrytis* species and can form the basis for future research on senescence in *Botrytis* species.

Keywords*: Senescence, Botrytis tulipae, Botrytis paeoniae, Botrytis cinerea, Reactive oxygen species (ROS), mitochondrial DNA (mtDNA).*

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1. Introduction

1.1 The evolution of senescence in fungi

Ageing and senescence in fungi has been studied for more than 50 years. Ageing and senescence are often considered to be equivalent. However, ageing is defined as the process of maturing during life, and senescence is defined as the process of deterioration of an organism during its life. During a lifetime a single organism has an increasing cumulative risk of dying. This is because of disease and predation, but also has other accidental causes. Traits that will only manifest themselves after the individual died will not be subject to natural selection. These traits can therefore still spread through the population. It can be said that ageing evolves in the shadow of natural selection. Ageing is not counteracted by natural selection. Paul Medawar came up with this theory, which is now called the mutation accumulation theory of ageing (Medawar 1952). Traits with a pleiotropic beneficial effect in early life may be favored by natural selection, even though this trait can have a negative effect on fitness later in life. This idea was also pointed out by Medawar, but was further worked out by George Williams and is now known as the antagonistic pleiotropy theory of ageing (Williams 1957). A trade-off between a beneficial effect in early life to a negative effect later is made by the organism.

Filamentous fungi are generally expected not to be subject to ageing and are considered to be immortal (Osiewacz 2002). This is thought since fungi are modular organisms and do not have a distinction between soma and germ line cell lines. Such distinction is essential for ageing according to the disposable soma theory (Kirkwood 1977). This theory is a special case of the mutation accumulation theory. The germ line is the cell line for sexual reproduction and is set aside from the soma of the organism. The germ line must be immortal, whereas the soma is disposable after successful reproduction and is allowed to senesce. This theory however applies to unitary organisms, like animals. Modular organisms have an iterative indeterminate growth.(Kirkwood 1977).

Nevertheless, fungi regularly stopped growing in laboratories and were cast away without looking any further to the causes of death. The first intensive study was done on the filamentous ascomycete *Podospora anserina,* which showed that all natural isolates of *P. anserina* have a strain specific limited lifespan and show distinctive signs of senescence (Rizet 1953). Now more then 50 years of research on several fungi, especially *P. anserina,* has shown us a lot about fungal senescence (Osiewacz 2002). Beside *P. anserina,* only some *Aspergillus* and *Neurospora* isolates have been shown to senescence (Griffiths 1992). Most *Neurospora* isolates tend to be immortal, for they show almost no senescence, or senescence is absent. However, a few *Neurospora* populations show polymorphism for senescence, for example the Hawaiian populations of *N. intermedia* and *N. tetrasperma* (Debets, Yang et al. 1995; Maas, van Mourik et al. 2005). Senescence has also been

found in sectors of the entomopathogenic fungus *Metarhizium anisoplia*, where sectorization has been seen as a sign of ageing (Wang, Butt et al. 2005).

1.1.1 Characteristics of senescence in fungi

Podospora anserina is the model organism for senescence in fungi. However, senescence-prone filamentous fungi have similar patterns in aging. The degradation in almost all these species is more or less the same. Fungi tend to have a period of reproduction, after which several typical signs of senescence appear. These typical signs of senescence are reduction in the production of hyphae, female sterility, growth decline, reduced sporulation and death (Bertrand 2000). This can be seen in a figure by Helmut Bertrand (Figure 1). A slightly different observation was found on the entomopathogenic fungus *Metarhizium anisopliae*. *M. anisopliae* tends to deteriorate by sectorization, by forming sectors containing fluffy-mycelial type growth without sporulation. It was found that this sectorization was characterized by the gene expression often associated with ageing. This sectorization is also linked with oxidative stress that causes due to the production of reactive oxygen species (ROS) (Wang, Butt et al. 2005; Li, Pischetsrieder et al. 2008). On molecular level senescence in *Podospora anserina* is associated with the accumulation of small circular mitochondrial derivatives, increased oxidative stress due to loss of specific regions of the mitochondrial genome, ROS production, loss of cytochrome oxidase activity and a switch to alternative oxidase (Osiewacz 2002; Maas, de Boer et al. 2004; Maas, Sellem et al. 2007). Calorie restriction is the only regimen known to extend lifespan in all aging organisms and it also postpones senescence in *P. anserina* (Maas, de Boer et al. 2004).

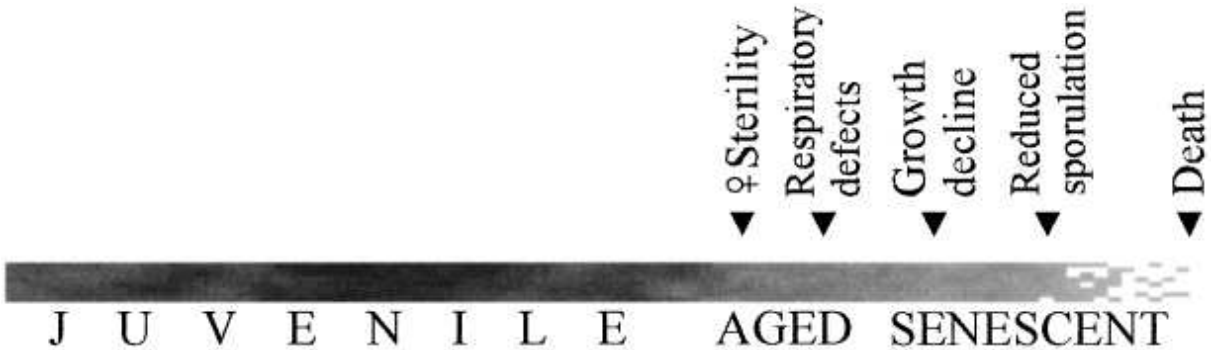


Figure 1: Life cycle of mycelia of senescence-prone fungi. The bar represents the density of hyphae from the point of inoculation to the dead frontier of the colony. The major phases of growth are indicated below the bar. The onset of each of the different aspects of senescence is indicated above the bar (Bertrand 2000).

1.2 The ecology of the plant pathogen Botrytis

1.2.1 The life cycle

Botrytis cinerea Pers. Fr. (teleomorph Botryotinia fuckeliana (de Bary) Whetzel) is a plant pathogen with a necrotrophic lifestyle. It attacks several crop hosts of agronomical importance by distributing its spores through the air (Williamson, Tudzynsk et al. 2007). *Botrytis cinerea* is the only

of the *Botrytis* species which affects multiple crop species. Other species have more specific hosts, or a small range of hosts. All species grow on the necrotic tissue of its host and can survive and sporulate as saprophytes. Host specific *Botrytis* species regularly cause primary lesions on a nonhost, but will not be able to expand these primary lesions (Prins, Tudzynski et al. 2000). For long-term survival sclerotia are formed. *Botrytis* species have several ways of infecting plant material. They can infect any part of a plant by any form of inoculums like, conidia, ascospores, sclerotia, chlamydospores and mycelia (Holz, Coertze et al. 2004). Sclerotia can be found on living crop, debris of the host on the ground, or buried in the soil. New crops are probably inoculated by old debris from previous crops lying on the ground or buried in the soil.

Co-evolution between a host and its pathogen is relatively normal and expected in relationships between an obligate biotrophic pathogen and its host. Obligate biotrophic pathogens do not kill the cells of the host, but live on living cells. However, *Botrytis* species are necrotrophic and therefore do not infect living cells, but kill them first, which makes it more difficult to imagine co-evolution taking place (Van Baarlen, Staats et al. 2004). A possible relationship between *Botrytis* species and its specific hosts was not detected during the study of the molecular phylogeny (Staats, van Baarlen et al. 2005).

1.2.2 Symptoms of Botrytis cinerea on its host

B. cinerea has a big effect on its host and has several different symptoms when infecting a host. Symptoms vary greatly among the different hosts, of which a few can be seen in figure 2. The most typical symptoms of *B. cinerea* are seen on leaves and soft fruits such as raspberry and strawberry (Figure 2a and 2b). Lesions can be seen on rose petals and give the petal a “pocked” appearance, although these lesions can also take the form of a full-scale soft rot (Figure 2b and 2c). Small lesions in the nodes on the stem of raspberry may lay dormant during wintertime and can form sclerotia, which may cause the formation of grey conidia spores during springtime, as can be seen on figure 2d and 2e (Williamson, Tudzynsk et al. 2007).

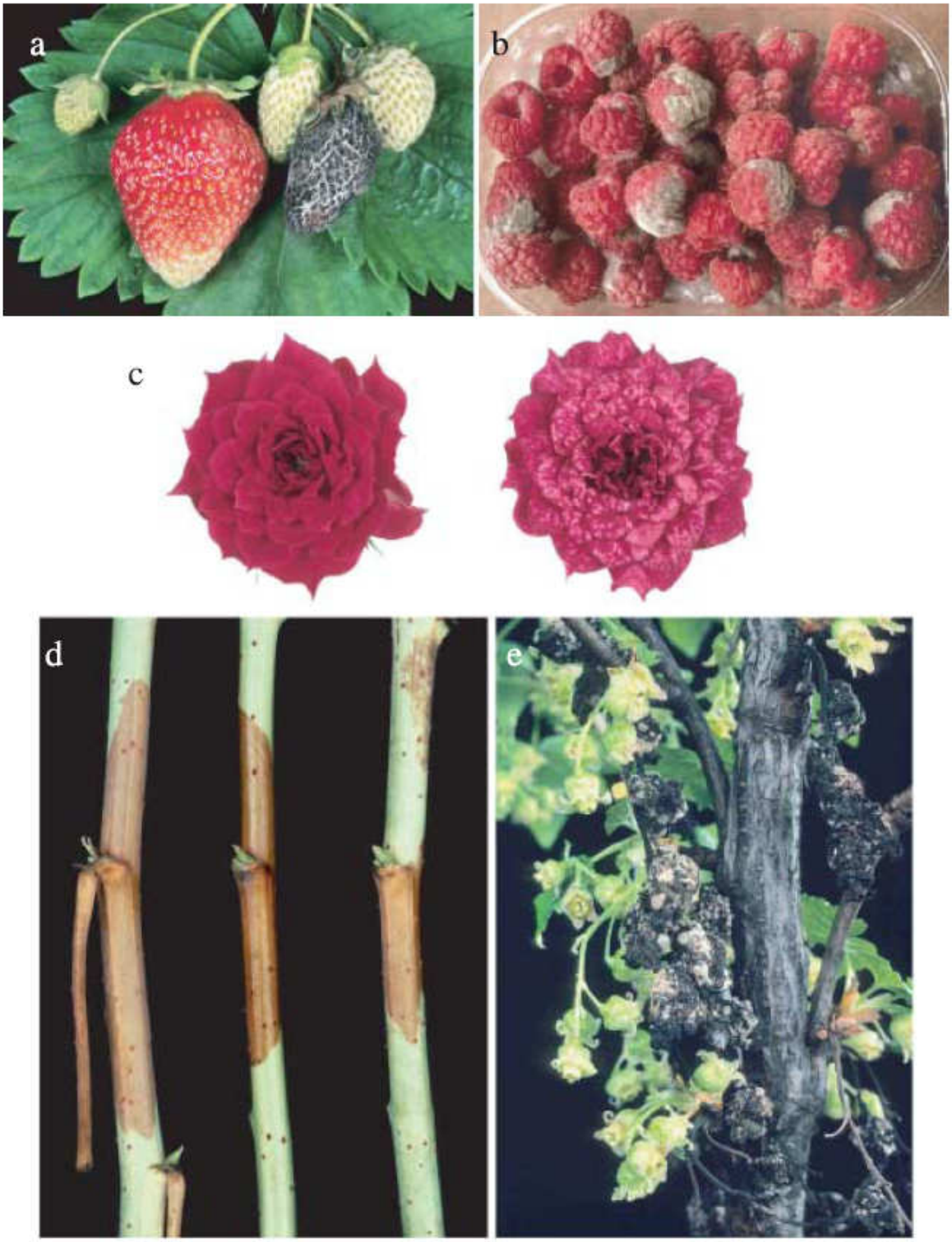


Figure 2: Several symptoms of infections by *Botrytis cinerea* on (a) strawberry, (b) raspberry and (c) rose petals. (d) Lesions arising at nodes following infection of raspberry leaves in autumn. (e) Mummified 1-year-old blackcurrant fruits attached to stem, releasing conidia between the newly opened flowers. Figure from (Williamson, Tudzynsk et al. 2007).

