

Nathalie Van Hese

**INTEGRATED CONTROL OF DOWNY MILDEW
(*BREMIA LACTUCAE*) ON LETTUCE
AND SIDE EFFECTS ON BASAL ROT CAUSED BY
BOTRYTIS CINEREA AND *RHIZOCTONIA SOLANI***

Thesis submitted in fulfilment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Geïntegreerde beheersing van valse meeldauw (*Bremia lactucae*) in kropsla en neveneffecten op smet, veroorzaakt door *Botrytis cinerea* en *Rhizoctonia solani*

Cover illustration

Bremia lactucae conidium, treated with calcofluor and visualized with a UV epi-fluorescence microscope.

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Dankwoord

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List of abbreviations

AG	anastomosis group
Avr	avirulence
Ca	calcium
CLP	cyclic lipopeptides
dbi	days before inoculation
dpi	days post inoculation
DMSO	dimethyl sulfoxide
EHM	extrahaustorial membrane
ET	ethylene
Eth	Ethephon
ETI	effector triggered immunity
ETS	effector triggered susceptibility
F10	fengycin 10 mg/L
HIGS	host-induced gene silencing
siRNA	small interfering ribonucleic acid
hpi	hours post inoculation
IBEB	International Bremia Evaluation Board
IPM	integrated pest management
ISR	induced systemic resistance
JA	jasmonic acid
LHP	lipid hydroperoxidase
LOX	lipoxygenase
M10	mycosubtilin 10 mg/L
MAMP	microbe associated molecular pattern
MeJA	methyl-jasmonate
MS-medium	Murashige and Skoog medium
NB-LRR proteins	nucleotide-binding leucine rich repeats proteins
NHR	non host resistance

List of abbreviations

NO	nitric oxide
pal	phenylalanine ammonia lyase
PAMP	pathogen associated molecular pattern
PDA	potato dextrose agar
PDB	potato dextrose broth
PGPR	plant growth-promoting rhizobacteria
pr	pathogenesis related
PRR	pattern recognition receptors
PTI	PAMP-triggered immunity
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
QTL	quantitative trait loci
S10	surfactin 10 mg/L
SA	salicylic acid
SAR	systemic acquired resistance

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Outline

Problem statement

Belgium is considered as one of the major lettuce producing countries (Subbarao 1998), making lettuce a very important export product. In the last decades, lettuce production has become an intensive practice. The majority of Belgian lettuce is year round produced in greenhouses in continuous monoculture. This intensification of the production led to a boost in the productivity, but also to an increased vulnerability of the crop to various diseases (Barrière et al. 2014). One of the most feared diseases for growers is downy mildew, caused by the oomycete *Bremia lactucae* (Verhoeff 1960; Barrière et al. 2014). When control measures fail, this disease can cause considerable yield losses as it can infect all growth stages of the lettuce plants and spread very fast due to the relatively short infection cycle and the production of an enormous number of spores. Since long time lettuce downy mildew is mostly controlled by applying fungicides on lettuce cultivars with resistance genes (Crute 1984). In Belgium only eight fungicides are registered for lettuce downy mildew control in 2015. A restricted number of fungicides and frequent applications induce selection pressure on the pathogen and loss of efficacy of fungicides has been reported several times (Wicks et al. 1994; Brown et al. 2004). Furthermore, genetic resistance in lettuce cultivars is usually based on gene-for-gene resistance, which is often not long lasting because small modifications in the virulence factors of the pathogen can inhibit the recognition by resistance gene products of the host plant. This prevents the initiation of defence responses normally following upon virulence factor recognition (Jones and Dangl 2006). These findings emphasize the need for a durable control strategy, which can be defined as resistance that remains effective over long periods of widespread agricultural use under conditions favourable to disease. Durable resistance does not have one genetic or molecular basis, but involves slowing the evolution of virulence characteristics of the pathogen by maximizing the required effort for the pathogen to overcome the resistance (Michelmore et al. 2013). The European guideline 2009/128/EG prescribes a more sustainable pesticide use (Het Europees Parlement en de Raad van de Europese Unie 2009) and is converted into a Belgian National Plan of Action. This guideline aims to implement integrated pest management (IPM) for pesticide reduction. IPM comprises prevention, monitoring and intervention (Vlaamse Regering 2013). The purpose of this project was the development of an integrated, sustainable control strategy for downy mildew. Therefore the potential sources of inoculum were evaluated, the efficacy of various resistance genes and factors was studied, the possibility to adapt the greenhouse climate to suppress the development of the pathogen, the performance of the registered fungicides and also the potential of some cyclic lipopeptides and glycolipids as future biofungicides were analysed. An integrated control strategy should not favour other diseases. Therefore it is important to evaluate the side effects of this strategy on other lettuce diseases. In this work the side effect of some aspects of the strategy on basal rot was evaluated. Basal rot in lettuce can be caused by several pathogens, but our study was limited to two frequently occurring causal agents: *Botrytis cinerea* and *Rhizoctonia solani*.

Thesis outline

In this project we studied existing and some potential future control measurements to contribute to the development of a sustainable control of lettuce downy mildew.

Chapter 1 gives an overview of lettuce production in Belgium and the relevance of lettuce production in the world. Furthermore lettuce downy mildew symptoms and the causal agent, *B. lactucae* are described. Plant-pathogen interactions in general are used to review the specific *B. lactucae* – lettuce interaction characteristics. Also the main disease management strategies are outlined. Because possible side effects of the developed integrated control strategy on basal rot need to be considered, the two most important basal rot pathogens, *Botrytis cinerea* and *Rhizoctonia solani*, are also described. Part of the integrated control strategy is focused on the development of potential biofungicides. In this work, two groups are considered. The first group consists of cyclic lipopeptides produced by *Bacillus subtilis* and the second group of glycolipids produced by yeasts or derived from yeast products.

As mentioned in the introduction, genetic resistance in lettuce cultivars forms an important part of current downy mildew control. The interaction between plant and pathogen is very specific, thus it is important to know the virulence characteristics of the pathogen to determine the most effective resistance genes for future breeding. A study to characterize Belgian *B. lactucae* isolates was carried out and described in **Chapter 2**.

A second aspect of lettuce downy mildew control involves fungicide treatment. Because fungicides are more effective when applied closer to the moment of infection, forecasting models were developed to estimate the risk of disease outbreak and allow an in-time fungicide application. In **Chapter 3** a decision support tool was developed, based on an epidemiological study of Belgian *B. lactucae* isolates, which suggests greenhouse climate adaptation as a first step in disease intervention. Because the majority of Belgian lettuce is grown in greenhouses, climate adaptation could be a tool to interrupt the development of the pathogen and in that way save fungicide treatments. This decision support tool was evaluated not only for the effectiveness of downy mildew control but studied also for possible side effect on basal rot, tipburn and yield. The developed decision support tool was transformed into a web application to make it user friendly and accessible for growers.

As an extension of fungicides, the potential of biofungicides was considered. The first studied group of potential biofungicides consisted of cyclic lipopeptides of *Bacillus subtilis*: mycosubtilin, surfactin and fengycin. In **Chapter 4** the effect of these three cyclic lipopeptides on downy mildew was evaluated and first steps were taken to determine if these cyclic lipopeptides have a direct effect against *Bremia lactucae* and/or influence the defence mechanism of the plant.

Because the three cyclic lipopeptides of *B. subtilis* had a reducing effect on lettuce downy mildew, **Chapter 5** was dedicated to study the effect of these cyclic lipopeptides on two other important lettuce pathogens: *Rhizoctonia solani* and *Botrytis cinerea*. The effect of the lipopeptides on mycelium growth, conidia germination, sclerotia viability and disease index was evaluated.

The second group of potential biofungicides, glycolipids produced by or derived from products of *Starmarella bombicola* were studied in **Chapter 6**. Similar experiments as with the cyclic lipopeptides

were performed to assess their effects against downy mildew or basal rot caused by *B. cinerea* or *R. solani*.

In the final **Chapter 7** general conclusions are provided, the significance of this thesis is discussed and suggestions for further research are given.

Chapter 1. Literature review

Lettuce is a popular, fresh vegetable which is often grown in monoculture in Belgium and therefore prone to several diseases. In this chapter, firstly lettuce is described, followed by a detailed characterization of downy mildew, which is considered as one of the most severe diseases of lettuce. The purpose of this PhD was to develop an integrated control strategy for this disease. Applying a control strategy for one disease sometimes causes or stimulates problems with other diseases; therefore possible side effects of the developed integrated control strategy on basal rot were evaluated. Basal rot is a common problem in Belgian lettuce and therefore an obvious choice to focus on in this work. In Belgium, basal rot is often caused by *Rhizoctonia solani* or *Botrytis cinerea*. Both are shortly discussed in this chapter. Two groups of biosurfactants are tested in this work for their potential as biofungicides. One group consists of cyclic lipopeptides produced by *Bacillus subtilis* and the other group contains glycolipids, produced by or derived from products of *Starmerella bombicola*. Both groups are described in the last section of this chapter.

1.1 Lettuce

Lettuce (*Lactuca sativa* L.), a member of the *Lactuca* genus in the Asteraceae (Compositae) family (Lindqvist 1960), is a popular, fresh vegetable and a typical cool-season crop with moderately high content of certain vitamins and minerals, principally vitamins A and C and calcium (Davis et al. 1997). First records of lettuce production as a vegetable dates from a tomb painting from 4500 B.C. in Egypt (Lindqvist 1960). This vegetable was popular in ancient Rome and is probably introduced into northern and western Europe by the Romans (Davis et al. 1997).

In statistics of the Food and Agriculture Organisation of the United Nations (FAO) reports for lettuce combine data of lettuce and chicory. Therefore data of both crops are also represented together in this chapter. Annual production of lettuce and chicory amounted to more than 24 million tons worldwide on about 1.15 million ha in 2013 (<http://faostat3.fao.org/download/Q/QC/E>). Nowadays the production is concentrated in Asia (Figure 1-1), where about two-third of the production area is situated, with China as the largest producer (<http://faostat3.fao.org/download/Q/QC/E>). Worldwide, the USA is the second largest producer, responsible for approximately 15% of total amount of produced lettuce and chicory in 2013 (<http://faostat3.fao.org/download/Q/QC/E>), as shown in Figure 1-1. Spain, Italy, Germany and France are the largest lettuce producers in Europe, as can be seen in Figure 1-2 (<http://faostat3.fao.org/download/Q/QC/E>).

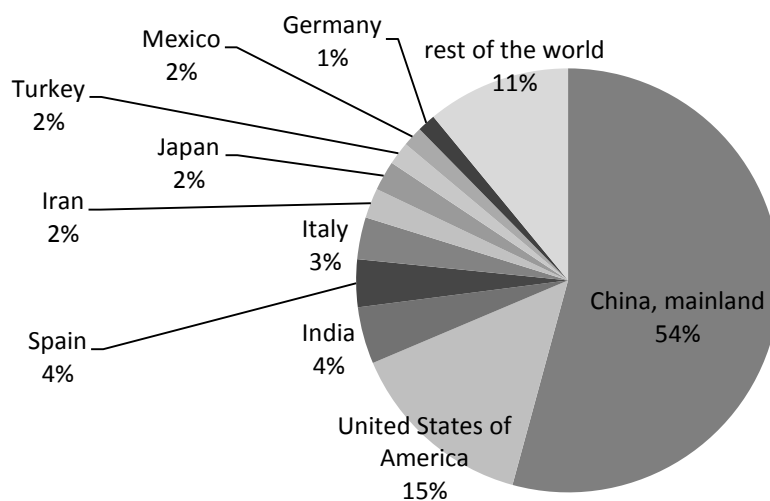


Figure 1-1: Countries with the largest production of lettuce and chicory worldwide in 2013 (<http://faostat3.fao.org/download/Q/QC/E>).

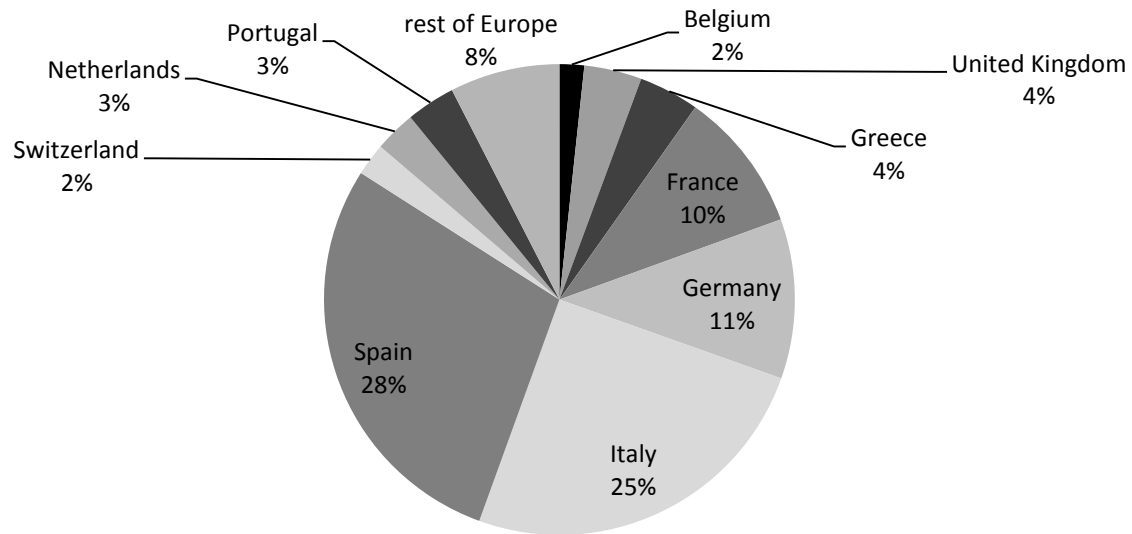


Figure 1-2: European countries with largest lettuce and chicory production in 2013 (<http://faostat3.fao.org/download/Q/QC/E>).

Lettuce, with butterhead lettuce being the most common type, is in Belgium economically the second most important vegetable crop after tomato (Appeltans 2014) with an annual production of 44 050 ton in 2013 (VLAM 2014). Butterhead lettuce distinguishes itself from other lettuce cultivars by its smooth texture. The colour is usually light to dark green outside and creamy yellow inside the head, yet some cultivars have red pigmentation (Davis et al. 1997). The majority of Belgian lettuce, 37 000 ton, was cultivated year round mainly in a continuous monoculture with up to six crops of lettuce per year in greenhouses on a total area of 179 ha. The remaining 7 050 ton came from open field lettuce production annually produced from spring to autumn on a total area of 62 ha (VLAM 2014). Of this annual production, 28 506 ton lettuce is exported, counting for 5% of the export value of fresh vegetables (VLAM 2014).

The majority of Belgian vegetable production is situated in Flanders. In open field approximately 67% of the area for vegetable production is established in Flanders and about 99% of the area for vegetable production in greenhouses (Figure 1-3 and Figure 1-4) (Bernaerts and Demuyck 2010).

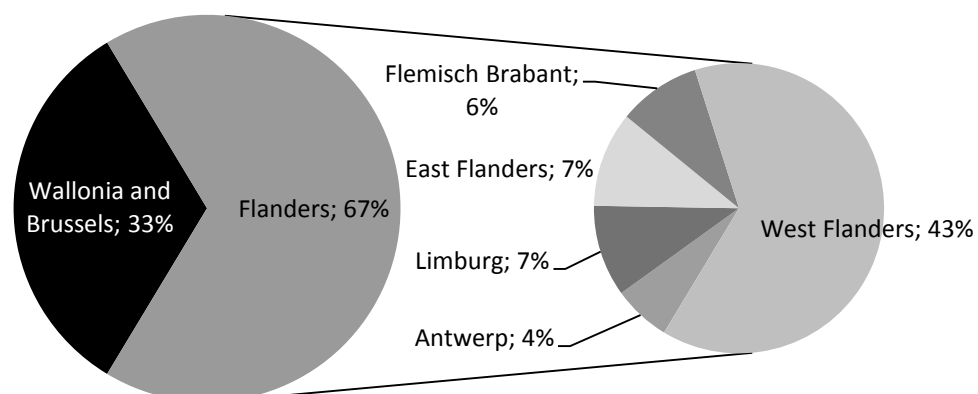


Figure 1-3: Distribution of vegetable production area in open field in Belgium in 2009 (Bernaerts and Demuyck 2010).

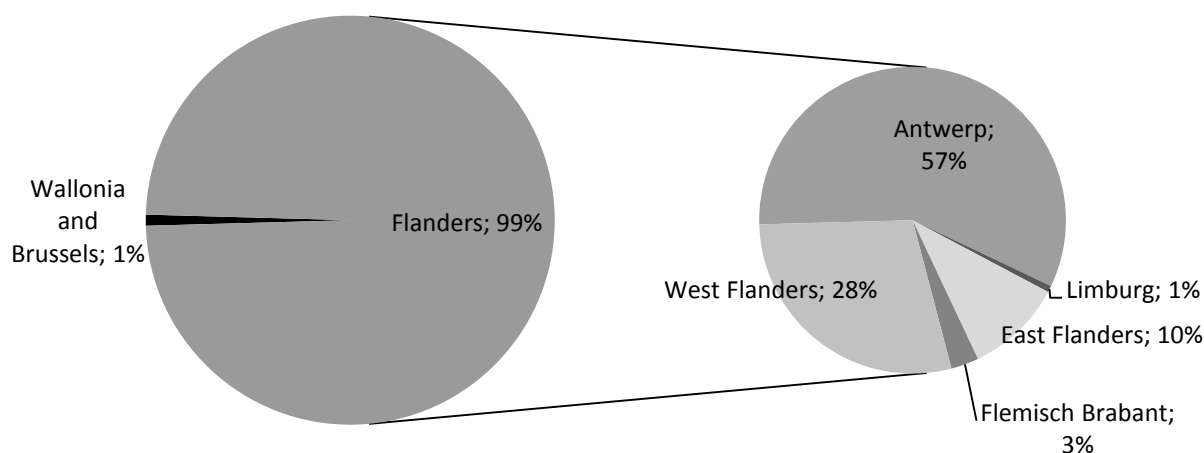


Figure 1-4: Distribution of vegetable production area in greenhouses in Belgium in 2009 (Bernaerts and Demuyne 2010).

Lettuce is sown in peat blocks of 3-5 cm and blocks are planted directly into soil when seedlings reach the 4-6 true leaf stage, spaced approximately 30 cm apart, with an average of 13 plants per m². Since the last decade, hydroponic systems are also used for lettuce production. In hydroponic systems there are approximately 20 lettuce heads per m². Furthermore the distance in between the plants is not fixed as in soil, but dependent on lettuce head size, improving the efficiency of use of space. Another advantage of hydroponic systems is that more cycles per year are possible: around 6 crop cycles per year in soil and 8 in hydroponic systems, yet the costs for a hydroponic system are much higher compared to cultivation in soil (Gfactueel 2003). Nutrient Film Technique (NFT) is a hydroponic system of the company Hortiplan NV, in which the plants are grown in gutters and a thin water film flows through the gutters, providing nutrients for the plants. The water is recycled after filtration and adaptation of the nutrient content (Hortiplan 2015).

Quality of Belgian lettuce is guaranteed by the Flandria, EurepGap and FlandriaGap labels (http://www.mijnflandria.be/Het_Flandria-label_1 , <http://www.reo.be/eng/eurepgap/wat.asp> , www.Flandriagap.be , <http://www.imik.org/lastenboek/FlandriaGAP.html>). Optimization of the yield, demands optimization of the cultivation conditions. In the greenhouse, climate can be adapted according to the needs of the plants. With a climate box and climate computer heating, ventilation and irrigation for instance can be automatized according to the climate conditions in the greenhouse and criteria chosen by the grower (<http://www.hortiplan.com/nl/klimaattechniek/> and <http://www.hortimax.nl/4/1/nl/solutions/klimaat-energie.html>).

Due to the intensive monoculture, lettuce production is threatened by various diseases including basal rot (by *Sclerotinia minor*, *S. sclerotiorum*, *Botrytis cinerea*, *Rhizoctonia solani* anastomosis groups AG1-1B, AG4 HGI, AG10, AG2-1, AG2-1 Nt and AG3 and *Pythium ultimum*, *P. cylindrosporum*, *P. (ir)regularare* and *P. sylvaticum*), downy mildew (by *Bremia lactucae*) and fusarium wilt (by *Fusarium oxysporum*) for Belgian lettuce (Van Beneden et al. 2009; Barrière et al. 2014). Furthermore midrib rot (caused by *Pseudomonas cichorii*) is another serious threat to the production of Belgian greenhouse butterhead lettuce (Cottyn et al. 2009; Pauwelyn et al. 2011).

Besides these diseases, tipburn can also reduce yield and is frequently occurring in Belgium. Tipburn is a physiological disorder, visible as brown or black necrosis along the margins of inner leaves, associated with lack of calcium (Ca) in these inner leaves. Ca follows the water flow in the plant, which is mainly mediated by transpiration. The middle and outer leaves of a plant have a higher transpiration rate than the younger inner leaves. A high relative humidity results in low leaf transpiration, which causes an increase in the concentration of mineral elements in the roots. This high mineral concentration results in plant demand for water from the soil and the absorption of water by their roots creates root pressure, which drives Ca transport to the younger leaves (Corriveau et al. 2012).

1.2 Downy mildew

Lettuce downy mildew is caused by the biotrophic oomycete *Bremia lactucae* Regel and is often considered to be one of the oldest, most frequent and most feared diseases affecting lettuce. The disease can cause considerable yield losses because lettuce at all growth stages can be attacked, the disease can spread exponentially under favourable, cool and humid climate conditions and the pathogen adapts rapidly to overcome the resistance in lettuce cultivars or to become resistant to frequently applied fungicides (Crute 1984; Verhoeff 1960; Brown et al. 2004; Blancard et al. 2006; Michelmore and Wong 2008).

1.2.1 Disease symptoms

A wide array of symptoms can occur in the interaction between *B. lactucae* and lettuce, depending on the ontogenetic stage of the plants and the degree of resistance (Lebeda and Sedlářová 2008).

B. lactucae can attack lettuce throughout its entire growing cycle, although seedlings are more susceptible than older plants. An infection of young plants results in covering of the cotyledons by many sporangiophores (Figure 1-5), followed by yellow discoloration of the leaves, which is never confined to defined spots. Soon after the discoloration of the leaves, the seedlings will die. In case of severe infection, the seedlings sometimes show stunting as a first symptom (Lebeda and Sedlářová 2008).



Figure 1-5: Cotyledons of 17-day old lettuce seedlings, covered with *B. lactucae* sporangiophores.

The outcome of a compatible interaction on older plants will first be visible as yellow spots, mainly on the outer leaves, which are delineated by the veins, followed by sporulation, usually on the underside of the leaves and visible as white powder on the leaf surface (Figure 1-6). When the plants grow very close to each other, the sporangiophores may be present on the upper side of the leaves too. Later on the lesions may become necrotic and parts of the leaf or the entire leaf may be killed (Verhoeff 1960; Ryder 1998; Blancard et al. 2006; Lebeda and Sedlářová 2008). Sporulation will be

rather limited on plants with incomplete resistance and abundant in full compatible interactions between host and pathogen (Lebeda and Sedlářová 2003). Chlorotic symptoms might result from an effect on the photosynthetic processes of the plant, as a linkage between the photosynthetic deterioration of the photosynthetic apparatus of the plant and compatibility of the interaction is shown (Lebeda and Sedlářová 2008). In incompatible reactions, no symptoms or, on the contrary, extensive necrosis will be visible.



Figure 1-6: Downy mildew symptoms: typical yellow spots delineated by veins and sporulation on the underside of the leaves.

1.2.2 The pathogen

Bremia lactucae was first described by Regel in 1843 (Regel 1843) and is considered as one of the most highly specialized biotrophic downy mildews not only because the asexual spores (usually) germinate directly and not via zoospores, but also because *B. lactucae* can penetrate directly through the plant cuticle and epidermal cells rather than through stomata. This pathogen has long been studied as a model for understanding biotrophy in the Oomycetes (Crute and Norwood 1986; Lebeda and Sedlářová 2008; Michelmore and Wong 2008). Different pathotypes have been described, defined by virulence profiles on a set of lettuce inbred lines (International Seed Federation 2014) and new pathotypes continue to appear.

1.2.2.1 Taxonomy

The species *Bremia lactucae* can be taxonomically categorized in the kingdom Stramenopila, the phylum Oomycota and the class Oomycetes. This pathogen is a member of the genus *Bremia*, within the *Peronosporaceae* family and the order of Peronosporales (Dick 2002; Michelmore and Wong 2008). Despite the morphological, physiological and ecological similarities to fungi, oomycetes do not belong to the kingdom Fungi, but to the kingdom Stramenopila (Dick 2002; Voglmayr 2008). The similarities between oomycetes and Fungi are an example of convergent evolution, i.e. the independent evolution of similar features in species of different lineages. Oomycetes are closer related to algae, while fungi share a common ancestor with animals (Latijnhouwers et al. 2003) (Figure 1-7). One of the most important morphological and physiological differences between oomycetes and fungi are the differences in ploidy. Oomycetes are diploid, while fungi are haploid or dikaryotic most of the time during their lifecycle. Furthermore many oomycetes are (partial) sterol auxotrophs and in their membranes sterols are replaced by lipids with long-chain fatty acids, whereas sterols form a major component of the mycelium membrane of fungi. Also small amounts of chitins are detected in the cell walls of only few oomycetes, while chitin is the major constituent of fungal cell walls. Cell walls of oomycetes consist mainly of 1,3- β -glucans, 1,6- β -glucans and 1,4- β -glucans (cellulose). Besides, some lysine synthesis is mediated by different pathways with oomycetes

using the α,ϵ -diaminopimelic acid pathway and fungi the α -aminoadipic acid pathway (Latijnhouwers et al. 2003). A more extended list of differences between oomycetes and fungi is given in Table 1-1.

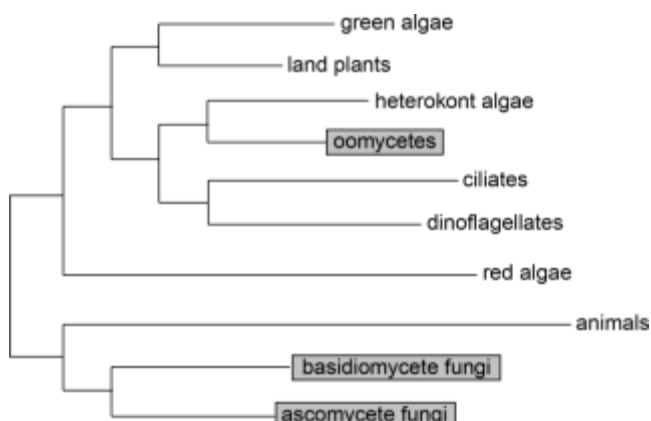


Figure 1-7: Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups. The Oomycetes and the fungi are marked in grey, showing the evolutionary distance between these two. Figure adapted from (Latijnhouwers et al. 2003) and (Kamoun et al. 1999).

Table 1-1: Differences between oomycetes and fungi.
Table adapted from Judelson and Blanco (Judelson and Blanco 2005)

Criterion	Oomycetes	Fungi
Hyphal architecture	Aseptate + coenocytic tubular hyphae	Single cell or septated hyphae, with one or more nuclei per compartment
Ploidy of vegetative hyphae	Diploid (at least), except for transient haploid nuclei in gametangia	Haploid or dikaryotic, often with a stable or semi-stable diploid stage following mating
Major glucans in cell wall	Celluloses and β -1,3- and β -1,6-linked glucose polymers	Usually chitin (β -1,4- linked glucosamine) often with other β -1,3- and β -1,6-,glucans
Pigmentation	Usually unpigmented	Very common in hyphae or spores, or secreted
Presence of motile asexual spores	Often biflagellate zoospores	Uncommon
Sexual spores	Oospores, formed on hyphae after fertilization of an oogonium by an antheridium	Various types, often formed in large numbers within complex enclosures (e.g. perithecia, mushroom caps etc.)
Type of mitochondria	With tubular cristae	With flattened cristae
Major energy reserves used by spores	Mycolaminarin and lipid	Glycogen and trehalose, also sugar alcohols and lipids
Lysine synthesis	Via α,ϵ -diaminopimelic acid pathway	Via α -aminoadipic acid pathway
Content of cell membrane	Lipids	(Ergo)sterols

The obligate biotrophic downy mildews, the *Peronosporaceae*, are subdivided into four subgroups based on morphological or ecological characteristics. One of these subgroups comprises the genera with vesicular to pyriform haustoria: *Basidiophora*, *Benua*, *Bremia*, *Paraperonospora*, *Plasmopara*, *Plasmoverna* and *Protobremia* (Voglmayr et al. 2004; Göker et al. 2007; Lebeda and Sedlářová 2008). Within the genus *Bremia* several genetically distinct species are described besides *B. lactucae*, including *B. lapsanae*, *B. sonchicola* and *B. taraxaci*, which are all host-specific species (Thines et al. 2010).

1.2.2.2 Host range

The host range of species within the genus *Bremia* is restricted to Asteraceae (Voglmayr et al. 2004). Because *Bremia* isolates collected from *Lactuca* are genetically different from isolates collected from other hosts, lack of interbreeding is suggested and therefore infected Asteraceae other than *Lactuca* are unlikely to be a source of inoculum for infections of *Lactuca* spp. (Ling and Tai 1945; Voglmayr et al. 2004; Thines et al. 2010). There are 100 wild *Lactuca* species described, of which only 14 are defined as natural hosts of *B. lactucae*, with *L. serriola* and *L. sativa* the most frequently occurring host plants (Thines et al. 2010).

L. serriola is thus considered as an alternative host for *B. lactucae* isolates infecting commercially grown lettuce, *L. sativa* (Lebeda 1984; Lebeda and Petrželová 2004; Lebeda et al. 2008). *L. serriola* is one of the four species within the serriola group of the *Lactuca* genus and has typical lobed leaves with blunt apex and prickles on stem and leaves (Lindqvist 1960) (Figure 1-8). This plant is a common weed in Europe, including the Netherlands (Hooftman et al. 2006) and Belgium (<http://wildeplanten.nl/kompassla.htm>). Flowering happens from July until September (<http://wildeplanten.nl/kompassla.htm>). It is a Mediterranean ruderal plant, which has expanded northwards since the beginning of the 19th century. With its short life cycle, strong self-fertilization ability, good adaptation for wind dispersal and quick germination it is a successful weed (D'Andrea et al. 2009). It is likely that domesticated lettuce originated from *L. serriola* because this plant is a primitive, nearly wild form of lettuce (Davis et al. 1997). Although *L. serriola* can be an important weedy host for *B. lactucae*, naturally infected plants are only described in Czech Republic (Lebeda et al. 2014) and Southern France (Maisonneuve et al. 2011). Figure 1-9 shows the diversity of symptoms that can be observed on *L. serriola* plants infected with downy mildew. Small yellow spots, delineated by the veins with sporulation on the down side of the leaf are described, but also discoloration without sporulation or necrosis (Lebeda et al. 2008).



Figure 1-8: Leaves and flower of *Lactuca serriola*. (Leaves, own picture, flower (<https://www.kuleuven-kulak.be/kulakbiocampus/images>)).

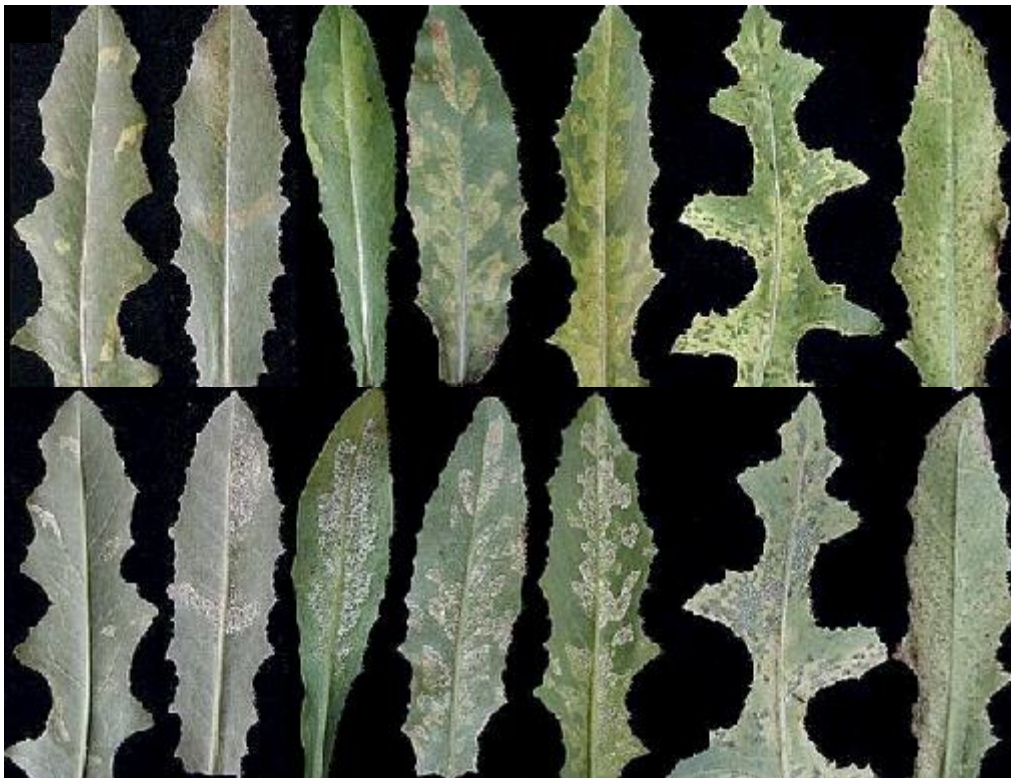


Figure 1-9: Pictures of symptoms of downy mildew on *Lactuca serriola* (Lebeda et al. 2008).

1.2.2.3 Life cycle

The asexual life cycle (Figure 1-10) starts with the direct germination of asexual spores or conidia although more common for *Peronosporaceae* members is germination via zoospore formation. Next the *B. lactucae* germ tubes directly penetrate the plant cell through the cuticle and epidermal cells, rather than entering via stomata (Michelmore and Wong 2008; Lebeda and Sedlářová 2008). It seems from the shape of the cuticle profile and the microfibrils of the epidermal wall during penetration that little or no mechanical force is used to enter the host, suggesting that penetration occurs following enzymatic dissolution of the cuticle and cell wall (Sargent et al. 1973). Once inside the plant

a primary vesicle is formed in the epidermal cell and subsequently a secondary vesicle (Sargent et al. 1973; Wu et al. 2002; Lebeda and Sedlářová 2008). The first vesicle is suggested to function as a “second spore” which supplies nutrition to the second vesicle which establishes an intimate relation with the host plant (Sargent et al. 1973). Afterwards, young non-septate mycelium will fill the substomatal cavity nearest to the entering place and spread to surrounding substomatal cavities, but not to other leaves via leaf base and stem of the plant. Plant cell walls are penetrated by the hyphae but the protoplast stays intact. Pyriform haustoria are developed, which invaginate the plant cell membrane and create an extrahaustorial membrane (Lebeda and Sedlářová 2008). Haustoria are thought to be the main sites for carbohydrate and amino acid uptake by the pathogen but also for the secretion of effectors (Whisson et al. 2007; Devdutta 2013). When a substomatal cavity is completely filled with mycelium, conidiophores will be produced and appear on the leaf surface, from which fresh conidia will be released (Verhoeff 1960). Duration of the life cycle is dependent on relative humidity and temperature conditions and varies between 4 days to approximately one month (Verhoeff 1960).

Besides asexual reproduction, which can result in a fast spread of the disease, oomycetes can also reproduce sexually (Figure 1-10). In oomycetes there are two types of sexual reproduction: heterothallism and homothallism. For heterothallism, the sexual reproductive cells, oogonia and antheridia are produced by two different organisms of two different mating types (B1 and B2), while in homothallic organisms, the two types of sexual reproductive cells are produced by the same organism. *B. lactucae* is mainly heterothallic (Michelmore and Ingram 1980; Michelmore and Ingram 1981), although secondary homothallism is also reported. These homothallic isolates behave predominantly as B2 mating type, but produce oospores when cultured together with a B1 mating type and oospores are also produced in low frequency when this isolate is cultured alone in high density, due to generation of B1 components at low frequency (Michelmore and Ingram 1982; Michelmore and Sansome 1982). Asexual reproduction is suppressed when hyphae of opposite mating type come into contact and sexual reproductive cells will be formed. Next the haploid gametes will be transferred from the antheridium to the oogonium, resulting in oospore formation (Michelmore and Ingram 1981). Germination tubes of oospores can infect lettuce plants and start a new disease cycle (Morgan 1978; Morgan 1983).