1.2.3 Reactive Oxygen Species

During a large part of its lifecycle, *Botrytis* species have to deal with reactive oxygen species (ROS). Plants produce ROS as a defense mechanism against pathogen attacks. This widespread defense mechanism is called the oxidative burst. Not only are ROS effective in harming cells, they

also induce other host defensive reactions. Reactions as such can be the cross-linking of cell-walls and a hypersensitive response (HR), which results in cell-death at the site of infection and gives rise to necrosis. Since *Botrytis* is a necrotrophic pathogen it benefits by this mechanism. Biotrophic pathogens, which depend on living host cells, are easily killed by this mechanism. As a model organism for necrotrophic fungi, research on *Botrytis cinerea* supports the fact that necrotrophic fungi benefit from the hypersensitive response (Rolke, Liu et al. 2004). Not only is *B. cinerea* extraordinarily good at scavenging ROS, inside as well as outside the cell, but it also stimulates this hypersensitive response in plants (von Tiedemann 1997). It has been found that *B. cinerea* even needs the HR of the host plant for achieving full pathogenicity and that it does so by producing an elicitor (Govrin and Levine 2000; Govrin, Rachmilevitch et al. 2006). Many more evidence has been found on the fact that *Botrytis cinerea* induces a response that leads to the production of ROS or other markers involved during oxidative stress (Deighton, Muckenschnabel et al. 1999; Muckenschnabel, Goodman et al. 2001; Muckenschnabel, Williamson et al. 2001; Muckenschnabel, Goodman et al. 2002; Muckenschnabel, Gronover et al. 2003).

1.2.4. Botrytis species in society

Most research is done on *Botrytis cinerea* since it has a diverse range of hosts of which a lot are of agronomical importance. Especially in the Netherlands is a lot of production of commercially flower bulbs, which are affected by several other *Botrytis* species. Fungicides are available to prevent *Botrytis* from causing a big economical problem on the flower bulb market in the Netherlands. However, more restrictions are put on the use of fungicides and the reliance on fungicides increases the risk of the development of resistance in *Botrytis*. The production of several fruits is also affected by *Botrytis* species, although in some cases it can even lead to beneficial products, but that is more an exception. Grapes, meant for the production of wine, affected by *Botrytis cinerea* in cold to moderate temperature conditions cause so-called noble rot, which gives rise to special sweet dessert wines. Perfect conditions for noble rot are not perfect conditions for *Botrytis cinerea*, but just right to grow and sustain (Elad, Williamson et al. 2004).

1.3 Expectations on the evolution of senescence in species of the genera *Botrytis*

Botrytis species tend to grow on ephemeral substrates such as fruits or flower bulbs, which is one of the main reasons to look for senescence in *Botrytis* species. For *Podospora anserina* it is hypothesized ageing occurs due to its ephemeral ecology. The life cycle is typically short on its natural substrate, herbivore dung, which lasts approximately two weeks. In several laboratories it was observed that some *Botrytis* strains were lost over time, which also evoked interest in more research. The reasons why some *Botrytis* strains get lost will be investigated during this study. This study

mainly investigates whether *Botrytis* species do senesce and if it is related to its ecology. Which Botrytis species are best for further research and what is the cause of this senescence?

Secondly, *Botrytis* is a necrotrophic plant pathogen and therefore has an ecology containing a lot of reactive oxygen species (ROS), produced by plants, but also by the *Botrytis* species when infecting a host. The formation of ROS in mitochondria, and the limitation for scavenging these ROS, plays a big role in senescence in *P. anserina*. *Botrytis* species are very well capable of scavenging external as well as internal ROS. The presence of a lot of ROS in the ecology could have an influence on the lifespan of the organism, which is also influenced by the array of ROS scavengers of *Botrytis* species. We studied several ROS scavenger mutants of *Botrytis cinere*, which were expected to have a lengthened or shortened lifespan when they show senescence.

Finally, both in *P. anserina* and *Neurospora* species mitochondrial DNA rearrangements were found in senescing material. *Botrytis* species may show similar rearrangements when senescing. Also ROS production is expected to play a big role in these rearrangements. We test this by comparing old senescent mitochondrial DNA (mtDNA) to young non-senescent mtDNA.

2. Materials and methods

2.1 Organisms and growth conditions

Strains were collected from the laboratory of phytopathology Wageningen University with help by Dr. Jan van Kan and were incubated at 18°C on Petri dishes containing Malt medium. A back-up collection was put in the cold room at 4°C. Strains used for the study of the molecular phylogeny had to be collected. Some strains used during the study for the molecular phylogeny could not be recovered and are missing in this study. Fresh isolated strains of *B. cinerea* strains were collected by Dr. Jan van Kan and added to the collection. Several *B. cinerea* mutants were collected by Dr. Jan van Kan in collaboration with Prof. Dr. Paul Tudzynski. A full list of strains and references can be found in Table 1. (Rolke, Liu et al. 2004; Doehlemann, Berndt et al. 2006; Rui and Hahn 2007; Segmuller, Ellendorf et al. 2007; Segmuller, Kokkelink et al. 2008)

During all experiments a 2% agar medium was used, with the addition of different nutrition and carbon sources. A list of the different media can be found in the appendix. Incubation was always at 18°C in dark conditions. The strains were only exposed to light during measurements and when they were transferred to new racetubes or Petri dishes. Racetubes are plastic or glass tubes, containing a medium on which fungi can grow from one side to the other. The plastic tubes are made of a disposable plastic 50ml pipette, while the glass racetube is made of specially constructed glass, solely for the purpose as a racetube.

2.2 Pilot

To check for senescence, six different strains were put on racetubes by transferring 1mm square piece of mycelium as a pilot experiment. Strains were chosen from different clades on the molecular phylogeny produced by Staats and his colleagues (Staats, van Baarlen et al. 2005): *B. cinerea* SAS 405, *B. aclada* PR 006, *B. elliptica* Be 9401, *B. narcissicola* MUCL 2120, *B. convoluta* 9801 and *B. porri* MUCL 3234. These strains were put on disposable 50ml pipettes containing 22.5 ml of either 2%, 0.2% or 0.02% glucose GB2 medium and on Malt medium containing 2% Glucose or no Glucose. Each Monday, Wednesday and Friday, the racetubes were marked at the growth front.

B. aclada PR 006 was put on Ø9cm and Ø14cm plates containing Malt medium to observe growth pattern. Wild-type strain, *B. cinerea B05.10*, was also put on several plates containing 3% Malt medium, since all available mutants for later experiments are mutants of this wild-type strain.

B. aclada showed senescence and the reproducibility of this senescence was tested by taking samples from *B. aclada* 10 and 5 days before death. These samples were put on racetubes to see whether they would senesce more or less at a predictable time.

2.3 Large Botrytis senescence experiment

After each experiment small other experiments were performed to justify results or for more interesting results. Several sizes Petri dishes were used for further experiments on found observations. Special 20cmx20cm plates were sterilized by using 70% alcohol, to get larger overviews of the growth pattern of senescent strains.

For the first experiment all available strains from the molecular phylogeny (Table 1, strains 1 to 31) were put in duplicate on glass racetubes, instead of the 50ml plastic racetubes used in the pilot. Two different media were chosen, 2%Glucose GB5 and 2%PGA GB5 medium. Since no difference in calorie restriction was detected in the pilot, calorie restriction was excluded from this experiment and left for further research.

B. cinerea did not show much sign of senescence neither during the pilot as during the first experiment, which could have been a result of using old laboratory strains. Therefore, younger strains (Table 1, strains 42 to 51) were obtained and put in duplicate on glass racetubes containing 2%Glucose GB5 or 2% PGA GB5 medium, similar to the first experiment. The host, from which each strain is isolated, is known and can also be found in Table 1. Next to these "younger" strains, new strains were freshly isolated from grapes, which have been called *B. cinerea* fs1 and *B. cinerea* fs2 (Table 1, strain 52 and 53), as can be seen on Figure 3.

Figure 3: Several fungi and bacteria species growing on grape, which was put on 2%Glucose GB5 medium, from which Botrytis cinerea fs1 and fs2 were isolated.

All *B. cinerea* mutants available, including several ROS mutants, (Table 1, strains 32 to 41) have been put on racetubes to observe any signs of senescence and were compared to the wild-type *B. cinerea* strains in the other experiments with special attention to the *B. cinerea* B05.10 strain, which is the wild-type of these mutants. Mutant strains were put on racetubes containing 2%PGA GB5 medium. These mutants were kept in the dark at 18°C for 2 weeks. After these 2 weeks, racetubes were marked and checked for senescence.

2.4 Mitochondrial DNA instability

There is not a clear visible difference between old (senescing mycelia) and young mycelia, compared to other senescing fungi, which have a much clearer pattern of senescence (Bertrand 2000). An assumption was made that more or less 1 cm before the end of growth, where the mycelia is considered dead, is old material. Young and old material was collected by inoculating several 1mm square pieces of young or old mycelium on Petri dishes containing the needed medium covered with cellophane. After incubation at 18°C for at least one week, the mycelium was collected by scraping it off. Total DNA including mitochondrial DNA (mtDNA) was isolated from the cells by using the phenol chloroform protocol (Appendix). Restriction enzymes were chosen for a regular CC/GG pattern, which should cut more regularly in genomic DNA and cut it in many small pieces. Mitochondrial DNA has more adenosine/tyrosine and will therefore be cut into larger pieces. HaeIII was chosen as a more regular cutter and EcoRI was chosen as a rarer cutter to obtain more different bands.

(mt)DNA was run on gel according to standard protocols and a picture was taken. The DNA was probed using a probe based on the *P. anserina* mtDNA. Pictures were taken and developed.

3. Results

Using plastic racetubes has its advantages over glass racetubes in some ways, since it does not need to be cleaned and can be easily cut to obtain mycelia half-way. One of the major disadvantages of plastic racetubes is the obscuration of the surface. Not only does the racetube contain lines for measuring, but they also tend to get a vague foggy appearance in time. Mycelium tends to grow on the surface of the racetubes due to the small layer of medium stuck to it. Observation of spore formation, mycelia, sclerotia or any other interesting structures are therefore hard.

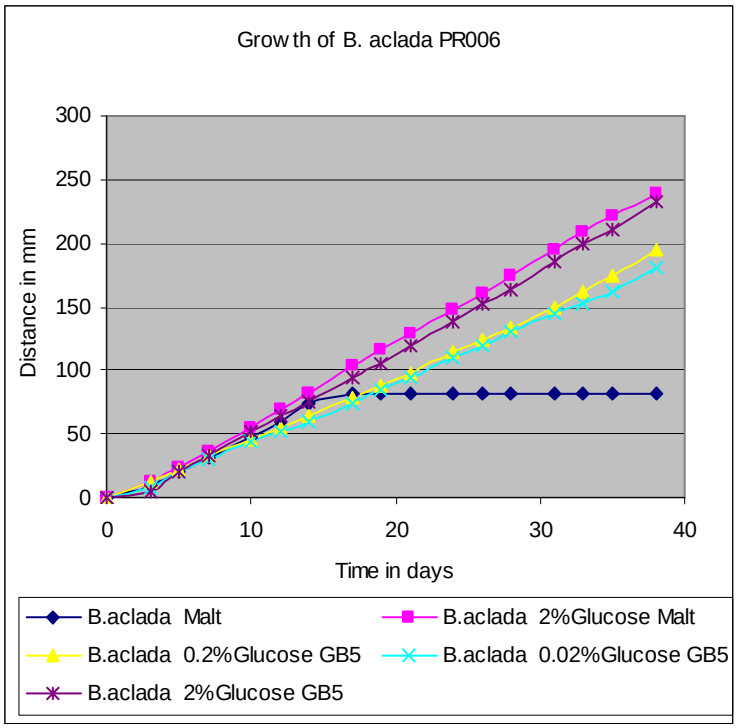


Figure 4: Graph showing growth of *B. aclada* on different media. Clearly *B. aclada* stopped growing on Malt medium.

During the first ageing experiments after more or less two weeks, the first signs of senescence were seen with the species *B. aclada* on Malt medium. This medium was rather rich in nutrients, although not as rich as the 2%Glucose Malt medium. The strain stopped suddenly growing without a very clear frontline. This B. aclada formed a patchy pattern before it suddenly stopped growing, as can be seen on the picture in figure 5. Its growth can be seen in figure 4, which shows that the other samples did not show any sign of senescence. The graph shows a sudden horizontal line for *B. aclada* on Malt medium after 17 days. Compared to the rich conditions, *B. aclada* grew less under the calorie restricted conditions.

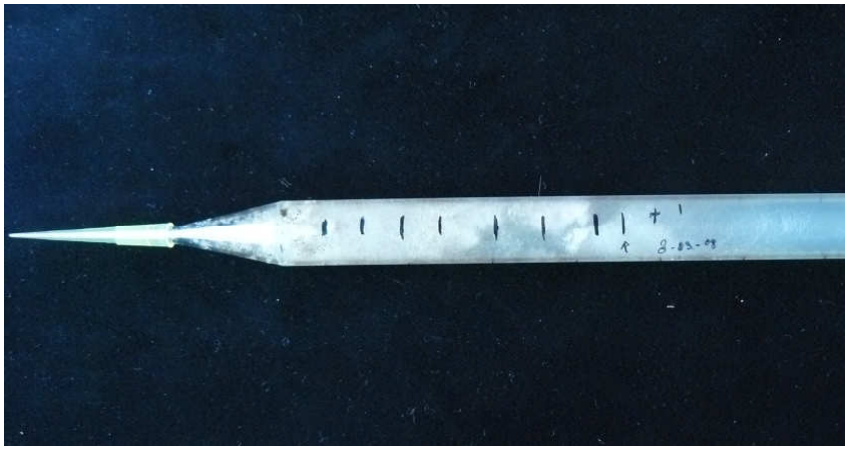
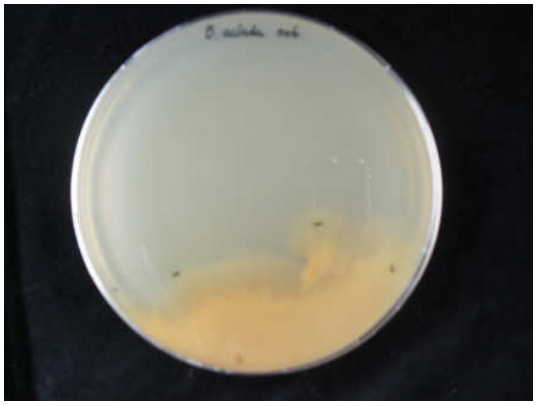


Figure 5: Picture of a disposable plastic 50ml racetube containing Malt medium, showing senescence in *B. aclada* PR006.

Just before the end of the period of the pilot in which it was acceptable to measure anything senescing, one more species showed signs of senescence: *B. porri* MUCL3234 on 2% Glucose GB5 medium stopped growing after two months, though after 3 days growth resumed.

Similar to the results of *B. aclada* on racetube containing Malt agar, senescence was clearly seen on plate. *B. aclada* stopped growing after a period of 2 to 3 weeks a. However, after a while, new formed mycelia could be seen, but also this growing stopped in time, as can be seen on the picture in figure 6. These periods of growing and motionlessness continued until the entire plate was overgrown. These periods more or less showed a patchy pattern.



B. cinerea B05.10 is a fast growing fungus and had to be transferred several times on new plates. Sclerotia could clearly be seen, and at times conidia were seen at the edge of the plate. This is probably due to more exposure to the outside atmosphere. However, this strain did not show any sign of ageing.

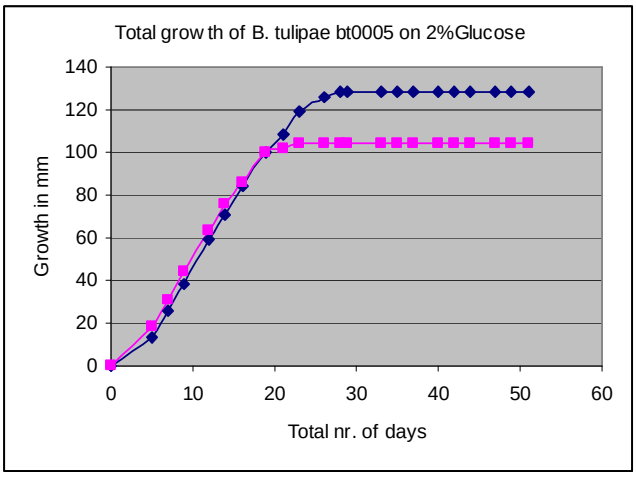
Figure 6: Picture of senescing *B. aclada* PR006 on plate containing Malt agar.

The predictability of senescence and corresponding stop of hyphal growth was tested by growing old material taken 5 or 10 days before the end of growth. However, as the samples were expected to live approximately 5 to 10 days, it took more time before senescence could be seen again. After transferring mycelia it always took some time for the mycelium to recover and start growing at a more normal pace. Although the predictability of senescence in days could not be determined, senescence was observed in the same material repeatedly.

We tested 31 different strains (Table 1, strains 1 to 31), belonging to 17 different Botrytis species for their lifespan in duplicate on 2 different media in glass racetubes: 2%Glucose GB5 and 2%PGA GB5 medium. Big differences between growth pattern and morphology could be seen between the different species, but also between strains of the same species on different media.

Though senescence was already seen after 2 weeks in *B. aclada* in the first experiment, this strain did not show senescence in the same time span. *B. polyblastis* seemed inapt to grow further than 2 cm, because of senescence. After 2 weeks the first signs of senescence were seen with *B. tulipae* showing less growth, as can be seen in figure 7a. Of the three different strains of *B. tulipae,* two died

2% PGA GB5 medium and one on 2%Glucose GB5 medium. Both duplicates of these three strains died shortly after each other. Remarkable is the formation of sclerotia just before the stop of growing at the frontline, as can be seen in the several pictures of senescing *B. tulipae* (Figure 9 and 10). Similar sclerotia formations are seen in senescing *B. paeoniae* strains a few weeks later, as can be seen in figure 7b. *B. paeoniae* not only forms more sclerotia, but also shows some pigmentation around these sclerotia (Figure 11).



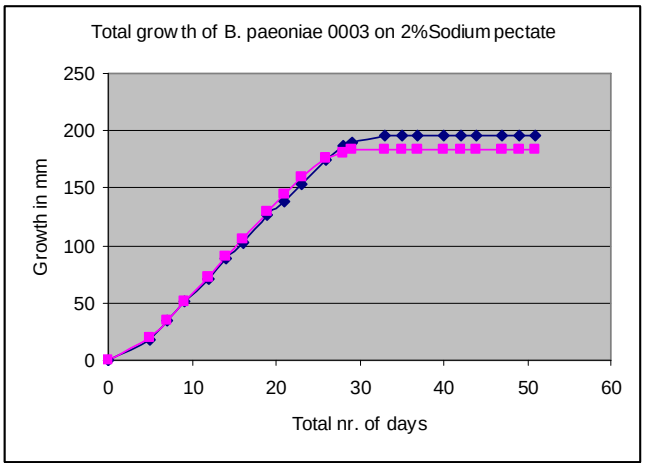


Figure 7a and 7b: Graphs of *B. tulipae* and *B. paeoniae* showing a decline in growth and eventually stop of growth.

Strains senescing on racetubes, were also tested on different sized plates: ø9mm, ø14mm and 20cm x 20cm plates, containing different media, such as 2%Glucose GB5, 2%PGA GB5 or Malt medium.

As a small sidestep, strains of *B. tulipae* were put on racetubes under calorie restricted conditions, 0.02%, 0.2% and 2% Glucose GB5 media. One of these strains died before reaching the end of the tube, which was on 0.2% Glucose GB5 medium.

Fresh isolates of *B. cinerea* were put on racetubes since most strains used in the former experiment were already old and kept in laboratories for a long time (Table 1, strains 42 to 53). Without knowing these strains could have been selected for not being able to age and live indefinitely. With freshly isolated strains including the 2 strains from grapes, this experiment was expected to give more signs of senescing. These strains of *B. cinerea* showed very much similarity to old lab strains. However, some of these strains eventually showed senescence and stopped growing, however never in duplicate, as can be seen in figure 8. Remarkably these strains produced a patch of a tremendous amount of conidiospores shortly before senescing and eventually death. In figure 12, this patch can be clearly seen in the aged strain *B. cinerea* ssp6A. Of the 15 natural strains put on racetubes, only 3 strains showed senescence and stopped growing, *B. cinerea* ssp6A, ssp12A and ssp21B.