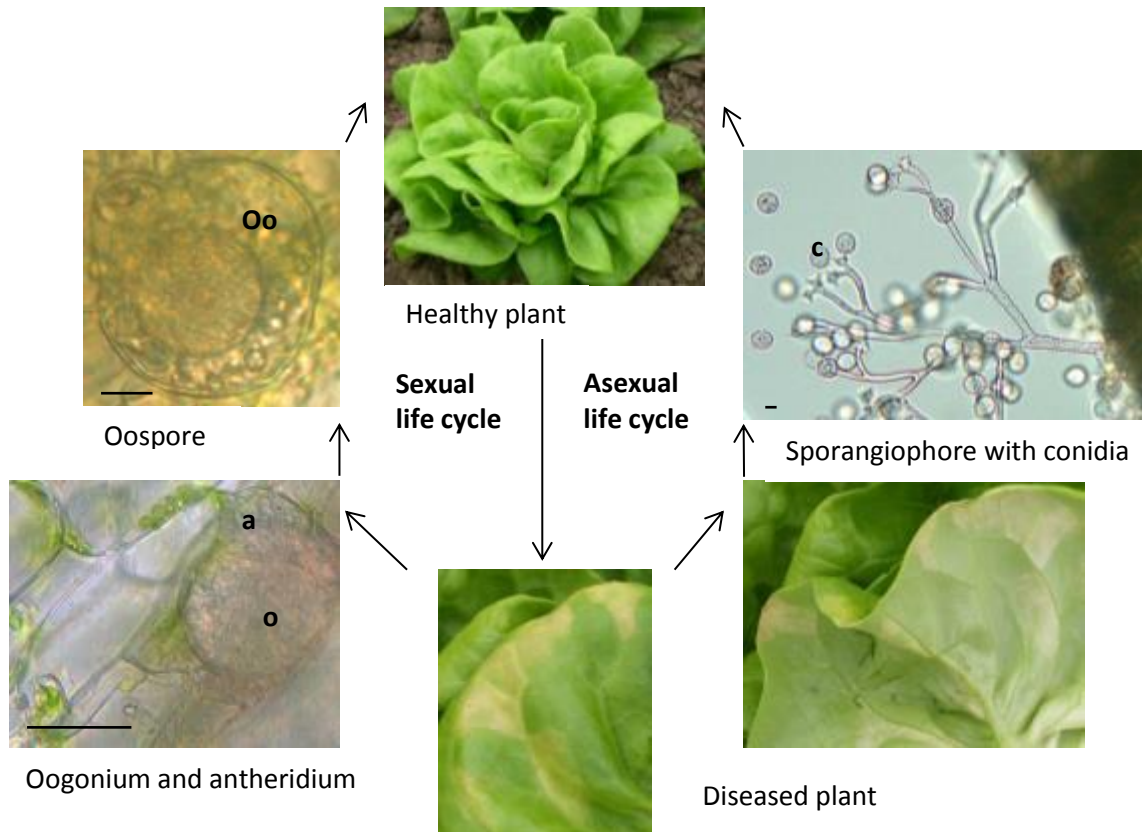


Figure 1-10: Sexual and asexual life cycle of *Bremia lactucae*. When hyphae of two different mating types grow in close contact, an antheridium (a) and oogonium (o) will be formed. Gametes can be transferred from the antheridium to the oogonium, resulting in formation of an oospore (Oo). This oospore can germinate and infect new lettuce leaves. In the asexual cycle, a new cycle starts with the spread of conidia (c), landing on a fresh lettuce leaf, where they germinate and penetrate the host. Inside the host tissue mycelium grows, and when substomatal cavities are filled, yellow spots appear. On the down side of the leaf new sporangiophores with conidia appear. Scale bars on the microscopic pictures represent 10 μm .

1.2.2.4 Influence of temperature, relative humidity, leaf wetness and solar radiation on disease development and dispersal

Throughout the growing season, airborne asexual conidia are the most important means of disease spread. The intensity of sporulation and the viability of the spores mainly depend on environmental conditions and concentration of primary inoculum (Lebeda and Sedlářová 2008). Sporulation occurs mostly at night (Carisse and Phillion 2002) or is retarded by light, according to other studies. This suppression by light is found to be dependent on temperature: with a higher temperature the sporulation reducing ability of light increases (Nordskog et al. 2007). Furthermore an influence of the light intensity and quality was noticed and light with wavelength of 400 to 450 nm leads to the strongest reduction (Nordskog et al. 2007). Light-dark regime is crucial for optimal sporulation as continuous darkness also reduces sporulation (Nordskog et al. 2007). Optimum sporulation temperature seems to be location dependent. In Canada and California sporulation was possible in a temperature range from 4 to 20 °C with an optimum of 15 °C (Su et al. 2004), while in Great Britain an optimum of 6 to 11 °C was observed (Powlesland 1954). Another climatologic requirement is a high relative humidity of at least 80% and remarkable increase of sporulation was noticed when the relative humidity reached above 90% (Su et al. 2004). Wind is a fourth factor affecting sporulation because from 0.1 m/s wind speed sporulation was significantly reduced and no sporulation was

observed at wind speeds of more than 0.5 m/s (Su et al. 2004). Some epidemiologic studies focus on leaf wetness rather than relative humidity. For instance in a study of Kushalappa sporulation was observed to start after 7 h leaf wetness, with an optimum of 10 h if the temperature was between 10 and 15 °C (Kushalappa 2001). After spores appeared on the leaf surface, they can be released and infect new plants or leaves.

Release and dispersal of conidia require a decrease of the relative humidity and both processes are triggered by light and an increasing temperature. In California optimum spore release was observed between 10 a.m. and 12 a.m. (Schermer and van Bruggen 1995). Too high values of relative humidity, near saturation, reduce spore release (Carisse and Pillion 2002). With rain splashes or wind, conidia can be spread over distances up to 3 km (Wu et al. 2001). If they land on a susceptible host, a new life cycle of the pathogen can start. Viability of the dispersed conidia is dependent on temperature, sun radiation and relative humidity. A study from the Netherlands showed that conidia stored at 2 °C and with a relative humidity of 70% could retain viability up to 140 days. Influence of UV light was not investigated in that study (Verhoeff 1960), however later it was shown that conidia rarely survive during dry and sunny days because of the negative effect of solar radiation, especially UV-B-light (290-320 nm) (Wu et al. 2000). A short period of sun light with UV-radiation of 0.5 MJ/m² can be sufficient to kill the majority of spores present, although conidia on lower leaves are protected by the shadow of other leaves and might survive (Wu et al. 2000).

Germination of conidia is mostly directly, zoospore formation is scarce (Blancard et al. 2006) if occurring at all (Valade 2012). Criteria for germination are leaf wetness, a high relative humidity and moderate temperatures (Davis et al. 1997). Reported minimum and maximum temperatures for germination are 5 °C and 30 °C, with an optimum of 10-15 °C. If a water layer on the leaf surface is present and relative humidity is nearly 100%, conidia can germinate within 2 to 3 hours after deposition on the leaf (Davis et al. 1997). It was reported in a Californian study that leaf wetness was the most important factor for germination, and optimally leaf wetness period should last for 4 h after sunrise (Schermer and van Bruggen 1994a). Also the period of 8 to 9 h after penetration is critical for the establishment of pathogenicity because active parasitism is not fully established yet in this first period after penetration and the pathogen used a large proportion of its energy reserves for germination and penetration, making it susceptible to environmental stress. If temperature during this post-penetration stage increases from 15 to 30 °C, the percentage infected plantlets decreased from 30 to 5% according to another Californian study (Wu et al. 2005).

The length of the incubation period is determined by temperature: with the shortest period of four or five days when the temperature was between 20 and 22 °C and the longest period of 24 to 34 days when the temperature was 6 °C (Verhoeff 1960). However, the influence of temperature on the length of the incubation temperature was found to be smaller for fluctuating temperatures (Schermer and van Bruggen 1994b).

Survival of the pathogen inside the soil in infected plant material, is not very likely because as an obligate pathogen, *B. lactucae* needs a living host and buried plant material will rot too fast (Verhoeff 1960). Survival as oospores is reported several times, depending on the surveyed region: in the Netherlands, New York (Yuen and Lorbeer 1987) and Sweden (Gustafsson et al. 1985) oospores would be an important source of primary inoculum, while in California the sexual reproduction is of minor importance (Illot et al. 1987). Oospore germination was found to be possible at 17 °C (Blok 1981) and the germination tube can directly infect lettuce plants (Morgan 1978).

Another possibility for the pathogen to survive periods without cultivated lettuce is to infect alternative hosts, such as *Lactuca serriola*. Conidia from this infection can start a new infection cycle on cultivated lettuce (Lebeda 1984; Yuen and Lorbeer 1987; Petrželová and Lebeda 2003; Lebeda and Petrželová 2004; Lebeda et al. 2008).

1.2.3 Plant-pathogen interaction

1.2.3.1 General plant-pathogen interactions

Unlike mammals, plants do not possess mobile defender cells or an adaptive immune system, but they rely on the innate immunity of each cell and on systemic signals emanating from the infection sites. Constitutive or preformed structures and components such as the cuticula and certain metabolites form a first protection layer for the plant which is able to offer resistance to the majority of pathogens (Hardham and Cahill 2010). Besides this first layer, the immune system of plants can be seen as a two-branched innate system. The first branch recognizes and responds to microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by means of transmembrane pattern recognition receptors (PRRs). This branch can lead to PAMP-triggered immunity (PTI). The response to new microbial patterns might be faster due to earlier MAMP or PAMP elicitations that “prime” further defence responses (Jones and Dangl 2006).

Typical examples of MAMPs are flagellin, peptidoglycans, lipopolysaccharides or chitin (Boller and Felix 2009). It has been shown that biosurfactants and more specific cyclic lipopeptides (CLPs) can act as determinants of PTI (De Vleeschauwer and Höfte 2009). CLPs are produced by fungi and various bacterial genera e.g. *Streptomyces*, *Pseudomonas* and *Bacillus* (Raaijmakers et al. 2010). For instance a CLP mixture produced by *Bacillus amylolequifaciens* was able to reduce infection caused by the rhizomania disease vector *Polymyxa betae* in sugar beet (Desoignies et al. 2013) and massetolide A, a CLP of *Pseudomonas fluorescens* was shown to be a powerful elicitor of local and systemic resistance of tomato against *Phytophthora infestans* (Tran et al. 2007).

Successful pathogens can interfere with PTI by deploying effector proteins (effectors) which manipulate plant structure and/or metabolism (Hardham and Cahill 2010). Fungal or oomycete effectors can act either in the extracellular matrix or inside the plant cell. They can have a structural role, for example in the extrahaustorial matrix, or they may promote nutrient leakage or pathogen dispersal. Some effectors mimic plant hormones and by doing so disturb the hormone household of the plant. Others play a role in the suppression of PTI or ETI (effector triggered immunity). ETI is a stronger and faster version of PTI and is established when effectors are recognized by specific disease resistance (R) genes. These effectors are called avirulence factors. Most R genes encode NB-LRR proteins, named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR). ETI is reached as a direct or indirect response to pathogen effectors. NB-LRR activation induces differential salicylic acid- (SA) and reactive oxygen species- (ROS) dependent responses, oxidative bursts and/or local and systemic changes in gene expression. Natural selection can drive pathogens to avoid ETI by shedding or diversifying the recognized effector gene or by acquiring additional effectors to suppress ETI (Jones and Dangl 2006). Because of the large battery of virulence proteins secreted by oomycetes, a natural defence for plants is the production of proteases to degrade the virulence

proteins. In response, oomycetes are predicted to secrete protease inhibitors targeted against the plant proteins (Jiang and Tyler 2012a).

Both PTI and ETI can lead to programmed cell death, which might be a good strategy of the plant to defend itself against biotrophic or hemibiotrophic pathogens (Dangl and Jones 2001). In Figure 1-11 an overview of the possible interactions between plants and oomycete pathogens is shown (Jiang and Tyler 2012).

Induction of resistance is mediated by signal transduction pathways in which the plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) function as key signalling hormones (Turner et al. 2002; Wang et al. 2002; Glazebrook et al. 2003; Lorenzo and Solano 2005; Grant and Lamb 2006; Jones and Dangl 2006; Loake and Grant 2007; Vleesschauwer and Höfte 2009; Dodds and Rathjen 2010; Robert-Seilaniantz et al. 2011). Upon pathogen recognition, plants produce a very specific mixture of SA, JA and ET, which induces the activation of different sets of defence-related genes (Glazebrook et al. 2003). The specificities of this mixture, the so-called signal signature regarding quantity, quality and timing, play an important role in the organisation of the defence responses (De Vos et al. 2005). It is clear that these signalling pathways interact both in antagonistic and synergistic manners. This crosstalk allows the plant to fine-tune the defence response to best suit a specific threat because different threats can occur at the same time, making a trade-off necessary (Kunkel and Brooks 2002; Rojo et al. 2003; Spoel et al. 2007; Koornneef and Pieterse 2008; Robert-Seilaniantz et al. 2011). Despite several mentioned exceptions and reported synergism between the pathways (van Wees et al. 2000; Bostock 2005; Asselbergh et al. 2007; Spoel et al. 2007; Truman et al. 2007; Thaler et al. 2010), it can be generally stated that SA promotes resistance against biotrophic pathogens, while JA and ET function as positive signals in defence response activation upon necrotrophic pathogens and herbivorous insects attack. Often antagonistic effects between SA and JA/ET-pathway have been reported (Thomma et al. 2001; Rojo et al. 2003; Glazebrook 2005). Mechanistic explanations of the crosstalk between the different pathways are complex, yet some transcription factors are described. For instance the *ETHYLENE RESPONSE FACTOR1 (ERF1)* belongs to a family of transcription factors binding to a GCC-box present in promoters of many ethylene-inducible, defence-related genes and thus a transcriptional cascade mediated by -amongst others- ERF proteins, leads to the regulation of ethylene controlled gene expression (Guo and Ecker 2004). Induction of signalling pathways leads to different reactions, including cell wall modifications such as strengthening through increased synthesis and deposition of hydroxyproline-rich glycoproteins, callose, lignin and other phenolic compounds, also production of pathogenesis related (PR) proteins (Dempsey and Klessig 1995; Łażniewska et al. 2010).

When the induced resistance in the plant is induced to subsequent microbial infection at the whole plant level by a localized pathogen inoculation, it is called systemic acquired resistance (SAR), while when the induced resistance is induced by non-pathogenic rhizobacteria, it is known as induced systemic resistance (ISR) (De Vleesschauwer and Höfte 2009).

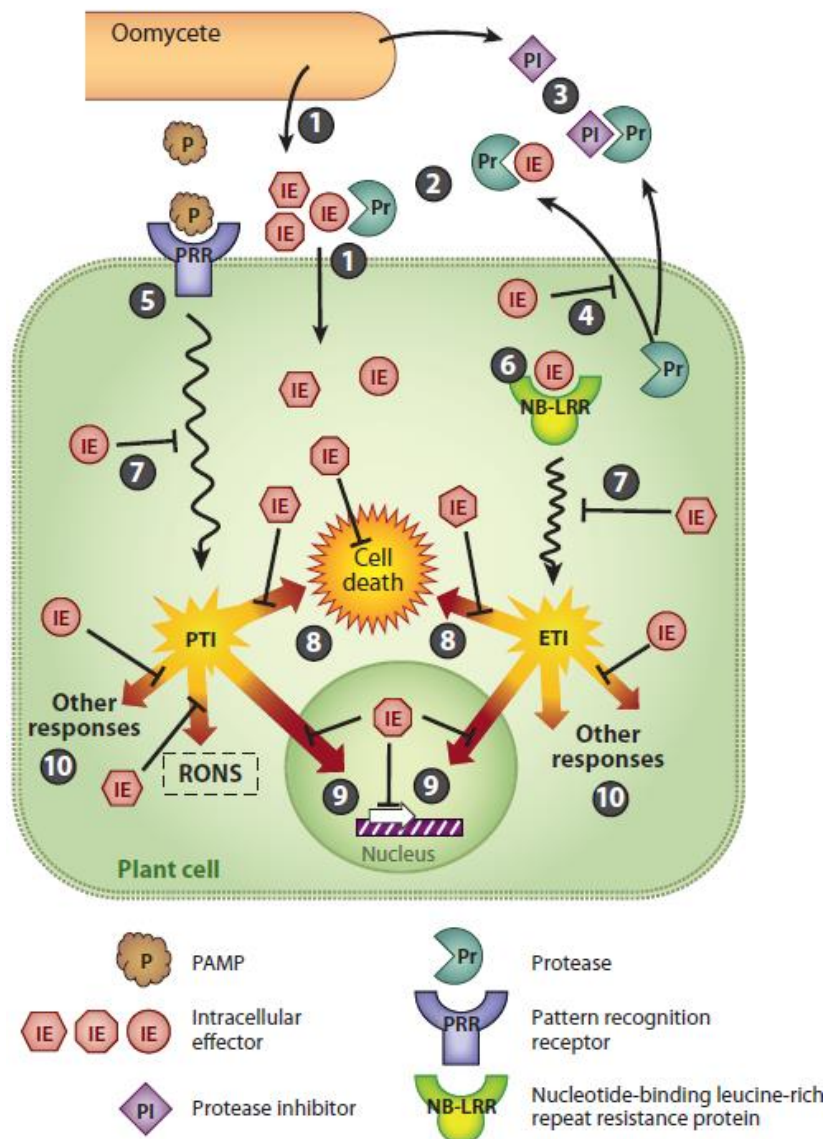


Figure 1-11: An overview of the possible interactions between plants and oomycete pathogens, as described and represented by Jiang and Tyler (Jiang and Tyler 2012).

Oomycete pathogens secrete intracellular effectors (IEs) of which some can enter the plant cytoplasm (1). Yet, plants may secrete proteases (Prs) that can degrade intracellular or extracellular effectors in the apoplast (2). Pathogens may secrete protease inhibitors (PIs) (3) that block those proteases or they may produce effectors that block secretion of the proteases (4). Recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) (5) results in production of signalling events, activating responses that lead to PAMP-triggered immunity (PTI). Recognition of intracellular effectors by nucleotide-binding site leucine-rich repeat receptors (NB-LRRs) (6) activates responses leading to effector-triggered immunity (ETI) (7). Both PTI and ETI can induce programmed cell death (8), however, effectors may inhibit the triggering of cell death or the cell death machinery itself. Both PTI and ETI involve transcriptional changes (9). Nuclear-targeted effectors may directly interfere with signalling within the nucleus or with transcriptional events. PTI and ETI also involve numerous other responses (10), amongst which the production of reactive oxygen and nitrogen species (RONS). Effectors may also interfere with those responses.

1.2.3.2 Lettuce-*Bremia lactucae* interaction

Compatible and incompatible interactions can be distinguished within 48 h post inoculation (hpi) by the differences in the development of *B. lactucae*. Whereas there is a prompt growth and fast development of primary infection structures of the pathogen in a compatible interaction, pathogen growth is retarded and/or completely blocked in an incompatible interaction (Lebeda and Sedlářová 2003) (Figure 1-12 (Kamoun et al. 1999)).

Plant disease resistance can be classified into qualitative resistance and quantitative resistance. Qualitative resistance is mediated by a single resistance gene leading to complete resistance, while quantitative resistance is conferred by multiple genes or quantitative trait loci (QTL), each providing partial resistance (Kou and Wang 2010). For resistance breeding in lettuce, research had been focusing on resistance genes of old German and French *Lactuca sativa* lines in the 1950s, genes originating from *L. serriola* and *L. saligna* and more recently also *L. virosa* were considered as sources for resistance (Lebeda et al. 2014).

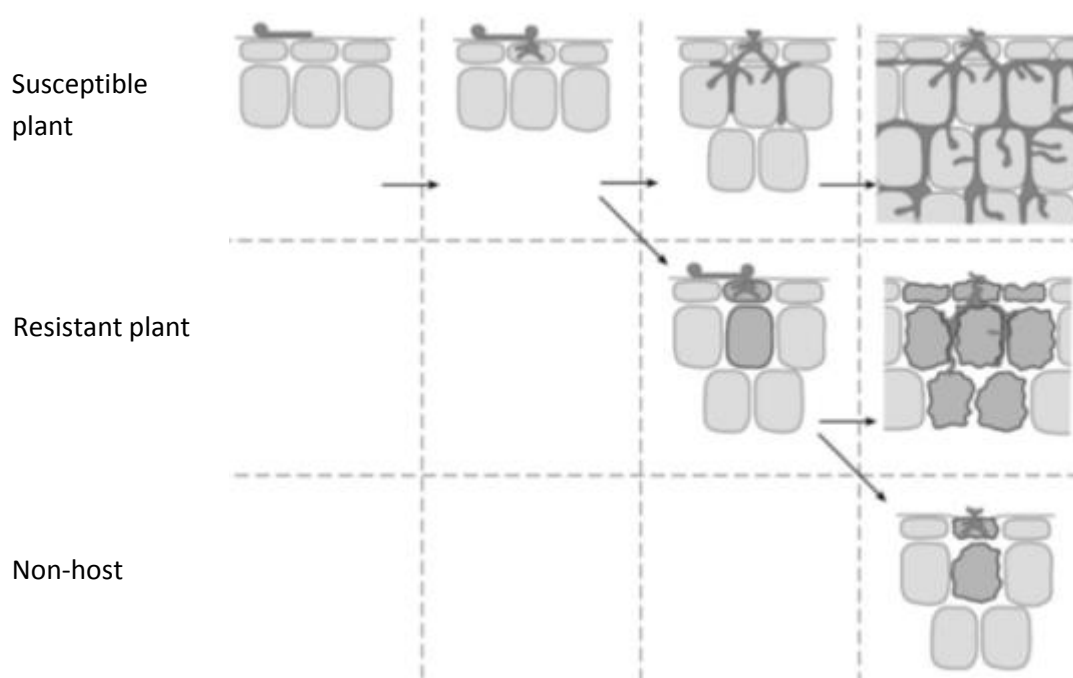


Figure 1-12: Schematic view of the early infection events in susceptible and resistance interactions between oomycetes and plants. Early stages are similar in all types of interactions. In susceptible plants, no visible defence responses occur (upper row), a primary and secondary vesicle develop, mycelium hyphae grow into intercellular space and form haustoria. In resistant plants, cells are activated after penetration by the pathogen (middle row). The hypersensitive response (HR) is induced and the pathogen is contained within a group of dead plant cells (middle row) or within the penetrated epidermal cell (lower row) depending on the genotypes of the interacting plant and pathogen. The HR lesions in the middle row are visible macroscopically as brownish–black spots. In many nonhost plants (lower row), the HR is induced extremely fast, and only one or two plant cells are sacrificed, with no macroscopically visible symptoms (Kamoun et al. 1999).

1.2.3.2.1 Host resistance

Race-specific resistance

The specificity of race-specific resistance is determined by dominant resistance genes and/or resistance-factors in the host, respectively *Dm* genes (Downy mildew resistance genes) and/or R-

factors (resistance factors) (Lebeda and Sedlářová 2008; Michelmore and Wong 2008). For resistance factors it is not (publicly) known yet if the resistance is mediated by one or more genes (Personal communication A. Van der Arend, Bayer, October 2014). These resistance genes or R-factors are matched by dominant factors of the pathogen. This type of resistance is well documented in cultivars of *L. sativa* as well as in wild *Lactuca* spp. Until now, more than 45 genes/factors and matching pathogen virulence genes are predicted and this number is expected to increase further (Lebeda and Sedlářová 2008). The *Dm* genes characterized in *Lactuca* spp. nowadays, are clustered in at least five distinct clusters within the lettuce genome (Lebeda and Sedlářová 2008; Michelmore and Wong 2008). The major cluster contains over nine genetically separable *Dm* specificities and also provides resistance to root aphid (Michelmore and Wong 2008). Some of these *Dm* genes originate from *L. serriola*. Likewise the R-factors R36 and R37 originate from *L. saligna* (Lebeda and Sedlářová 2008). Most of the currently identified *Dm* genes confer high levels of resistance. These genes are the ones identified and used by breeders. Other *Dm* genes, for instance *Dm* 6, confer incomplete resistance phenotypes. The phenotype of the interaction depends on the gene and on the environment as resistance allocated by several *Dm* genes becomes less effective at lower temperatures. Furthermore, different isolates of *B. lactucae* can present different levels of incompatibility to the same *Dm* gene (Michelmore and Wong 2008).

Dm 3 encodes a nucleotide binding site and leucine-rich repeat (NBS-LRR) protein, similar to genes cloned from other species offering resistance to downy mildews and other pathogens. *Dm* 3 contains nearly double the number of LRRs compared to proteins characterized in other species and is a member of the large *Resistance Gene Candidate2* multigene family, which can vary in copy number from 12 to over 30. Within this family there are two patterns of evolution. *Dm* 3 belongs to type 1, which means this gene occurs rarely in nature. Type 1 genes are chimeras resulting from frequent sequence exchange between paralogues. Type 2 genes, on the other hand, are more frequently observed in nature and sequence exchanges only rarely occur between individual lineages (Michelmore and Wong 2008). Race-specific gene *Dm* 11 originates from interspecific hybrid between *L. sativa* and *L. serriola* and was determined at the end of the 1960s. Also *Dm* 5, *Dm* 5/8, *Dm* 6, *Dm* 7, *Dm* 10, *Dm* 11, *Dm* 13, *Dm* 15 and *Dm* 16 are derived from *L. serriola* (Lebeda et al. 2014). Occurrence of race-specificity in other wild *Lactuca* species, other than *L. serriola* and *L. saligna* are not studied in detail yet, however race-specific responses of *L. viminea*, *L. tatarica*, *L. quercina*, *L. indica* and *L. biennis* has been reported (Lebeda et al. 2002; Lebeda et al. 2014).

Lebeda and Sedlářová (Lebeda and Sedlářová 2008) summarized host-resistance defence reactions of lettuce when challenged by *B. lactucae*, describing the importance of nitric oxide, peroxidase and rutin. Nitric oxide (NO) mediates the transcription of specific genes during pathogenesis. Besides that, synchronized formation of NO and H₂O₂ regulates programmed cell death and the phenylpropanoid pathway. Increase of peroxidase activity was linked to race specific resistance in *L. sativa* and *L. virosa*. The hypersensitive reaction is the most important feature in race-specific resistance of lettuce to *B. lactucae* (Lebeda and Sedlářová 2008).

One of the first reactions of the plant on a pathogen invasion is the reorganization of the cytoskeleton. In case of a compatible interaction, the cytoskeleton may support the development of infection structures, whereas in an incompatible interaction the cytoskeleton can participate in blocking the penetration. Another specific feature of incompatible interactions is the accumulation of phenols around primary infection structures and near the cell wall of the infected plant cell. Although

H₂O₂ accumulation in the periplasmic space occurs in both compatible and incompatible interactions, it is much more intensive in the latter (Lebeda and Sedlářová 2003). H₂O₂ plays a role as an antimicrobial agent and causes localized membrane damage (Bestwick et al. 1997). Membrane damage in penetrated cells is considered as a key signalling event leading to widespread activation of defence responses (Bennett et al. 1996). In addition, the hypersensitive reaction is a characteristic of incompatible host- and non-host-pathogen interactions, which limits the further spread of the pathogen in the plant tissue and occurs prehaustorially or posthaustorially, depending on the gene-for-gene combinations (Bennett et al. 1996; Lebeda and Sedlářová 2003).

Field resistance

Field resistance or horizontal resistance is a complex epidemiological phenomenon, expressed by reduced susceptibility of mature plants, grown in fields with natural infection of *B. lactucae* and susceptible younger lettuce plants (Grube and Ochoa 2005; Lebeda and Sedlářová 2008). Field resistance is described for several crisphead lettuce cultivars grown in experimental plantings within commercial lettuce fields in New York in 1980-1981 (Yuen and Lorbeer 1984). Also for lettuce cultivars Grand Rapids and Iceberg, field resistance was suggested to be responsible for the reduced disease susceptibility (Grube and Ochoa 2005). Furthermore significant differences in level of field resistance were observed in accessions with *L. serriola*, in a study were 31 accessions of four *Lactuca* species (*L. serriola*, *L. saligna*, *L. aculeate*, *L. indica*/syn. *L. squarrosa*) and one *L. serriola* x *L. sativa* hybrid were studied (Lebeda et al. 2014).

1.2.3.2.2 Non-host resistance

Non-host resistance (NHR) can be defined as resistance of an entire plant species to all isolates of a microbial species. This type of resistance is known as the most durable form and the basis of this resistance consists of reciprocity of inducible plant defence responses and constitutive barriers (Nürnberg and Lipka 2005). A bunch of complex and sometimes opposing mechanisms is involved in NHR. Because of its durability in nature and effectiveness against broad ranges of potential pathogens, NHR is receiving much attention as a potential source of resistance mechanisms for improving crop plants (Fan and Doerner 2012). Data from a Dutch study suggest that nonhost resistance in *L. saligna* is the result of cumulative effects of several QTLs, operating at different development stages of the crop (Zhang et al. 2009). Three combined QTLs of this nonhost have proven to be sufficient to provide complete resistance against lettuce downy mildew (Zhang et al. 2009).

1.2.3.2.3 Virulence of downy mildews

B. lactucae possesses different features to successfully infect *Lactuca* plants. As an obligate plant pathogen, *B. lactucae* needs to penetrate and grow within the host, modify host cell structure, reprogram host metabolism and reproduce prolifically together with successfully evading the host immune system. It is known that genomes of these obligate pathogens have specific adaptations: a reduced number of secreted degradative enzymes is noticed in comparison with necrotrophic pathogens to avoid defence responses of the host, the genome is configured for rapid evolution with a large percentage of repeated elements and there is the deployment of large arrays of secreted

effector proteins. Furthermore, downy mildew pathogens have lost enzymes for assimilation of inorganic nitrogen and sulfate (McDowell 2011).

Biotrophic pathogens need to minimize the disruption of the host cell because integrity of the plasma membrane and homeostasis of cellular processes are crucial for plant functionality. Initiation of intercellular growth of the pathogen, after the formation of the primary and secondary vesicles, is usually linked to membrane damage and accumulation of autofluorescent phenolics. In incompatible interactions, pathogen recognition results in irreversible loss of membrane integrity and initiation of hypersensitivity response (Lebeda and Sedlářová 2008). In compatible interactions, the intercellular growing hyphae form haustoria.

Downy mildew genomes have large arrays of secreted effector proteins that act within and outside host cells to suppress the immune system and perhaps facilitate other processes that are integral to survival within a host (McDowell 2011). Pathogens and plants had a dynamic coevolution which resulted in structurally and functionally diverse families of effectors and resistance genes, encoded by rapidly evolving genes. Oomycetes have genome-wide remarkable large and diverse classes of effectors, which indicate they have evolved sophisticated pathogenicity mechanisms (Bozkurt et al. 2012).

Some oomycete effector proteins act in the apoplast and can interfere there with apoplastic plant proteins involved in pathogen defence and surface receptors of the host cells. Other oomycete effectors, the cytoplasmic effectors, translocate inside host cells where they target different subcellular compartments (Kamoun 2006; Kamoun 2007; Schornack et al. 2009). Diversification of effectors during evolution has mostly targeted the C-terminal effector domain, rather than the N-terminal domain functioning in secretion and translocation (Allen et al. 2004; Schornack et al. 2009; Vleeshouwers and Oliver 2014). Some of the cytoplasmic effectors are defined by an N-terminal motif: RXLR (arginine, any amino-acid, leucine, arginine), which helps for translocation into the host (Kamoun 2007; Whisson et al. 2007; Dou et al. 2008). This RXLR-dependent entry does not require the presence of the pathogen (Dou et al. 2008), but would probably be mediated by binding to phospholipids, specifically phosphatidylinositol-3-phosphate, and via lipid raft-mediated endocytosis (Kale and Tyler 2011; Kale 2012; Tyler et al. 2013). The occurrence of RXLR effectors is restricted to the Peronosporales clade, more specifically *Phytophthora* and downy mildews. In a large number of RXLR effectors the WY domain, a conserved alpha-helix protein fold, has evolved as a conserved but adaptable protein fold that supports diversification of effectors. This diversification may support the gain of new effector functions and the evasion of host immunity pathways (Bozkurt et al. 2012).

Massively parallel sequencing of cDNA derived from *Bremia lactucae* spores and infected lettuce seedlings resulted in 78 protein models with RXLR effector features (Stassen et al. 2012). Out of these protein models, 34 potential RXLR-like effector proteins were selected and tested for specific recognition within a collection of *B. lactucae*-resistant *Lactuca* lines. Two of these effector candidates triggered a hypersensitive response: BLG01 and BLG03. Lettuce cultivars displaying *Dm 2*-mediated resistance to *B. lactucae* were responsive to BLG03, suggesting that this effector is a candidate Avr2 protein (Stassen et al. 2013). By *Agrobacterium*-mediated transient expression of effector genes in lettuce leaf discs and subsequent inoculation of the leaf discs with *B. lactucae*, the contribution of potential effectors was investigated. Two effector genes *BLR16* and *BLR27* could significantly increase host susceptibility while a third, *BLR03*, significantly reduced host susceptibility (Stassen 2012; Stassen et al. 2013)

1.2.4 Disease management

1.2.4.1 Lettuce cultivars breeding for downy mildew resistance

The substantial impact of downy mildew on European lettuce production motivated the establishment of the International Bremia Evaluation Board (IBEB) in 1998. The board is a joint effort of French and Dutch lettuce breeding companies, a Dutch inspection service (Naktuinbouw) and the French National Seed Station (GEVES) which identifies and nominates new downy mildew races or pathotypes forming a threat to the European lettuce industry. The identification and nomination are based on the most frequently occurring virulence characteristics in the pathogen populations, collected in lettuce culture (Van Ettehoven and van der Arend 1999; van Treuren et al. 2011). Until now the IBEB denominated 30 *B. lactucae* races, referred to as “Bl:” followed by a space and the race number (International Bremia Evaluation Board 2013; Board 2015). Modifications in the avirulence (avr) factors of the pathogen, will obstruct the recognition mediated by *Dm* genes or R-factors and thus resistance response of the plants (Michelmore and Wong 2008; Gijzen et al. 2014). Because of the frequent modifications of *B. lactucae* virulence characteristics, continuous efforts to discover new resistance genes to compensate for the virulence shifts in the pathogen population are necessary. A good knowledge of the diversity and relative frequencies of the virulence characteristics over space and time can help breeders to select the most effective *Dm*-genes and R-factors (International Seed Federation 2014). Plants in commercial lettuce culture usually possess two or more resistance genes which offer resistance to many European *B. lactucae* races (Maisonneuve et al. 2011).

As hundreds of RXLR effector gene candidates are found in the genome sequence of *Phytophthora sojae*, *Phytophthora ramorum*, *Phytophthora infestans* and *Hyaloperonospora arabidopsis*, while only small complements of bacterial effectors are required for infection, there is a potential for functional redundancy (Birch et al. 2008; Hein et al. 2009). This means that the pathogen can have lost the expression of an effector without an apparent cost to their fitness, which could explain how for instance *P. infestans* can rapidly overcome deployed resistance genes. To breed cultivars with a more durable disease resistance, resistance genes able to recognize the effector genes essential for virulence should be selected (Birch et al. 2008).

1.2.4.2 Fungicides

Fungicides can be systemic and/or direct antifungal. Systemic fungicides are often site-specific making them not likely to be phytotoxic. However, even a small mutation in the pathogen, can disturb this site-specific mode of action, resulting in resistant isolates (Isaac 1999). Outbreaks of *Bremia lactucae* insensitivity to fungicides have been reported several times (Crute 1984; Wicks et al. 1994; Isaac 1999; Brown et al. 2004). Resistant pathogen strains might be able to bypass a block in metabolism and circumvent the action of a fungicide or may compensate via production of unusually large amounts of specific enzymes (Isaac 1999).

In Belgium eight fungicides are registered for lettuce downy mildew control in 2015 (<http://www.fytoweb.fgov.be/indexNL.asp>) (Table 1-2). The active ingredient mancozeb is a contact fungicide (Gisi 2002), mandipropamid will be absorbed by the wax layer of the plant surface (Gisi 2002; Lamberth et al. 2008) and metalaxyl-M, dimethomorf, propamocarb, fenamidone, fosetyl-Al

and azoxystrobin are systemic fungicides (Gisi 2002). Mancozeb belongs to the group of dithiocarbamates which have three complementary modes of actions: inhibition of glucose oxidation, nucleic acid synthesis, and fatty acid degradation (Barrière et al. 2014). Both dimethomorph and mandipropamid affect pathogen cell wall synthesis (Gisi 2002; Lamberth et al. 2008; Blum et al. 2010). Propamocarb disturbs pathogen plasma membrane permeability, which disrupts mycelium growth, germ tube elongation and sporulation. Azoxystrobin and fenamidone inhibit mitochondrial respiration at enzyme complex III in the respiratory pathway, which affects spore germination and spore release. Metalaxyl-M prevents ribosomal RNA-synthesis, disturbing hyphal growth, haustorium and spore formation. The primary target of fosetyl-AI is not known yet, but the effect would be twofold: a direct antifungal effect and an indirect effect via enhancement of the host plant resistance (Gisi 2002).

Table 1-2: List of registered chemicals against lettuce downy mildew in Belgium in 2015.