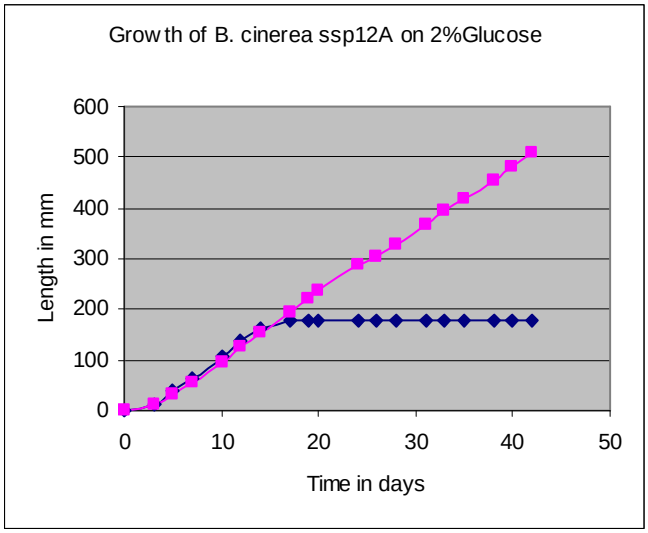


Figure 8: Graph showing the growth of *B. cinerea* ssp12A on 2%Glucose GB5 medium in duplicate. One strain stopped growing and showed senescence, while the other kept on growing indefinitely.

Figure 9: Plate containing 2%PGA GB5 medium showing senescence in *B. tulipae* Bt9830, with a rim containing relatively more sclerotia.

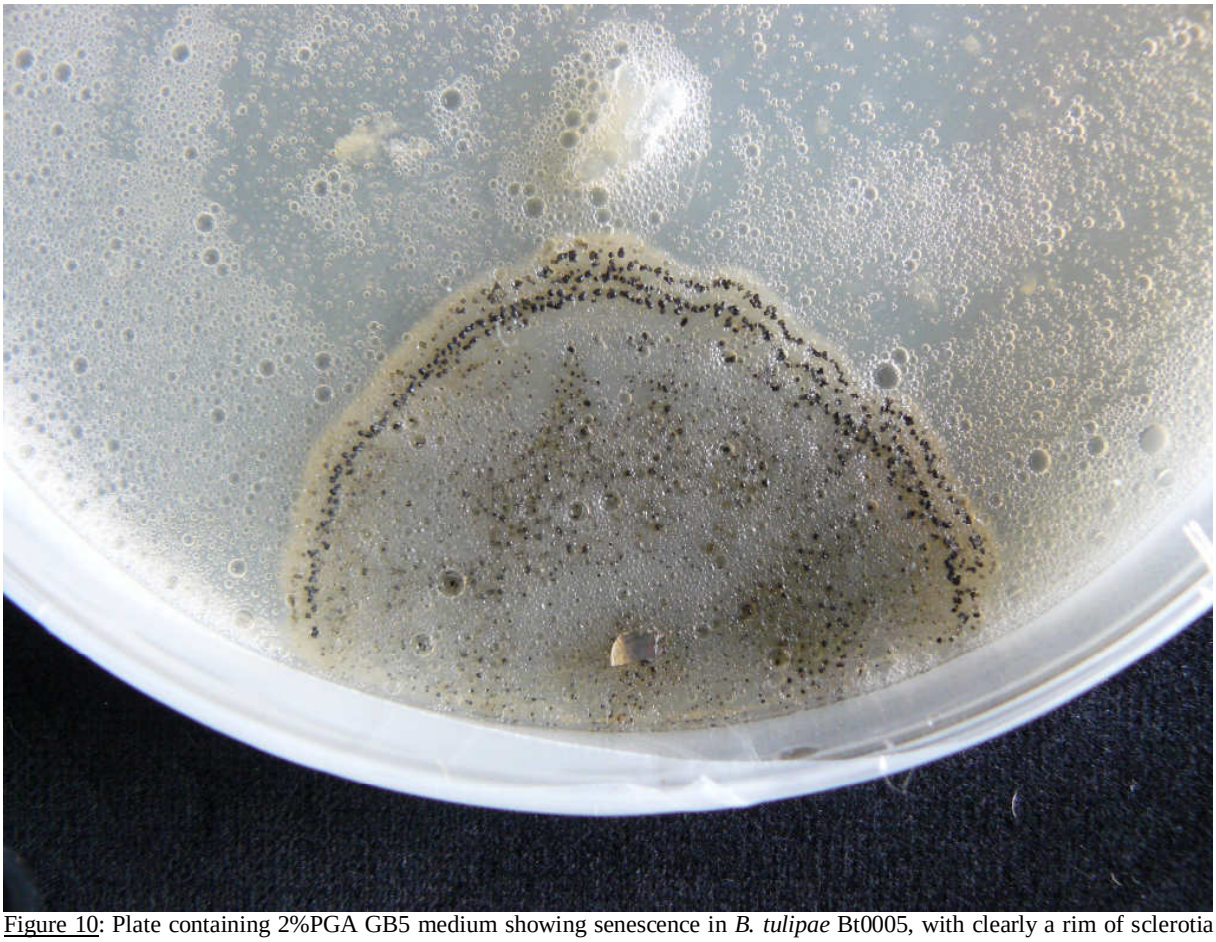


Figure 10: Plate containing 2%PGA GB5 medium showing senescence in *B. tulipae* Bt0005, with clearly a rim of sclerotia formation.

Figure 11: Picture showing senescence in *B. paeoniae* 0003 on 2%Glucose GB5 medium. Formation of sclerotia could be seen a few cm before stop of growth. Same could be seen on 2%PGA GB5 medium, available in the appendix.

Figure 12: Racetubes containing 2%PGA GB5 medium showing senescence in *B. cinerea* ssp6A, with a patch of conidia spore formation shortly before death.

After 2 weeks of incubation in the dark at 18°C, one strain showed a decrease in growth compared to most strains and especially the duplicate. The strain completely stopped growing and showed the same patch of conidiospore formation as seen with other senescent *B. cinerea* strains. This particular strain was Δbcg3 and is known to have a mutation in the cAMP pathway. This strain is known to have trouble germinating and is known to be less able in the production of spores (Doehlemann, Berndt et al. 2006). As can be seen on the picture in figure 13, Δbcg3 produced a lot of conidiospores shortly before senescing.

Figure 13: Racetubes containing *B. cinerea* Δbcg3 showing senescence relatively short after inoculation, more or less 2 weeks after inoculation. (racetube marked with a cross) Lots of conidiospores can be seen just before the stop of growth

No other mutant strains showed any sign of senescence, which is not entirely as expected. All mutants and results can be found in Table 1, strains 32 to 41.

For the mitochondrial DNA instability experiment the digestion of mtDNA gave only 2 clear bands in each sample, because the probe was unspecific. The pictures were not conclusive and are not included in this report.

Table 1: Species used during this study, with their collection number, year of isolation, sexual stage, lifespan on different media, substrate, and geographic origin.

	Species	Collection number	Year of isolation	Sexual stage	Lifespan (Gluc)	Lifespan (PGA)	Substrate	Geographic origin
1	*B. aclada* (Fresen.) Yohalem	PRI006	\	No	17 days (Malt)	\	Bulbs of onion, garlic and leek	\
2		MUCL3106	1961	No	\	\	Bulbs of onion, garlic and leek	USA
3		MUCL8415	1965	No	\	\	Bulbs of onion, garlic and leek	Germany
4	*B. calthae* Hennebert	MUCL2830	1961	Yes	\	\	Stem of marsh-marigold	USA
5	*B. convolute* Whetzel and Drayton	MUCL9801	1998	Yes	\	\	Rhizomes of cultivated iris	The Netherlands
6		MUCL11595	1968	Yes	\	\	Rhizomes of cultivated iris	USA
7	*B. cinerea* Pers. / *B. fuckeliana* (de Bary) Whetzel	SAS56	\	Yes	\	\	Fallen leaves, fruits and flowers	Italy
8		SAS405	\	Yes	\	\	Fallen leaves, fruits and flowers	Italy
9		B05.10	1994	Yes	\	\	Fallen leaves, fruits and flowers	\
10	*B. croci* Cooke and Massee	MUCL436	1968	No	\	\	Leaves of cultivated crocus	The Netherlands
11	*B. elliptica (*Berk.) Cooke	BE9401	\	Yes	\	\	Leaves, stems and flowers of cultivated lilies	\
12	*B. fabae* Sardina	CBS 109.57	1957	No	\	\	Leaves of bean	The Netherlands
13		98	1929	No	\	\	Leaves of bean	Spain
14	*B. ficariarum* Hennebert	CBS 176.63	1960	Yes	\	\	Buttercup	Belgium
15		MUCL376	1957	Yes	\	\	Buttercup	Belgium
16	*B. galanthina* (Berk. and Br.) Sacc.	MUCL435	1958	No	\	\	Snowdrop	The Netherlands
17		MUCL3204	1963	No	\	\	Snowdrop	The Netherlands
18	*B. gladiolorum* Timm. / *B. draytonii* (Budd. and Wakef.) Seaver	9701	1997	Yes	\	\	Stems of cultivated gladiolus	\
19		MUCL3865	1963	Yes	\	\	Stems of cultivated gladiolus	The Netherlands
20	*B. hyacinthi* Westerd. and Beyma	0001	1999	No	\	\	Leaves of hyacinth	The Netherlands
21	*B. narcissicola* Kleb. Ex Westerb. and Beyma	MUCL2120	1961	Yes	\	\	Bulbs of narcissus	Canada
22	*B. paeoniae* Oud.	MUCL16084	1970	No	\	44 days	Stems of cultivated peonies	Belgium
23		0003	2002	No	37-40 days	33-35 days	Stems of cultivated peonies	The Netherlands
24	*B. pelargonii* Roed	CBS 497.50	1949	Yes	\	\	Leaves of geranium	Norway
25		MUCL1152	1960	Yes	\	\	Leaves of geranium	Norway
26	*B. polyblastis* Dowson	PPO0804	\	Yes	9 days	6-9 days	Leaves of narcissus	\
27	*B. porri* Buchw.	MUCL3234	1926	Yes	38+ days	\	Bulbs of garlic, leek	\
28		MUCL3349	1963	Yes	\	\	Bulbs of garlic, leek	Belgium
29	*B. tulipae* Lind	BT9830	2000	No	\	21 days	Leaves, stems, and flowers of cultivated tulips	The Netherlands
30		BT0005	\	No	26-29 days	37-44 days	Leaves, stems, and flowers of cultivated tulips	\
31		BT9901	2000	No	\	\	Leaves, stems, and flowers of cultivated tulips	The Netherlands
32	*B. cinerea* Pers. / *B. fuckeliana* (de Bary) Whetzel	Δbcg1	\	Yes	\	\	Fallen leaves, fruits and flowers	\

33	*B. cinerea* Pers. / *B. fuckeliana* (de Bary) Whetzel	Δbcg3	\	Yes	\	14 days	Fallen leaves, fruits and flowers	\
34		ΔbcnoxA	\	Yes	\	\		\
35		ΔbcnoxB	\	Yes	\	\		\
36		ΔbcnoxR	\	Yes	\	\		\
37		Δbmp3	\	Yes	\	\		\
38		Δbcsak1	\	Yes	\	\		\
39		Δbap1	\	Yes	\	\		\
40		Δsod1	\	Yes	\	\		\
41		Δgod1	\	Yes	\	\		\
42	*B. cinerea* Pers. / *B. fuckeliana* (de Bary) Whetzel	ssp2A	2008	Yes	\	\	Cucumber	The Netherlands
43		ssp6A	2008	Yes	\	33	Cyclamen	The Netherlands
44		ssp9A	2008	Yes	\	\	Lettuce	The Netherlands
45		ssp12B	2008	Yes	19	\	Tomato	The Netherlands
46		ssp14A	2008	Yes	\	\	Lettuce	The Netherlands
47		ssp15A	2008	Yes	\	\	Cucumber	The Netherlands
48		ssp21B	2008	Yes	38	\	Lettuce	The Netherlands
49		ssp29A	2008	Yes	\	\	Lettuce	The Netherlands
50		ssp34B	2008	Yes	\	\	The common bean	The Netherlands
51		ssp35A	2008	Yes	\	\	The common bean	The Netherlands
52		fs1	2008	Yes	\	\	Grape	The Netherlands
53		fs2	2008	Yes	\	\	Grape	The Netherlands

4. Discussion

Senescence was seen in different Botrytis species. A list of all senescent strains can be seen in Table 2. Some strains showed senescence unpredictable and erratic: *B. aclada*, *B.cinerea* and *B. porri*. Two strains were good to predict and showed senescence on a more regular basis, these strains were *B. paeoniae* and *B. tulipae*. The Δbcg3 mutant of B. cinerea showed senescence in 2 weeks.