Fungicide	Active ingredient
Fenomenal	fenamidone
	fosethyl
Fubol Gold	mancozeb
	metalaxyl-M
Amistar	azoxystrobin
Mirador	azoxystrobin
Ortiva	azoxystrobin
Paraat	dimethomorf
Previcur Energy	fosethyl
	propamocarb
Proplant	propamocarb
Revus	mandipropamid

1.2.4.3 Forecasting models

To increase the efficacy and reduce the number of fungicide applications, treatments should take place close to the moment of pathogen infection. Therefore different advice models were developed to assess risk for disease outbreak.

In the first model, which was developed in California, morning leaf wetness is forecasted to predict outbreaks. Based on these predictions, indications for the optimal fungicide application moment are given. Based on this model fungicide should be applied when morning leaf wetness lasts longer than 10 a.m., starting from 6 a.m. (Scherm and van Bruggen 1995; Scherm et al. 1995). Based on later studies the thresholds of this model were modified to a shorter period of morning leaf wetness and incorporated the effect of high temperatures (Wu et al. 2001). Another model is BREMCAST, a Canadian model created in 2001 based on the Californian model with adaptations to Canadian circumstances and with incorporation of sporulation criteria. BREMCAST wants to give growers information about risk of a downy mildew outbreak to allow timely intervention before symptoms

are observed (Kushalappa 2001). Later on the model was extended with degree-day calculations post a so called sporulation-infection period (Hovius et al. 2007).

Furthermore, three models were developed in Europe. The first one is the Swedish model Modell-Analys, which adapted the Californian model to Scandinavian circumstances, estimating the risk for disease outbreak based on conditions favourable for sporulation and infection (Jönsson et al. 2005). The second one is Plant Plus which was developed by the Dutch company Dacom Plant service B.V. and uses climate data and data input from the grower such as planting date, lettuce cultivar, growth and infection pressure, to advice fungicide applications. Initially Plant Plus was developed to control *Phytophthora infestans* outbreaks. It has been used on-farm since 1994. Later on Dacom has extended the PLANT-Plus system with a large range of disease models for other crops (Raatjes et al. 2003). Good results were obtained with this model both for downy mildew control in open field grown lettuce and greenhouse grown lettuce at the research centre Productschap Tuinbouw in the Netherlands (Raatjes et al. 2003; Oostingh 2004). A third model is used for the web application VIPS in Norway. Based on weather forecasting risk for sporulation and inoculation are evaluated and the output is represented with a colour code to show the risk of infection in a certain area (<http://www.vips-landbruk.no>). Criteria of the different models and the subsequent advice they provide for growers are listed in Table 1-3.

Table 1-3: Criteria of forecasting models. Abbreviations used in the table: LW: leaf wetness, T: temperature, RH: relative humidity

Model	Criterion for germination/ penetration	Criterion for sporulation	Advice
Californian model, 1 st version (Schermer et al. 1995)	LW ended late in the morning: after a period of 4 h, starting from 6 a.m. until 10 a.m.	Not included in this model	Fungicide application if morning LW ended after 10 a.m. Minimal time interval of 10 days between two applications
Californian model, 2 nd version (Wu et al. 2001; Wu et al. 2002)	Period of minimum 3 h with LW and a maximum T of 20 °C. The 3 h period starts at sunrise, starting when measured solar radiation exceeded 8 W/m ² . The period should be followed by a period of 4 h (post-penetration period) with a maximum T of 22 °C.	Not included in this model	Fungicide application if the criterion for germination/ penetration is fulfilled.
BREMCAST (Kushalappa 2001)	Infection values are calculated based on duration of morning (6 a.m. – 1 p.m.) LW and average T, with actual risk for infection starting from a period of 3 h with LW if T is maximum 15 °C and a period of 4 h for higher T.	Sporulation occurs only in dark conditions after 7 h of LW, with T ranging from 5 to 25 °C. Spores are released in the morning when RH drops and T increases. A sporulation value was calculated based on T, nightly RH and LW.	Based on the presence of inoculum, the infection value and sporulation value a disease severity value is calculated and the grower is supposed to make the right decisions based on these predictions and disease severity value.

Model	Criterion for germination/penetration	Criterion for sporulation	Advice
Modell-Analys (Jönsson et al. 2005)	There is a risk for infection if LW period lasts at least 3.5 h.	LW or a RH above 90% for more than 4 h gives a risk for sporulation.	Based on the criteria for sporulation and inoculation, infections are predicted and the model advises to apply fungicides as close as possible to the moment of infection. Which fungicide should be used, depends on the T. An information pack that supplements the model informs growers about the persistence of fungicides and the impact of pathogen development within the lettuce field.
Plant-Plus (Raatjes et al. 2003; Provinciaal Proefcentrum voor de Groenteteelt (PCG) 2003; Oostingh 2004)	Based on climate data of the lettuce field and weather forecasts, risk for inoculation is predicted (details not published).	Based on climate data of the lettuce field and weather forecasts, risk for sporulation and dispersal of spores is predicted (details not published).	The risk assessment for inoculation and sporulation is combined with information about leaf stage of the plants and the growth rate to predict the risk of downy mildew disease outbreak. This combined risk assessment is represented with a value and from value 200 a fungicide application with a specific type of fungicide is advised.
VIPS (http://www.vips-landbruk.no)	A period of at least 3 h with leaf wetness can be sufficient for germination and penetration.	There is a risk for sporulation if RH at night is at least 4 h minimum 93%.	Based on the criteria for sporulation and infection, which were evaluated for two subsequent days (current day and day before), risk is assessed and indicated per location which informs growers about the risk in the environment of their fields/greenhouses.

1.3 Basal rot

Besides lettuce downy mildew, basal rot is a major threat in lettuce cultivation. This disease can be caused by several pathogens amongst which *Botrytis cinerea* and *Rhizoctonia solani* (Nordskog et al. 2008; Van Beneden et al. 2009; Barrière et al. 2014). A 3-year study with 150 isolates in 56 Belgian greenhouses revealed that *B. cinerea* was the most common pathogen in winter, whereas *R. solani* was most frequently detected in summer (Van Beneden et al. 2009). Therefore these two pathogens were chosen to be studied in this work.

1.3.1 *Botrytis cinerea*

1.3.1.1 Disease symptoms

Basal rot caused by *B. cinerea* is also known as grey mould (Davis et al. 1997) and is characterized by water-soaked brownish grey, soft rot of damaged or senescent leaves and stems of lettuce plants, with prolific grey conidiophores and (macro)conidia. Black sclerotia can be formed on infected tissue. Plants will wilt and collapse. Symptoms are shown in Figure 1-13 (Blancard et al. 2006). *B. cinerea* is often a secondary pathogen, or associated with other pathogens such as *Bremia lactucae*, *Rhizoctonia solani*, *Sclerotinia* spp. or *Pythium* spp. (Davis et al. 1997).



Figure 1-13: Symptoms caused by *Botrytis cinerea* (Blancard et al. 2006).

a) A few brown spots on leaves in contact with the soil. b) The leaves are covered with grey mycelium and conidiophores. c) Rotting spreads and destroys completely the head of the crop. Some black sclerotia are formed (sc). d) Damage of the vascular system by the pathogen. e) The lettuce plant wilts.

1.3.1.2 The pathogen

Botrytis cinerea Pers (teleomorph *Botryotinia fuckeliana*) (Elad et al. 2007; Williamson et al. 2007) belongs taxonomically to the phylum Ascomycota (Williamson et al. 2007) (Figure 1-7). It is a necrotrophic pathogen, which means after infection host cells are killed and the fungus can survive and sporulate as saprophyte on the necrotic tissue. This pathogen can also produce survival structures in living plants or in plant debris in or on the soil. Survival structures of this fungus are sclerotia but each part of the fungus thallus can serve as survival structure. Furthermore, *B. cinerea* has a very wide host range (Elad et al. 2007), attacking over 200 crop hosts, mainly dicotyledonous plants, worldwide (Williamson et al. 2007). In spring, sclerotia start growing in temperate regions, producing conidiophores and multinucleate conidia, which can serve as primary inoculum. Also mycelium which survives in crop debris can produce conidia and initiate infections in a new crop. Besides this asexual inoculum, the pathogen can also produce microconidia, functioning as

spermatia. Spermatization of sclerotia leads to production of apothecia and asci with ascospores. Conidia can be dispersed by air currents or water droplets, or even by insects (Williamson et al. 2007). The life cycle of this pathogen is shown in Figure 1-14.

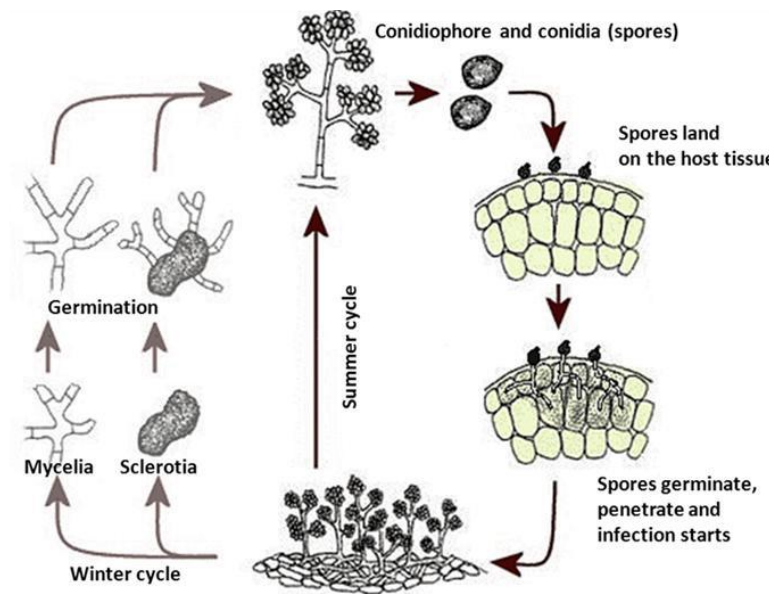


Figure 1-14: Life cycle of *Botrytis cinerea* (<http://www.thewinedoctor.com/author/sweetnoble.shtml>).

1.3.1.3 Disease control

Fungicide control often fails because of the genetic plasticity of this pathogen. Furthermore, control is difficult because *B. cinerea* has a variety of modes of attack, diverse hosts and can easily survive as mycelium, conidia and/or sclerotia (Williamson et al. 2007).

Registered fungicides in Belgium in 2015 against *B. cinerea* in lettuce are summarized in Table 1-4 (<http://www.fytoweb.fgov.be/indexNL.asp>).

Table 1-4: In 2015 registered fungicides in Belgium against *Botrytis cinerea* on lettuce (<http://www.fytoweb.fgov.be/indexNL.asp>).

Fungicide	Active ingredient
Foram 80 WG	thiram
Grisu	iprodione
Hermosan 80 WG/ Garden	thiram
Internum WG	boscalid and pyraclostrobin
Karva 500 SC	iprodione
Luna privilege	fluopyram
Pomarsol WG	thiram
Prestop	gliocladium and catenulatum
Rovral SC/WG	iprodione
Serenad ASO	<i>Bacillus subtilis</i> strain QST 713
Signum	boscalid and pyraclostrobin
Switch	cyprodinil and fludioxonil
Teldor	fenhexamid

Cultural practices that can help reduce *B. cinerea* infection, are reduction of leaf wetness duration, by avoiding sprinkler irrigation, crop rotation to reduce primary inoculum, minimize crop residues after harvest and reduce humidity by ventilation of the greenhouse (Davis et al. 1997).

1.3.2 *Rhizoctonia solani*

1.3.2.1 Disease symptoms

Favoured by warm and wet conditions, this pathogen causes most damage in summer (Davis et al. 1997; Van Beneden et al. 2009). Lesions typically appear at first at the lower leaves, which are in direct contact with the soil and are initially visible as small, rust-brown spots on the down side of the leaf midribs. Under favourable conditions, the disease can expand very fast, leading to quickly rotting midribs and leaves (Figure 1-15 (Blancard et al.2006)). Sometimes amber-coloured droplets can be observed on the lesions. Wilting of the outer leaves is followed by decaying heads which are at first slimy and brown and become almost black as they collapse and dry. During later stages of the disease, dark brown sclerotia are produced (Davis et al. 1997; Grosch et al. 2004).



Figure 1-15: Symptoms caused by *Rhizoctonia solani* (Blancard et al.2006).

a) Several small, elongated, reddish to rust-brown lesions appear on the leaves and principal veins. b) Dark brown damaged spots are visible on some veins and also an unobtrusive mycelium web. c) Broad necrotic areas can be observed. d) If the principal vein is severely damaged, the leaves wilt and decompose. e) In humid conditions and in presence of bacteria, the rotting is dark brown to black.

1.3.2.2 The pathogen

Rhizoctonia solani Kühn (telemorph *Thanatephorus cucumeris* (Frank) Donk) (Ogoshi 1987; Davis et al. 1997; Grosch et al. 2004; Van Beneden et al. 2009) belongs to the phylum of Basidiomycota (Zheng et al. 2013) (Figure 1-7). It is a soilborne fungal species complex that can be subdivided into 13 anastomosis groups (AGs): AG1 to AG13, based on hyphal anastomosis (Ogoshi 1987; Carling et al. 2002). AG1-1B1B was shown to be one of the *R. solani* AGs causing bottom rot in lettuce (Van Beneden et al. 2009), besides AG2 and AG4 (Grosch et al. 2004). The pathogen survives periods without host plants as melanised mycelium or sclerotia in soil (Takashi and Tadao 1978) and these

sclerotia remain viable for several years, forming an important source of primary inoculum. The life cycle of this pathogen is shown in Figure 1-16. When basidiospores or sclerotia germinate and mycelium is formed, the pathogen can enter the plant via stomata or via direct penetration, followed by inter- and intracellular colonization of the host tissue. Under favourable conditions (temperature between 25 and 27°C) the period between infection and initial symptoms can be as short as 36 to 48 hours.

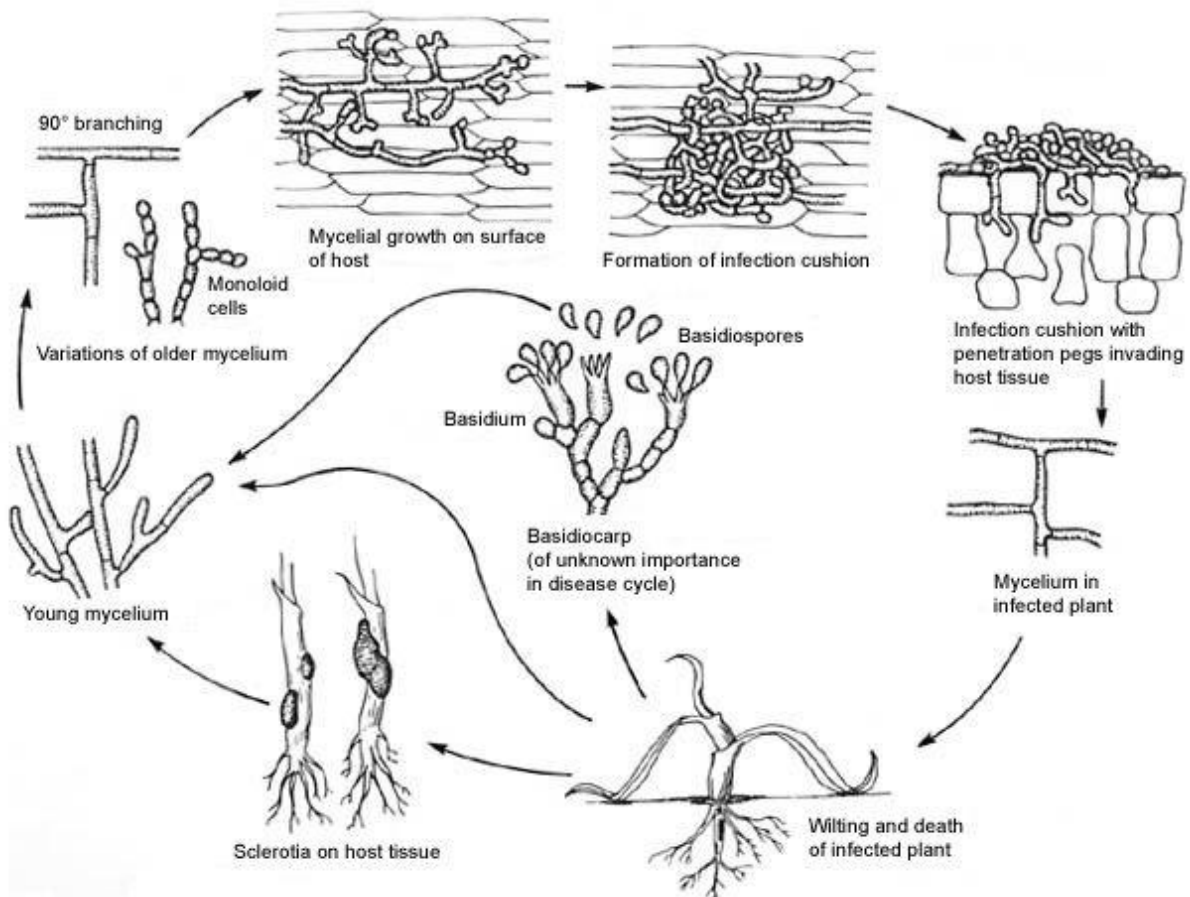


Figure 1-16: Life cycle of *Rhizoctonia solani*

(<http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/Rhizoctonia.aspx>).

1.3.2.3 Disease control

Registered fungicides in Belgium in 2015 against *R. solani* in lettuce are summarized in Table 1-5 (<http://www.fytoweb.fgov.be/indexNL.asp>).

Table 1-5: Fungicides registered in Belgium in 2015 against *Rhizoctonia solani* in lettuce (<http://www.fytoweb.fgov.be/indexNL.asp>)

Fungicide	Active ingredients
Amistar	azoxystrobin
Cavron	iprodione
Internum WG	boscalid and pyraclostrobin
Karva 500 SC	iprodione

Fungicide	Active ingredients
Mirador	azoxystrobin
Ortiva	azoxystrobin
Rizolex 500 SC	tolclofosmethyl
Rovral WG	iprodione
Rovral SG	iprodione
Signum	boscalid and pyraclostrobin

Besides fungicides some cultural practices can be used to reduce disease severity/incidence. For instance ploughing can reduce the viability of sclerotia because the viability decreases with increasing depth. Furthermore crop rotation can also reduce the inoculum potential. Limiting contact between leaves and soil, for instance by planting the peat blocks not totally buried or by using a hydroponic cultivation system, can also be effective (Davis et al. 1997).

1.4 *Bacillus subtilis* cyclic lipopeptides

Bacillus subtilis is a Gram positive, endospore forming bacterium and can be isolated from many environments, which might be due to a remarkable ability to adapt to different conditions within the biosphere or because of the potential of endospores to migrate long distances via airflows and wind (Earl et al. 2008). *B. subtilis* is often found to grow in close association with plant root surfaces (Pandey and Palni 1997; Bais et al. 2004; Cazorla et al. 2007; Earl et al. 2008) and there is evidence that through these associations the bacterium promotes plant growth via outcompeting other microbes in the rhizosphere, via induction of the plant defence system or by making certain nutrients more readily available to the plants (e.g. phosphorus and nitrogen) (Wipat and Harwood 1999; McSpadden Gardener 2004; Rudrappa et al. 2007; Earl et al. 2008). Because of these characteristics, *B. subtilis* strains showing biocontrol activities are frequently found (Ongena et al. 2005; Cazorla et al. 2007; Nihorimbere et al. 2010; García-Gutiérrez et al. 2013). Some of the strains are commercially available e.g. *B. subtilis* strain QST 713 (Bayer Crop Science, <http://www.cropscience.bayer.com/en/Products-and-Innovation/Brands/Fungicides.aspx>), BASF, http://www.agro.basf.com/agr/AP-Internet/en_GB/function/conversions:/publish/content/news_room/news/downloads/P-09-193_serenade.pdf) or *B. subtilis* strain MBI 600 (Becker Underwood, USA, www.beckerunderwood.com, <http://www.agproducts.basf.us/products/label-and-msds/subtilex-ng-product-label.pdf>) (Ongena and Jacques 2008).

Lipopeptides are compounds consisting of a short peptide chain conjugated with an acyl chain. The cyclic lipopeptides (CLPs) produced by *B. subtilis* are biosurfactants, which are surface-active agents because they are amphiphilic molecules able to reduce the interfacial tension between liquids, solids and gases. Physicochemical characteristics defining surfactants are the ability to enhance the apparent water solubility of hydrophobic compounds, to form water-hydrocarbon emulsion and to reduce surface tension (Sobéron-Chávez and Maier 2011; Tran et al. 2012). *B. subtilis* CLPs can be classified in three families: surfactin, iturin and fengycin (Figure 1-17). The surfactin group comprises

heptapeptide variants, which forms a cyclic lactone ring structure and of which the peptide moiety is linked to a β -hydroxyl fatty acid with 12 to 16 carbons. Surfactins are powerful biosurfactants with strong emulsifying and foaming properties, known to display haemolytic, antiviral, anti-mycoplasma and antibacterial activities. Members of the iturin family are heptapeptides linked to a β -amino fatty acid chain with a length of 14 to 17 carbons. Mycosubtilin is one of the members of this family. Besides strong haemolytic activities, iturins also possess antifungal and limited antibacterial activities. Fengycins or plipastatins form the third family, typically lipodecapeptides with an internal lactone ring in the peptide moiety and with a β -hydroxy fatty acid chain with 14 to 18 carbons. Fengycins also display a strong fungitoxic activity but are less haemolytic than the other families (Ongena and Jacques 2008).

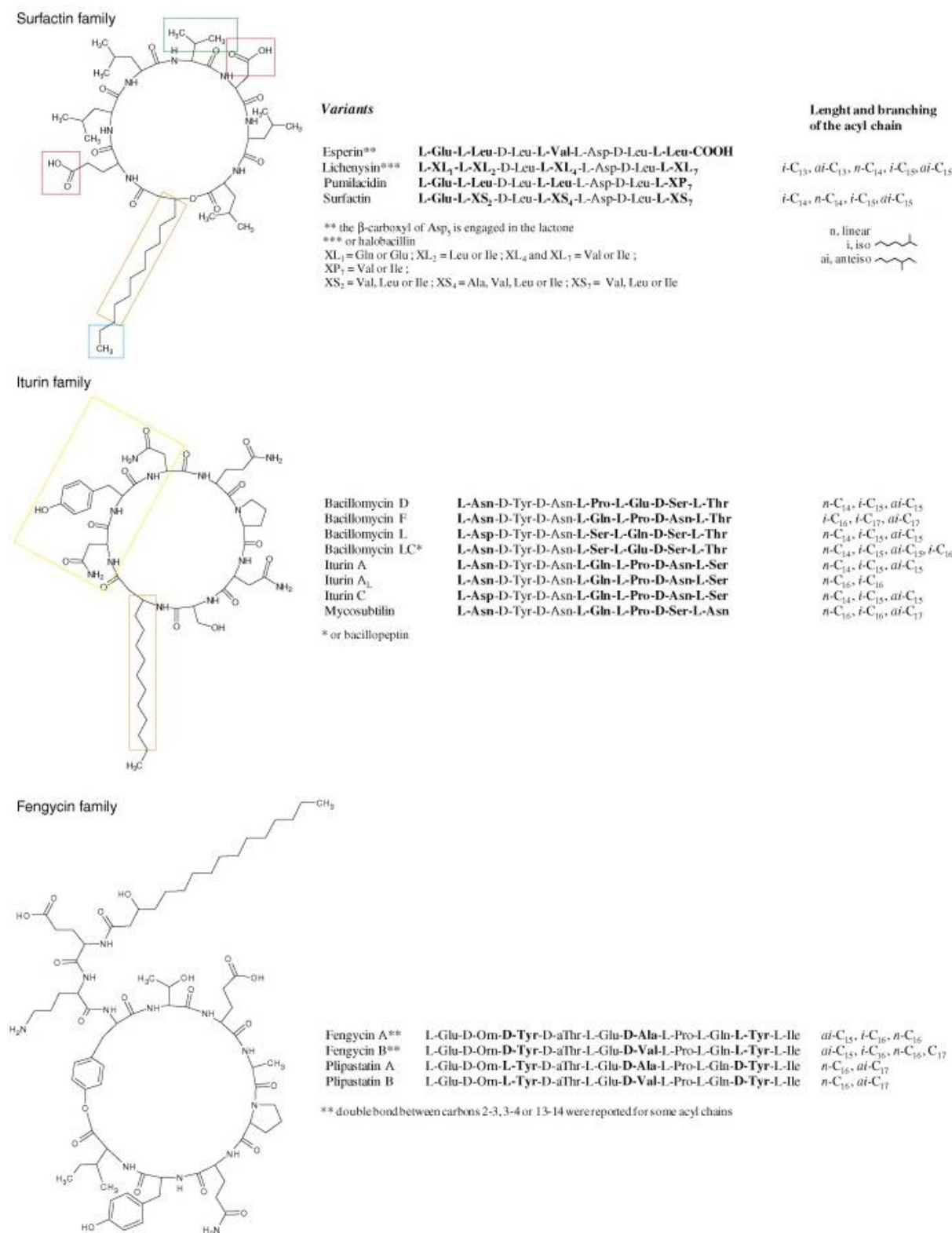


Figure 1-17 (Ongena and Jacques 2008): Structures of representative members of the three cyclic lipopeptides families of *Bacillus* species. Boxed structural groups were shown to be particularly involved in interaction with membranes and/or supposed to be important for biological activity of the bacteria in addition to the cyclic nature of the compounds (Peypoux et al. 1999; Bonmatin et al. 2003).

The CLPs of *B. subtilis* have a low ecotoxicity, which was tested with three types of tests: the phytotoxic effect was determined on garden cress (*Lepidium sativum*), the Microtox test with *Vibrio*

fischeri and the *Daphnia magna* (freshwater crustacean) immobilization test. The phytotoxicity test showed low phytotoxic effect for the three measured parameters (seed germination, relative root growth and other phytotoxic reactions) and the other two tests allowed to rank mycosubtilin and fengycin among the least toxic chemical pesticides on which these tests were performed (Deravel et al. 2014). The CLPs had also shown to exert prophylactic effects (Figure 1-18). For instance, surfactins are known to trigger immunity in bean, melon, tomato, tobacco and grapevine, whereas fengycins elicit defence responses in potato, tomato and tobacco (Ongena et al. 2005; Ongena et al. 2007; Ongena and Jacques 2008; García-Gutiérrez et al. 2013; Farace et al. 2015). Mycosubtilin on the other hand, was recently found to trigger defence signalling mechanisms in grapevine (Farace et al. 2015).

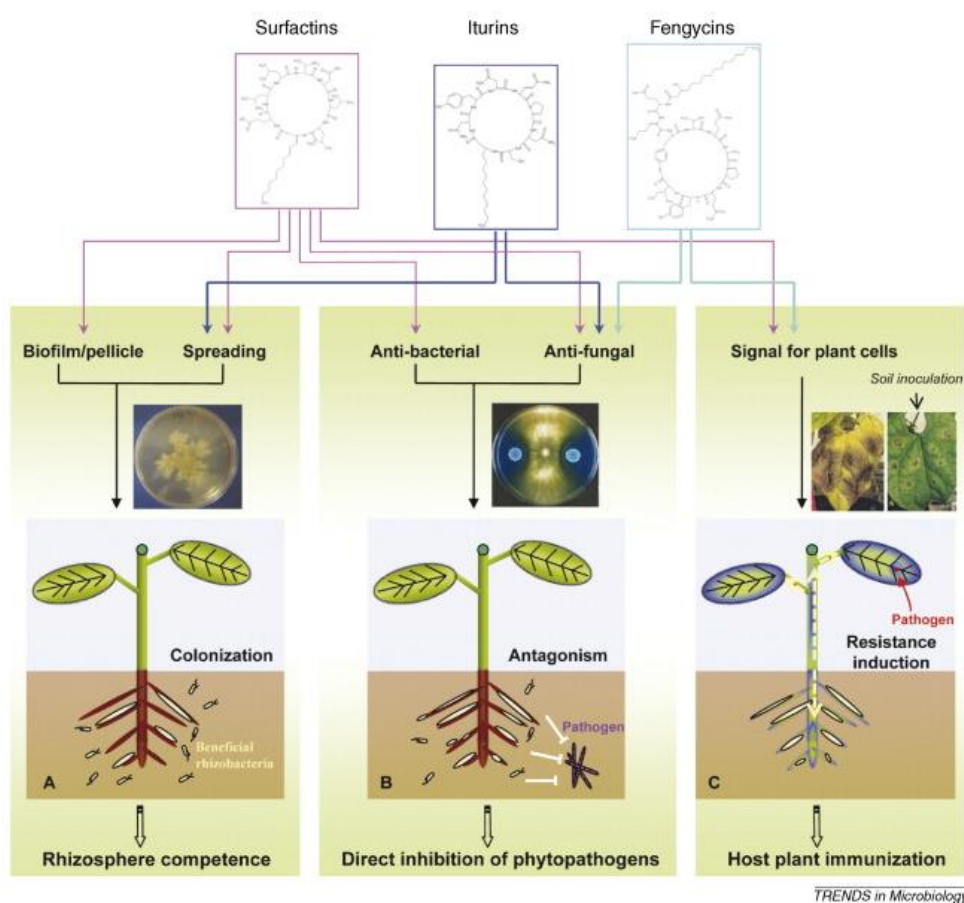


Figure 1-18: Overview of different interaction mechanisms of the *Bacillus subtilis* cyclic lipopeptide interactions in the context of biological control of plant diseases. A and B represent direct antibiosis and C illustrates the emission of a signal following perception of the lipopeptides at root level of the plants, leading to systemic induction of defence response at sites of infection. Figure from Ongena and Jacques 2008.

1.5 Glycolipids

At the department InBio of the faculty Bioscience Engineering of Ghent University (<http://www.inbio.ugent.be/>) research is ongoing for the development of natural surfactants to replace synthetic surfactants, used in households, laundry detergents, applications in the medical world, cosmetics, crop protection, pharmaceuticals, bioremediation and paper and pulp industry (Roelants et al. 2014). A lot of these surfactants inevitably end up in aquatic systems (Sobéron-

Chávez and Maier 2011). Because of the growing environmental awareness, bio-accumulation and eco-toxicity of synthetic surfactants became a major concern. Consequently, biosurfactants gained attention as environmentally-friendly alternatives due to their relatively fast degradation and because they can be produced from renewable resources by plants, animals or microorganisms such as yeasts, bacteria and fungi (Sobéron-Chávez and Maier 2011; Tran et al. 2012). The biosurfactants tested in Chapter 6 are glycolipids, which are compounds containing saccharides residues and long-chain aliphatic acids or hydroxylaliphatic acids (Tran 2012). In this work three types of glycolipids are used: cellobiose lipid (Figure 1-19a), glucolipid (Figure 1-19b) and bola sophorolipid (Figure 1-19c).

Cellobiose lipids consist of a residue of the disaccharide cellobiose and a fatty acid residue (Kulakovskaya and Kulakovskaya 2013; Christie 2014). They are secreted by a small number of basidiomycetous yeasts, mostly *Ustilaginales* (Roelants et al. 2014), by *Pseudozyma flocculosa*, *Pseudozyma fusiformata*, *Pseudozyma graminicola*, *Cryptococcus humicola* (Tran 2012) Some cellobiose lipid are reported to have antimicrobial activities (Shah et al. 2007; Golubev et al. 2008; Mimee et al. 2009; Kulakovskaya et al. 2010; Hammami et al. 2011; Teichmann et al. 2011; Trilisenko et al. 2012). Glucolipids consist of one glucose moiety linked to a fatty acid tail (Tran 2012). The glucolipid used in the experiments in chapter 6 is produced by the yeast *S. bombicola* (Saerens et al. 2011; Van Bogaert et al. 2013). Glucolipids are intermediates formed during the biosynthesis of sophorolipids (Saerens et al. 2011) and are interesting building blocks for the synthesis of new glycolipids with increased biological activity (Tran 2012). Sophorolipids consists of sophorose, linked to a fatty acid and these glycolipids are produced by *Starmerella* species (Christie 2014). Some sophorolipids possess antifungal, antiviral and spermicidal properties (Christie 2014). Also some sophorolipids, produced by *S. bombicola*, are claimed to have mild antimicrobial activities, yet, it is likely that the predominant reason for the yeast to produce these secondary metabolites is extracellular carbon storage (Van Bogaert et al. 2007; Christie 2014). Sophorolipid yields can be as much as 300 g/L (Christie 2014).

The bola sophorolipid used in chapter 6 were produced by a newly at InBio developed *S. bombicola* strain (Sophie Roelants, InBio, personal communication on 27th of June 2014). Bola sophorolipids or tetraglucolipids are molecules with two hydrophilic end groups connected by a hydrophobic linker, which can promote phospholipid translocation across vesicle membranes. Bola amphiphiles often self-assemble in water, forming monolayer lipid membranes that are more tightly packed and less permeable than analogous bilayer membranes, which makes them interesting structures for the fabrication of highly stable lipid membranes in various membrane mimetic devices (Forbes et al. 2006).

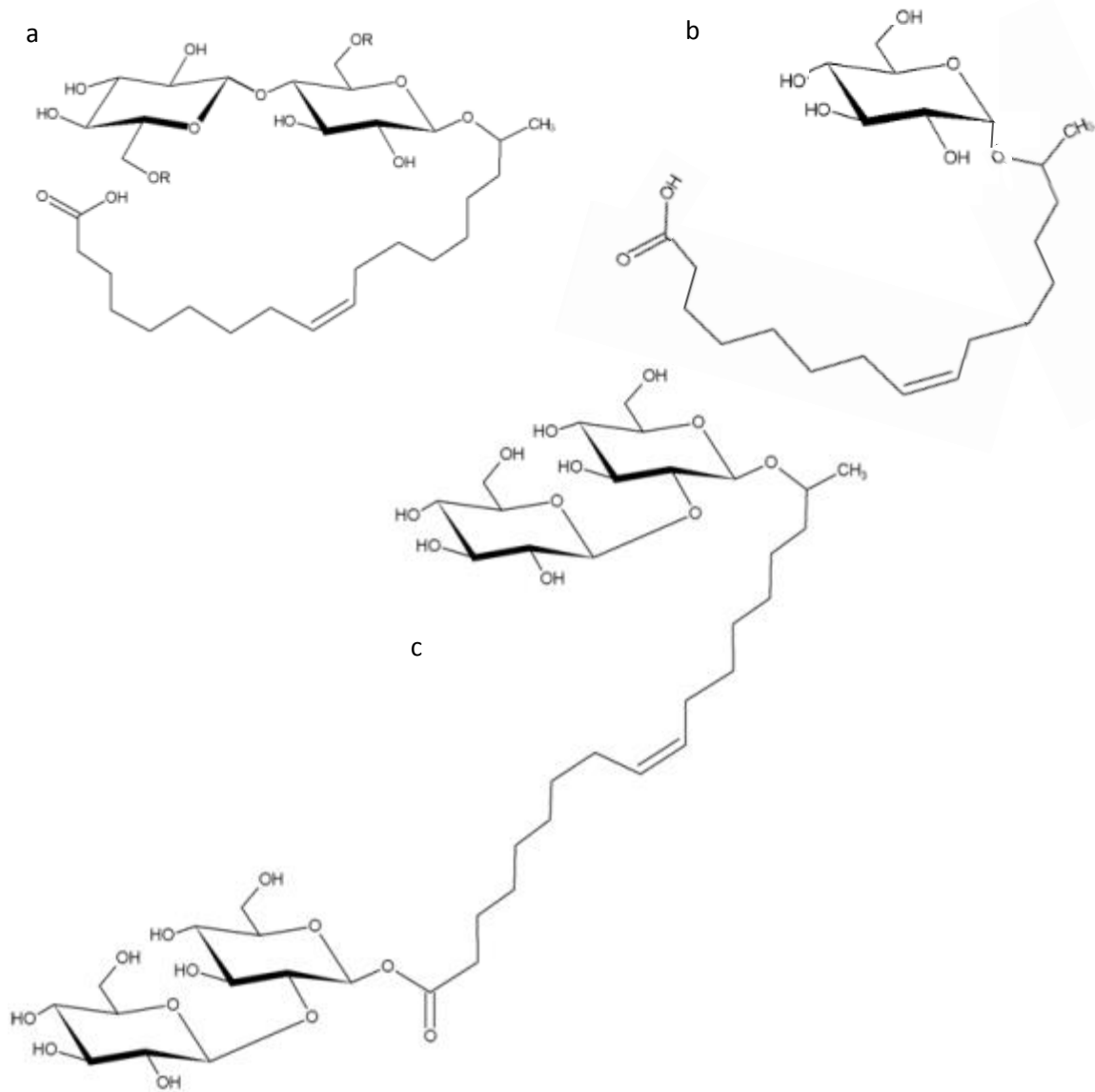


Figure 1-19: Structure of cellobiose lipid (a) (Roelants et al. 2013), glucolipid (b) and bola sophorolipid (c) (personal communication S. Roelants, Inbio, June 2014).

Chapter 2. Virulence variability and evolution of the *Bremia lactucae* population in Belgian lettuce

One aspect of a sustainable control strategy for downy mildew is the use of resistant lettuce cultivars. As resistance of lettuce cultivars is based on gene-for-gene interaction and offers mostly short-term protection, a lot of effort is put in breeding new resistant lettuce cultivars. For efficient breeding, it is important to know the virulence characteristics of *B. lactucae* populations in order to select lettuce cultivars carrying the most effective resistance genes for production. In this study 55 isolates of *B. lactucae* were collected from Belgian lettuce culture and their virulence characteristics were analysed. Results showed that the resistance genes with the highest resistance efficacy to the *B. lactucae* isolates are *Dm 15*, *Dm 17* and the resistance factors of Balesta, Bedford and Bellissimo, while *Dm 1*, *Dm 4*, *Dm 5/8*, *Dm 10* and *Dm 12* were least effective. There was a lot of variability in virulence within the *B. lactucae* populations, with 41 of the 55 characterized isolates having a unique virulence-phenotype. The results of this Chapter provide important information for selection and future breeding of resistant lettuce cultivars, especially for Belgian lettuce producers.

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2.1 Introduction

Genetic resistance of lettuce plants to downy mildew, should be the first step of an integrated control strategy because commercially cultivated lettuce usually possesses two or more resistance genes and it was shown that these can offer resistance to many European *Bremia lactucae* races (Maisonneuve et al. 2011). Selection of the resistance genes which are most effective against current, local *B. lactucae* races is thus essential.

To allow selection of the most effective resistance genes for future breeding of lettuce for Belgian cultivation, *Bremia lactucae* isolates were collected in Flanders, where most lettuce is grown in Belgium (Bernaerts and Demuyck 2010). In this chapter virulence characterization of the collected isolates is described as was done in similar studies in Norway (Nordskog et al. 2014), Czech Republic (Petrželová et al. 2013), France (Maisonneuve et al. 2011; Valade et al. 2011), the Netherlands (International Seed Federation 2014), Germany (Lebeda and Zinkernagel 2003), Israel (Sharaf et al. 2007), Brazil (Braz et al. 2007; Castoldi et al. 2012) and Australia (Trimboli and Pijnaker 2000; Trimboli and Nieuwenhuis 2011). The results of similar studies are used by the International Bremia Evaluation Board, to identify and nominated official *Bremia lactucae* races for which resistance genes will be used by breeding new lettuce cultivars. From 2003 until 2010 virulence characteristics of 3754 isolates were analysed in cooperation with the IBEB. The majority of these isolates were characterized in France, the Netherlands and Germany. The geographical origin of the analysed isolates is shown in Figure 2-1.

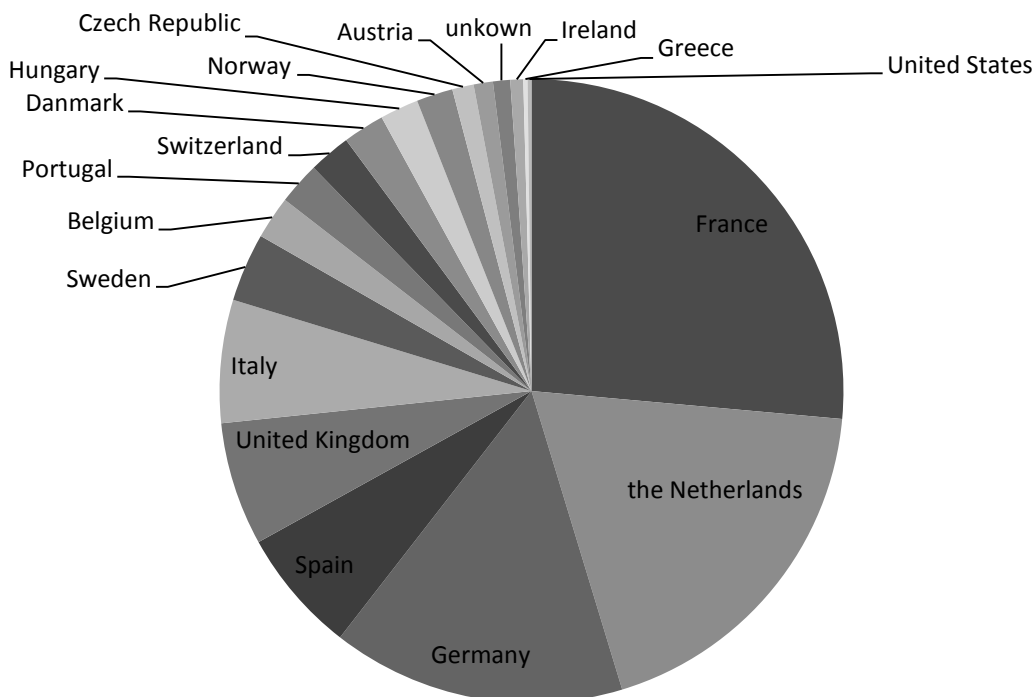


Figure 2-1: Geographic origin of 3754 isolates of which virulence characteristics are analysed and reported to the IBEB from 2003 until 2010 (International Seed Federation 2014).

Since 1999 there were 30 official races identified and nominated by the IBEB (Table 2-1) (International Seed Federation 2014).

Table 2-1: Official races identified and nominated by the IBEB and matching sextet-codes. Note that Bl: 8 and Bl: 9 do not exist (International Seed Federation 2014; Board 2015).

Race	Sextet-code	Race	Sextet-code	Race	Sextet-code
Bl: 1	11-58-00-00	Bl: 13	21-63-00-00	Bl: 23	63-31-02-01
Bl: 2	63-58-00-49	Bl: 14	63-62-00-00	Bl: 24	59-31-10-01
Bl: 3	56-59-01-32	Bl: 15	31-31-00-00	Bl: 25	59-31-42-00
Bl: 4	27-59-00-00	Bl: 16	63-31-02-00	Bl: 26	63-31-58-01
Bl: 5	05-27-01-00	Bl: 17	22-59-41-08	Bl: 27	63-63-13-19
Bl: 6	27-62-00-00	Bl: 18	59-31-10-00	Bl: 28	59-31-10-05
Bl: 7	47-59-00-00	Bl: 19	63-62-00-01	Bl: 29	59-63-47-03
Bl: 10	63-59-00-00	Bl: 20	63-31-10-00	Bl: 30	59-63-13-03
Bl: 11	57-59-03-00	Bl: 21	63-31-51-00	Bl: 31	63-63-12-06
Bl: 12	57-63-03-00	Bl: 22	59-63-09-16	Bl: 32	61-31-03-00

Based on the results of the virulence characterization of our collected isolates, an overview of the most effective and least effective resistance genes is given in this Chapter.

Furthermore the virulence characteristics were compared for three sampling regions in Flanders and for different sampling years. Also the virulence complexity of the *B. lactucae* population was determined and compared with other European countries.

2.2 Materials and methods

2.2.1 Collection of *B. lactucae* isolates

Between 2008 and 2013 55 samples of lettuce downy mildew were collected in greenhouses and fields in three areas in Flanders, the northern part of Belgium: the first in the West of Flanders, the second in the East of Flanders and the third in the centre of Flanders (Figure 2-2). The distance between the centres of region one and three is approximately 40 km, between region two and three it is about 100 km. Outbreaks of downy mildew were reported by growers to the research centre PSKW. Naturally infested plots on the fields of the research centres PSKW, PCG and Inagro were also included in the sampling. Spores of one lesion were defined as one isolate. Yet, sometimes sporulation was limited in which case the spores from leaves from one or two adjacent plants were defined as one isolate (Sharaf et al. 2007; Nordskog et al. 2014) because this did not influence the

outcome of our study (determining the most frequently occurring avirulence factors and mating types).

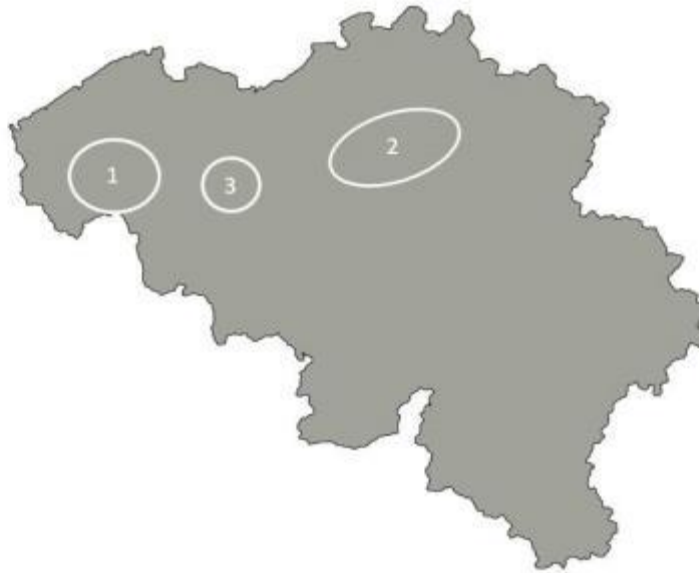


Figure 2-2: Map of Belgium with indication of region 1, 2 and 3. The first region is situated in the western part of Flanders, region two in the eastern part and region three in the centre of Flanders.

2.2.2 Plant materials and pathogen maintenance

The collected isolates were maintained and multiplied on seedlings of *L. sativa* cv. Cobham Green (Michelmore and Crute 1982) or cv. Green Towers. Plants were sown on wetted cotton wool and a layer of filter paper in plastic transparent boxes. The plants were grown in a growth chamber at 17.5 °C and day/night regime of 16 h light, 8 h dark. Fresh spores were washed off from infected leaves with sterile water. The concentration of the spore suspension was adapted to 5×10^4 spores/mL and applied as a foliar spray on seven-day-old seedlings in two leaf stage until running-off. Boxes with inoculated seedlings were incubated under growth chamber conditions (day/night regime of 16 h light/8 h dark and 17.5 °C, relative humidity above 90%). Under these conditions new spores appeared 5 to 6 days after inoculation, and sporulation was maximal at 10 days after inoculation. Cobham Green was used to maintain and multiply isolates because this variety was considered as the universal susceptible control of the first differential set defined by the IBEB. When it became clear that Cobham Green also possesses a resistance factor, this cultivar was replaced by Green Towers in 2010 as universal susceptible control (Lebeda and Petrželová 2004; Maisonneuve 2011). Isolates collected after this replacement were maintained and multiplied on cv. Green Towers.

2.2.3 Assessment for the presence and absence of virulence-factors

The IBEB defined a set of lettuce cultivars with distinct major resistance genes (Table 2-2) to assess the virulence of *B. lactucae* isolates. A first set was defined in 1999 and a second set in 2010 with three replacements in comparison with the first set and five additions. Replacements were needed due to problems with seed quality and progress in breeding knowledge resulted in additions (International Seed Federation 2014). The cultivars of the differential sets are ranked in four groups of each six varieties, with a specific value (1, 2, 4, 8, 16, 32) associated to the position of the variety within this group. The values of the cultivars susceptible to the specific isolate are summed per group, and the combination of these four ranked sums comprise a “sextet code” defining the virulence phenotype (v-phenotype) (Michelmore and Crute 1982; Van Etteken and van der Arend 1999; International Seed Federation 2014). The seeds for the differential sets were obtained from Naktuinbouw (www.Naktuinbouw.com). Fifteen seeds of each cultivar were sown in transparent trays filled with a layer of moistened soil and covered with transparent plastic foil. Lettuce cv. Cobham Green was initially used as universal susceptible and from 2010 on this cultivar was replaced by Green Towers as universal susceptible control (Lebeda and Petrželová 2004; Maisonneuve 2011). The interaction was evaluated 10 days after inoculation and plants were seven days old at the moment of inoculation.

The interaction of the cultivars with an isolate is marked with “+” if it is a susceptible interaction with sporulation and without necrosis, “(+)” if almost all tested plants were susceptible or a lot of necrosis and abundant sporulation was observed, “m” if it was a mixed reaction, “(-)” for an almost resistant reaction with some sporulation on necrotic spots and “-” for a resistant reaction without sporulation. To determine the sextet code, “+”, “(+)” and “m” are counted as susceptible interactions (van der Arend et al. 2011).

Table 2-2: Set of differential lettuce lines used for detection of virulence factors of *Bremia lactucae*.

Lettuce cultivar (first set, defined in 1999)	Lettuce cultivar (second set, defined in 2010)	Resistance genes and factors
Lednický	Lednický	<i>Dm</i> 1
UC DM2	UC DM2	<i>Dm</i> 2
Dandie	Dandie	<i>Dm</i> 3
R4T57 D	R4T57 D	<i>Dm</i> 4
Valmaine	Valmaine	<i>Dm</i> 5/ <i>Dm</i> 8
Sabine	Sabine	<i>Dm</i> 6
LSE 57/15	LSE 57/15	<i>Dm</i> 7
UC DM10	UC DM10	<i>Dm</i> 10
Capitan	Capitan	<i>Dm</i> 11
Hilde II	Hilde II	<i>Dm</i> 12
Pennlake	Pennlake	<i>Dm</i> 13
UC DM14	UC DM14	<i>Dm</i> 14
PIVT 1309	NunDm15	<i>Dm</i> 15
LSE/18	CG Dm16	<i>Dm</i> 16
LS-102	NunDm17	<i>Dm</i> 17
Colorado	Colorado	<i>Dm</i> 18
Ninja	Ninja	R36
Discovery	Discovery	R37
Argelès	Argelès	R38
	RYZ2164	"Silvinas" *
	RYZ910457	"Murai" *
	Bedford	monogenic *
	Balesta	monogenic *
	Bellissimo	monogenic *

* The resistance genes or R-factors offering resistance in the cultivars RYZ2164, RYZ910457, Bedford, Balesta and Bellissimo, are not yet public knowledge. From the last three it is known the resistance is based on one gene.

2.2.4 Virulence diversity and complexity assessment

Virulence complexity was expressed as the mean number of *Dm*-genes or R-factors ineffective for resistance per isolate (C_i) and per v-phenotype (C_p). C_i is calculated as $\sum_j p_j v_j$ with $j = 1 \dots N_p$, N_p is the number of v-phenotypes identified in the studied population based on the virulence test of the IBEB, p_j is the frequency of the j^{th} phenotype and v_j is the number virulence factors of v-phenotype j . C_p is calculated as $(\sum_j v_j)/N_p$ (Andrison 1994). We consider the number of virulence factors of v-phenotype j as the number of *Dm*-genes and R-factors ineffective against an isolate of v-phenotype j .

The similarity between isolates x and y was calculated as $S_{xy} = 2a / (2a+b+c)$ with a is the number of *Dm*-genes or R-factors ineffective for resistance to both isolates x and y , and b and c are the number of *Dm*- genes or R-factors only ineffective for resistance to isolate x and isolate y respectively (Dice 1945).

Bremia lactucae populations were compared across region and time using the Rogers index of proportional overlap (H_R): $H_R = 0.5 \sum_j |p_{j1} - p_{j2}|$ where p_{j1} and p_{j2} are the frequencies of v-phenotype j in sample populations 1 and 2, respectively. The Rogers index of proportional overlap can vary between 0, meaning that all v-phenotypes are present in both populations at equal frequencies, and 1, indicating that the two populations do not share any v-phenotypes (Andrison 1994).

2.3 Results

In this study 55 isolates were collected and characterized (Table 2-3). The number of collected isolates per year varied between 3 and 29 with the highest number collected in 2009. In the 2009 characterization of Flemish *B. lactucae* isolates the main focus was to determine if the population in Flanders was more or less uniform. This was a reasonable hypothesis because Flanders is a relatively small area in comparison with other lettuce producing region.

Genes *Dm* 15, *Dm* 17 and the resistance factors of lettuce cultivars Bedford, Balesta and Bellissimo showed the highest efficacy of resistance to the collected isolates over the entire sampling period, marked in Table 2-3 with dark grey. The efficacy of the resistance factor of Balesta was very high over the entire period with only one isolate from 2012 able to infect plants expressing this resistance factor. Plants possessing *Dm* 12, in contrast, were susceptible for all the isolates we collected in this study. Also resistance to our isolates mediated by *Dm* 1, *Dm* 4, *Dm* 5/8 and *Dm* 10 was limited, light grey marked in Table 2-3. *B. lactucae* isolates unable to cause disease on lettuce cultivars possessing *Dm* 4 and *Dm* 5/8 were only detected in 2011. Resistance based on *Dm* 1 and *Dm* 13 was observed only with *B. lactucae* isolates found in 2008 and 2009, respectively. Whereas plants possessing *Dm* 16 were resistant to some isolates collected in 2010 or earlier, they were not resistant to any of the 14 isolates collected in 2011 or later. In 2008 and 2011 none of the collected isolates were able to infect plants possessing *Dm* 3.

Values associated with resistance genes or R-factors (Table 2-3) were used to determine a sextet code for each isolate, defining a virulence-phenotype (v-phenotype). We found a high degree of virulence diversity in the Flemish *B. lactucae* populations. The virulence complexity of the isolates (C_i) was highest in 2010 and 2013, 15.4 and 19 respectively. The higher C_i , the more virulent the isolates were, or the lower the number of *Dm*-genes or R-factors that were effective for resistance to the

collected isolates. The virulence complexity of the v-phenotypes (C_p) was often similar to C_i , indicating that all v-phenotypes were equally frequently detected (Table 2-3).

Table 2-3: Number of collected isolates per year (expressed as fraction of the total number of isolates collected that year) for which resistance was observed, provided by the different resistance genes or factors (*Dm*-genes or R-factors). On the right, the summed amounts are given for the entire sampling period, along with the relative resistance frequency. The *Dm* genes and R-factors showing the least effective resistance are marked in light grey, whereas those showing the most effective resistance are marked in dark grey. On the bottom, the total number of characterized isolates per year is summed as well. The virulence complexity is expressed per isolate (C_i) and per virulence phenotype (C_p). For calculation of C_i and C_p , the number of *Dm*-genes or R-factors unable to provide resistance to, respectively, an isolate or phenotype, is averaged. On the left resistance-genes (R genes) or R-factors and sextet code values per cultivar are shown.

R genes/ factors	Sextet code value	2008*	2009*	2009	2010	2011	2012	2013	TOTAL	Resistance frequency (%)
<i>Dm</i> 1	1	1/7	0	0	0	0	0	0	1/55	1.8
<i>Dm</i> 2	2	1/7	0	0	1/5	4/6	1/5	0	7/55	13
<i>Dm</i> 3	4	7/7	2	9/25	2/5	6/6	3/5	1/3	30/55	55
<i>Dm</i> 4	8	0	0	0	0	1/6	0	0	1/55	1.8
<i>Dm</i> 5/8	16	0	0	0	0	2/6	0	0	2/55	3.6
<i>Dm</i> 6	32	1/7	0	3/25	0	4/6	2/5	0	10/55	18
<i>Dm</i> 7	1	0	0	3/25	0	2/6	2/5	1/3	8/55	15
<i>Dm</i> 10	2	0	0	1/25	0	0	0	0	1/55	1.8
<i>Dm</i> 11	4	0	0	0	0	3/6	3/5	0	6/55	11
<i>Dm</i> 12	8	0	0	0	0	0	0	0	0	0
<i>Dm</i> 13	16	0	2/4	2/25	0	0	0	0	4/55	7.3
<i>Dm</i> 14	32	4/7	4/4	14/25	3/5	4/6	4/5	1/3	34/55	62
<i>Dm</i> 15	1	5/7	4/4	20/25	5/5	5/6	4/5	2/3	45/55	82
<i>Dm</i> 16	2	1/7	0	5/25	1/5	0	0	0	7/55	13
<i>Dm</i> 17	4	6/7	4/4	23/25	5/5	5/6	5/5	1/3	49/55	89
<i>Dm</i> 18	8	0	0	0	1/5	1/6	1/5	0	3/55	5.5
R 36	16	6/7	2/4	11/25	2/5	4/6	3/5	0	28/55	51
R 37	32	5/7	2/4	8/25	2/5	1/6	1/5	0	19/55	35
R 38	1	1/7	0	5/25	0	1/6	2/5	0	9/55	16
"Silvinas" *	2			20/25	5/5	6/6	5/5	0	36/55	82
"Murai"*	4			18/25	2/5	4/6	3/5	2/3	29/55	66
monogenic*	8			24/25	5/5	6/6	4/5	2/3	41/55	93
monogenic*	16			25/25	5/5	6/6	4/5	3/3	43/55	98
monogenic*	32			25/25	4/5	6/6	4/5	2/3	41/55	93
C_i		13.6	14.0	15.4	15.4	12.2	13.8	19.0	23.0	
C_p		13.7	14.0	15.2	15.4	12.2	13.8	19.0	19.8	

* The resistance genes or R-factors offering resistance in the cultivars RYZ2164, RYZ910457, Bedford, Balesta and Bellissimo, are not yet public knowledge. From the last three it is known the resistance is based on one gene.

The majority of the isolates (21) originated from a region in the West of Flanders, while 15 isolates were collected in the East of Flanders and 19 in the centre of Flanders (Table 2-4). The number of isolates collected per region per year ranged from 0 to 13. Most of the isolates were collected after spontaneous disease outbreaks in the research centres. However, 23 of the isolates came from growers, 13 of which are located in region 2.

Of the 55 analysed isolates, 41 had a unique v-phenotype with a unique sextet code. The sextet codes of nine isolates were identical to races denominated by the IBEB. Race Bl: 24 was found twice in 2008, race Bl: 26 was found four times in 2009 and race Bl: 28 was found twice in 2009 and once in 2010 (Table 2-4).

Based on the dice coefficients for similarity, the isolates that differed most from the other isolates were isolates BmVL138a and BmVL138b with average similarity coefficients of 0.65 and 0.68 respectively. Compared to the other characterized isolates, more *Dm*-genes and R-factors were effective for resistance to these isolates. In general the collected isolates were not very different from one another with most dice coefficients for similarity higher than 0.8.

Table 2-4: Characteristics of collected *Bremia lactucae* isolates.

Isolate	Sampling site ^a	Year	Lettuce cultivar and resistance to IBEB <i>B. lactucae</i> races ^b	Sextet-code	IBEB race	Dice coefficient
BmVL004	1	2008	Gardia (Bl: 1-27.29.30)	59-31-10-01*	Bl: 24	0.86
BmVL009	3	2008	Sartre (Bl: 1-26. 28)	27-63-14-01*		0.80
BmVL010	3	2008	Gardia (Bl: 1-27.29.30)	59-31-10-01*	Bl: 24	0.86
BmVL011	3	2008	Gardia (Bl: 1-27.29.30)	59-63-43-00*		0.82
BmVL012	3	2008	Neil (Bl: 1-31)	56-63-10-01*		0.78
BmVL013 ^(x)	3	2008	Jumbis (Bl: 1-24. 27. 28. 30. 31)	59-31-08-01*		0.82
BmVL016	2	2008	Natalia (Bl: 1-26. 28. 31)	59-31-59-01*		0.86
BmVL017 ^(x)	1	2009	Brigade (Bl:1-17. 21. 23)	59-15-10-01*		0.82
BmVL018 ^(x)	1	2009	Brigade (Bl:1-17. 21. 23)	63-31-58-01*	Bl: 26	0.87
BmVL019 ^(x)	1	2009	Hertog (Bl: 1-17. 21. 23)	59-15-10-01*		0.82
BmVL020	1	2009	Tribore (BL1-25. 27. 28. 30. 31)	63-31-58-01*	Bl: 26	0.87
BmVL047a	2	2009	Mondai (Bl: 1-16. 21. 23)	63-31-62-07		0.82
BmVL047b	2	2009	Mondai (Bl: 1-16. 21. 23)	63-31-58-01	Bl: 26	0.87
BmVL047c	2	2009	Mondai (Bl: 1-16. 21. 23)	59-31-14-07		0.80
BmVL070a	1	2009	Alexandria (Bl: 1-23.25. 31)	59-31-42-01		0.87
BmVL070b	1	2009	Alexandria (Bl: 1-23.25. 31)	59-31-43-01		0.85
BmVL070c	1	2009	Alexandria (Bl: 1-23.25. 31)	59-31-43-01		0.85
BmVL071	2	2009	Flandria (Bl: 1-17. 21. 23)	59-31-10-05	Bl: 28	0.85
BmVL073a	1	2009	Cosmopolia (Bl: 1-25. 27. 28. 30. 31)	63-63-58-01		0.87
BmVL073b	1	2009	Cosmopolia (Bl: 1-25. 27. 28. 30. 31)	63-63-58-01		0.87
BmVL085a	2	2009	Red Oak lettuce	59-63-08-02		0.77

Isolate	Sampling site ^a	Year	Lettuce cultivar and resistance to IBEB <i>B. lactucae</i> races ^b	Sextet-code	IBEB race	Dice coefficient
BmVL085b	2	2009	Red Oak lettuce	63-63-58-00		0.84
BmVL085c	2	2009	Red Oak lettuce	59-31-10-05	Bl: 28	0.85
BmVL091a	3	2009	Troubadour (Bl: 1-17. 21. 23)	63-31-58-05		0.86
^(x) BmVL091b	3	2009	Troubadour (Bl: 1-17. 21. 23)	63-62-59-10		0.78
BmVL091c	3	2009	Troubadour (Bl: 1-17. 21. 23)	27-63-08-05		0.76
BmVL092a	3	2009	Gardia (Bl: 1-27.29.30)	27-63-08-05		0.78
BmVL092b	3	2009	Gardia (Bl: 1-27.29.30)	63-63-08-02		0.77
BmVL107a	3	2009	Jumbis (Bl: 1-24. 27. 28. 30. 31)	63-63-10-00		0.87
^(x) BmVL107b	3	2009	Jumbis (Bl: 1-24. 27. 28. 30. 31)	63-63-58-01		0.82
BmVL110	2	2009	Troubadour (Bl: 1-17. 21. 23)	63-63-58-01		0.83
BmVL111	2	2009	Mariken (Bl:1-25. 27. 28. 30. 31)	63-31-59-01		0.86
BmVL116a	1	2009	Troubadour (Bl: 1-17. 21. 23)	63-14-58-01		0.81
BmVL116b	1	2009	Troubadour (Bl: 1-17. 21. 23)	63-31-58-01	Bl: 26	0.87
BmVL119a	1	2009	Troubadour (Bl: 1-17. 21. 23)	31-12-56-01		0.70
BmVL119b	1	2009	Troubadour (Bl: 1-17. 21. 23)	63-31-59-01		0.86
BmVL126	1	2010	Neil (Bl: 1-31)	61-31-58-01		0.84
BmVL130	3	2010	Troubadour (Bl: 1-17. 21. 23)	59-31-10-05	Bl: 28	0.85
BmVL131a	3	2010	Troubadour (Bl: 1-17. 21. 23)	63-31-58-05		0.86
BmVL131b	1	2010	Fenston (Bl: 1-28)	59-63-58-37		0.83
BmVL132	1	2010	Lambor (Bl: 1-24. 27. 28. 30. 31)	63-63-00-01		0.78
BmVL135	2	2011	Gardia (Bl: 1-27.29.30)	17-31-42-05		0.76
BmVL136a	2	2011	Gardia (Bl: 1-27.29.30)	57-27-42-01		0.80

Isolate	Sampling site ^a	Year	Lettuce cultivar and resistance to IBEB <i>B. lactucae</i> races ^b	Sextet-code	IBEB race	Dice coefficient
BmVL136b	2	2011	Gardia (Bl: 1-27.29.30)	59-63-42-05		0.86
BmVL137	2	2011	Satine (Bl: 1-28. 30. 31)	09-63-58-01		0.77
BmVL138a	2	2011	Satine (Bl: 1-28. 30. 31)	09-26-11-01		0.65
BmVL138b	3	2011	Hofnar (Bl1-23. 25. 31)	27-26-54-00		0.68
BmVL139a	3	2012	Hofnar (Bl1-23. 25. 31)	31-27-58-01		0.82
BmVL139b	1	2012	Gardia (Bl: 1-27.29.30)	63-31-58-00		0.85
BmVL141	3	2012	Romaine lettuce	27-58-03-01		0.69
BmVL144	3	2012	Romaine lettuce	57-26-42-12		0.70
BmVL146	1	2012	Hofnar (Bl1-23. 25. 31)	59-31-42-53		0.81
BmVL147	1	2013	Hofnar (Bl1-23. 25. 31)	59-62-63-03		0.79
BmVL148	1	2013	Gardia (Bl: 1-27.29.30)	63-31-58-07		0.84
BmVL152	3	2013	Flandria (Bl: 1-17. 21. 23)	63-63-62-43		0.86

^a The sampling sites in Flanders are divided in three regions: region 1 is located in the West part of Flanders, region 2 in the East part and region 3 in the centre of Flanders.

^b For all lettuce cultivars except Romaine lettuce and Red Oak lettuce, the resistance to the official IBEB *B. lactucae* races is indicated in the list. Bl: a-b means this cultivar is resistant to *B. lactucae* race a until b. For the isolates isolated from Romaine lettuce and Red Oak lettuce, the exact cultivar and therefore the resistance to IBEB *B. lactucae* races is unknown.

* Sextet codes determined with the first IBEB set. The other isolates were determined with the second set.

The calculated Rogers indices (H_R) showed there is not a clear overlap between the populations collected in the different years of the sampling period, as can be seen in Table 2-5a. The H_R -values for all isolates collected from 2008 until 2013 and characterized with the first 19 *Dm*-genes, are very high. The isolates of 2008 and 2009 have one v-phenotype in common, also in 2008 and 2010, 2010 and 2013, 2009 and 2012 and 2012 and 2013 one v-phenotype was in common. The isolates of 2009 and 2010 had two v-phenotypes in common. Between the isolates collected in the period 2009-2013 and characterized with the 24 *Dm*-genes and R-factors, only in 2009 and 2010 there were three v-phenotypes in common, resulting in an H_R -value of 0.8. These results indicate that the v-phenotypes are not stable across time.

The different investigated sampling regions did not share many v-phenotypes either (Table 2-5b). Based on the characterization with 24 *Dm* genes and R-factors (set 2), there were two v-phenotypes in common between region 1 and 2 (Bl: 26 and sextet code 63-63-58-01); three common v-phenotypes in region 1 and 3 (Bl: 24, sextet code 63-63-58-01 and sextet code 63-31-58-05) and two were detected both in region 2 and 3 (Bl: 28 and sextet code 63-63-58-01). Only one v-phenotype (sextet code 63-63-58-01) was detected in all three regions. Comparison of the frequency of isolates for which resistance was provided by the different resistance genes or R-factors, also shows an equal tendency for the three sampled regions (Figure 2-3). The only noteworthy differences between the regions are that *Dm* 1 could only provide resistance to an isolate collected in region 3, while resistance mediated by *Dm* 4 and *Dm* 5/8 was only observed for one and two isolates, respectively, collected in region 2. Furthermore, although all isolates collected in region 2 and region 3 could infect lettuce plants containing only *Dm* 13, four isolates collected in region 1 could not. RYZ 910457 offered resistance to 81% of the isolates collected in region 1 and to 57% of isolates collected in region 2 and region 3.

Table 2-5: Rogers indices showing overlap between the isolate populations of different sampling years from 2008 until 2013 (a) determined with the first 19 *Dm* genes and R-factors (upper part) or based on the reaction to the 24 *Dm* genes and R-factors (lower part). Rogers indices showing overlap between the isolate populations of the different sampling regions (b) with sextet-code determination based reaction to the first 19 *Dm* genes and R-factors (upper part) or based on the reaction to the 24 *Dm* genes and R-factors (lower part). Index 0 indicates that all the v-phenotypes are represented in both populations, while Rogers index 1 indicates there are none in common.

a)	2009	2010	2011	2012	2013
2008	0.93	0.80	1	1	1
2009		0.83	1	0.97	0.90
2010	0.80		1	1	0.80
2011	1	1		1	1
2012	1	1	1		1
2013	1	1	1	1	

b)	Region 1	Region 2	Region 3
Region 1		0.77	0.79
Region 2	0.90		0.83
Region 3	0.90	0.86	

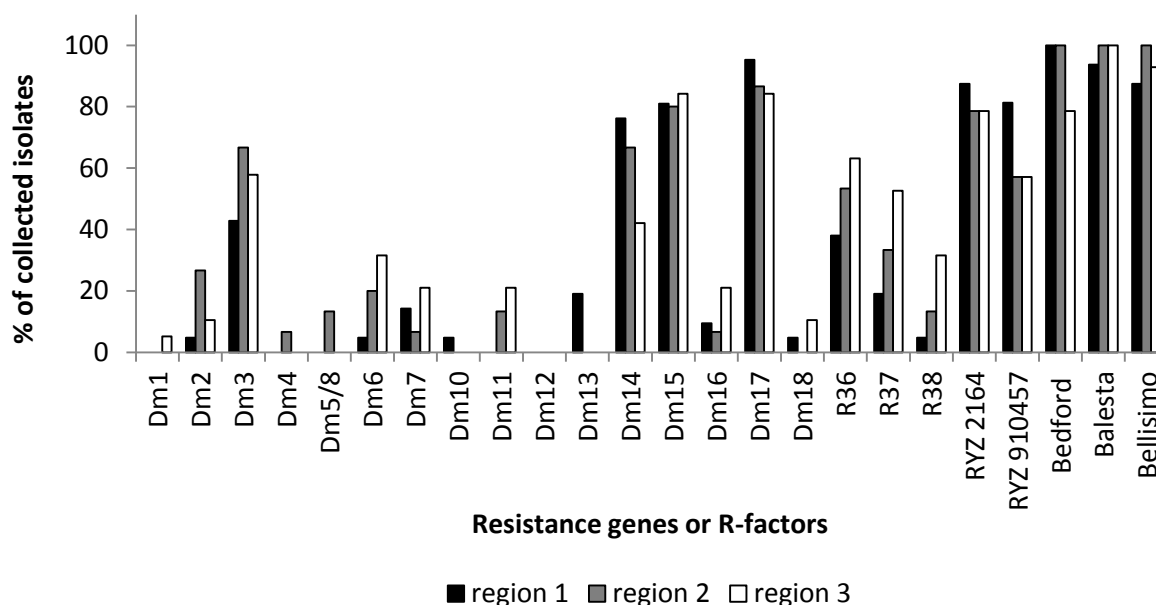


Figure 2-3: Percentage of collected isolates per region for which the different resistance genes or factors (*Dm* genes or R-factors) provided resistance. Twenty-one isolates were collected in region 1 of which 5 were characterized with the first differential set and 16 with the second. Fifteen isolates were collected in region 2 of which one was characterized with the first differential set, the other 14 with the second set. Nineteen isolates were collected in region 3 of which 5 were characterized with the first differential set and 14 with the second set.

2.4 Discussion and conclusions

In this study, we aimed to investigate the virulence characteristics of the *B. lactucae* populations in Belgium and provide information for lettuce breeders. We collected downy mildew samples in the period 2008-2013. Of 55 collected isolates, 41 had a unique v-phenotype (74.5%), which is comparable to the results found in Norway where 63.7% of the isolates had a unique v-phenotype (Nordskog et al. 2014).

Nine of our 55 analysed isolates were identical to three officially denominated races of the IBEB: Bl: 24, Bl: 26 and Bl: 28. The same three races were detected in the Netherlands, the northern neighbour of Belgium. Bl: 24 was first detected in 2002; while Bl: 26 and Bl: 28 were nominated in 2008 and March 2011 respectively (International Seed Federation 2014). In southern France, samples were collected in the period 2006 to 2011 and Bl: 22, Bl: 24 and Bl: 25 were determined (Maisonneuve et al. 2011). In Norway, samples were collected from 2001 until 2006, comprising Bl: 17, Bl: 18, Bl: 22 and Bl: 24 (Nordskog et al. 2014). In 2013 three new races, Bl: 29, Bl: 30 and Bl: 31, were denominated by the IBEB with sextet codes 59-63-47-03; 59-63-13-03 and 63-63-12-06 respectively. In 2014 one new isolate was denominated by the IBEB: Bl: 31 with sextet code 61-31-03-00 (Board 2015). Bl: 29, Bl: 31 and Bl: 32 were also found in Belgium (International Bremia Evaluation Board 2013; Board 2015). However, we did not identify these new races, which may be due to the fact that in 2013 we only characterized three isolates.