Table 2
Strains showing senescence

Species	Collection nr.	Senescence	Predictability	Sexual stage	Medium
B. alcada	PR006	rarely seen	Hard, 2+ weeks	no	Malt
B. cinerea	ssp6A	rarely seen	Hard, 2+ weeks	yes	2% Sodium pectate
	ssp12A	rarely seen	Hard, 2+ weeks	yes	2% Glucose
	ssp21B	rarely seen	Hard, 2+ weeks	yes	2% Glucose
B. paeoniae	0003	regularly seen	Good, 3-4 weeks	no	2% Sodium pectate and 2% Glucose
	MUCL 16084	regularly seen	Good, 3-4 weeks	no	2% Sodium pectate
B. porri	MUCL 3234	rarely seen	Very hard, 8+ weeks	yes	2% Glucose
B. tulipae	Bt 0005	regularly seen	Good, 2-3 weeks	no	All
	Bt 9830	regularly seen	Good, 2-3 weeks	no	All

For the first growth experiment it was clear that, although observation of the fungi in the disposable plastic racetubes was difficult, many interesting observations were made. *B. aclada* clearly showed senescence, since it stopped growing after more or less 2 weeks, which is more or less around the same time as *P. anserina* dies of senescence (Maas, Sellem et al. 2007). However, no clear pigments are formed before stop of growth or any other clear signs shown by *P. anserina*. It is strange that *B. aclada* only showed senescence on Malt medium and not on any other medium. Since Malt medium can be very different for each batch made, it will be hard to repeat the exact experiment and therefore hard to proof the cause of death. Malt medium of course is a very rich environment and can be of great influence on senescence in this species. On the disposable plastic racetubes and on Malt medium, *B. aclada* never produced big amounts of conidiospores, while *B. aclada* produced a lot of conidiospores on the glass racetubes with 2%Glucose GB5 or 2%PGA GB5 medium. Glass racetubes are better ventilated and *B. aclada* produced a lot of conidiospores on both media. This production of conidiospores could have influenced the detection of senescence, if conidiospores inoculated the still running racetube.

B. aclada was put on new racetubes to see if it would senesce again at a predictable time. This did not happen since it took longer for senescence was shown. For both the 5 days before senescence and 10 days before senescence samples, senescence was seen at almost the same time. After transferring mycelium, it usually takes some time for the fungus to recover and continue growth. This does not count for each species, since some recover faster as others. Although the exact time was not as good to predict, they still stopped growing, which proofs the fact *B. aclada* ages.

Senescence of *B. aclada* was shown on racetubes as well as on plate, which seems to take more or less 2 weeks. After some time *B. aclada* always was able to recover, or mycelia from behind the frontline overtook the frontline and continued growing. In time the whole plate would be covered with continuously repeated moments of recover and senescence.

During the first experiments for senescence *B. porri* showed senescence after a month, while it continued its growth after 3 days. This is an interesting observation, but hard to use for future research. *B. porri* shows senescence erratically and is a fast growing fungus with a lot of aerial mycelia formation, which could explain this erratic behavior. Young hyphen can by-pass the frontline by aerial hyphen.

The racetubes were kept in the dark and were only exposed to the light during measurements. For some species, particularly *B. cinerea*, these periods of exposure to light triggered the formation of sclerotia. Even a small difference in growth could be seen on days with more light compared to days with less light, for instance during the weekends. On the average the racetubes were more exposed to light during the week, as during the weekends. It is known that light has a big effect on *Botrytis* species, since it is also used to maintain control over *Botrytis* in greenhouses, without having to use fungicides (Islam, Honda et al. 1998).

Glass racetubes are much better for observation and are better ventilated as mentioned earlier for *B. aclada*. There are several advantages and disadvantages for using glass racetubes instead of disposable plastic racetubes. The main advantage of having glass racetubes is the clear vision. The better ventilation of the racetubes has both advantages and disadvantages, since good ventilation causes the fungi to grow at optimal conditions, which also causes some fungi to form conidiospores. These spores can fall ahead of the frontline, or just start growing at the place they sprouted from. This interferes with the experiment since young material is added at a later time. With *B. aclada*, this is probably the case. *B. aclada* clearly showed senescence during the pilot experiment on tube as well as on plate. However *B. aclada* on glass racetubes was always producing spores, which might have caused a false observation. Since not all species were put on the disposable plastic racetubes, it could be that other species had the same problem, although compared to *B. aclada*, no species produced as much conidiospores.

The asexual *B. tulipae* is the first clear *Botrytis* species showing senescence. It produces a lot of sclerotia during its growth, and has a rim of extra sclerotia production just before death. Somehow *B. tulipae* is able to detect its downfall and reacts by forming more survival structures in the form of sclerotia. This is remarkable since it is not seen in any other fungi showing senescence at this point.Bertrand describes several senescing fungi, but so far none was able to produce sclerotia, or had a similar survival mechanism (Bertrand 2000). The formation of sclerotia and the formation of this rim of sclerotia can be clearly seen in figure 9 and even clearer in figure 10.

The asexual *B. paeoniae* is the second clear *Botrytis* species showing senescence. Similar to *B. tulipae* do both duplicates more or less start showing senescence at the same time. Also on plates they

showed senescence at a similar time compared to the racetube experiment. Interestingly, sclerotia were formed just before the stop of growth in both *B. tulipae* as *B. paeoniae*. Sclerotia are survival structures, so it seems logic and natural for an organism to develop survival structures if it senses ageing and senescence becoming tremendously dangerous.

Remarkably the strains of ageing *B. tulipae* and *B. paeoniae* aged at a predictable time and length. *B. paeoniae* tends to grow a bit longer and further as *B. tulipae* and is therefore more difficult as a model organism for researching senescence. *B. tulipae* seems to be a fairly good model organism for researching senescence in asexual ascomycetes.

Strains of *B. tulipae* were put on racetubes containing calorie restricted media. The results from these were interesting, since senescence was only seen under high calorie conditions and low calorie conditions. Compared to P. anserina this is an interesting observation, since calorie restriction normally lengthens lifespan (Maas, Sellem et al. 2007). However, this experiment was rather small and more a sidestep from the original experiment.

Fresh strains of *B. cinerea* did show senescence, only erratically for some strains. This suggests that the old labstrains are selected for extended lifespan and that young strains are needed for more research. Only 3 strains, ssp6A, ssp12A and ssp21B showed senescence erratically. The two freshly isolated strains fs1 and fs2 did not show any signs of senescence, which confirms senescence is a trait found erratically in *B. cinerea*. The strains that did show senescence had a remarkable production of conidiospores shortly before death, which suggests some response to ageing. This production could be a response to ageing to let the species survive. It could also be that ageing is a result of this need for energy for this sudden production of conidiospores.

Of the several *B. cinerea* mutants only one showed senescence: *B. cinerea* Δbcg3. Similar to other senescing *B. cinerea* strains did *B. cinerea* Δbcg3 show a production of conidiospores before death. It is hard to tell why this particular mutant showed senescence, while other mutants expected to show senescence did not. *B. cinerea* Δbcg3 has a mutation involved in the signaling pathway, which could suggest that the surrounding environment has a big influence on senescence. However, the duplicate of *B. cinerea* Δbcg3 did not show any signs of senescence. Especially mutants concerning the production and scavenging of ROS were expected to give interesting results. Only a small part of *B. cinerea* strains showed senescence and therefore not much can be said about the outcome of the experiment with the different mutants. In particular *B. cinerea* B05.10 did not show any signs of senescence, which makes comparison to the mutant strains difficult.

The digestion of (mt)DNA did not show any clear results. The probe of *P. anserina* mtDNA was unspecific and therefore only 2 bands for each strain were seen. The picture was not added to this report since it had no further value. There were some differences between strains of old and young material, but very small and insignificant. A much more specific probe should be used in future experiments, since the rest went as expected. Bands of mtDNA were detected, however not all bands.

The different senescent species of *Botrytis* are compared and checked for any relationships, such as sexual or asexual reproduction and position in the molecular phylogeny. The senescent species are spread all over the molecular phylogeny, although *B. aclada* and *B. paeoniae* are sister groups and form subclade 2a (Figure 15). *B. aclada* seemed to senesce on rare occasions, but could well be an interesting organism for further research. The fact that *B. aclada* produces a large amount of spores could have made false observations in glass racetubes and *B. aclada* might as well be similar in senescence to *B. paeoniae*.

The most obvious species showing senescence are *B. tulipae* and *B. paeoniae*, which both are asexual species. With these little results it is hard to proof any relationship between asexual reproduction and senescence, especially since several sexual species showed senescence in some cases.

Although only a small number of fresh *Botrytis cinerea* strains showed senescence it can be well concluded that most lab strains of *Botrytis cinerea* are selected for living indefinitely. Obtaining fresh material and new wild type species is important for more research. Known senescent strains can be used for further analyses to the cause of senescence, but new strains can give more information about the natural ecology and the relationship of this ecology to the evolution of senescence in *Botrytis* species.

5. Conclusion

Senescence is a trait that occurs in several fungi growing on ephemeral substrate. Senescence does occur differently among species and only for a select few. For only 2 asexual *Botrytis* species the signs of senescence were really clear and fairly predictable after a few observations. *B. tulipae* and *B. paeoniae* were predictable on racetube as well as on plate, however not all strains died. For further research I would suggest using these species, although *B. paeoniae* takes longer and therefore could be inefficient for using as a model organism.

Calorie restriction does not seem to have an influence on the lifespan of *Botrytis tulipae*, although it is known to be the only regimen to extend lifespan in nearly all organisms. The results of the small experiment are short and not conclusive to exclude the effect of calorie restriction on *Botrytis* species. It would be interesting to repeat this experiment and use calorie restricted conditions for several strains of *Botrytis tulipae*.

Each species grows differently on a different substrate; therefore a graph was made to show the difference between growth on 2%Glucose GB5 and 2%PGA GB5 medium. The difference in the speed or length of growth could mean several things and it is hard to tell which media is preferred. It could however have influence on the media used for further research, since some strains died a bit earlier on one of the media, which might be practical for using less material and time (Figure 14).

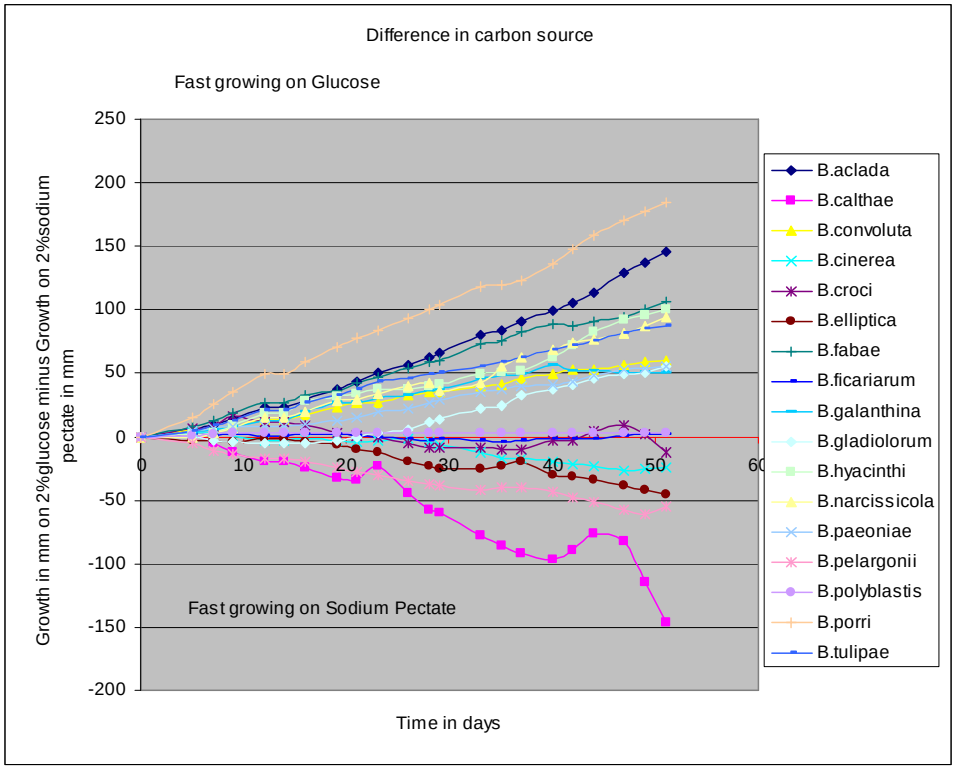


Figure 14: Graph showing all species and the difference in average growth between 2%Glucose GB5 and 2%PGA GB5 medium. When a species reaches a positive number it is faster growing on Glucose, while a negative number represents a faster growth on Polygalacturonic acid.

Fungal senescence has always been described as gradually degradation of the mycelium leading to sterility and death, which can be seen on figure 1. A sudden increase of survival structures as sclerotia and conidia spores formation is a total new observation. This sudden burst of sclerotia formation or spore formation can be seen as a reaction to senescence or senescence could be a result of the energy needed for this formation. The former seems more logical since survival structures are triggered by stress and senescence results in stress. *Botrytis cinerea* shows a tremendous production of conidia spores shortly before dying, which is a reaction to stress caused by senescing.

Even though the mtDNA instability digestion did not show any clear differences between young and old, because the probes were unspecific, future experiments look promising. For each lane 2 bands were visible and more or less at the same location, but could just as well have had small differences. Using the same restriction enzymes, but using a more specific probe could give more conclusive bands and could have interesting results.

This research gives more new insights in fungal senescence and can form a basis for further research. More research is needed to find the mechanism or causes behind senescence in *Botrytis*.

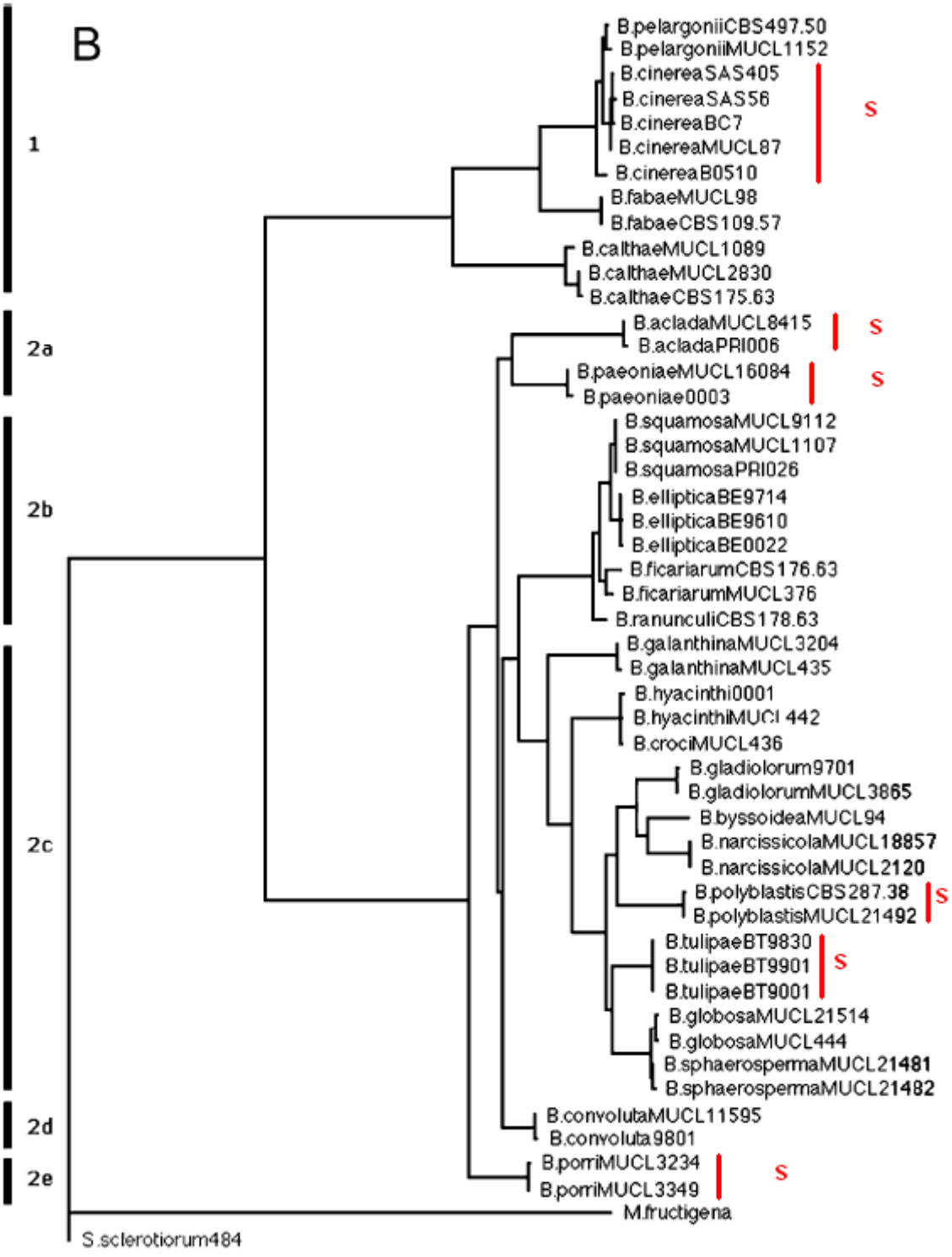


Figure 15: Senescence seen in several assigned species shown in the molecular phylogeny with red lines. Senescence was detected in not always the same strain as shown, but it was detected in that particular species. *B. tulipae* and *B. paeoniae* strains showed senescence on a regular predictable basis, in contrary to *B. cinerea*, which showed in some cases senescence. *B. aclada*, *B. polyblastis* and *B. porri* strains only had some rare strains that irregularly stopped growing and showed senescence. Figure adjusted from published molecular phylogeny (Staats, van Baarlen et al. 2005).

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8. Appendix

8. Appendix

Table of contents

Protocols

Media

800ml bottle

3% Malt medium (Malt medium)
- 24g Malt (Oxoid)
- 16g Agar

2% Glucose Gamborg B5 medium (2%Glucose GB5 medium)
- 1.25g Gamborg B5
- 16g Glucose
- 16g Agar

2% Polygalacturonic acid Gamborg B5 medium (2%PGA GB5 medium)
- 1.25g Gamborg B5
- 16g Polygalacturonic acid (Sigma)
- 16g Agar

0.2% Glucose Gamborg B5 medium (0.2% Glucose GB5 medium)
- 1.25 Gamborg B5
- 4ml 40% Glucose solution
- 16g Agar

0.02% Glucose Gamborg B5 medium (0.02% Glucose GB5 medium)
- 1.25 Gamborg B5
- 0.4ml 40% Glucose solution
- 16g Agar

2% Glucose Malt medium (2% Glucose Malt medium)
- 24g Malt (Oxoid)
- 16g Glucose
- 16g Agar

Phenol/Cholorform DNA isolation of *Podospora* adjusted for *Botrytis*

This method is used for the isolation of both nuclear and mitochondrial DNA.

1 Grow mycelium on Malt or 2% PGA GB5 medium + cellophane for 7 days at 18 ºC
2 Harvest the mycelium, avoiding as much as possible sclerotia formed
(This can be stored at -20 or -80ºC until further use)
3 Freeze the mycelium with liquid Nitrogen and add ± 5 glass beads (approx 2-3 mm diameter)
4 Grind the mycelium for 10 seconds with a bead beater machine
5 Freeze again in liquid nitrogen and add again ± 5 glass beads
6 Grind the mycelium for the second time for 10 seconds with a bead beater machine
7 Add 1 ml of LETS buffer and mix well (vortex)
8 Centrifuge for 5 minutes at 13.000 rpm
9 Transfer 700 µl of the supernatant into a fresh 2 ml eppendorf tube
10 Add 5 µl of proteinase K (stock 20 µg/µl is kept at -20 ºC) and incubate for 1 hour at 37 ºC
11 Add 300 µl phenol and 300 µl SEVAG and mix well (vortex)
12 Centrifuge for 15 minutes at 13.000 rpm
13 Transfer 600 µl of the supernatant to a clean 1.5 ml eppendorf tube
14 Add 300 µl of SEVAG, mix and centrifuge for 15 minutes at 13000 rpm
15 Transfer 500 µl of the supernatant to a clean 1.5 ml eppendorf tube
16 Add 300 µl (0.6 x volume) isopropanol, mix gently, and store this mixture for 15 minutes at -20 ºC or overnight at 4 ºC
17 Centrifuge nucleic acids out of suspension in an eppendorf centrifuge for 15 minutes at 13.000 rpm (mark the orientation of the tubes in the rotor to find your pellet)
18 Discard the supernatant with a micro-pipet and wash the pellet carefully (pellet must stay in place) using 100 µl of icecold 70% ethanol from the -20 ºC freezer
19 Centrifuge 1 minute at 13.000 rpm in an eppendorf centrifuge
20 Carefully discard (decant or use a micro-pipet) the supernatant and dry the pellet under vacuum
21 Dissolve overnight in 50 µl of Milli-Q at 4 ºC
22 Mix solution using a pipet and store at -20 ºC

N.B. For PCR purposes the DNA solution can de diluted 10-100 fold. For restriction analyses used undiluted.