In this study resistance was most frequently observed based on *Dm* genes 15 and 17 and on the resistance factors of Bedford, Balesta and Bellissimo, whereas resistance based on *Dm* genes 1, 4, 5/8, 10 and 12, in contrast, was the least effective. These results were very similar to the results of a study performed with samples collected from 1999 until 2011 in the Czech Republic (Petrželová et al. 2013). Petrželová and colleagues could not detect isolates able to infect plants expressing *Dm* 17. What is more, very few of their isolates were neither able to infect plants possessing *Dm* genes 15 or 18, nor plants with R-factors 36 or 38. By contrast, efficacy of *Dm* genes 1, 2 or 12 for resistance to the Czech Republican isolates was very low (Petrželová et al. 2013). Also in Norway, Israel and Germany, plants possessing *Dm* 1 and *Dm* 12 had the lowest resistance to *B. lactucae* isolates (Lebeda and Zinkernagel 2003; Sharaf et al. 2007; Nordskog et al. 2014). Furthermore, resistance provided by *Dm* 17 and resistance factors of Bedford, Balesta and Bellissimo were also effective against the newly denominated races of the IBEB (Bl: 29, Bl: 30 and Bl: 31), while plants possessing only *Dm* 15 were susceptible to these three races (International Seed Federation 2014). Outside of Europe, a study on *B. lactucae* isolates collected from São Paulo state, Brazil, from 2008 and 2009 showed the lowest frequency of resistance mediated by *Dm* genes 1, 2, 3, 17 and 18 (Castoldi et al. 2012). In contrast, the analysis performed in Australia showed that the described virulence patterns do not resemble any of the patterns of races denominated by the IBEB (Trimboli and Nieuwenhuis 2011). The similar occurrence of v-phenotypes in European countries suggests that long-distance transport of airborne sporangia would be possible, as was also suggested by Nordskog et al. (2014). It is reported that the influence range of downy mildew incidence at one location on other locations is estimated to be between 80 m and 3000 m (Valade 2012). Yet, a study with genetic markers would be useful to give more specific information taking into account wind and weather conditions. We hypothesize that the pathogen survives in warmer regions of Europe and in spring spores are

transferred by wind to our country. To confirm this hypothesis, we should sample with spore traps in different fields spread over Belgium at the end of the winter to detect where spores are firstly detected. This should be done for several years to allow strong conclusions. Based on the results of these sampling, it would be possible to describe trajectories of spore flights as has been done for cucumber downy mildew in Norway (Nordskog et al. 2008).

An improvement of the differential lettuce set of IBEB was suggested as it came clear that there were some restrictions in the current set. One of them was that the cultivars Ninja and Discovery of the differential set were not monogenic. The resistance factor R 36 of Ninja turned out to be a combination of *Dm 3*, *Dm 4*, *Dm 11* and a resistance factor from *L. saligna*, called *Rsal-1*. Similarly the resistance factor R 37 of Discovery is a combination of *Dm 7* and *Rsal-1* (Maisonneuve 2011). Therefore it would not be possible to have a susceptible reaction on Ninja or Discovery if a resistant reaction was noticed mediated by one of the *Dm* genes their resistance factor consists of. However for ten of our isolates this contradiction was noticed. Probably this is due to the sampling method. As mentioned before, if sporulation was not abundant, spores of one or two leaves of one or two adjacent plants were considered as one isolate, making it possible that what is considered as one isolate is a mixture of isolates, which could explain the unexpected susceptibility reactions on Ninja and Discovery. We were aware of this restriction of our sampling method, but decided to stick to this method as it was described before (Sharaf et al. 2007; Nordskog et al. 2014) and conclusions concerning which *Dm* genes and R-factors are most effective for resistance to Belgian *B. lactucae* isolates would not be influenced.

Our data show that the v-phenotypes of our *B. lactucae* isolates are not very stable over the years. During the sampling period some fluctuation in meteorology data of the different years was recorded, however, comparison of these data with the variability of the v-factors over the years did not result in any clear relationship (data not shown). These data may suggest that climate change is not a major factor to cause variation of *B. lactucae* populations in Flanders, Belgium. On the contrary the infected lettuce cultivars from which *B. lactucae* isolates were sampled, changed over the years. It is known that each time new lettuce cultivars are released, only the few isolates able to overcome the resistance factors of the new lettuce cultivars can infect these cultivars. As downy mildew is an exponentially spreading disease, these isolates can spread very fast and can probably be candidates of newly denominated races. Therefore the resistance of new lettuce cultivars is often not long lasting. Many cultivars from which we sampled in 2008 and 2009, for instance Neil, Natalia, Brigade, Hertog, Troubadour, Fenston and Mariken are no longer commercially cultivated in Belgium.

The variation of the v-phenotypes of the isolates we described in this paper was also mentioned in other reports (Lebeda and Zinkernagel 2003; Petzelová and Lebeda 2003; Sharaf et al. 2007; Nordskog et al. 2014). One possible explanation for this variation could be sexual reproduction, as suggested in a study from isolates in New York (Yuen and Lorbeer 1987). This possibility will be discussed in Chapter 3. In addition to sexual reproduction, asexual genetic variation may result in virulence variation of *B. lactucae* populations. *B. lactucae* is a diploid oomycete (Tommerup et al. 1974) and the alleles of the two identified effectors in *B. lactucae* isolates, BLG01 and BLG03 show high diversity. This diversity was suggested to be possibly due to gene duplication, polyploidy or nonsense mutations (Stassen et al. 2013). In our study, Belgian *B. lactucae* populations are very variable and most isolates do not belong to the denominated races. We suggest that asexual genetic

variation could be an important factor in causing Belgian *B. lactucae* populations displaying highly diverse virulence profiles.

To keep a more durable resistance to downy mildew in lettuce, it has been recommended to alternate varieties with different resistance factors (Hägnefelt 2000). Also field resistance or partial resistance can help to lower the population density of the pathogen and reduce the risk of further distribution of the disease because the mature plants show reduced susceptibility (although the seedlings of these cultivars are readily infected) and this reduced susceptibility is marked by fewer and smaller lesions on fewer affected leaves, a slower rate of disease progression and a longer latent period compared to susceptible plants (Yuen and Lorbeer 1984; Hägnefelt 2000; Grube and Ochoa 2005). Genes involved in quantitative resistance are not that widely used yet in breeding, but might be an interesting pool for new resistant lettuce cultivars (Michelmore 1995). In future it will be important to characterize the effectors of *B. lactucae* and select the effectors indispensable for pathogenicity. Resistance genes for these effectors will provide a more sustainable resistance. Furthermore, breeders used pyramiding of major resistance genes to reduce selection pressure on *B. lactucae*. Yet, these combined resistance genes had often been used before alone and have been broken down before. A more durable breeding strategy would be to use pyramiding of major resistance genes that have not been broken down before (Valade 2012). A possible biotechnological approach to engineer a disease-resistant lettuce cultivar might be interfamily transfer of pattern-recognition receptors (PRRs). PRRs are employed by plants for sensitive and rapid detection of potential pathogens and pests. Some PRRs are plant-family specific, however transferring them to other plant families has been demonstrated to result in increased disease resistance (Zipfel 2014).

Recently transgenic plants were constructed, in which small interfering RNAs (siRNAs) are produced in the host plant and which subsequently move into the pathogen to silence pathogen genes (Govindarajulu et al. 2014; Knip et al. 2014) such as *Highly Abundant Message #34 (HAM34)* or *Cellulose Synthase (CES1)* genes. These plants showed greatly reduced growth and sporulation ability of *B. lactucae*. Pathogen control mediated by host-induced gene silencing (HIGS) is very likely to be more durable than classical resistance conferred by major resistance genes, as approximate 400 base pairs (bp) of genes, which are vital to the pathogen, are targeted and thus a major change in the pathogen is required to overcome the HIGS mediated resistance. HIGS is also likely to be accepted by the public because no new proteins are made, in contrast with other transgenic strategies (Govindarajulu et al. 2014). Moreover, the availability of host genome sequence data helps to ensure that the constructs does not target or negatively affect the host. The ability to identify pathogen targets allows to make the construct very specific and helps to develop environmental safe transgenic plants (Nunes and Dean 2012). However further research is needed because for instance the form and mechanism of the movement of the silencing signal from plant to pathogen is still unknown (Nunes and Dean 2012; Govindarajulu et al. 2014; Knip et al. 2014).

In conclusion of the results in this chapter, we can state that the variability of the Belgian *B. lactucae* populations reflects the known genetic flexibility of the pathogen to adapt to changes in host plants and surrounding conditions. *Dm 15*, *Dm 17* and the R-factors of the lettuce cultivars Bedford, Balesta and Bellissimo can offer the most efficient protection, based on virulence characterization of the collected isolates in this study.

Chapter 3. Decision support tool

As a second part of the integrated control strategy for downy mildew which is studied in this work, climate adaptation and optimal fungicide application are considered. Although timing of fungicide spraying is often following a calendar based schedule, applications closer to the moment of pathogen inoculation are more effective. Sometimes fungicides are applied without actual risk for downy mildew outbreak. Forecasting models were developed to assess disease outbreak risk, determine the optimal fungicide application moment and avoid unnecessary treatments. Inspired by these models, we constructed a decision supporting tool in which risk assessment, based on temperature predictions and relative humidity measurements, is used to advice greenhouse climate adaptations to suppress pathogen development. Furthermore, accurate suggestions for fungicide applications are advised in situations in which climate adaptation is not feasible. The construction of this tool was based on an epidemiological study of the pathogen. Furthermore, we converted the tool into a web application, making it accessible and user friendly for growers. In greenhouse experiments it was demonstrated that lettuce cultivation based on this strategy required a reduced number of fungicide applications in comparison with a standard cultivation with calendar based fungicide applications, with a similar level of downy mildew disease control, except for experiments with very high disease pressure. Crop yield and basal rot were also monitored during cultivation based on the advice of the tool. Both were not significantly different in comparison with standard fungicide applications. Also some preliminary tests were performed to evaluate if a compromise between measures to avoid tipburn and criteria to inhibit downy mildew infection could be found. All together the experiments described in this chapter show that the developed decision support tool can be a valuable part of an integrated control strategy.

Results of this chapter were obtained as a cooperation between:

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3.1 Introduction

An almost inevitable part of downy mildew control consists of fungicide applications. In the past, several models were developed to predict disease outbreaks and these predictions were used to determine the optimal moment for fungicide applications, improving their efficiency (Scherm et al. 1995; B.M. Wu, Subbarao, et al. 2001; Kushalappa 2001; Wu et al. 2002; Provinciaal Proefcentrum voor de Groenteteelt (PCG) 2003; Raatjes et al. 2003; Oostingh 2004; Jönsson et al. 2005) (<http://www.vips-landbruk.no>). Improving the effectiveness of fungicides can slow down resistance occurrence against these fungicides, which is an often described problem (Crute 1984; Wicks et al. 1994; Isaac 1999; Brown et al. 2004). Timing of fungicide application is often based on a calendar schedule. In Belgian lettuce cultivation, the first application is usually foreseen in the first week after planting, a second in the second week, a fourth just before leaves of adjacent plants touch each other, a third in between the second and fourth and two weeks before harvest the last fungicide treatment should be applied (personal communication with Isabel Vandevelde, PSKW, crop consultant). In other regions, slightly different schedules can be used (Blancard et al. 2006).

As part of the integrated control strategy developed in this work, a decision support tool was constructed in analogy with the existing models. The purpose was to develop a decision support tool, helping the grower to take decisions in order to keep his plants free of downy mildew. Advice from this tool suggests the optimal moment for fungicide applications to improve their effectiveness, but also suggests the most accurate fungicide at each moment relatively to the moment of application relatively to the moment of inoculation. Moreover, the first action of intervention suggested by the tool would be to adapt the greenhouse climate in order to reduce the number of fungicide applications and thereby reduce the development of pathogen resistance against fungicides. The development of fungicide resistance can be decelerated by moderating the fungicide application number and/or doses, applying multisite fungicides or by applying a mixture of substances with different modes of actions (Isaac 1999; Blancard et al. 2006; Savary et al. 2006; Barrière et al. 2014). Climate adaptation has been reported before to be an appropriate tool for disease control (Morgan 1984). Moreover, the majority of Belgian lettuce is produced in greenhouses (VLAM 2014), making greenhouse climate adaptation a possibly useful tool to suppress disease development.

To develop this decision support tool, several possible sources of inoculum needed to be studied and an epidemiological study of the pathogen was required to estimate the impact of relative humidity and temperature on the different life stages of the pathogen. As possible sources of inoculum, other than airborne conidia from cultivated lettuce plants, oospores and conidia from infected *Lactuca serriola* plants were considered. For the production of oospores usually the presence of two mating types is required because *Bremia lactucae* is predominantly heterothallic. Also the optimal application moment for the various fungicides needed to be determined. The results of the epidemiological study and analyses of the fungicide performances could then be used as a basis for criteria to estimate disease outbreaks, determine the optimal moment for fungicide application and formulate advice for greenhouse climate adaptation. At the end of this chapter the decision support tool was used to cultivate lettuce and the effect on the number of fungicide applications and downy mildew disease control was evaluated. Furthermore side effects of cultivation with the decision

support tool were assessed on basal rot and yield. Final question was if a compromise could be found between downy mildew control and tipburn control because tipburn can be reduced by creating humid conditions, which might favour downy mildew.

3.2 Materials and methods

3.2.1 *Plant materials and pathogen maintenance*

Bremia lactucae isolates obtained from Rijk Zwaan (Fijnaart, the Netherlands) or collected from infected plants in fields and greenhouses in Flanders, Belgium, were maintained and multiplied on seedlings of *L. sativa* cv. Green Towers. For lab experiments the plants were sown on wetted cotton wool and a layer of filter paper in plastic transparent boxes. The plants were grown in a growth chamber at 17.5 °C and day/night regime of 16 h light, 8 h dark. Fresh spores were washed off from infected leaves with sterile water. The concentration of the spore suspension was adapted to 5×10^4 spores/mL and applied as a foliar spray on the seven-day-old seedlings at the two-leaf-stage until running-off. Boxes with inoculated seedlings were incubated under growth chamber conditions (day/night regime of 16 h light/8 h dark, 17.5 °C and relative humidity above 90%). Under these conditions new spores appeared 5 to 6 days after inoculation, and sporulation was maximal at 10 days after the inoculation. For long term storage, leaves with sporulation were frozen at -20 °C.

3.2.2 *Search for Lactuca serriola plants, naturally infected with Bremia lactucae*

To investigate the importance of *Lactuca serriola* as primary source of inoculum, we searched for infected plants on and near the research centres Inagro (West-Flanders), PSKW (Antwerp) and PCG (East-Flanders). We also posted calls on the web sites of the three research centres to report infected plants. In these calls we described that this plant is a pioneer plant, mostly growing at open spots on eutrophic soil (sand, clay, marl or stony ground). Therefore this plant can most frequently be found along roads, on reclaimed land, coastal dunes, along railways, between paving stones at industrial sites, but also in ruderal places, gardens, waterfronts. We provided determination steps and pictures (Figure 3-1) to help detection of the plant.

Determination steps:

- 1) It is a large plant, often more than 1 meter and a typical member of the Asteraceae (Compositae) family with composed flowering heads.
- 2) Flowers are yellow.
- 3) The plant contains latex, visible when the plant is damaged.
- 4) At the downside of the leaves, spines (≥ 2 mm) are visible.
- 5) Often the leaves are quarter turn turned, with the top side vertically erected.



Figure 3-1: Leaves and composite flower of *Lactuca serriola*. (Leaves, own picture, composite flower (<https://www.kuleuven-kulak.be/kulakbiocampus/images>)).

3.2.3 Collection of *Bremia lactucae* isolates and evaluation of oospore presence

From 2008 until 2014 samples with *B. lactucae* symptoms were collected. For each of these samples, part of the infected leaf was discoloured in 100% ethanol and presence of oospores in the leaf was examined with a light microscope. We chose a part of the infected leaf which was yellow or brown but did not show abundant sporulation as we noticed and read before that at sites where oospores were formed, asexual sporulation was suppressed (Morgan 1978; Morgan 1983). From each sample, the pathogen was isolated and chronologically numbered. Some abundantly sporulating isolates were used for virulence characterization (Chapter 2) and the most abundantly sporulating isolates were used for mating type analysis (section 3.2.4).

3.2.4 Mating type analysis

Dutch *B. lactucae* races NL5 and NL15 were obtained from the research department of the breeding company Rijk Zwaan (<http://www.rijkszwaan.nl/>). NL5 was defined as mating type B2 and NL15 as mating type B1 (Michelmore and Ingram 1980; Lebeda and Blok 1990). A conidial suspension of NL5 or NL15 was mixed with our isolate of interest and this mixture was sprayed on seven-day-old seedlings until running-off (Yuen and Lorbeer 1987). A mixture of NL5 and NL15 spores was used as a positive control for oospore formation. As a negative control, lettuce seedlings were inoculated with NL5 or NL15 only. For investigation of secondary homothallism (Michelmore and Ingram 1982), lettuce seedlings were separately inoculated with each *B. lactucae* isolate of interest. After 14 days of incubation in standard conditions, the presence of oospores on inoculated leaves was observed under a light microscope after discoloration of the leaves in 100% ethanol. Three repetitions of ten seedlings were inoculated with the different inoculum mixtures. Five leaves of each repetition were microscopically analysed.

3.2.5 Spore sampling

Spore concentrations were monitored using a 7-day volumetric spore sampler (Burkhard Manufacturing Company, Rickmansworth Herfordshire, UK, www.burkard.co.uk) from ILVO (Institute for Agriculture and Fisheries Research, Melle, Belgium). The sampler was placed in the middle of a lettuce field (open field or inside a greenhouse) at PCG (Figure 3-2) or Inagro, or on an open spot outside the greenhouses of Inagro. Air was sampled at 10 L/ min. Airborne spores and particle matter were impacted on adhesive coated transparent tape, supported on a clockwork-driven drum. Tapes were removed at 7-day intervals, cut into segments corresponding to the desired period. The first 30 days of sampling, tapes were divided into pieces corresponding to the first six hours after sunrise, the period from six hours after sunrise until sunset, and the night (from sunset until sunrise), for each day. This division was chosen to analyse in which part of the day most spores could be detected. On the other sampling days, the tapes were divided in periods per 24 hours. DNA was isolated from the tapes and based on the calculation of the amount of DNA yield per spore, the number of spore equivalents could be calculated by real time-PCR analyses. A spore suspension with a known concentration was used to construct a standard curve, which was the basis to determine the number of spore-equivalents trapped with the Burkard spore sampler. Primers for the PCR analyses were kindly provided by the Norwegian Institute of Bioeconomy Research (NIBIO), via Dr. May Bente Brurberg. Primer sequences are currently unpublished and will therefore not be shown here.



Figure 3-2: Volumetric spore sampling (Burkhard) set up in open field at PCG to monitor presence of *B. lactucae* conidia (picture from Aaike Bogaert).

3.2.6 Oospore germination

Three different methods were used to stimulate germination of the oospores produced during the tests for mating type analysis. Firstly, oospores were buried in soil in which we sowed *L. sativa* cv. Cobham Green and we investigated the plants weekly for symptoms during two months.

Secondly, we used the method described by Blok (Blok 1981). Briefly, after the formation of oospores we kept the seedlings containing oospores for two months until all leaf tissue was decayed and there were no viable conidia left. Then, we placed the tissue paper on which the seedlings with conidia had been growing in a glass jar on a layer of soil. We tried both sterilized and non-sterilized soil. Lettuce seeds of cv. Cobham Green were sown around the filter papers and the jar was incubated for two

months at 16 °C. All plants were checked weekly for symptoms of downy mildew (Blok 1981). Assessment of disease incidence and index is described in section 3.2.7.

The third method was a modification of several protocols (Morgan 1978; Morgan 1983; Sung and Clerjeau 1988; Van der Gaag and Frinking 1997a; Van der Gaag and Frinking 1997b; Spring and Zipper 2000; Vercesi et al. 2010). Leaves containing oospores were put in nylon bags and stored in soil at 15°C or at 4°C for one month. After incubation, the leaves were ground with a blender after adding an amount of sterile water, sufficient to submerge the leaves. The mixture was filtered through two sieves. The upper sieve had a pore size of 100 µm and the second sieve had a pore size of 20 µm to harvest oospores of *B. lactucae* which have an average diameter of 36 µm. The remaining parts on the second sieve were resuspended in sterile water. To avoid contamination, 100 µg/mL ampicillin and 10 µg/mL rifampicin was added to the oospore suspension in some experiments, as was done in assays on the germination of the oospores of *Peronospora viciae* or *Plasmopara halstedii* (van der Gaag and Frinking 1996; Van der Gaag and Frinking 1997b; Spring and Zipper 2000). The suspension was divided in three parts. One part was dropped on leaves of seven-day-old seedlings. Another part was dropped on 1% water-agar plates. Next to the droplets some seeds of *L. sativa* cv. Cobham Green were positioned. The rest of the suspension was transferred on a microscopic slide. To avoid mechanical stress through pressure, small spots of Vaseline were placed at each corner of the cover slip. The slides were placed in 90 mm Petri dishes. The Petri dishes were put in a moisture transparent plastic box and incubated at 16 °C in darkness or in an incubator with a day/night regime of 16 h light/8 h dark.

3.2.7 Epidemiological study

For the epidemiological study of the pathogen, inoculations were done with *Bremia lactucae* isolate BmVL144, an isolate collected in 2012 in Sint-Katelijne-Waver on Romaine lettuce (*Lactuca sativa* var. *longifolia*). This isolate was chosen because it was abundantly sporulating. Sextet-code (57-26-42-12) and mating type (B1) of this isolate were determined.

To divide the life cycle of the pathogen in different stages a bright-field microscopic study with an Olympus BX-51 microscope was used. Images were captured with a Colour View III camera and edited with the software package CELL-F (Olympus Soft Imaging Solutions, Münster, Germany). Seven-day-old plants were inoculated with BmVL144 and plants were incubated under growth chamber conditions (17.5 °C and relative humidity above 90%, day/night regime 16 h light, 8 h darkness). It was noticed that in these conditions the pathogen developed well. (as described in 3.2.1). Samples were taken 1.5 hours post inoculation (hpi), 3 hpi, 24 hpi, 5 days post inoculation (dpi), 6 dpi and 10 dpi. Inoculated leaves were cut off the plantlets and cleared and fixed in 100% ethanol. Fungal structures and dead cells were stained with 0.1% trypan blue in 10% acetic acid for 2 minutes. After staining and washing, the leaves were mounted in 50% glycerol.

To assess the influence on different pathogen development stages, temperature or relative humidity was adjusted during the stage of interest and kept at standard settings (17.5 °C and relative humidity above 90%, day/night regime 16 h light, 8 h darkness) during the rest of the incubation period. Tested temperatures during germination and penetration period and sporulation phase were 6 °C, 12 °C, 17.5 °C, 24 °C, 26 °C and 30 °C. During incubation temperature was adapted to 6 °C, 12 °C, 17.5 °C,

23 °C and 28 °C. Relative humidity was only adapted during the germination and penetration stage of the pathogen. The effect of a relative humidity of minimum 90% was compared to a relative humidity below 90% (85% on average). Disease incidence was evaluated 10 dpi, expressed as the percentage infected leaves on the total number of inoculated leaves. Disease index was not evaluated in these experiments because preliminary experiments showed no statistical differences, probably because plants were still very small during the experiments. Statistical analyses to assess the effect of the treatments on disease incidence were performed using binary logistic regression analysis ($p=0.05$).

3.2.8 Lab experiment for determination of the optimal fungicide application

Seven-day-old plants in two-leaf stage were inoculated with *Bremia lactucae* isolate BI: 26 (4.8×10^4 spores/mL) and inoculated plants were incubated under growth chamber conditions (17.5 °C and relative humidity above 90%, day/night regime 16 h light, 8 h darkness). Plants were treated four days after inoculation with Previcur N, Fenomenal or Fubol Gold.

3.2.9 Greenhouse experiments

During greenhouse experiments the climate in the greenhouse is monitored with the climate box of which data are sent to the web application. The relative humidity is determined via psychrometric measurement.

3.2.9.1 Downy mildew

Inoculations in greenhouse experiments were done with inoculum collected from an infected field at PCG in the spring of 2010, which was recollected after each experiment to be used as fresh inoculum in the next greenhouse experiment. Recollected samples were stored at -20°C until the next greenhouse experiment. Leaves with sporulation were mixed with water. The spore solution was filtered to remove plant debris and the concentration was adjusted to $1-5 \times 10^4$ spores/mL. Next the spore solution was sprayed over the plants.

In greenhouse experiments, disease incidence and/or disease index were determined. Disease incidence indicates the percentage of infected plants, while disease index indicates the severity of the disease.

$$disease\ incidence\ (\%) = \frac{\#\ of\ plants\ with\ downy\ mildew\ symptoms}{total\ \#\ of\ inoculated\ plants} \times 100$$

To determine the disease index, symptoms are divided in four classes (Figure 3-3):

Class 0= no disease symptoms

Class 1= one spot

Class 2= few spots, delineated by the veins

Class 3= spots not delineated by the veins

Class 4= (almost) the entire leaf is yellow or brown

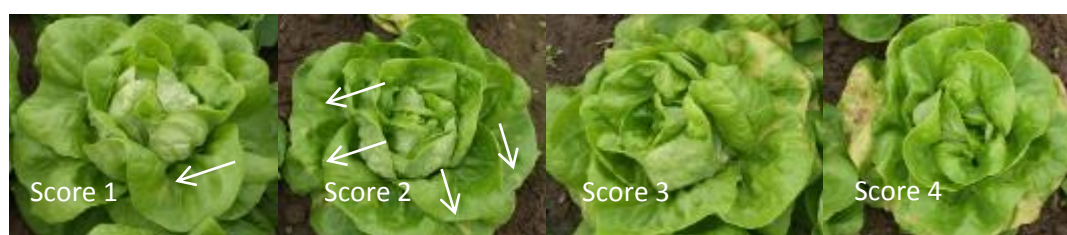


Figure 3-3: Score of lettuce downy mildew symptoms.

The disease index is calculated according to following formula:

$$\text{disease index (\%)} = 100 \times \frac{(C1) \times 1 + (C2) \times 2 + (C3) \times 3 + (C4) \times 4}{\text{total \# of inoculated plants} \times 4}$$

With C1, C2, C3 and C4 are the number of plants with symptoms of class 1, class 2, class 3 or class 4, respectively.

3.2.9.1.1 Determination fungicide applications

Lettuce plants of cv. Flandria (Bl: 1-17, 21-23) was planted on the 3rd of August 2011. Plants were treated with Paraat or Fubol Gold one day before inoculation or one week after inoculation. The experiment was set up as a random block design with 20 plants per repetition per treatment and each treatment was repeated three times.

Plants were artificially inoculated on the 11th of August with an inoculum concentration of 4×10^4 spores/ mL.

3.2.9.1.2 First experiment at Inagro to evaluate the definitive version of the decision support tool

Lettuce plants of cv. Hofnar (Bl: 1-23, 25, 31) were sown on the 26th of August 2013 and plants were planted on the 12th of September. On the 14th of October, plants were inoculated with an inoculum concentration of 3×10^4 spores/mL.

The number of fungicide applications and the level of downy mildew control were compared between three cultivation methods: control plants, on which no fungicides were applied, a group of plants which received fungicide applications on a calendar based schedule and the plants which treatments were prescribed by the decision support tool.

Experimental set up was a randomized block design with three blocks and three treatments per block. In each treatment, 700 plants were evaluated, being 230 plants in two blocks and 240 in the third block.

The plants with fungicide applications according to a calendar based schedule were treated on the 17th of September with Fenomenal, the 23rd of September with Previcur Energy, the 26th of September with Paraat and on the 1st of October with Proplant. The decision support tool prescribed fungicide applications on the 17th of September with Proplant, on the 2nd of October with Proplant and on the 18th of October with Fenomenal. Fungicides were applied in the legally allowed doses.

3.2.9.1.3 Second experiment at Inagro to evaluate the definitive version of the decision support tool

Plants of lettuce cultivar Flandria (Bl: 1-17, 21-23) were sown on the 27th of February 2014, planted on the 20th of March and harvested on the 5th of May. The greenhouse climate was adjusted according to the advice of our model. The plants were divided into four groups: the first group of plants was covered with transparent, plastic foil during the first 24 h after inoculation to create a high relative humidity, favourable for germination and penetration of *B. lactucae*. This allowed us to evaluate the effect of step 2 of our model (avoiding germination and penetration) on the disease development. This adaptation of the relative humidity is the only difference with the second group of plants. Both groups consisted of untreated plants. Timing of fungicide applications for the third group of plants was determined by a calendar based schedule while for the fourth group timing of fungicide application was based on the advice of the decision support tool. The experimental set up of the experiment was a randomized block design with four blocks, each containing the four treatments. In total four times 44 plants were evaluated for each treatment.

Standard fungicide applications were performed on March the 23rd, March the 28th, April the 7th, April the 15th and April the 22nd; with Fenomenal, Previcur Energy, Previcur Energy, Proplant and Revus respectively. Fungicides were applied in the legally allowed doses.

The plants of the four groups were artificially inoculated twice to make sure sufficient *B. lactucae* spores were present in the greenhouse. The first inoculation was on the 31st of March and the second on the 11th of April. Both inoculations were performed with a mixture of *B. lactucae* isolates, collected from a naturally infested field at PCG, with a concentration of 5×10^4 spores/mL for the first inoculation and 1.8×10^4 spores/mL for the second inoculation.

3.2.9.2 Basal rot

Basal rot was evaluated in these experiments with a score from 1 to 9, with score 1 for plants with no symptoms of basal rot and 9 for plants with most severe symptoms. Each experiment was performed with at least three repetitions and minimum 20 plants per repetition for each treatment.

3.2.9.2.1 Experiment at PSKW

Lettuce cultivar Cosmopolia (Bl: 1-25, 27, 28, 30, 31) was sown on the 7th of April 2014, planted on the 29th of April at the greenhouse and harvested on the 10th of June. Three groups were compared: untreated plants, plants with standard fungicide application and plants which received fungicide treatments based on advice of our model. Each group consisted of four repetitions with 40 plants per repetition. Standard fungicide applications were Fenomenal on the 30th of April, Previcur Energy on the 9th of May, Proplant on the 16th of May and the 19th of May and Revus on the 30th of May. Based on the advice of the decision support tool Proplant was applied on the 5th of May and on the 19th of May. Fungicides were applied in legally prescribed doses. Plants were harvested on the 10th of June.

3.2.9.2.2 Experiment at PCG

Lettuce plants of cultivar Flandria (Bl: 1-17, 21, 23) were sown on the 13th of August 2013, planted on the 29th of August and harvested on the 10th of October. Fungicide applications of the calendar based

schedule were Previcur Energy on the 10th of September, Paraat on the 18th of September, Proplant on the 24th of September and Revus on the 3rd of October. The first application of this calendar based schedule with Fenomenal which should have taken place in the first week after planting, was forgotten. According to the advice of the decision support tool, Proplant was applied twice, once on the 3rd of September and once on the 18th of September. In this experiment there was no control group with untreated plants.

3.2.9.3 Effect on average lettuce head biomass

3.2.9.3.1 First experiment at PCG

Lettuce cultivars Flandria (Bl: 1-17, 21, 23) and Cosmopolia (Bl: 1-25, 27, 28, 30, 31) were sown on the 26th of March 2014, planted on the 16th of April 2014 in the greenhouse of PCG and harvested the 4th of June. The plants of each cultivar were divided into three groups: an untreated group, a group with standard fungicide application and a group with model advised fungicide applications. The climate in the greenhouse was adjusted according to the advice of the decision support tool and was the same for all plants because all were cultivated in the same greenhouse compartment to keep the majority of factors (e.g. solar radiation, orientation and water supply) the same for all plants. Each treatment was repeated three times and each repetition consisted of 21 plants per treatment. The group with standard fungicide applications was treated the 22nd of April with Fenomenal, the 28th of April and the 5th of May with Previcur Energy, the 12th with Proplant and the 16th of May with Revus. Based on the advice of the decision support tool, only two treatments were applied: on the 25th of April Previcur Energy was applied and the on 12th of May, a treatment with Proplant was done. Fungicides were applied in the legally allowed doses.

3.2.9.3.2 Second experiment at PCG

lettuce cv. Flandria (Bl: 1-17, 21, 23) and Cosmopolia (Bl: 1-25, 27, 28, 30, 31) were sown on the 26th of May 2014, planted the 12th of June and harvested the 21st of July. According to the calendar based schedule, 5 fungicide applications were performed: Fenomenal on the 16th of June, Previcur Energy on the 19th of June, and on the 26th of June, Proplant on the 30th of June and Revus on the 8th of July. Based on the advice of the decision support tool two applications were performed, both with Proplant, one on the 16th of June and one on the 30th of June. Each treatment was repeated three times with 21 plants per repetition per treatment.

3.2.9.4 Compromise between downy mildew and tipburn control

To evaluate the effect on tipburn, a tipburn index was calculated:

$$\text{tipburn index} = \frac{F \times 1 + M \times 2 + S \times 3 + VS \times 4}{P \times 4} \times 100$$

with F, M, S and VS are the numbers of plants with, few, medium severe, severe and very severe tipburn symptoms, respectively, and P is the total number of plants. To determine the category of symptoms on a plant, the number and size of spots is taken into account, as shown in Table 3-1.

Table 3-1: Classification of plants with tipburn symptoms based on the number and size of tipburn spots.

Number of spots	Size of spots [mm]			
	<1	1-3	3-6	>6
1	Few symptoms	Few symptoms	Medium severity	Medium severity
2-5	Few symptoms	Medium severity	Medium severity	Severe symptoms
6-10	Medium severity	Medium severity	Severe symptoms	Very severe symptoms
More than 10	Medium severity	Severe symptoms	Very severe symptoms	Very severe symptoms

Statistical analyses to assess the effect of the treatments on disease incidence were performed using binary logistic regression analysis ($p=0.05$). To assess the effect on disease index, Kruskal-Wallis and Mann-Whitney U analyses were used ($p=0.05$).

3.2.9.4.1 First experiment at Inagro

Lettuce cultivars Hofnar (Bl: 1-23, 25, 31) and Gardia (Bl: 1-27, 29, 30) were sown on the 7th of December 2012 and planted on the 31st of January in a hydroponic system (Nutrient Film Technique) at greenhouse compartments of Inagro. Hofnar is known to be more sensitive for tipburn than Gardia. Three treatments were compared for the effect on tipburn and downy mildew, and each treatment was performed on 160 plants. The first treatment consisted of an increase of the relative humidity in a period from 8 p.m. until 12 p.m. The second treatment was a control treatment without influencing the relative humidity and the third treatment was an increase of the relative humidity during the entire night. Relative humidity increase was obtained by nebulizing water every 15 minutes (during 35 seconds) underneath the gutters, which resulted in a 5% increase during the period of water spraying. The plants were artificially inoculated with *B. lactucae* on the 25th of March (4×10^4 spores/mL).

3.2.9.4.2 Second experiment at Inagro

Increasing the relative humidity was obtained in this experiment by nebulizing water every 15 minutes (during 35 seconds) over the plants. Plants of lettuce cultivars Cosmopolia (Bl: 1-25, 27, 28, 30, 31) and Gardia (Bl: 1-27, 29, 30) were sown on the 16th of June 2014 and planted in a hydroponic NFT system on the 1st of July. The experimental set up consisted of four plots with each 385 plants. The first plot consisted of plants grown in circumstances with an increased relative humidity starting 2 hours before sunset until 2 hours post sunset. For the second plot, relative humidity was increased from 2 hours before sunset until 4 hours after sunset. The relative humidity was not increased for plants in the third plot and for plants in the fourth plot, relative humidity was increased during six hours, starting from sunset. Plants were inoculated the 10th of July with *B. lactucae* (3.8×10^4 spores/mL)

3.3 Results

3.3.1 Epidemiological study as basis for decision support tool

3.3.1.1 Inoculum sources

The considered potential primary inoculum sources for downy mildew outbreaks are oospores and airborne conidia originating from infected lettuce fields or alternative host plants, predominantly *Lactuca serriola* (Blancard et al.2006).

Oospores

Oospores were microscopically detected in 14 of the 153 collected samples. Oospores were found in samples from fields with very severely infected crops and often in lettuce tissue close to the main vein (Figure 3-4). Information of the samples and isolates determined from the samples is given in Table 3-2. Two samples were collected from infected crops in open field, the others from greenhouse grown crops. Four samples originated from fields which were artificially inoculated with IBEB race Bl: 26 on one of the research centres. All samples in which oospores were found are taken in 2009, which is not surprisingly because the vast majority of samples were taken in 2009: 103 of the 153 samples. In 2009 the main focus of the study was the characterization of Flemish *B. lactucae* isolates.



Figure 3-4: Severely infected lettuce head with indication (arrow) of the tissue where *B. lactucae* oospores were mostly found.

As a second approach to evaluate the importance of oospores as primary inoculum in Belgium, mating type analyses were performed. Because two mating types are needed for oospore production, frequency of occurrence of both was estimated with mating type analyses of 18 isolates. From the 18 analysed isolates, 17 were of mating type B1, only BmVL132 was B2. No secondary homothallism was observed.

Table 3-2: Isolates determined from samples in which oospores were detected.

Isolate	Lettuce cultivar	Greenhouse or open field	Sextet-code	IBEB race
BmVL013	Jumbis (Bl: 1-24, 27, 28, 30)	Open field	59-31-08-01*	
BmVL017	Brigade (Bl: 1-17, 21, 23)	Greenhouse	59-15-10-01*	
BmVL018	Brigade (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-01*	Bl: 26
BmVL019	Hertog (Bl: 1-17, 21, 23)	Greenhouse	59-15-10-01*	
BmVL021 ^x	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-01*	Bl:26
BmVL036	Mariken (Bl: 1-25, 27, 28, 30,31)	Greenhouse	nd	
BmVL037 ^x	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-01*	Bl: 26
BmVL038 ^x	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-01*	Bl: 26
BmVL039	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	nd	
BmVL040	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	nd	
BmVL054 ^x	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-01*	Bl: 26
BmVL091a	Troubadour (Bl: 1-17, 21, 23)	Greenhouse	63-31-58-05	
BmVL106	Hilton (Bl: 1-25, 27, 28)	Greenhouse	nd	
BmVL107b	Jumbis (Bl: 1-24, 27, 28, 30)	Open field	63-63-58-01	

^x: isolates are isolated after artificial inoculation with IBEB race Bl: 26.