* LETS-buffer:

  0.1 M LiCl
  10 mM EDTA pH=8.0
  10 mM Tris pH=8.0
  0.5 % SDS

* SEVAG:

  isoamylalcohol:chloroform 1:24

Agarose Gel electrophoresis

Making the Agarose gel:
1. Determine what concentration of agarose you need. Detecting fragments between 100 and 1500 bp in size a 1% agarose will be fine. Detecting larger fragments you may lower the concentration of agarose, detecting smaller fragments a higher amount of agarose will give a better resolution.

2. Determine what gel volume you need for all your samples. Remember that you will also need some place for you molecular weight markers or other markers. In Table 1 you can find the sizes and maximum number of samples you can put on a certain type of gel.

Table 1. The three different horizontal gel electrophoresis equipment in our lab.

	Mini Sub Cell	Wide Mini Sub Cell	Sub Cell 192
Tray	7 x 10 cm	15 x 10 cm	25 x 25 cm
Gel Volume	50 ml	100 ml	375 ml
Volume Buffer	250 ml	500 ml	3 l
Well per comb	8 (30 µl)	15 (26 µl)	26 (30 µl)
(max volume per slot)	or 15 (18 µl)	20 (28 µl)	51 (15 µl))
		26 (18 µl)	
		30 (18 µl)	
Max number of combs/tray	1	2	3
Max Voltage	75 V	75 V	75 V
Migration of Bromophenol blue dye at 75 V (around 400 bp dsDNA)	4.5 cm/hr	4.5 cm/hr	4.5 cm/hr

3. Start wearing nitril/latex gloves and protective clothing. Read the Ethidium Bromide protocol for why this is.

4. Decide whether you want to use 0.5xTBE (Tris Borate-EDTA buffer) or 1xTAE (Tris-Acetate-EDTA buffer) as buffer. 0.5xTBE is more stable than 1xTAE and has to be changed less frequent, but is less good in detecting smaller fragments. Use the same buffer for making your gel as you use for running buffer in the gel container. Be careful as the buffer in the container often already contains ethidium bromide or other staining agent.

5. Weigh the right amount of agarose (e.g. 1 gram of agarose for a 1% 100 ml agarose gel). You can weigh this in a small tray or directly in one of the Erlenmeyer flasks used for preparing gels. These flasks as they may be contaminated with Ethidium Bromide may never leave the area reserved for preparing the gels.

6. Add the right amount (e.g. 100 ml) of the preferred buffer to the agarose in the Erlenmeyer flask. Never make your gel with water as this will yield very blurry gels.

7. Melt your agarose solution in the microwave oven in the gel electrophoresis area. For one 100 ml 1% agarose gel this takes about 2 minutes. (the on/of button on this microwave is on the bottom of the machine).

8. Leave the molten agarose in the fume hood until cooled to approximately 55□C (you don't burn your fingers anymore) or cool it under a tap with running water until that temperature.

9. Prepare your gel tray. Close both sides of your tray with painter's tape and hang in one or more of the desired combs. Put it on the special wooden horizontal tray in the fume hood for pouring.

10. Check your gloves and protective clothing. Time to add a stain to your agarose mixture in the Erlenmeyer flask.

A. Collect the Ethidium Bromide from the common fridge. In the Fume Hood a special pipet just for handling ethidium bromide is available. For a 100 ml gel you add 10µl of the stock solution.
B. If you stain you gel with Gel Red (and thus also have some GelRed in your running buffer) you add 10 µl of the stock solution. The GelRed is more thermostable and the stock solution (Eppendorf) can be found in the fume hood.

N.B. Do not combine the two stains and make sure you do not use Ethidium Bromide gels in a cell with buffer with GelRed and vice versa.

11. Pour the mixture in the gel and allow it to solidify for approximately 30 minutes. Remove any bubbles by moving the comb or with a disposable pipettip. A solid gel is less shiny than a non-solidified gel and often you can see some curvy lines on the surface. Softly shaking the tray will prove it is really solidified.

Wash and rinse the used Erlenmeyer flask well at the sink in the EtBr area of the lab. Leave it to dry on the rack for next use.

12. Remove the painter's tape from both end, remove the comb(s), and carefully put your tray in the right cell. Depending on your preferences you can put the cobs more to the back side of the cell or to the front. This is important when you want to start your gel. Your gel is now ready for loading. When you keep it well flooded with buffer (or store it in a container with buffer in a fridge) you can keep it for a day or more.

Preparing your samples

13. Decide how much sample you want to put on gel. For further blotting and hybridization, isolation of fragments from gel or good RFLP patterns you may want to use 20 µl or as much as you have. For checking if a PCR reaction worked 5 to 10 µl is often sufficient.

14. Add the right amount of 6x loading buffer (door common fridge) to your sample. AT least one sixth of the final volume should be loading buffer. Some amounts of water can be added if the volumes to load on gel are very small.

N.B. I prefer to put a piece of parafilm on a small Eppendorf tray and with (gloved) hands push small dips in the parafilm. This prevents the samples from rolling over the parafilm and mixing. The tray underneath makes it easier to transport. In the dips I first pipet for all my samples the amount of loading buffer I want to use. Then I add the different samples. Other people just use parafilm to mix samples, use eppendorf tubes for mixing or whatever.

N.B.2 The loading buffers we use are either bought or home made. All contain 30% of glycerol which ensures that your DNA won't float, but sinks inside the well. Buffer's can contain different combinations of color markers like xylene cyanol (comparable to approx. 1500 bp DNA), bromophenol blue (approx 400 bp DNA), and orange G (comparable to approx 50 bp DNA). If you may have faint bands of DNA or RNA to detect or want to see complex patterns on good photographs you may want to avoid interfering colours.

15. Decide on what markers you want to use. Commercially available markers like the 100bp-ladder still have to be mixed with loading buffer. The home-made lambda-digestions (see fridge for fragment sizes) are already mixed with loading buffer. For most markers 5µl gives (more than) enough signal on your gel to be useful.

Running the gel

16. Put your samples on gel as fast as you can (especially if you have many samples). Putting two markers on either side of a series of samples helps to correct later on for strange running patterns.

17. Nucleic acids have a negative charge so they will run to the positively charged pole. Check if all connections are in the right positions and switch on the transformator.

18. Check that you do not apply more than 75 Volt on your gel. Check if there is a current going through your gel (there should be a lot of Amperes on the gel) and check if you see bubbles formed at the anode and cathode in the gel as indication that indeed your gel is running. After a few minutes check if the dye of the loading buffer is going in the right direction (else changes poles).

N.B. Check your gel regularly to make sure you samples don't run of. A lower voltage corresponds to a longer running time, so you can play with it to avoid having your gel interfering with your other experiments or coffee break.

Detection

19. Whether you used Ethidium Bromide or GelRed to (pre) stain your gel, the following procedure is the same and the most difficult part is working half with gloves and half without to avoid contamination of surfaces.
Switch off the transformator. Put on gloves on both hands and get a plastic tray for carrying your gel to the UV-computer set up for photography. Carefully lift of the lid of the electrophoresis cell and lift out your gel with tray. Very slightly tilt your gel to pour off the excess of buffer, without having your gel sliding of the tray. Put your gel in the carrying tray. If you had to touch the gel, change glove(s) immediately.

20. For transporting your gel remove one glove: this is the hand you use for touching all surfaces that should not be contaminated with Ethidium Bromide. With the gloved hand you handle the gel and other possible contaminated surfaces.
Go to lab 1034 (banana), in the window tables there is a camera set up connected to an always switched on computer.

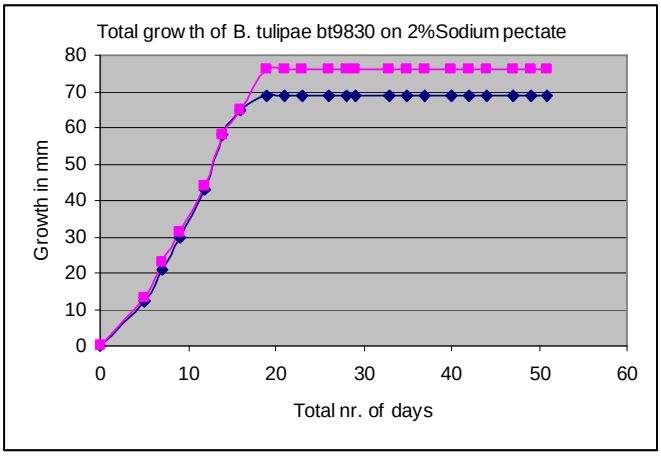
21. Carefully with you gloved hand (opening the door or drawer of the camera set-up with your ungloved hand) arrange your gel in the middle of the UV-light box. The gel-tray is UV-permeable so you can decide to leave it on the tray. Close the doors. Swith on the Quantity One program for a new GelDoc. Switch on the normal light and focus your gel in the middle of the picture (life/focus button). Size adjustments can be made with the diaphragm of the camera sticking out above the UV tower (careful: ungloved hand!). Swith off the normal light and the UV on. The auto-expose button will often give a first reasonable view of your DNA/RNA signal. Via the manual expose you can correct for over or under exposure. You need to save (in your own directory) the geldoc before you are able to print.

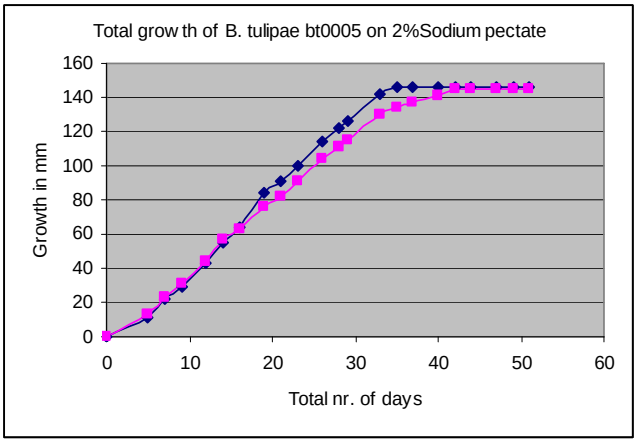
N.B. the Quantity One has many possibilities to enhance the quality of your picture. Try what is best for your gel.