*: sextet-codes are determined with the first IBEB determination set. The other isolates were determined with the second IBEB determination set (Table 2-2).

'nd' means the sextet code is not defined

With the oospores formed during mating type analyses (Figure 3-5), several attempts with different experimental methods were undertaken to stimulate oospore germination and infection of lettuce seedlings. Yet, these attempts were not successful.

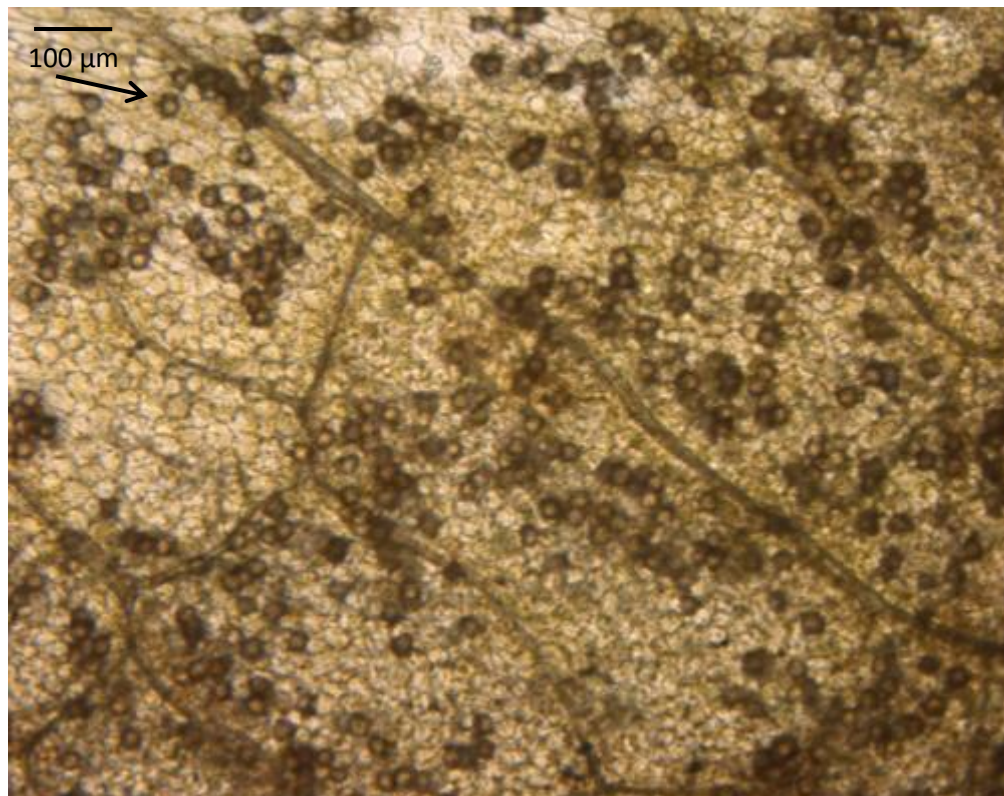


Figure 3-5: Leaf tissue with *B. lactucae* oospores (The arrow points at one oospore).

Conidia from *Lactuca serriola*

Another possible explanation for the noticed variability within the *B. lactucae* population, could be conidia from isolates on *L. serriola*. During our study, approximately 100 plants were found, mostly around train rails. Most of the plants had no symptoms resembling downy mildew symptoms. Approximately 10 plants were taken to the lab with symptoms similar to downy mildew symptoms. Yet microscopic verification pointed out that the causal agent of these symptoms was not *Bremia lactucae*. From our calls via the websites of the research centres, we received one report of *L. serriola* from Westerlo (Antwerp) but also this plant was not infected with downy mildew.

Seeds from wild *L. serriola* plants were collected and the seedlings of both *L. serriola* and *L. sativa* cv. Green Towers were inoculated with the in Flanders frequently detected *B. lactucae* race Bl: 26. The *L. serriola* plants were as susceptible to Bl: 26 as *L. sativa* cv. Green Towers plants (data not shown). These results together with the fact that *L. serriola* plants infected with *B. lactucae* were found in France (Maisonneuve et al. 2011), lead to the conclusion that conidia from *L. serriola* cannot be neglected as a potential source of inoculum.

Forecasting presence of airborne spores

With the Burkhard spore sampler airborne spores were captured at the end of 2012, the beginning of 2013 and in the spring of 2014. An overview of the sampling period is given in Table 3-3.

Table 3-3: Period and location of spore sampling with indication of visible sporulation symptoms of downy mildew and division of the spore sampler tape.

Tape number	Start date sampling	End date sampling	Sampling site	Visible downy mildew symptoms nearby sampler	Division of the tape
1	18/10/2012	24/10/2012	Open field	Severely infected lettuce heads in field	3 parts/day
2	25/10/2012	30/10/2012	Greenhouse	Symptoms on lettuce heads outside greenhouse (minimal sporulation)	3 parts/day
3	30/10/2012	06/11/2012	Greenhouse	Symptoms on lettuce heads outside greenhouse (minimal sporulation)	3 parts/day
4	8/11/2012	15/11/2012	Greenhouse	Symptoms on lettuce heads outside greenhouse (minimal sporulation)	3 parts/day
5	16/11/2012	22/11/2012	Greenhouse	No symptoms	3 parts/day
6	29/01/2013	07/02/2013	Greenhouse	Few symptoms in field	3 parts/day
7	28/02/2013	06/03/2013	Greenhouse	Symptoms in adjacent greenhouse compartment	3 parts/day
8	07/03/2013	12/03/2013	Greenhouse	No symptoms	3 parts/day
9	02/05/2014	08/05/2014	Greenhouse	Symptoms, first 3 days, next 4 days no symptoms	Per day
10	08/05/2014	14/05/2014	Greenhouse	No symptoms	Per day
11	15/05/2014	28/05/2014	Greenhouse	No symptoms	Per day
12	22/05/2014	28/05/2014	Outside greenhouse	No symptoms	Per day
13	28/05/2014	05/06/2014	Outside greenhouse	No symptoms	Per day
14	05/06/2014	12/06/2014	Outside greenhouse	No symptoms	Per day
15	12/06/2014	19/06/2014	Outside greenhouse	No symptoms	Per day

Sporulation is known to happen predominantly during the night (Su et al. 2004; Blancard et al.2006; Nordskog et al. 2007) and spore release to be triggered by light (Scherm and van Bruggen 1995; Su et al. 2000; Kushalappa 2001). Furthermore spore survival is reduced by UV radiation (Wu et al. 2002). In a study from Su and colleagues it was shown that release of spores was maximal at one to three hours after light initiation, and the number of detected spores was very low at 6 hours after light initiation (Su et al. 2000). Therefore it was expected to detect most spores during the first 6 hours after sunrise. Yet, 40 days sampling with a Burkhard spore sampler in the period from the 19th of

October 2012 until the 21st of November 2012 and from the 31st of January until the 3rd of March 2013 showed that there was no significant difference in the number of spore equivalents detected during the first 6 hours after sunrise, in the afternoon or at night (Figure 3-6). A comparison between the number of spore equivalents and meteorological data (average rainfall) revealed that on rainy days none or few spores were detected (Figure 3-7).

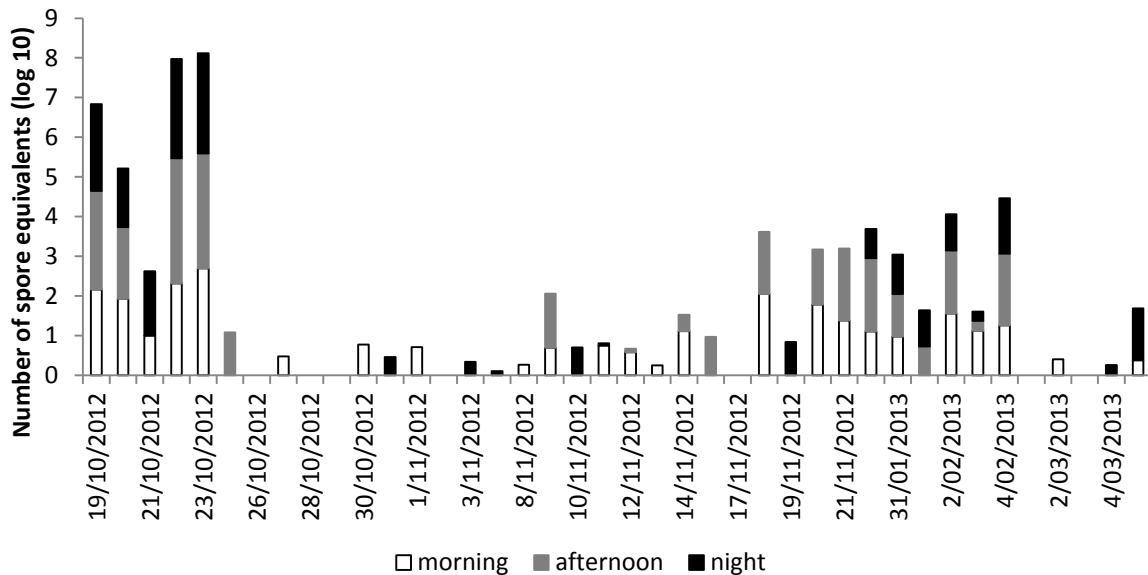


Figure 3-6: Number of *B. lactucae* spore equivalents (log 10). Sampling days were divided in three periods: the first six hours after sunrise (morning), the period from six hours after sunrise until sunset (afternoon) and the period from sunset until sunrise (night). On 24/10/2012, on 5/11, 6/11, 7/11 and 15/11/2012 and from 22/11 until 29/11/2012 the spore sampler was not operative, thus no data are shown for this dates. No significant difference could be found between the numbers of detected spore equivalents in the three sampling periods for the 40 sampling days.

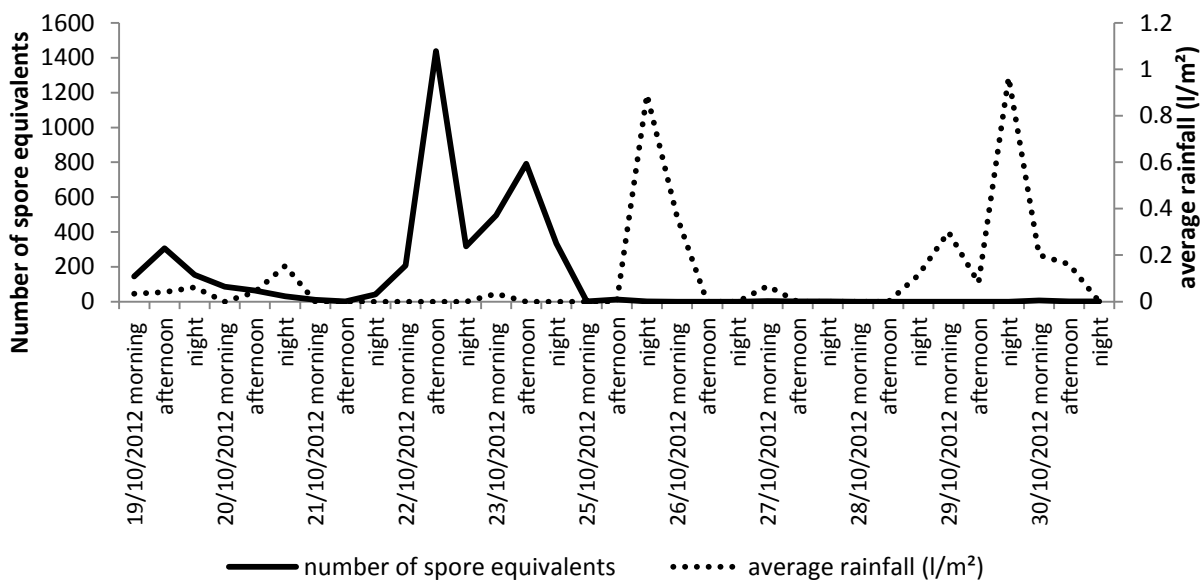


Figure 3-7: Number of *B. lactucae* spore equivalents detected with the Burkhard spore sampler and average rainfall (l/m²) in the sampling period 19/10/2012 until 30/10/2012. On 24/10/2012 the spore sampler was not operative, thus no data are shown for this date. The sampled days were subdivided in three periods: the first 6 h after sunrise (morning), the period from 6 h after sunrise until sunset (afternoon) and the period between sunset and sunrise (night).

3.3.1.2 Influence of relative humidity and temperature on life stages of *Bremia lactucae*

Although several epidemiological studies had been performed before, we decided to analyse the epidemiology of *B. lactucae* isolates collected in Belgium because in the previous studies different climatological optima for different life stages of the pathogen were described, depending on the region of sampling (Powlesland 1954; Verhoeff 1960; Scherm and van Bruggen 1994a; Scherm and van Bruggen 1994b; Davis et al. 1997; Kushalappa 2001; Carisse and Phillion 2002; Su et al. 2004; Wu et al. 2005; Nordskog et al. 2007).

In the microscopical study performed to divide the life cycle in different stages, we observed that some spores started germination process at 1.5 h post inoculation (hpi) and at 3 hpi the majority of the spores had germinated. A mycelium network was developed at 24 hpi and sporangiophores appeared at 6 days post inoculation (dpi). At 10 dpi abundant sporulation was visible (Figure 3-8). Therefore we defined the first 24 hpi as the germination and penetration period, the period 1-5 dpi as the incubation period and 6-10 dpi as the sporulation period.

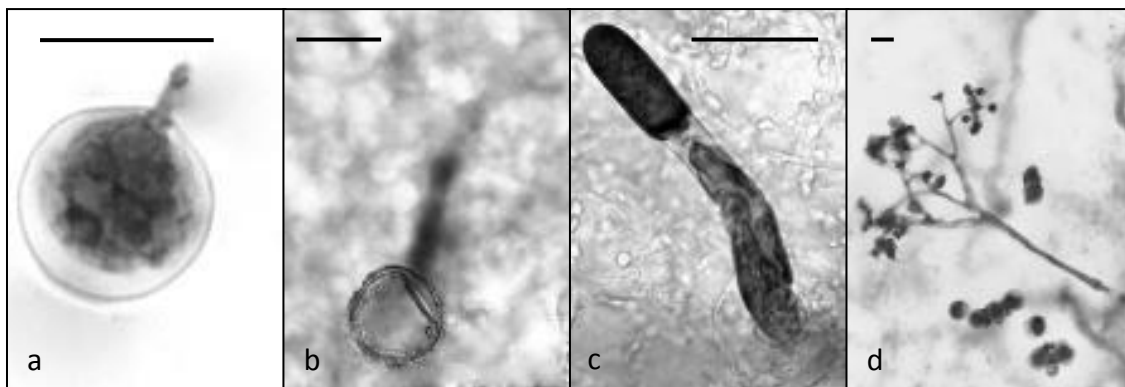


Figure 3-8: Asexual development of *B. lactucae*. a) 2 hpi: start germination b) 3-24hpi: penetration and development of mycelium inside host tissue c) 6 dpi: start development sporangiophores d) 10 dpi: sporulation. The scale bars in each picture represent 25 μ m.

We assessed the influence of temperature and relative humidity in the different periods of the asexual development of the pathogen (Figure 3-9). During all life stages, 17.5 °C was found to be the optimal temperature because at that temperature, symptoms appeared earliest. Reducing this temperature during germination and penetration period could reduce the disease incidence significantly with approximate 18% when the temperature was reduced to 6 °C. Raising the temperature during this period to 23 °C could significantly reduce the disease incidence from 83% to 52% and at 30 °C the pathogen was not able to infect any plants (Figure 3-9A). Also the relative humidity plays a key role during germination and penetration (Figure 3-9B) because keeping the relative humidity below 90% during these first 24 hpi, reduced the disease incidence with 94%. In experiments in which the relative humidity was kept below 90%, the relative humidity was on average 85%. Figure 3-9C shows that temperature during incubation period has also an influence on the development of the pathogen, resulting in a reduction of the disease incidence from 91% of plants grown at 17.5 °C to 6.4%, 4.9% and 0% for plants grown during this period at 6 °C, 23 °C and 28 °C respectively. Temperature effect during sporulation period was very similar to the effect during germination and penetration period (Figure 3-9D): relatively small reduction in disease incidence by lowering the temperature from 6 to 10 dpi to 6°C, strong reduction by raising the temperature to

26°C and no infection at 30°C. The lowest temperature of our growth chamber in the lab was 6°C, yet by sampling with the spore sampler in the greenhouse, we observed that in a field with visible sporulation, no spores were detected when temperature was beneath 0°C (data not shown).

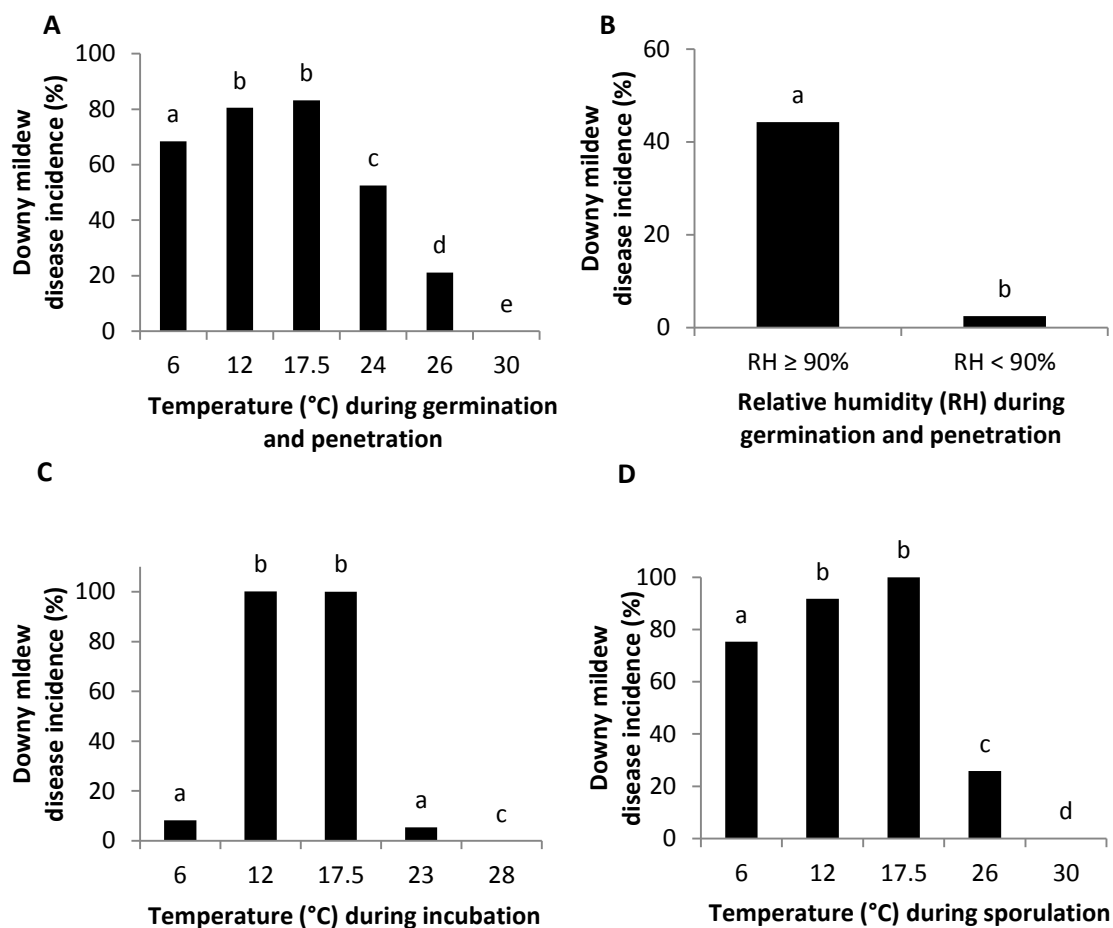


Figure 3-9: Influence of temperature and relative humidity during different periods of the disease development on the downy mildew disease incidence. A: influence of temperature during germination/penetration (first 24 hpi) period. **B:** Influence of relative humidity (RH) during germination and penetration period. **C:** Influence of temperature during incubation (1 to 5 dpi), **D:** Influence of temperature during sporulation period (6 to 10 dpi). During the experiments to assess the influence of the temperature, the relative humidity was minimum 90%, and during the experiment for relative humidity influence evaluation, temperature was 17.5°C. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

In search for relative humidity criteria for sporulation, we analysed the relative humidity in different experiments with successful infection from 2010 and 2011 that were performed at the three research centres Inagro, PCG and PSKW. Based on the results summarized in Table 3-4 it can be hypothesized that a period of minimum 5 hours with a relative humidity of at least 85% was the minimum requirement, preceding sporulation in all experiments (indicated in grey in Table 3-4). Experiments were performed in different seasons, indicating that this criterion was independent of temperature.

Table 3-4: Indication of periods (in hours) with different relative humidity values during the night before first downy mildew symptoms were detected in 13 greenhouse experiments in 2010 and 2011 in the three research centres Inagro (I), PCG and PSKW. The night is defined as the period from sunset, which is the moment measured solar radiation equals 0 W/m^2 , until the moment of sunrise, when solar radiation is measured. Experiments were performed in different seasons: S = Summer, Sp= Spring, A = Autumn and W = Winter.

Experiment number	1	2	3	4	5	6	7	8	9	10	11	12	13
Season	Sp	S	W	S	W	Sp	A	A	W	A	A	A	A
Research centre	I	I	I	I	PCG	PCG	PCG	PCG	PCG	PSKW	PSKW	PSKW	PSKW
H with RH < 80%	0.2	2.5	2	0	0	1	0.5	0	0	0.2	0	0	0
H with 80% ≤ RH < 85%	3.7	3	2	0	0.5	1.5	1.5	0	0	0.3	2	0.75	0
H with 85% ≤ RH < 90%	2.6	5	5	0.33	3.5	0.5	2	6	0	0.2	5	3	0
H with RH ≥ 90%	2.5	0	0.5	9.33	2	6	6.5	6	14	9.5	0	2.5	13

Because in none of the greenhouse experiments could be avoided that the relative humidity raised above 85% for at least 5 hours during an entire experiment, there is no direct proof of this hypothesis. Yet, we never had an experiment with a shorter period with a relative humidity above 85% followed by sporulation. In the epidemiological background and establishment of criteria of BREMCAST is described that sporulation was observed at relative humidity values about 90%, yet this model uses the criterion of 7 hours of leaf wetness as minimal requirement for sporulation (Kushalappa 2001, Table 1-3). Furthermore in Modell-Analys the relative humidity sporulation criterion is 90% during at minimum four hours (Jönsson et al. 2005, Table 1-3), which differs 5% with our criterion (85%). This difference might be due to the sensor position in the greenhouse or to differences in the pathogen characteristics in different regions. We used criteria based on information gathered with the climate box, which is positioned approximately one meter above the crop. In a few experiments we compared relative humidity measurements with a sensor with capacitive measurement at leaf height with measurements of the climate box and found a difference of 5 to 10% (Figure 3-10).

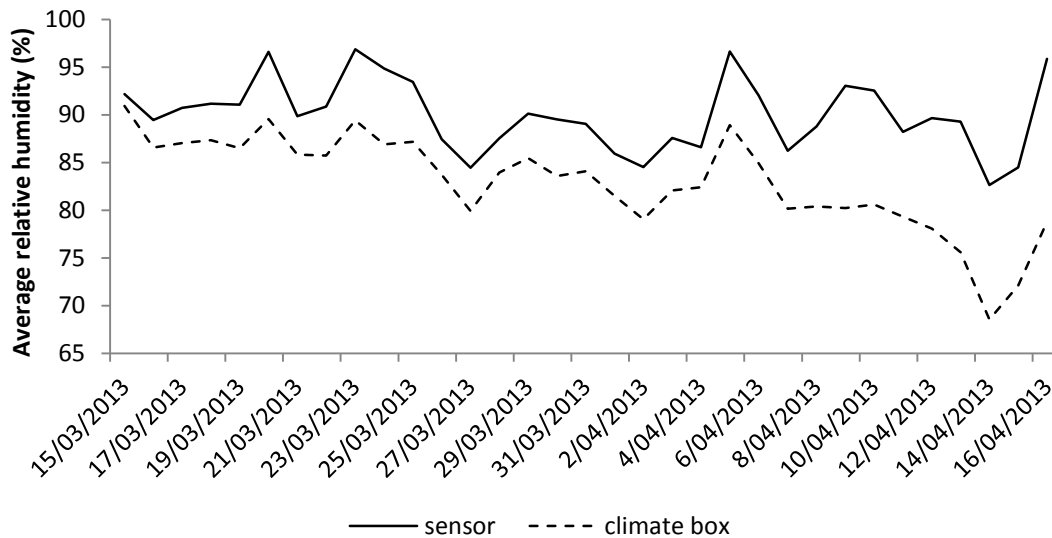


Figure 3-10: Difference in average relative humidity measured by the climate box and by a sensor positioned at crop level.

3.3.2 Determination of optimal fungicide applications

Keeping the relative humidity low by all means, is not economically feasible. Fungicide applications are thus necessary. Therefore we tested fungicides to determine the optimal fungicide choice on different moments relative to the inoculation moment. In all of these experiments, fungicides were applied in the legally prescribed dose. In total we did seven greenhouse experiments (2 at Inagro, 2 at PCG and 3 at PSKW) and three lab experiments to evaluate the performance of the fungicides, focussing on the optimal fungicide for different application moments after inoculation.

In a lab experiment, lettuce plants of cv. Green towers were treated four days after inoculation with Previcur N, Fenomenal and Fubol Gold. Plantlets were inoculated with *B. lactucae* BI: 26 (4.8×10^4 spores /mL) when the plants were seven days old and in two-leaf stage. The results of this experiment are shown in (Figure 3-11). Application of Previcur N 4 days after inoculation, could not significantly reduce the disease incidence of the plants. Approximately 3% of the inoculated leaves of plants treated with Fenomenal or Fubol Gold showed sporulation.

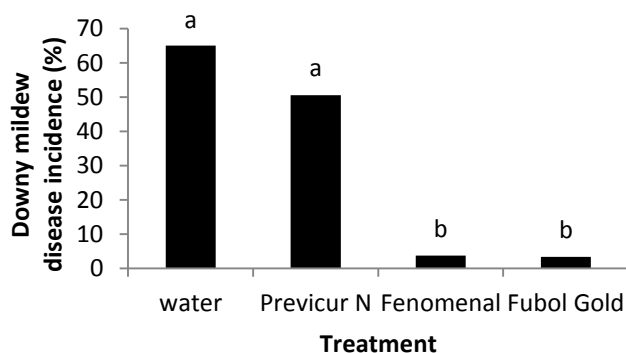


Figure 3-11: Downy mildew disease incidence, determined 10 days post inoculation, of plants treated 4 days after inoculation with fungicides or water (control).

In the summer of 2011 in an experiment at Inagro lettuce cv. Flandria (Bl: 1-17, 21-23) was planted on the 3rd of August. Plants were treated with Paraat and Fubol Gold 1 day before inoculation and 1 week after inoculation. The plants were inoculated on the 11th of August. Each treatment was repeated three times. The experiment was set up as a random block design, with 20 plants in each plot (repetition of each treatment). In this experiment these application moments were chosen in addition to other experiments, where other application moments closer to the moment of inoculation were tested. One day before inoculation was considered in this experiment as the positive control, of which was known from previous experiments that fungicide applications at this time point provide good downy mildew control. Purpose of this experiment was to analyse how long the effect would last and application one week after inoculation was chosen to determine how long after inoculation the fungicides could still be applied and offer sufficient protection against downy mildew. Symptoms appeared 8 days after inoculation, on the 19th of August, thus the incubation period was very short. At 8 dpi and 15 dpi disease incidence and disease index were determined, which is shown in Figure 3-12. Applied one day before inoculation, both Fubol Gold and Paraat offered very effective protection against downy mildew, and this effect was still very strong 15 days after inoculation. Applications one week after inoculation were too late for this experiment with such a short incubation period and Paraat and Fubol Gold did not have an effect on sporulation.

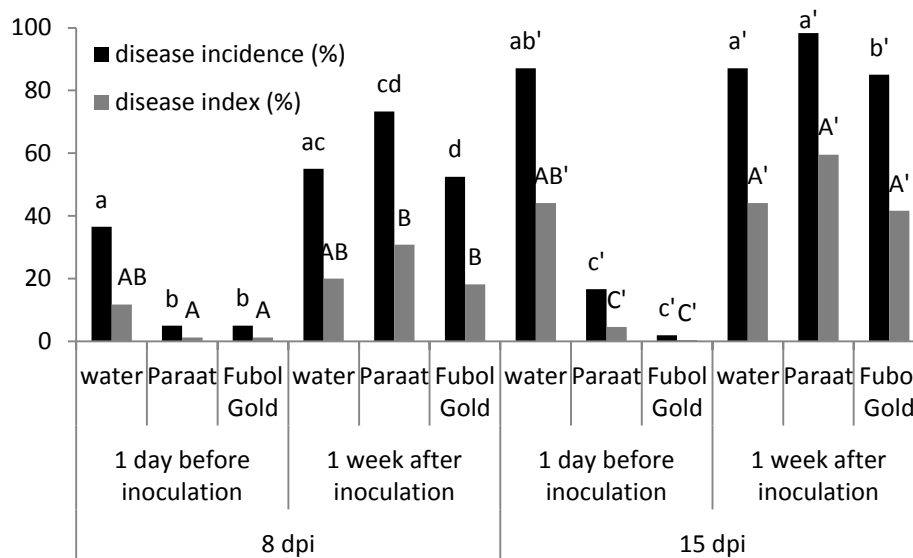


Figure 3-12: Downy mildew disease incidence and disease index at 8 days and 15 days after inoculation (8 dpi and 15 dpi) upon treatment with water (control), Paraat or Fubol Gold 1 day before inoculation or 1 week after inoculation. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses for disease indices and binary logistic regression analysis for disease incidence, $p = 0.05$). Note that no error bars are shown because data were not normally distributed.

Similar experiments were performed on the three research centres. The different experiments could not be pooled because different fungicide treatments were tested in different experiments and the disease incidence of the control group of plants varied strongly between the experiments (Table 3-6). An influence of the growth season on the length of the incubation period was noticed by analysing the greenhouse experiments of 2010 and 2011 (Figure 3-13): the shortest incubation period, 10 days, was observed in summer, when the average temperature of the incubation period was approximately 20 °C and the longest incubation period was observed in winter, 26 days, with an

average temperature of 7 °C during this incubation period. Therefore applying fungicides one week after inoculation may have a different effect when the incubation period is one week (and the fungicide should thus affect the sporulation to be most effective) in comparison with applications in seasons when the incubation period is approximately one month (and the fungicide should thus affect the mycelium growth to be most effective). The results obtained with the experiments to evaluate the fungicides were thus divided according to the seasons in three groups: Winter, Autumn + Spring, and Summer.

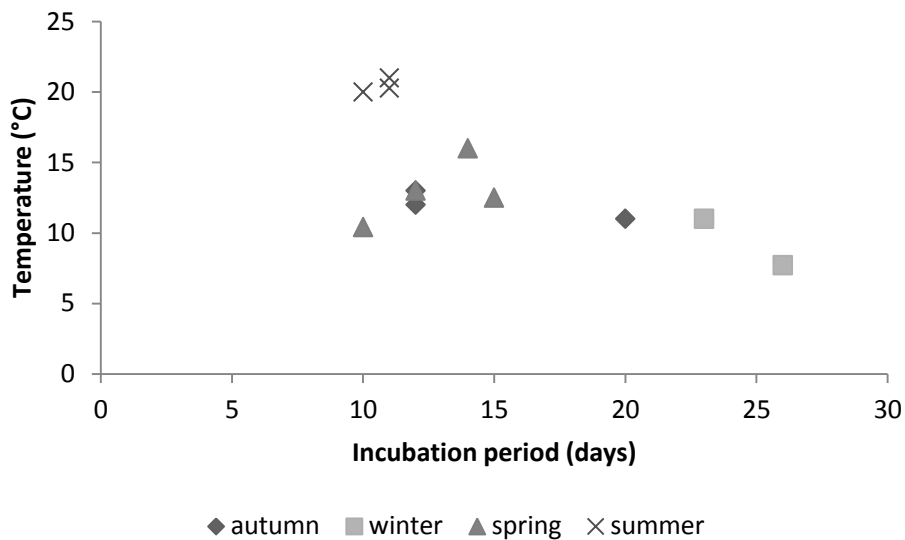


Figure 3-13: Influence of the average temperature and cultivation season on the length of the incubation period of *B. lactucae*.

In Table 3-5 the division of the cultivation seasons is shown, depending on the moment of planting (personal communication Isabel Vandeveld, PSKW, crop consultant).

Table 3-5: Division of cultivation rounds in seasons, based on moment of planting of the lettuce plants.

Season	Date of planting
Spring	27 th of December – 22 nd of April
Summer	23 rd of April – 29 th of August
Autumn	30 th of August – 2 nd of November
Winter	3 rd of November – 26 th of December

Table 3-6: Overview of fungicide experiments performed in the laboratory or in the greenhouses of Inagro, PCG or PSKW varying the application moments relatively to the moment of inoculation, expressed as days post inoculation (dpi) and the cultivation season.

A.m.	Cultivation season	Experiment performed by	Untreated control	fenamidone + fosethyl	mancozeb + metalaxyl-M	dimetho-morf	propamo-carb	mandipro-pamid	azoxy-strobin	fosethyl + propamocarb
1 DPI	Lab	UGent	82.5%	2.0%	3.0%	-	0%	11.0%	12.0%	12.0%
1 DPI	Autumn	PCG	11.1%	0%	2.8%	0%	2.8%	-	-	-
1 DPI	Autumn	PSKW	98.7%	10%	10%	86.7%	0%	-	-	-
1 DPI	Summer	PCG	19.4%	0%	0%	2.8%	0%	0%	-	-
1 DPI	Winter	Inagro	19.4%	-	-	-	7.0%	8.3%	2.8%	2.8%
2 DPI	Autumn	PSKW	98.7%	70%	50%	93.3%	0%	-	-	-
2 DPI	Winter	Inagro	27.8%	-	-	-	-	13.9%	16.7%	6.5%
3 DPI	Autumn	PCG	11.1%	0%	2.8%	0%	8.3%	-	-	-
3 DPI	Lab	UGent	96.2%	3.7%	3.3%	71.7%	50.5%	-	-	-
3 DPI	Lab	UGent	66.7%	53.7%	0%	1.1%	0%	-	-	-
3 DPI	Winter	PSKW	100%	0%	0%	-	-	100%	-	0%
5 DPI	Winter	Inagro	13.9%	-	-	-	-	5.6%	5.6%	16.7%
6 DPI	Autumn	PCG	11.1%	0%	8.3%	2.8%	0%	-	-	-
6 DPI	Summer	PSKW	91.7%	76.7%	76.7%	60%	66.4%	80%	-	-
7 DPI	Autumn	PSKW	98.7%	96.7%	88.9%	100%	73.3%	-	-	-
7 DPI	Summer	Inagro	36.5%	-	52.5%	73.3%	-	-	-	-
7 DPI	Summer	PCG	19.4%	2.8%	25.0%	16.7%	2.8%	16.7%	-	-

Based on the results summarized in Table 3-6, the fungicides are ranked as is shown in Table 3-7. Because some of the tested fungicides are no longer allowed in Belgium we represented the results with the active ingredients of the fungicides because in our results fungicides with the same active ingredient gave similar levels of protection against downy mildew. The moments of application are expressed as days post inoculation, which refers to the moments inoculum could have been present and germination and penetration could have been possible, based on the climate conditions.

Table 3-7: Ranking the different tested fungicides against *B. lactucae* from first choice to fifth choice for different application moments relatively to the inoculation moment and dependent on the cultivation season. "A.m." is the application moment expressed in days post inoculation (dpi). "nd" means this choice is not defined.

A. m. (dpi)	Cultivation season	1 st choice	2 nd choice	3 rd choice	4 th choice	5 th choice
1	Winter	azoxystrobin or fosethyl + propamocarb	propamocarb	mandipropamid	nd	nd
	Autumn/ spring	propamocarb	fenamidone + fosethyl	azoxystrobin or fosethyl + propamocarb	mandi- propamid	dimetho- morf
	Summer	propamocarb	fenamidone + fosethyl	mandipropamid or mancozeb + metalaxyl-M	azoxystrobin, fosethyl + propamocarb or dimetho- morf	nd
2-4	Winter	fosethyl + fenamidone or mancozeb + metalaxyl-M	fosethyl + propamocarb	azoxystrobin	mandi- propamid	nd
	Autumn/ spring/ Summer	fenamidone + fosethyl	propamocarb	mancozeb + metalaxyl-M	dimethomorf	nd
	Summer	mancozeb + metalaxyl-M	propamocarb	dimethomorf	fenamidone + fosethyl	
5-7	Winter	azoxystrobin or mandipropamid	nd	nd	nd	nd
	Autumn/ spring	propamocarb	fenamidone + fosethyl	dimethomorf	mancozeb + metalaxyl	
	Summer	propamocarb	fenamidone + fosethyl	mandipropamid	nd	nd

To avoid sporulation we selected fungicides which are known to have an effect against sporulation and/or germination (personal communication with phytopharmacy companies via Aaike Bogaert) : dimethomorf, propamocarb and fenamidone both are effective to suppress sporulation and germination, while metalaxyl-M suppresses sporulation and mandipropamid and azoxystrobin both inhibit germination. Therefore the first three are preferred and based on the results of the fungicide experiments, they are ranked:

1st choice: propamocarb

2nd choice: a fungicide based on fenamidone and fosethyl

3rd choice: dimethomorf

4th choice: mandipropamid

5th choice: metalaxyl-M

Both for applications to prevent germination and penetration as to prevent sporulation, a new fungicide application is only possible if the legal restrictions concerning the period between two applications, the maximum number of applications during one cultivation cycle or per year and the period between the last treatment and harvest are taken into account. These restrictions are summarized in Table 3-8.

Table 3-8: Legal restrictions for downy mildew fungicide applications in Belgium.

Product	Interval between 2 treatments (days)	Period between last treatment and harvest (days)	Maximum number of applications per year (y) or per cultivation cycle (cc)
Amistar/Ortiva	7	30	2x/cc and 4x/y
Proplant	10	Only apply before leaves of adjacent plants touch each other	3x/cc
Previcur Energy	14	14	2x/cc
Fenomenal	10	Application from 7 days post planting until lettuce head volume is 20% of lettuce head volume at harvest	2x/cc
Paraat	10	14	2x/cc
Revus	7	7	1x/cc
Fubol Gold	10-14	28	2x/cc
Mirador	7-14	14	2x/cc

3.3.3 Development of the decision support tool

The decision support tool consists of advice to reduce sources of inoculum, adaptation of the greenhouse to suppress the development of the pathogen and suggests most effective fungicide applications.

3.3.3.1 Inoculum reduction

Avoid sources of primary inoculum

Oospores were not very frequently found, only in 10% of the samples. Furthermore, predominantly occurring mating type was B1, with only 1 of the 18 analysed isolates being B2. Moreover, attempts

to stimulate oospore germination and infection of lettuce seedlings were not successful. These findings might indicate that oospores are probably not the most important source of primary inoculum. Still, because oospores were found and both mating types were determined, it should not be ruled out either. Therefore to reduce sources of primary inoculum, lettuce plant debris should be removed from the greenhouse or field after harvest.

Lactuca serriola was often found and although none of the plants were naturally infected with *Bremia lactucae*, artificial inoculation with BI: 26 was successful, therefore, to reduce this potential source of inoculum, *L. serriola* plants should be removed from (the surrounding of) the field or the greenhouse. Most ideal would be to remove all *L. serriola* plants in a circle of 3000 m around the lettuce field because the influence range of downy mildew incidence at one location on other locations is estimated to be between 80 m and 3000 m (Valade 2012).

Estimate risk for airborne conidia

Based on sampling with the spore trap (Figure 3-6 and Figure 3-7) and the results of the epidemiological study (Figure 3-9), the conclusion is that there is an actual risk for airborne conidia on predominantly dry days or nights with temperatures between 6 °C and 23 °C. These criteria for temperatures are for inside the greenhouse. Yet, to use weather forecasts for risk assessment, these criteria were changed to a minimum of 0 °C and a maximum of 23 °C outside the greenhouse, in open air. The maximum temperature was based on the epidemiological study with a very significant reduction of the disease incidence when temperature was 23 °C during incubation (Figure 3-9C) and if temperature outside the greenhouse is 23 °C, temperature inside the greenhouse will be 23 °C or higher. Because the possible minimum temperature attainable with the growth chamber for the epidemiological study was 6 °C, the minimum temperature for outside the greenhouse, was determined based on observations with the spore sampler. It was observed that no spores were detected inside the greenhouse if temperatures outside the greenhouse were 0 °C or lower even when plants with sporulation were present in the greenhouse. Therefore, 0°C was chosen as minimum temperature.

3.3.3.2 Adaptation of the greenhouse climate

After the epidemiological study of the pathogen, it was decided to focus on adaptation of the relative humidity in the greenhouse instead of the temperature because effect of a reduced relative humidity was shown to be more effective (Figure 3-9).

To avoid germination and penetration, relative humidity should be kept below 90% (Figure 3-9B). When airborne spores might be present, a period of at least 2 hours with a relative humidity of minimum 90% would be sufficient to allow successful germination and penetration because in the microscopic study it was observed that a number of conidia had already germinated and penetrated the host within 2 hours after inoculation (Figure 3-8). To avoid sporulation, relative humidity should stay below 85%. If the relative humidity was at least 5 hours minimum 85%, there might be a risk for sporulation (Table 3-4) if plants were infected. First sporulation symptoms might appear 5 days post inoculation in optimal circumstances (Figure 3-8) and the longest observed incubation period in all performed greenhouse experiments was 32 days.

The different aspects to limit inoculum, reduce the development of *B. lactucae* and apply fungicides as efficient as possible are combined as four steps of a decision support tool (Figure 3-14).

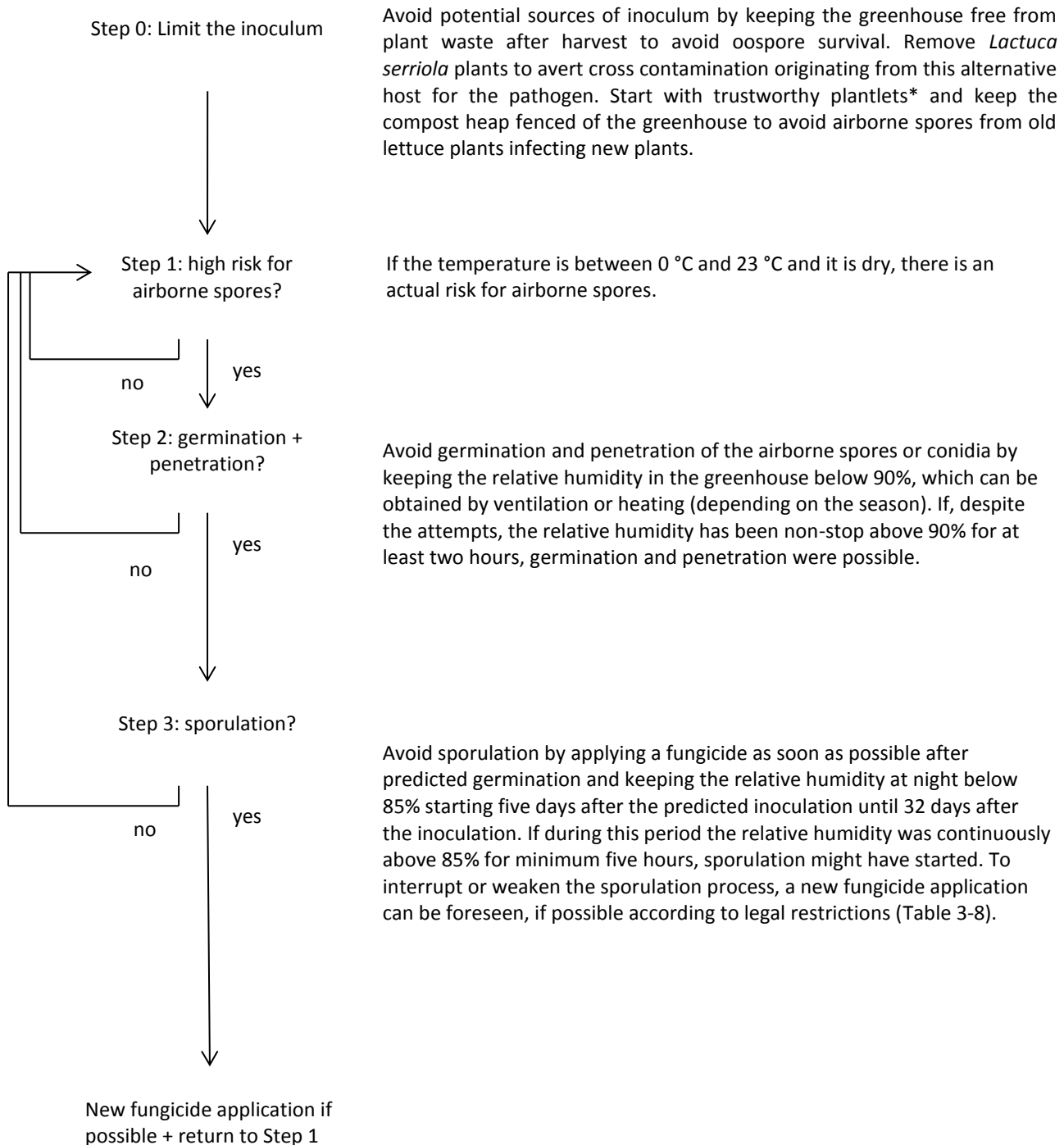


Figure 3-14: Flowchart of the different steps of the decision support tool, starting with advice to avoid inoculum, risk assessment and advice for greenhouse climate adaptations, supplemented with advice for fungicide treatments. * In this study no research for the prevalence of *B. lactucae* originating from plantlets infected before purchase was done, yet this has been determined as a possible source of inoculum (Isabel Vandevelde, PSKW crop consultant).

3.3.4 Development of the web application

In cooperation with network engineers at Inagro, a web application was developed to make the decision support tool user friendly. The application is part of an agriculture application platform, available online via <http://lap.inagro.be>. This platform collects the different models or applications developed in cooperation with the research centres PCG, PSKW and Inagro for an integrated management of various pests and diseases, which helps growers to find advice for their different crops. A grower should subscribe to one of the research centres to get access to the applications.

At first use of this application for downy mildew control, a grower fills in the address of his greenhouses because the weather forecast of step 1 is location dependent. The grower needs to input planting and harvest dates for each new crop cycle to determine the beginning and end of the crop cycle in the application. All warnings and advices are sent in short version as a text message to the growers mobile phone and a more extended version is sent via e-mail. Meteorological data of the royal meteorological institute of Belgium (<http://www.meteo.be/meteo/view/en/65239-Home.html>) form the input for estimating the risk of airborne spores. The climate inside the greenhouse is measured with a climate box which is usually used by Belgian lettuce growers. These greenhouse climate data are used to estimate pathogen development in the greenhouse. Dates of fungicide applications should be registered in the web application for their influence on future fungicide advice.

3.3.5 Evaluation of the decision support tool

3.3.5.1 Effect on number of fungicide applications and downy mildew control

During the development of the decision support tool, the effect on the number of fungicide applications and downy mildew disease control was tested in greenhouses of the three research centres. After each experiment the results of the three research centres were compared and the decision support tool and web application were fine tuned. At Inagro two experiments were performed with the final version of the decision support tool.

First experiment at Inagro with the final version of the decision support tool

First symptoms were observed on the 21st of October and plants were harvested on the 30th of October. Because plants were harvested earlier than expected, the fifth fungicide application of a standard, calendar based schedule, which was foreseen for the 23rd of October, was not applied. The process of the experiment is shown in Figure 3-15.

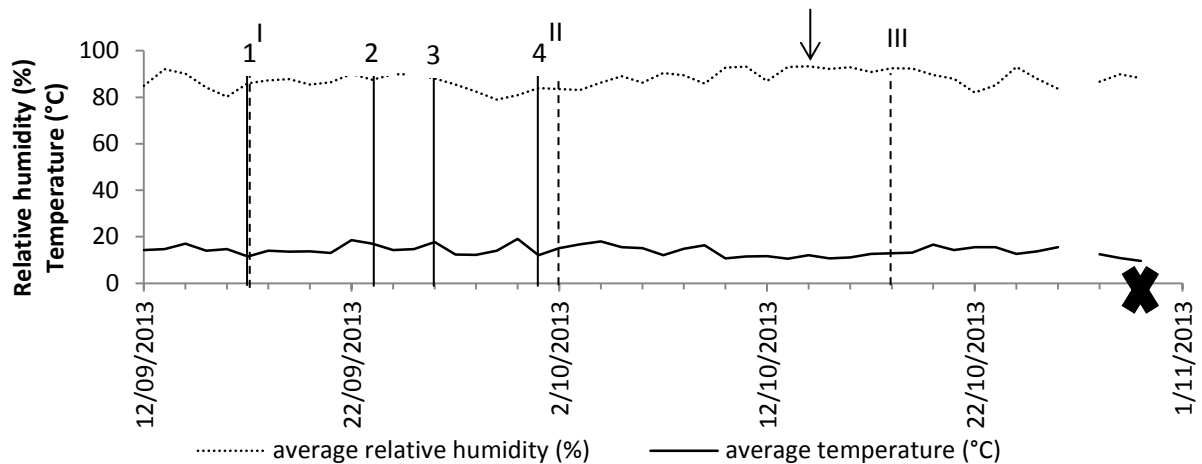


Figure 3-15: Temperature and relative humidity during the experiment. Plants were planted the 12th of September and harvest the 30th of November (indicated with cross). The vertical lines indicate the moments of fungicide applications: the full lines are the applications in the standard calendar based schedule and the dotted lines are the moment of applications as prescribed by the decision support tool. Calendar based applications: 1: Fenomenal, 2: Previcur Energy, 3: Paraat, 4: Proplant. Applications according to the decision support tool: I: Proplant, II: Proplant, III: Fenomenal. The arrow indicates the moment of inoculation with *B. lactucae*. On the 27th of October there was a technical problem and no measurements are recorded for that date.

Disease incidence and index were evaluated at the moment first symptoms were observed and the results are shown in Figure 3-16. The main conclusion of this experiment is that when fungicide applications are performed when prescribed by the decision support tool, less applications are done in comparison with a calendar based schedule, resulting in a downy mildew control which is at least as effective as supplied control by fungicide applications on a calendar based schedule. In this experiment the disease incidence and disease index were significantly lower for the plants that were treated according to the advice of the decision support tool, yet distribution in time of the fungicide applications on a calendar based schedule was not representative for practice because the third application was too soon after the second. Based on the results obtained with the decision support tool, the second and third application based on the calendar schedule were not necessary. The second and third application prescribed by the decision support tool were the reason of the better disease control obtained with the tool in comparison with the calendar based schedule.

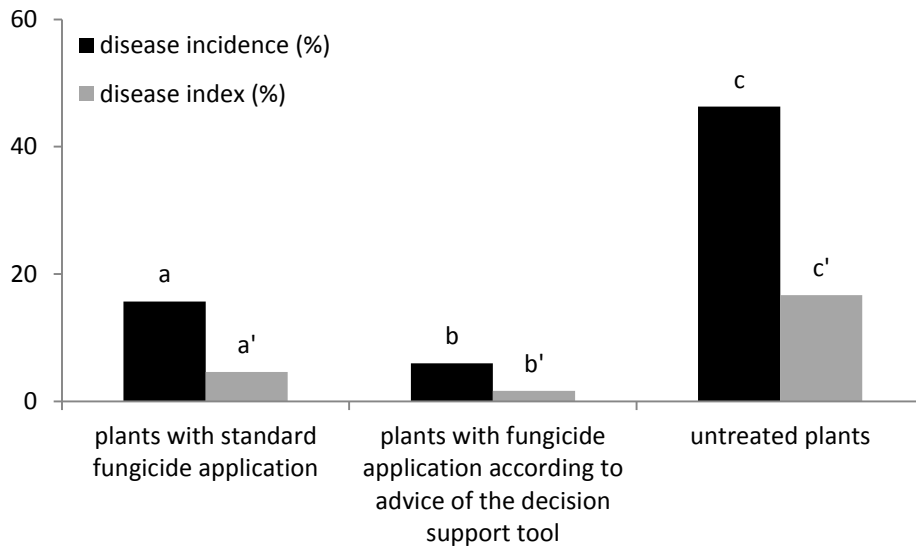


Figure 3-16: Downy mildew disease incidence (%) and disease index (%) determined 10 days post inoculation, of plants which received fungicide applications on a calendar based schedule, only when advised by the decision support tool or which were untreated during the entire experiment.

Second experiment at Inagro with the final version of the decision support tool

The process of the experiment is represented in Figure 3-17.

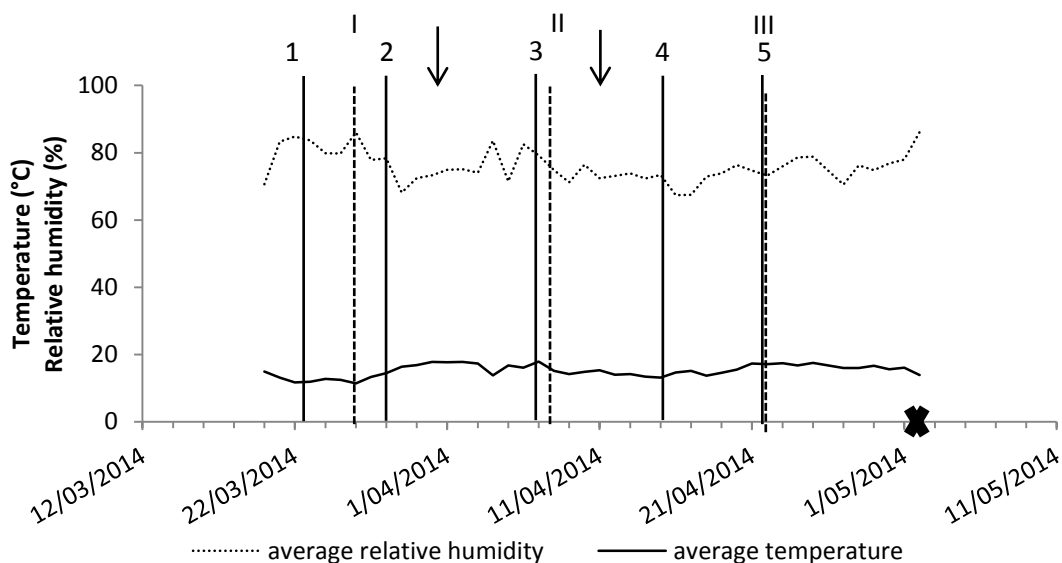


Figure 3-17: Temperature and relative humidity during the experiment. Plants were planted the 20th of March and harvested the 5th of May 2014 (indicated with cross). The vertical lines indicate the moments of fungicide applications: the full lines are the applications in the standard calendar based schedule and the dotted lines are the applications as prescribed by the decision support tool. Calendar based applications: 1: Fenomenal, 2: Previcur Energy, 3: Previcur Energy; 4: Proplant, 5: Revus; I: Proplant, II: Previcur Energy, III: Revus. The arrows indicate the moments of inoculation with *B. lactucae*.

During this experiment, average daily temperature was between 11 and 18 °C and there was not much rain fall predicted or registered, resulting in an actual risk for airborne spores. Therefore a reduction of the relative humidity below 90% was advised and attempted from the start of the crop

cycle to prevent spore germination and penetration. Yet, fungicide treatments were required according to the decision support tool on March the 26th, April the 8th and April the 22nd with Proplant, Previcur Energy and Revus, respectively. Concentrations of the used fungicides were the same as for the standard fungicide applications. The standard fungicide schedule prescribed five fungicide treatments, the decision support tool three. The first symptoms were noticed on the 2nd of May and disease incidence and index were then evaluated.

The effects of the different applications schedules and of the climate adaptations on the disease incidence and disease index are shown in Figure 3-18. The disease incidence of plants with standard fungicide applications was not significantly different from the disease incidence of plants with fungicide applications advised by the decision support tool, 21.9% versus 22.6% respectively, but the disease incidence of the untreated plants was significantly higher (35.8%). Increasing the relative humidity during the germination and penetration period (first 24 hpi) resulted in a disease incidence of 97.4%, which is significantly higher than for the untreated plants without increased relative humidity. The results for the disease index were completely following the same tendency. Disease index of untreated plants without increased relative humidity was approximately 36% versus a disease index of 60% for untreated plants with increased relative humidity. Disease indices of plants which had fungicide applications based on a calendar schedule or based on advice of the decision support tool were significantly lower than the index of plants without fungicide treatment and were not significantly different from each other: 7% and 6.7% respectively. Keeping the relative humidity below 90% is thus an important step in the disease control. Because symptoms were firstly observed on the 2nd of May, the artificial inoculation of the 31st of March was probably not successful and the perceived symptoms are presumably caused by the second artificial inoculation.

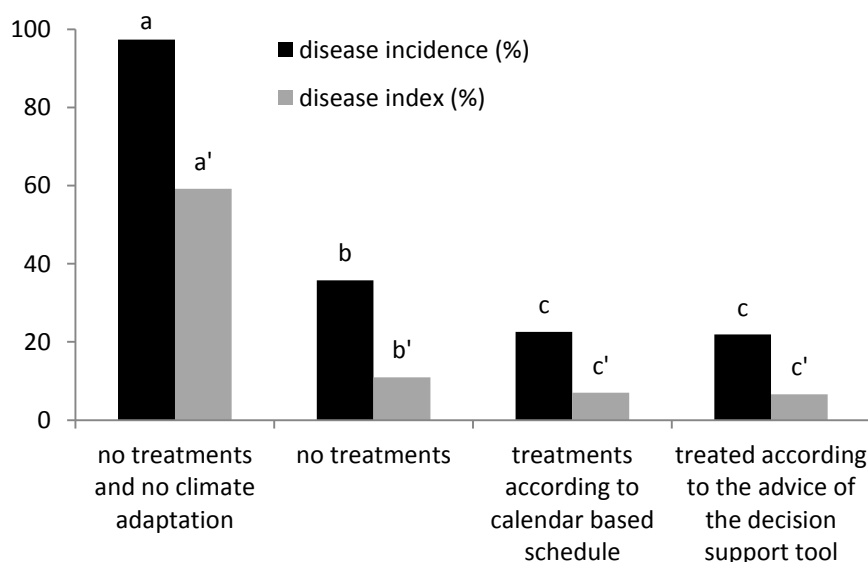


Figure 3-18: Difference in downy mildew disease incidence of plants with standard fungicide application, plants with model advised fungicide application, untreated plants and untreated plants with increased relative humidity during germination and penetration. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Also at PCG and PSKW experiments with the final version of the decision support tool were performed at the end of 2013 and 2014. In each experiment minimum three repetitions with at least

20 plants per repetition for each treatment were foreseen. In the experiment of PCG three applications less were prescribed by the decision support tool in comparison with the calendar based schedule, yet disease control could not be compared because there was no infection. At PSKW in the first experiment one application less was foreseen for the plants treated according to the decision support tool in comparison with the plants treated according to the calendar based schedule. In this experiment the disease control obtained with the decision support tool was less effective for the most sensitive lettuce cultivar. However, also from the plants treated according to the calendar based schedule 75% were infected with downy mildew, indicating that disease pressure was extremely high in this experiment. In the second experiment at PSKW, three applications less were prescribed by the decision support tool in comparison with the calendar based schedule, resulting in a downy mildew disease incidence and index which were not significantly different from the results obtained with the calendar based schedule. The results are summarized in Table 3-9.

Table 3-9: Experiments with the final version of the decision support tool performed at PCG and PSK. ‘Standard’ refers to fungicide application with a calendar based schedule. ‘dst’ is the abbreviation for decision support tool.

Research centre	Lettuce cv(s).	Date of				N° of fungicides based on		Downy mildew disease incidence (%)		Downy mildew disease index (%)	
		planting	inoculation	1 st symptoms	harvest	standard	dst	standard	dst	standard	dst
PSKW	Hofnar	2/10/2013	25/10	4/11	9/12	5	4	75	99.2*	31.5	75.2*
	Halewyn							0	0	0	0
PCG	Brighton	29/10/2013	16/12	/	28/1	5	2	0	0	0	0
PSKW	Flandria	29/4/2014	14/5	3/6	10/6	5	2	3.13	7.5	0.78	2.03
	Cosmopolia		22/5					0.63	1.25	0.16	0.31
			28/5								

Resistance of used lettuce cvs.: Hofnar: Bl: 1-23, 25, 31, Halewyn: Bl: 1-23, 25, 31, Brighton: Bl: 1-31, Flandria: Bl: 1-17, 21, 23, Cosmopolia: Bl: 1-25, 27, 28, 30, 31.

* indicates the results are significantly different from the results obtained with standard cultivation method.

3.3.5.2 Effect on basal rot

Because the number of fungicide applications can be reduced by implementing the decision support tool, it was likely that the crop would be more susceptible to diseases such as basal rot, since some of the registered fungicides against downy mildew, are also effective against some basal rot pathogens. Proplant, Previcur Energy and fungicides based on fenomenal or metalaxyl-M are effective against *Pythium* sp., while Amistar, Mirador and Ortiva are effective against *Rhizoctonia solani* and/or *Botrytis cinerea* or *Sclerotinia* sp. and fungicides based on mancozeb are also used to control *B. cinerea* (<http://www.fytoweb.fgov.be/indexNL.asp>). Therefore the effect of cultivation with the decision support tool on basal rot was evaluated at PCG and PSKW. These experiments were considered as preliminary to evaluate if isolation and determination of the causal agent of basal rot would be useful in future.

Experiment at PSKW

One of the experiments was performed in the spring of 2014 at PSKW. The results of this experiment are shown in Figure 3-19. There was no significant difference in the disease severity of basal rot between the conventional spraying schedule, with score 3.5 and the basal rot score of plants treated according to the advice of the decision support tool with score 4.5, whereas disease severity of the untreated control group was significantly worse with score 6.5.

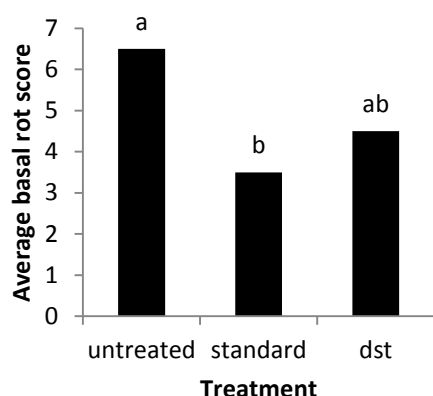


Figure 3-19: Comparison of the score for basal rot between untreated plants, plants with standard fungicide applications ('standard') and plants with fungicide applications based on advice of decision support tool (dst). Score for basal rot here is 1 for almost no symptoms to 9 for most severe symptoms. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Experiment at PCG

In an experiment of PCG, few symptoms of basal rot were observed. The results of the average basal rot scores of this experiment are shown in Figure 3-20.

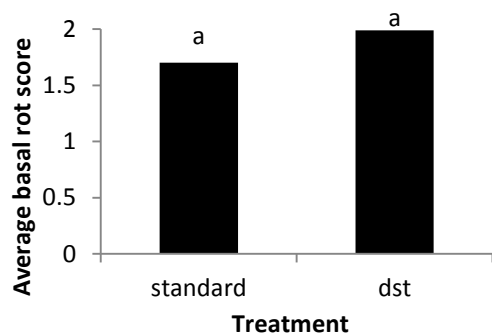


Figure 3-20: Comparison of the score for basal rot between untreated plants, plants with fungicide applications on a calendar based schedule (standard) and plants with fungicide applications based on advice of the decision support tool (dst). Score for basal rot here is 1 for almost no symptoms to 9 for most severe symptoms. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

3.3.5.3 Effect on average lettuce head biomass

First experiment at PCG

The effect of cultivation with the decision support tool was also evaluated for the yield, expressed as average lettuce head biomass. In this experiment the average lettuce head biomass of a Cosmopolia plant was 452.5 g and 464.2 g when the plants received standard fungicide applications and applications based on the decision support tool, respectively. The average lettuce head biomass of a Flandria plant was 456.7 g and 483.3 g, for a plant with fungicide applications based on a calendar schedule and applications based on the advice of the decision support tool, respectively. No significant differences between plants treated according to the two fungicide application strategies were observed (Figure 3-21), yet the average lettuce head biomass was slightly higher for lettuce heads with fungicide applications based on the advice of the decision support tool.

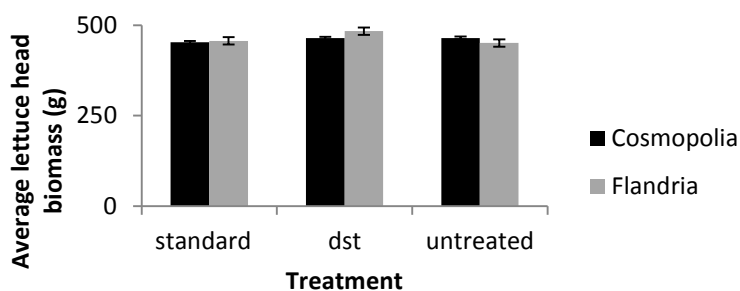


Figure 3-21: Difference in average lettuce head biomass between plants treated with fungicides based on a calendar schedule and plants treated with fungicides according to the advice of the decision support tool (dst), compared to untreated plants. Statistical analysis was done with One Way Anova and Tukey analyses, $p = 0.05$. Error bars represent standard errors.

Second experiment at PCG

The average lettuce head biomasses of the three groups were not significantly different from each other in this experiment (Figure 3-22).

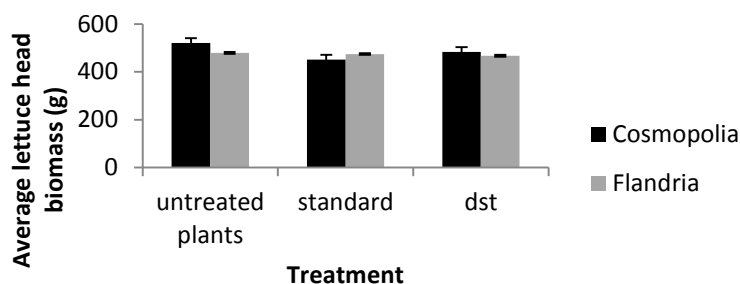


Figure 3-22: Difference in average lettuce head biomass between plants treated with fungicides based on a calendar schedule (standard) and plants treated with fungicides according to the advice of the decision support tool (dst), compared to untreated plants. Statistical analysis was done with One Way Anova and Tukey analyses, $p = 0.05$. Error bars represent standard errors.

3.3.6 *Compromise between lettuce downy mildew and tipburn control*

Because tipburn control demands a high relative humidity at night, which should be avoided for downy mildew control, a compromise to allow control of both is needed. More specific it is defined in the decision support tool that a high relative humidity (above 85%) at night during 5 hours is the minimum requirement for sporulation. Therefore it was chosen to increase the relative humidity in the two preliminary experiments described here during 4 hours because this would be insufficient for abundant sporulation of *B. lactucae* and thus might be a compromise between lettuce downy mildew and tipburn control.

First experiment at Inagro to search for a compromise between downy mildew and tipburn control

The first treatment consisted of an increase of the relative humidity in a period from 8 p.m. until 12 p.m. The second treatment was a control treatment without influencing the relative humidity and the third treatment was an increase of the relative humidity during the entire night. The first downy mildew symptoms were noticed on the 8th of April, one day before harvest. From the 11th of March until harvest, no fungicides were applied.

Downy mildew disease incidence and index were determined on the 8th of April and results are shown in Figure 3-23. Increasing the nightly relative humidity for 4 hours, did not result in a significant increase of the downy mildew disease incidence or index of Hofnar plants in comparison with the plants without nightly increased relative humidity at night, while an increase in both disease incidence and index was noticed for plants grown under conditions with a relative humidity which was increased during the entire night. Gardia plants, which were apparently more susceptible for the used inoculum, had a significant higher disease incidence and index if relative humidity was increased during the entire night. No significant difference was noticed between groups of plants grown in conditions without increased relative humidity at night and plants grown under conditions with increased relative humidity during 4 hours (Figure 3-23).

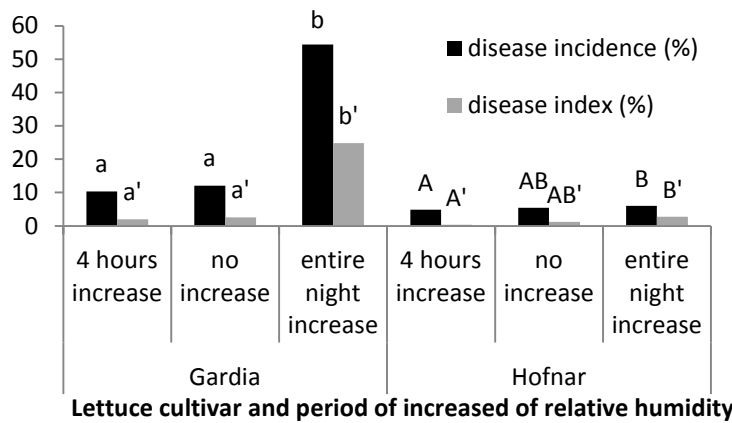


Figure 3-23: Downy mildew disease incidence (%) and disease index (%) determined on the 8th of April of plants with 4 hours, entire night or no period with increased relative humidity. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses for disease indices and binary logistic regression analysis for disease incidence, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Increasing the relative humidity during the night could affect tipburn development (Figure 3-24). For Gardia plants most severe symptoms were observed when the relative humidity was not increased, with an index of 100% one day before harvest. Increasing the relative humidity during the entire night could avoid symptoms of tipburn until one week before harvest and four hours increased relative humidity slowed down the tipburn symptoms development, with an index of 15% at one week before harvest, but an index of 72% at one day before harvest. Hofnar plants, which are more susceptible for tipburn, had a significant lower tipburn index for plants with an increased nightly relative humidity. No difference was observed between four hours increase or increase during the entire night, at the first observation, however tipburn indices were already 26% and 28% respectively. One week before harvest indices were 89% for plants with increased relative humidity during the entire night and 96% for plants without increased relative humidity. One day before harvest indices were 100%, without significant difference between the three groups.

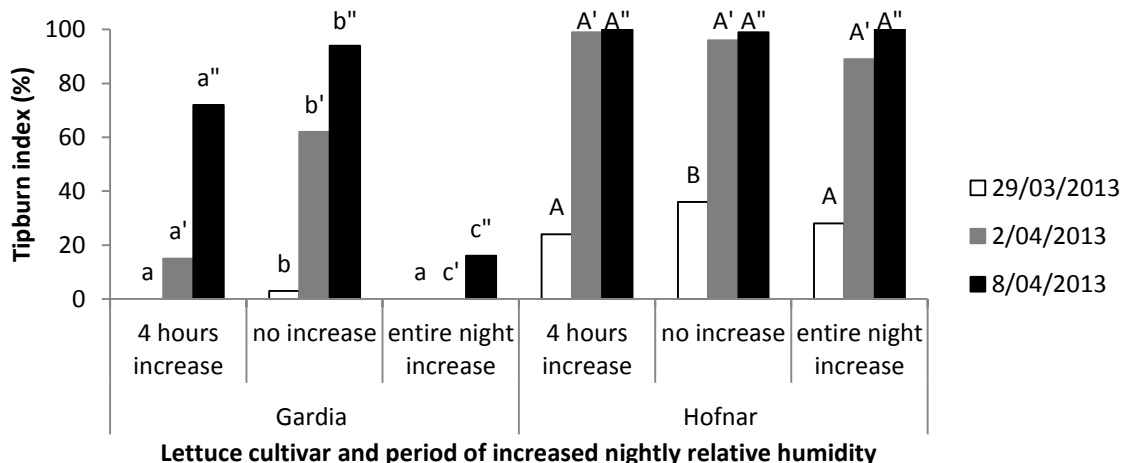


Figure 3-24: Tipburn index for Gardia and Hofnar plants without increased nightly relative humidity and with increased nightly relative humidity during 4 h or during the entire night. Indices were determined on 29/3/2013, 2/4/2013 and 8/4/2013. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

From this experiment increasing the relative humidity at night during four hours seems to be a good compromise to keep both tipburn and downy mildew under control.

Second experiment at Inagro to find a compromise between downy mildew and tipburn control

In order to reduce the negative effect of high relative humidity during darkness on sporulation, the increase in relative humidity started before sunset. Effect of increasing the relative humidity on the downy mildew disease incidence and index is shown in Figure 3-25. There is a clear link between the period with increased relative humidity and the disease incidence and index: from 0% disease incidence for Cosmopolia plants without increased relative humidity, to 4.4% disease incidence for Cosmopolia plants with 4 hours increased relative humidity, and 11.5 and 19.5% disease incidence for plants with 6 hours increased relative humidity (start of period with increased relative humidity before and after sunset, respectively). A similar trend for Gardia plants and for disease index for both cultivars was observed. Shifting 2 hours of the 6 hours of increased relative humidity to the day period, could decrease downy mildew incidence significantly. In this experiment increasing the relative humidity during 4 hours resulted in a higher disease incidence and disease index in comparison with the control plants grown without increased relative humidity. This might be due to the wetting technique, being irrigation on top of the plants, which a leaf wetness period longer than 4 hours.