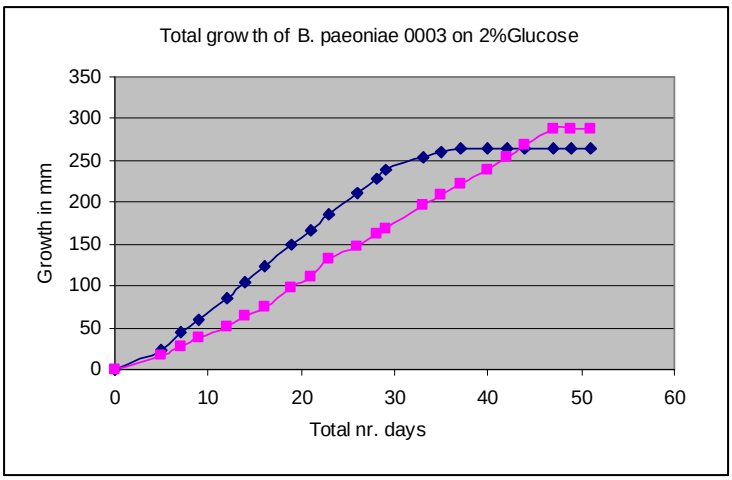
22. Disposal. Switch of UV (and Quantity One program). Carefully remove your gel from the set-up. Use towels to dry the (contaminated) surface of the UV-box. Dispose of gel and used towels in our own lab in the special blue containers. Rinse all the other used items (combs, trays, everything if you had not already done it) at the sink in the EtBr area of the lab.

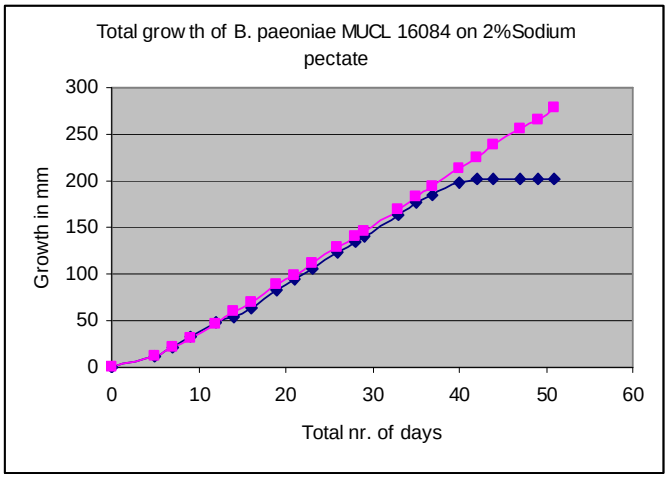
Additional Graphs

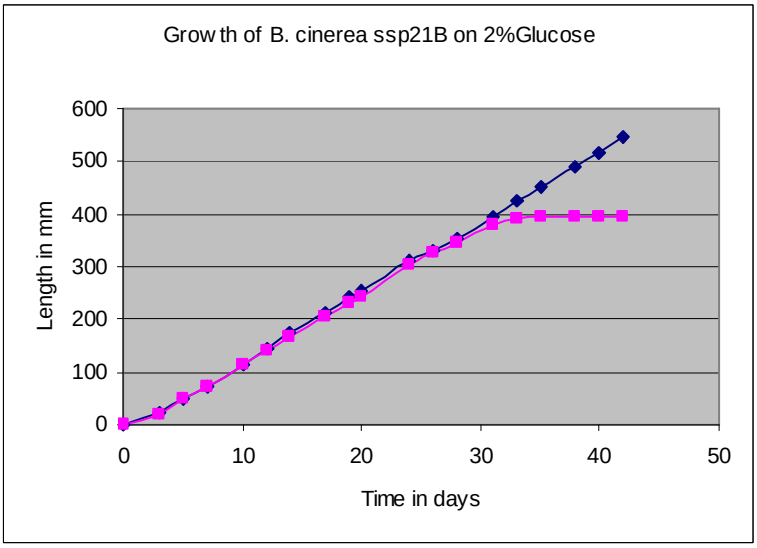
Graphs showing senescence in different *B. tulipae* and *B. paeoniae* strains on 2%Glucose GB5 or 2%PGA GB5 medium.





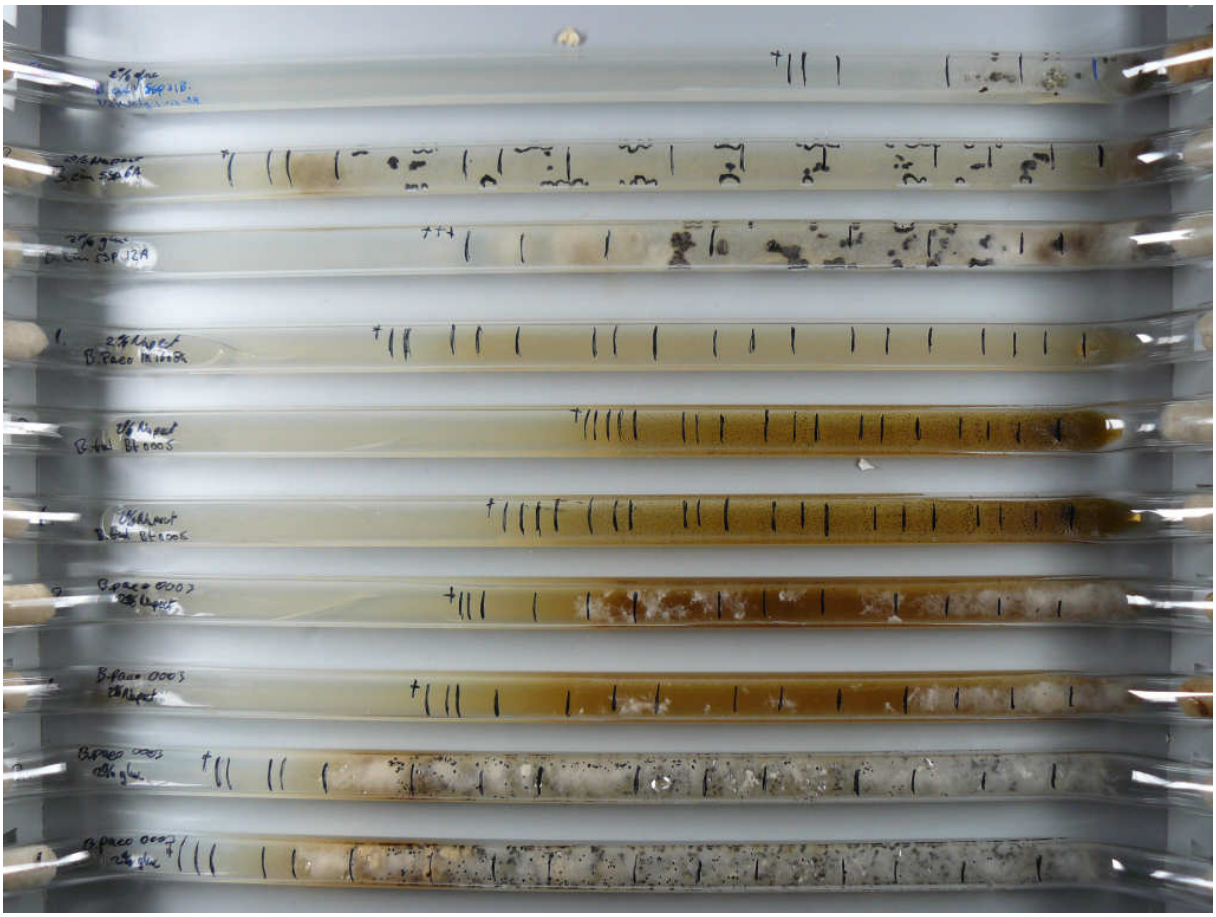




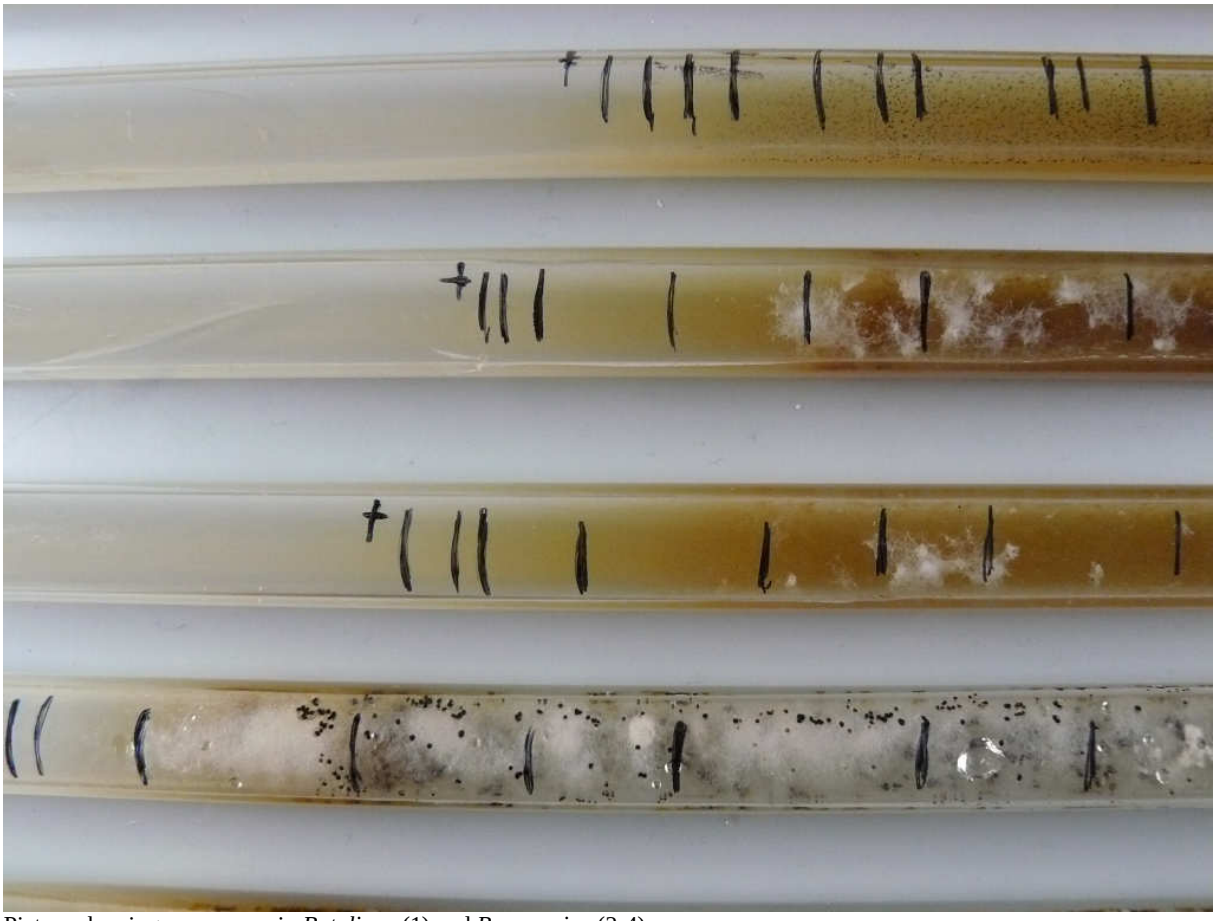


Graph showing senescence in one of the duplicates of B. cinerea ssp21B on 2%Glucose GB5 medium.

Additional Pictures



Picture showing several strains showing senescence: *B. cinerea*, (1-3), *B. paeoniae* (4, 7-10) and *B. tulipae* (5 and 6).



Picture showing senescence in *B. tulipae* (1) and *B. paeoniae* (2-4).