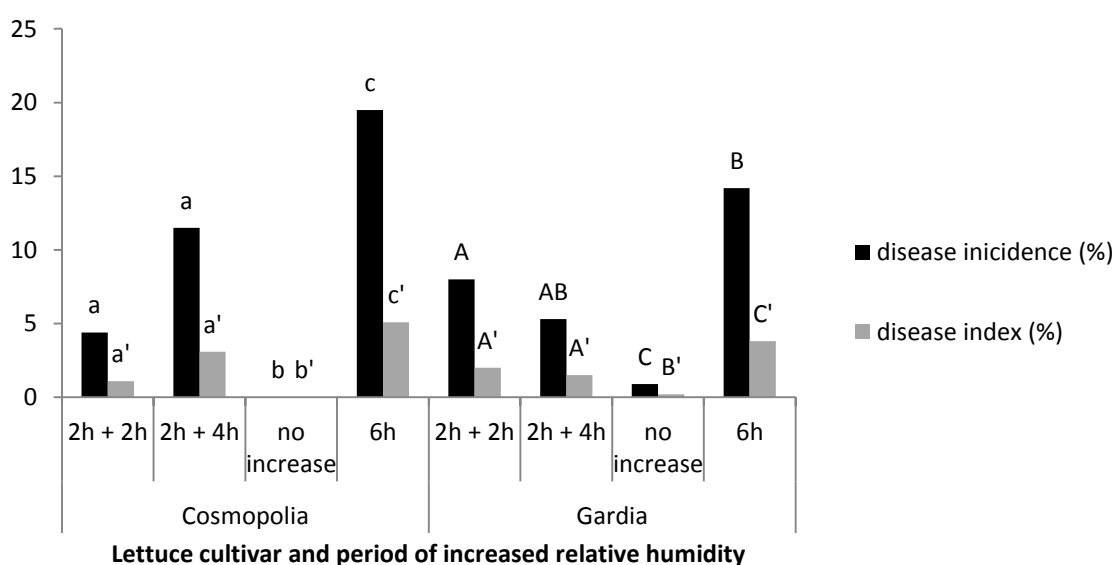


Figure 3-25: Downy mildew disease incidence (%) and disease index (%) of plants with 4 hours (2 hours before sunset until 2 hours post sunset, 2h+2h), six hours (2 hours before sunset until 4 hours post sunset, 2h+4h or six hours starting from sunset, 6h) or no increased relative humidity. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses for disease indices and binary logistic regression analysis for disease incidence, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Effect of the increased relative humidity on tipburn index is shown in Figure 3-26. It is clear that without increased relative humidity the tipburn symptoms are more severe. An increased relative humidity during 6 hours, starting from sunset, resulted in the lowest tipburn index. For Cosmopolia there was no significant difference between a period of 6 or 6 hours of increased relative humidity. For Gardia plants which are more sensitive for tipburn, a period of 6 hours starting from sunset gave lower tipburn index in comparison with a period from 2 hours before sunset until 4 hours after sunset. For Gardia plants there was no significant difference between a period with increased relative humidity of 4 or 6 hours (2h+4h).

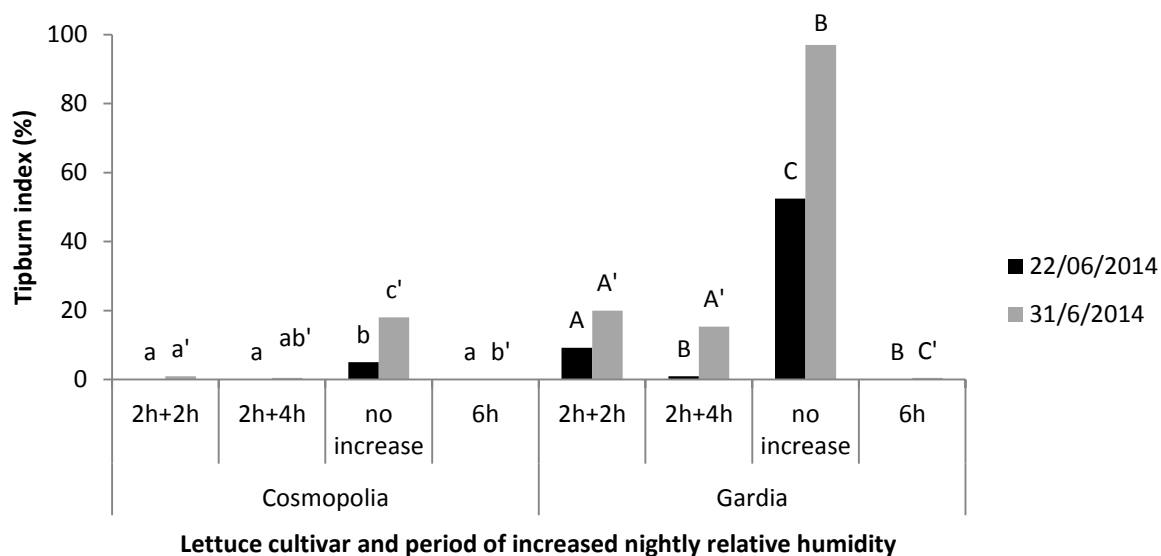


Figure 3-26: Tipburn index for Cosmopolia and Gardia plants without increased nightly relative humidity or with increased nightly relative humidity during 4 h (2 hours before sunset until 2 hours post sunset) or during six hours (2 hours before sunset until 4 hours post sunset or 6 hours starting from sunset). Indices were determined on the 22nd of June and the 31st of June 2014. Different letters indicate statistically significant differences (Kruskal-Wallis and Mann-Whitney U analyses, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

These two experiments show that a compromise can be found in greenhouse climate adaptation to provide control of both downy mildew and tipburn by limiting the period of increased relative humidity during the night to 4 hours.

3.4 Discussion and conclusion

3.4.1 Sources of primary inoculum

In our study, oospores were found in 14 of the 153 collected samples, whereas oospores were profusely found in one of three investigated fields in Norway (Nordskog et al. 2008). In a French study it was concluded that probably sexual recombination is more important in the centre-west region of France, compared to the southeast because in the southeast lettuce is cultivated throughout the year, whereas in the centre-west lettuce production is interrupted for four to five months each year. During this period, the pathogen could use oospore formation to survive until the next cultivation period, while in the southeast fresh host plants are year round available (Valade 2012). In Belgium, lettuce production is predominantly situated in Flanders, where it is cultivated year round, mainly in greenhouses. This means that host plants are available year round and this supports the hypothesis that sexual recombination is probably not that important in Belgium, at least in Flanders, the region this study focused on. It might be interesting to collect *B. lactucae* samples from lettuce cultivated in Wallonia and compare the virulence characteristics, mating types and oospore prevalence with samples collected in Flanders. Furthermore, we analysed the mating types of 18 isolates: 17 isolates were B1 and only one was B2. In comparison, of the 39 investigated isolates of a study of Michelmore and Ingram (Michelmore and Ingram 1980), 29 were mating type B2, 3 B1

and 7 were predominantly B2 although also B1. Moreover, germination of *B. lactucae* oospores turned out to be difficult, despite several attempts with various methods. Together these results suggest that sexual reproduction may not be the most important cue for the variation of *B. lactucae* in Belgium, as was also concluded by Verhoeff for the Netherlands (Verhoeff 1960).

Conidia production on *Lactuca serriola* was studied as another possible source of primary inoculum. During this study, we did not find downy mildew-infected *L. serriola*. However, the possibility that wildy grown *L. serriola* can be infected with downy mildew cannot be excluded because seedlings of *L. serriola* inoculated with BI: 26 were as susceptible as *L. sativa* cv. Green Towers plantlets (data not shown). Moreover, *L. serriola* plants infected with downy mildew were reported in France (Maisonneuve et al. 2011), therefore it is not unlikely that this might be a source of primary inoculum in Belgium as well. Further study is thus necessary to investigate isolates on *L. serriola* as a possible source of sexual compatible types which can bring new variation in the *B. lactucae* population in Flanders. Therefore more plants should be evaluated and sampled. Furthermore it might be interesting to artificially inoculate *L. serriola* plantlets before winter and evaluate if the pathogen can survive in this host even during freezing periods.

Primers used for the analyses with the spore sampler are species specific, however conidia with reduced or lost viability could not be distinguished from viable spores. Furthermore also conidia of *B. lactucae* isolates unable to infect cultivated lettuce because of resistance genes in the plant, could not be kept apart from the conidia of isolates virulent for the cultivated lettuce. Development of more specific primers might give more detailed information in future. Until the development of this kind of primers, growing lettuce plants without resistance genes along with commercially grown lettuce cultivars with resistance genes at spots where spores are sampled with a sporetrap, can help to distinguish the *B. lactucae* races.

3.4.2 Epidemiological study

Some of the disease forecast criteria used in the decision support tool are different from the criteria in previously developed models. For instance leaf wetness duration is considered as the most informative parameter in BREMCAS (Kushalappa 2001) and Modell-Analys (Jönsson et al. 2005), while our decision support tool is focused on relative humidity. However, predictions of leaf wetness duration gave occasional problems in the evaluation of the Californian model with wrong predictions for leaf wetness during the critical period (Scherm et al. 1995; Wu et al. 2001). Furthermore, previous studies at the research centres have shown that leaf wetness measurements were very inconsistent (personal communication, project IWT phytomonitoring).

3.4.3 Optimal moment for fungicide application

According to the information provided by the phytopharmacy companies (personal communication Aaike Bogaert) the mode of action of dimethomorf, mandipropamid, fenamidone and azoxystrobin is to avoid germination and of mancozeb and azoxystrobin to avoid penetration into the host. Inhibiting mycelium growth would be the mode of action of metalaxyl-M, dimethomorf and mandipropamid, but also of propamocarb and fosethyl. Metalaxyl-M, dimethomorf, propamocarb and fenamidone are described to reduce sporulation. Only fosethyl-Al is said to stimulate the defence of the plant.

According to this information, dimethomorf, mandipropamid, fenamidone, mancozeb, azoxystrobin or fosethyl should be applied at one day after inoculation to inhibit germination and penetration which can happen in the first 24 hours after inoculation and to stimulate the defence of the plant before inoculation occurs. Metalaxyl-M, dimethomorf, mandipropamid, mancozeb, azoxystrobin propamocarb or fosethyl should be applied at two to four days after inoculation to stop further development of the pathogen. Probably the conidia have germinated already, yet not all germtubes will have penetrated the plant and the isolates that have penetrated the plant already can be stopped by suppressing the mycelium growth. Starting from five days after the inoculation, sporulation can occur in optimal circumstances. Therefore applications at five to seven days post inoculation should be done with metalaxyl-M, dimethomorf, propamocarb or fenamidone.

Yet based on our experiments also propamocarb is advised to be applied at one day after inoculation because good results were obtained with this fungicide. Also in the list of fungicides suggested to be applied five to seven days after inoculation, azoxystrobin and mandipropamid are not known to suppress sporulation.

The ranking of the fungicides is based on a limited number of fungicide experiments. Therefore, to obtain stronger conclusions, more experiments should be performed. Also, during this study some of the fungicides for downy mildew control were no longer legally registered in Belgium. Previcur N is no longer allowed in Belgium since the 30th of March 2013 (www.fytoweb.fgov.be/NL/DOC/opgebruik.xls). Because the active ingredient of this fungicide (propamocarb) was still allowed, active ingredients were used in the summarizing table for the decision support tool. However, it is clear that it is important to update the decision support tool on a regular basis.

3.4.4 Comparison of the decision support tool with other models

In this chapter the development and validation of the decision support tool is described. This decision support tool distinguishes itself from the already existing models by the first step to intervene with pathogen development which is not fungicide application but greenhouse climate adaptation.

It was chosen to base criteria on the macroclimate because it is more homogenous than the microclimate (Jewett and Jarvis 2001). To get information of the macroclimate, climate boxes were used because they are well known and frequently used by Flemish growers. Yet, using the climate boxes requires adaptations of the climate box software to make the data available for the web applications. This is costly and therefore it might be useful to consider the use of other sensor types that are positioned at the same height as the climate box.

A more detailed estimation of inoculum presence would further improve disease forecast (Scherm et al. 1995) because the current prediction in step 1 of our decision support tool is based on a risk averse attitude. In future it would be an advantage if growers and the three research centres would input downy mildew outbreaks in the application. This information could be anonymously used by the application to give an improved estimation of the risk for surrounding growers. Risk for downy mildew outbreaks could be indicated with a colour code on a map and this could be integrated in the instrument in step 1 to predict the presence of airborne spores. The colour code would be green if

there is no risk, orange for medium risk based on weather forecast and red if there is a real outbreak mentioned. This map could be shown on the web application as it gives a fast overview of the downy mildew outbreak risk in a farmer's neighbourhood, similar to what is done on the Norwegian application, VIPS (<http://www.vips-landbruk.no>). Depending on the weather conditions the possible influence zone of a *B. lactucae* infection in one spot on the map can be estimated (Wu et al. 2001; Paul et al. 2012).

Until now the decision support tool started intervention before or during the first life cycle of the pathogen because lettuce downy mildew is an exponential spreading disease. Other models, however, determine an inoculum threshold for disease outbreaks and disease control measurements are only advised when this threshold is exceeded. For instance, the model for downy mildew on artichoke (*Cynara scolymus*), also caused by *B. lactucae*, only suggests intervention when three continuous life cycles of the pathogen occurred (Monot et al. 2012). Furthermore in the extended version of BREMCAST, it was suggested to include thresholds levels for lettuce downy mildew, and delay the first spray until the inoculum in the field trespasses these thresholds (Hovius et al. 2007). At the onset of the design of the decision support tool, it was decided not to include such a threshold, as the principle of zero tolerance was preferred. Yet, sampling with the Burkhard spore trap revealed that an inoculum threshold might be a possible extension of the decision support tool. Using this spore trap, between 13 and 7736 spore equivalents per day were detected in greenhouse compartments containing visually diseased plants. When the spore sampler was positioned in a greenhouse compartment without any visually diseased plants, but adjacent to a greenhouse compartment or field with diseased plants, between 0 and 250 spore equivalents per day could be detected. Only between 0 and 10 spore equivalents per day were detected in greenhouses without visually diseased plants in this greenhouse or the surrounding greenhouse compartments or fields, with five exceptions. On these five days, between 85 and 816 spore equivalents were detected. Because this number is too high to have an inoculum source outside the greenhouse compartment, it can be assumed that the inoculum source was inside the greenhouse compartment. However, no symptoms were observed, which suggests that the spores were released after a very weak infection, which did not result in yield loss. Taking these results into consideration, it might be useful to test this hypothesis and maybe reconsider adding an inoculum threshold to optimize the decision support tool.

3.4.5 Future of the web application platform

In future, our application platform 'LAP' will enclose more applications, for different crops, but also for different diseases of the same crop, similar to the Norwegian IPM technology platform 'VIPS' (<http://www.vips-landbruk.no/>). In order to avoid contradictory advice, we want the different applications to be interconnected. Therefore, the decision support tool was evaluated for the effect on lettuce downy mildew control, but also on basal rot and average lettuce head biomass.

During the development of our model and web application, user friendliness was the important priority to facilitate application by growers. Therefore input asked from growers was minimized, all the advices given by the application are explained and used terminology was chosen to be known by growers (Gent et al. 2013).

3.4.6 Evaluation of the decision support tool

In the two experiments performed at Inagro with the latest version of the decision support tool, one or two fungicide applications less were prescribed by the decision support tool in comparison with the calendar based fungicide schedule. In the first experiment better downy mildew control was provided by the decision support tool in comparison with the calendar based cultivation method, yet in that experiment the timing of the fungicide applications based on the calendar schedule was not optimal. In the second experiment two applications less were needed for the same level of downy mildew control. In this experiments it is likely that the first and fourth treatment prescribed by the calendar based schedule were not necessary. From the experiment at PCG in which the infection failed, probably due to too high temperatures in the period after inoculation, level of downy mildew disease control could not be evaluated. However, the decision support tool prescribed two fungicide applications, while five applications were foreseen with the calendar based fungicide schedule, indicating again that using the decision support tool can reduce the number of fungicide applications. In the first experiment performed at PSKW, one fungicide application less was performed according to the decision support tool, however, disease incidence was significantly higher for plants treated according to the decision support tool. Yet, the disease incidence of plants treated according to the calendar based fungicide schedule was also very high (75%). Thus in this experiment, with such a high disease pressure, the standard cultivation method with fungicides applied according to a calendar based schedule was not sufficient to provide decent downy mildew control either. In the second experiment, disease pressure was rather low and there was no significant difference between disease incidence and index of plants treated according to the calendar based schedule and plants treated according to the advice of the decision support tool. Yet three fungicide applications less were prescribed by the tool in comparison with the calendar based schedule.

Because some fungicides used against downy mildew also affect basal rot control, reducing the number of fungicides can increase basal rot problems. In our experiments the basal rot indices were not significantly different between crops with fungicide applications based on a calendar schedule or crops with fungicide applications advised by the decision support tool. However, despite the differences were not statistically significant, in some experiments the basal rot index was higher for plants treated according to the decision support tool. Therefore it might be useful in future to isolate the pathogens causing basal rot from plants of both fungicide schedules to determine which pathogen is causing the higher basal rot score. Probably this will be *Botrytis cinerea* because this pathogen was mostly found in winter, conditions favourable for downy mildew, while *Rhizoctonia solani* was more frequently detected in summer (Van Beneden et al. 2009). Yet other pathogens such as *Pythium* and *Sclerotinia* spp. cannot be ruled out either.

No difference was observed concerning average lettuce head biomass due to different number and/or different choices of fungicides. Effect of climate adaptation is more difficult to test: different compartments needed and effect of other factors (solar radiation, soil, ...) should be taken into consideration.

Furthermore, also some experiments were performed to find a compromise between downy mildew and tipburn control by evaluating the effect of a periodically increased relative humidity at night. The results of these experiments revealed that the advice of the decision support tool is not too rigorous

to cause problems with tipburn control and a compromise can be found. Increasing the nightly relative humidity during four hours can reduce tipburn development without increasing the disease incidence of downy mildew. Moreover, two periods with increased relative humidity, interrupted by a drier period, might be promising to give an even better control of tipburn without stimulating downy mildew development. In future, more experiments should be performed to find the optimal greenhouse climate conditions and determine the optimal method to increase the relative humidity to avoid downy mildew development and control tipburn. Yet, forecasting which of these two diseases is more likely to occur might also be an important step in finding a compromise between the two control strategies, because tipburn mostly occurs in dry periods (summer) and downy mildew is more frequently occurring in cooler and more wet seasons such as autumn and spring.

Chapter 4. The complex role of mycosubtilin and surfactin in the control of lettuce downy mildew

Because fungicides were considered in Chapter 3 as a part of the integrated control strategy studied in this work, biofungicides can be seen as an extension of this part. *Bacillus subtilis* are often studied for their biocontrol effect on many plant-pathosystems. Frequently it has been demonstrated that the cyclic lipopeptides produced by *B. subtilis* play an important role in the biocontrol activities. The effect of mycosubtilin and surfactin, two types of *B. subtilis* cyclic lipopeptides was shown to be promising in a first study. Yet, the working mechanisms behind the observed effects were still vague. Because knowledge of the working mechanism helps determining the application method, taking the first steps in unravelling the mode of action of mycosubtilin and surfactin but also fengycin, was the main purpose of this chapter. First an effect of mycosubtilin, surfactin but also fengycin on the germination of *B. lactucae* conidia was demonstrated. Next a dose dependent effect of the cyclic lipopeptides was observed for foliar applications close to the moment of inoculation, with better prophylactic effects for higher doses. For foliar applications at four days before inoculation, an opposite dose-dependent effect was observed, with only an effect on the disease incidence for lower doses of mycosubtilin and surfactin and no effect of fengycin. Furthermore synergism was observed for foliar applications of fengycin and mycosubtilin. This dose dependent effect of foliar application four days before inoculation, might suggest that mycosubtilin and surfactin affect stress related lettuce genes, resulting in enhanced defence of the plants against downy mildew. Therefore the effect of foliar application four days before inoculation on expression of five stress related genes, probably involved in the salicylic acid, jasmonic acid and ethylene pathway was analysed. Upon surfactin treatment, expression of none of these genes was up-regulated, suggesting that if surfactin induces defence mechanisms in the plant, they might be mediated by another signalling pathway. Mycosubtilin treatment seemed to have an effect on the salicylic acid pathway. To gain more insight in which pathways are involved in resistance of lettuce against downy mildew, the plants were treated with ethephon, methyl jasmonate and a salicylic-analogue (BTH). Methyl jasmonate and/or BTH or the combination of ethephon with BTH or methyl jasmonate could enhance the resistance of the plants.

4.1 Introduction

Search for potential biofungicides can contribute to the development of an integrated, sustainable control strategy because these biofungicides can be used as replacement or an addition for the conventional fungicides. By replacing fungicides or adding biofungicides with different mode of action, the development of resistance of the pathogen can be reduced (Isaac 1999; Blancard et al. 2006; Savary et al. 2006; Barrière et al. 2014). Biofungicides are pest management agents based on living micro-organisms or natural products (Chandler et al. 2011).

Many studies report about the biocontrol activities of *Bacillus subtilis*. *B. subtilis* belongs to a group of plant-growth promoting rhizobacteria (PGPR), known to have biocontrol activities in several plant-pathosystems, in which cyclic lipopeptides produced by these bacteria often play a role (Leclère et al. 2005; Ongena et al. 2005; Ongena and Jacques 2008; Desoignies et al. 2013).

A first study described that *B. subtilis* cyclic lipopeptides (CLPs) mycosubtilin and surfactin could reduce lettuce downy mildew infection. It was shown that 100 mg/L mycosubtilin or surfactin applied as a foliar spray, reduced downy mildew incidence when employed one day before inoculation. Combination of these two CLPs resulted in an even stronger reduction (Deravel et al. 2014). The mode of action of the cyclic lipopeptides can be a direct antagonistic effect against a pathogen or induction of resistance responses in the plant, dependent on the structure of the lipopeptides and on the plant-pathosystem (Ongena and Jacques 2008; Raaijmakers et al. 2010). A direct effect of mycosubtilin, enhanced by surfactin and an indirect effect via induced systemic resistance (ISR) was suggested as mode of action of the cyclic lipopeptides against lettuce downy mildew. Fengycin was not tested in this study (Deravel et al. 2014).

Levels of plant defence are influenced by systemic signals, mediated by the plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which are key hormones in the regulation of downstream defence genes (Robert-Seilaniantz et al. 2011). Interactions between these hormones, result in activation and/or modulation of an appropriate spectrum of defence responses of the plant, including plant cell strengthening with physical barriers and production of antimicrobial compounds (Dodds and Rathjen 2010). Early release of preformed phenolics and their intensive production upon stimulation of the phenylpropanoid pathway, are known as parts of disease resistance reactions, with demonstrated importance in incompatible *B. lactucae*–lettuce interactions (Sedlářová and Lebeda 2001).

In this chapter the effect of the *B. subtilis* CLPs was studied in more detail to unravel if the mode of action of the CLPs is a direct antagonistic effect against *Bremia lactucae* or an indirect effect via enhancement of the defence mechanism of lettuce plants. Therefore the effect of the CLPs on germination of *B. lactucae* conidia was studied. Furthermore the lipopeptides were applied in various concentrations one day and four days before inoculation. Also the effect of foliar application of the CLPs on the expression of stress related lettuce genes was analysed. Based on a study in which lettuce gene expression in a compatible *B. lactucae* interaction was studied, we chose genes of which some were up-regulated upon *B. lactucae* infection and others which expression was not influenced by the infection (De Cremer et al. 2013). Furthermore we selected a lipoxigenase-encoding and a phenylalanine lyase-encoding gene, because synthesis of these two enzymes has several times been

reported to be activated upon treatment with *B. subtilis* CLPs (Ongena et al. 2005; Ongena et al. 2007; Ongena and Jacques 2008; Jourdan et al. 2009; Chandler et al. 2015). To have a better understanding on which hormone signalling pathways are involved in defence of lettuce plants against downy mildew and which pathways the studied stress related genes are associated with, plants were treated with hormone solutions: methyl-jasmonate (naturally occurring derivative of jasmonic acid), ethephon (ethylene releasing compound (Wang et al. 2002)) and benzothiadiazole-S-methyl ester (BTH (Thaler et al. 2010)), known to induce the salicylic pathway (Dempsey and Klessig 1995; Pieterse and Van Loon 1999; Grant and Lamb 2006; Pré et al. 2008; Cohen et al. 2009; De Coninck et al. 2015). Both the effect of the hormone solutions on downy mildew disease incidence and on expression of the stress related genes was studied.

4.2 Materials and methods

4.2.1 Plant material

Lettuce plants of the susceptible variety Green Towers, of which seeds were kindly provided by Diederik Smilde from Naktuinbouw (<http://www.naktuinbouw.com/>) were used for all experiments. Approximate 20 seeds were sown on wetted cotton wool and a layer of filter paper in a plastic transparent box. The plants were grown in a growth chamber at 17.5 °C and day/night regime of 16 h light, 8 h dark.

4.2.2 Pathogen maintenance and multiplication

Bremia lactucae isolate BmVL144 was used for all inoculations. This isolate was collected from Romaine lettuce (*Lactuca sativa* var. *longifolia*) in Sint-Katelijne-Waver in 2012 and maintained and multiplied on seedlings of *L. sativa* cv. Green Towers. Fresh spores were washed off with sterile water from infected leaves. The concentration of the spore suspension was adapted to 5×10^4 spores/mL and applied as a foliar spray on seven-day-old lettuce seedlings in two-leaf-stage until running-off. Boxes with inoculated seedlings were incubated under growth chamber conditions (day/night regime of 16 h light/8 h dark, 17.5 °C and relative humidity of minimum 90%). Under these conditions new spores appeared 5 to 6 days after inoculation and sporulation was maximal at 10 days post inoculation, which was the moment the disease incidence was determined in all experiments. Disease incidence was calculated as the percentage of leaves with sporulation on the total number of inoculated leaves.

4.2.3 Cyclic lipopeptides

All lipopeptides used in this study, i.e. fengycin, mycosubtilin and surfactin were produced and purified by the ProBioGem team from different strains of *B. subtilis* using an integrated bioprocess as recently described (Coutte et al. 2013; Farace et al. 2015). Structure formulas of the lipopeptides used for our experiments are for mycosubtilin: $C_{55}H_{86}N_{14}O_{16}$, for surfactin: $C_{53}H_{93}N_7O_{13}$ and for fengycin: $C_{72}H_{110}N_{12}O_{20}$.

4.2.4 Antibiosis assays

Antibiosis assays on *Bremia lactucae* conidia germination, were conducted by collecting fresh conidia at 10 days post inoculation. Conidial suspensions were mixed with the cyclic lipopeptides at 100 mg/L suspended in 0.1% DMSO. This concentration was chosen because this concentration was tested before against lettuce downy mildew with the potential to reduce downy mildew incidence (Deravel et al. 2014). The spore suspension was applied as foliar spray on seven-day-old seedlings at the two leaf stage. Plants were incubated as described above. Control plants were inoculated with a spore suspension suspended in 0.1% DMSO. Germination was assessed at 24 hours post inoculation by adding 20 μ L of 0.02% calcofluor (Fluorescens brightener 28, Sigma Aldrich) to each leaf and germination was visualized with a UV epi-fluorescence Olympus BX-51 microscope. Images were captured with a Colour View III camera and edited with the software package CELL-F (Olympus Soft Imaging Solutions, Münster, Germany). Records were made from 100 spores per repetition, and each treatment was repeated two or three times. Binary logistic regression analysis to assess the effect of the CLPs on the number of germinated conidia was used ($p=0.05$).

4.2.5 Foliar applications of cyclic lipopeptides

Plants were treated with CLPs, applied as foliar spray until running-off, four days or one day before inoculation. The CLPs were solved in dimethyl sulfoxide (DMSO) and 0.1% DMSO was used as control treatments in all experiments. It was shown before that 0.1% DMSO had no significant effect on downy mildew development in lettuce (Deravel et al. 2014). Concentration of CLPs applied one day before inoculation was 100 mg/L in experiments with a relatively low disease pressure. In experiments with higher disease pressure also 50 mg/L was tested. Concentration of CLPs applied four days before inoculation was 10 mg/L or 100 mg/L in experiments with relatively low disease pressure and 10 or 50 mg/L in experiments with higher disease pressure. Experiments with a disease incidence of the control plants treated with 0.1% DMSO above 50% were considered as experiments with high disease pressure, experiments with a disease incidence of the control plants of maximum 50% were considered as experiments with relatively low disease pressure. For all experiments each repetitions was performed with at least 20 plants. Statistical analyses to assess the effect of the treatments on disease incidence were performed using binary logistic regression analysis ($p=0.05$).

4.2.6 Foliar application of hormone homologues

A foliar spray of BTH (benzo-thiadiazole-7-carbothioic acid S-methyl ester, 500 μ M (Si-Ammour et al. 2003; Cohen et al. 2007)), ethephon (Eth, 500 μ M) or methyl jasmonate (MeJA, 100 μ M) or combinations (BTH+Eth, 500 μ M + 500 μ M; Eth+MeJA, 500 μ M + 100 μ M; BTH+MeJA, 500 μ M + 100 μ M) was applied on six-days-old seedlings. BTH is a functional salicylic acid (SA)-analogue, which is often used to activate resistance via SA mediated defence signalling pathways (Friedrich et al. 1996; Lebeda et al. 2002; Si-Ammouret al. 2003; Glazebrook 2005; Cohen et al. 2007; Thaler et al. 2010; Maffei et al. 2012; Desoignies et al. 2013; Gamir et al. 2014). Ethephon is a compound releasing ethylene (Dempsey and Klessig 1995; Wang et al. 2002; Pré et al. 2008; Cohen et al. 2009), yet it is also reported that ethephon-mediated pathogenesis related gene expression appears to use a SA-dependent pathway (Dempsey and Klessig 1995). Methyl jasmonate is a plant growth regulator

derived from the octadecanoid signalling pathway, elicited by biological stress (Bender et al. 1999). Methyl jasmonate is an ester of jasmonic acid (Dao et al. 2011), of which has been demonstrated that it is an important signal molecule in plant defence systems (Kunkel and Brooks 2002; Tierranegra-García et al. 2011). One day later the plants were artificially inoculated with BmVL144 (5×10^4 spores/mL) and ten days after inoculation, disease incidence was assessed. On average 28 plants per treatment per repetition were treated and inoculated, and each treatment was repeated twice. Statistical analyses to assess the effect of the treatments on disease incidence were performed using binary logistic regression analysis ($p=0.05$).

4.2.7 RNA extraction and real-time PCR analysis

Plant samples treated with CLPs, 0.1% DMSO or hormone homologues as foliar spray and/or challenged with *Bremia lactucae* infection 4 days or 1 day after treatment, were taken at two days post inoculation (2 dpi), at 5 dpi or at 4 dpi and 6 dpi. Sampling point 2 dpi was chosen because at 2 days after inoculation differences between compatible and incompatible interactions can be seen. Sampling point 4 days post inoculation was chosen as the middle of the incubation period between inoculation and sporulation, while at 6 dpi first symptoms start to appear. Because in the experiment with hormone homologue treatments the different treatments and repetitions required a lot of plant material, it was chosen to reduce the number of sampling points and samples were taken at 2 dpi and 5 dpi and not at 4 dpi and 6 dpi as was done in the experiment with CLP treatment.

Plant samples were immediately snap-frozen and crushed in liquid nitrogen and stored at -80°C until RNA extraction was performed. RNA was isolated from frozen plantlets using TRI reagent (Sigma) and subsequently treated with Turbo DNase according to the manufacturer's instructions (Ambion/Applied Biosystems). First-strand cDNA was synthesized from 1 μg of total RNA using GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions. Quantitative PCR amplifications were conducted on optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green Master Mix (Stratagene/Bio-Connect). Relative induction ratios of treated samples compared with mock-treatment (0.1% DMSO and water treatment without inoculation for the experiments with CLPs and for the experiments with hormone homologues, respectively) were calculated based on the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen 2001). The thermal profile of the analyses consists of a first cycle of 5 minutes at 95°C , 40 cycles of 15 seconds at 95°C , 30 seconds at 59°C , 30 seconds of 72°C and a last cycle of 1 minute at 95°C , 30 seconds at 59°C and 30 seconds at 95°C .

β -tubulin, Lsa017612.1 was used as normalizing gene (Argyris et al. 2008; De Cremer et al. 2013). Sequences of defence gene primers used for RT-qPCR were previously described (De Cremer et al. 2013) and sequences are listed in Table 4-1. We used *OPR3* (12-oxophytodienoate reductase 3) (Díaz et al. 2012) as marker involved in the jasmonic acid (JA)-pathway (Schaller and Stintzi 2009). Oxophytodienoate reductase 3 is known to be involved in the biosynthesis of jasmonic acid from linolenic acid (Schaller and Stintzi 2009; Díaz et al. 2012). *PRB1*, coding for a basic pathogenesis related protein 1 was selected as marker for the salicylic acid (SA)-regulated marker (Loake and Grant 2007) as SA results in activation or accumulation of different pathogenesis related proteins which contribute to systemic acquired resistance (SAR) or are known to have antifungal or antibacterial activities (Dempsey and Klessig 1995; Loake and Grant 2007). Furthermore, Cohen and colleagues

showed major accumulation of PR-1a upon treatment with BTH (Cohen et al. 2009). *EDS1* (*enhanced disease susceptibility 1*) is a gene described to be involved in the biosynthesis of salicylic acid (Kunkel and Brooks 2002). *ERF1*, an ethylene responsive factor, was chosen as ethylene (ET) and jasmonic acid-regulated marker because activation by ethylene and/or jasmonate of *ERF1* was described (Wang et al. 2002; Bostock 2005). Furthermore we analysed the expression of *phenylalanine ammonia lyase1*, *PAL1*, and *LOX*, coding for a lipoxygenase. Phenylalanine ammonia lyase is known to be involved in the biosynthesis of cinnamic acid, which represents the entry of the phenylpropanoid pathway (Jourdan et al. 2009). The oxylipin pathway, which leads to the biosynthesis of biologically active secondary metabolites of which some have antifungal properties, is initiated by lipoxygenase (Blée 2002; Ongena et al. 2009). Both *PAL* and *LOX* are described to be JA- and ET-responsive (Pieterse et al. 2000).

Table 4-1: Primers used for qRT-PCR analysis, with BLAST description of the encoded genes, lettuce identity and forward and reverse primers (De Cremer et al. 2013).

BLAST2GO description and gene abbreviation	Lettuce ID	Forward primer (5' → 3')	Reverse primer (5' → 3')
enhanced disease susceptibility 1 (<i>eds1</i>)	Lsa005556.1	ACCTTGAGGAATACACGCGATCCA	AAGGCGTGTGTGAATATCCGGTCA
ethylene-responsive element binding protein1 homolog (<i>erf1</i>)	Lsa016859.1	TCGCCGGTGATGTCCAGTTATCAA	TGTTTCCCTCTCTGCTGGTTCACA
lipoxygenase (<i>lox</i>)	Lsa036946.1	GCAACTAAGCGTGCTTCACCCAAT	TGCCTCAAGAAGACCTCCACCATT
12-oxophytodienoate reductase 3 (<i>opr3</i>)	Lsa040211.1	CACCGCCGTCTGCCGACAAT	CCACCGGCGGTTGCTCTCTG
pathogenesis-related protein 1 (<i>prb1</i>)	Lsa018589.1	ATGGGACAGTCGTGTGGCTAGTTT	TGTTACAGCATCTACACCGGTCA
phenylalanine ammonia lyase (<i>pal1</i>)	Lsa044239.1	TGGCCCCACCGGAGAAGTCC	GGAAGCCATCCCGGACCCCA
tubulin beta-2/beta3 chain (β - <i>tubulin</i>)*	Lsa017612.1	TAGGCGTGTGAGTGAGCAGT	AACCCTCGTACTCTGCCTCTT

* housekeeping gene

4.3 Results

4.3.1 Effect of the cyclic lipopeptides on germination of *Bremia lactucae* conidia

Figure 4-1 shows the germination of a *Bremia lactucae* conidium. Although all three CLPs reduced the number of germinated conidia, the reduction by fengycin was strongest: 40.4% less conidia had germinated when mixed with 100 mg/L fengycin (F100) in comparison with the control group of conidia mixed with 0.1% DMSO. Mixed with 100 mg/L mycosubtilin (M100), 26.4% conidia had germinated 24 hours post inoculation and 42.9% of the conidia mixed with 100 mg/L surfactin (S100), while in the control group 50.4% of the conidia had germinated. The number of germinated conidia mixed with mycosubtilin was not significantly different from the germinated percentage of the conidia mixed with surfactin or fengycin. The effect of surfactin was significantly weaker than the effect of fengycin (Figure 4-2).



Figure 4-1: Germinating *B. lactucae* conidium.

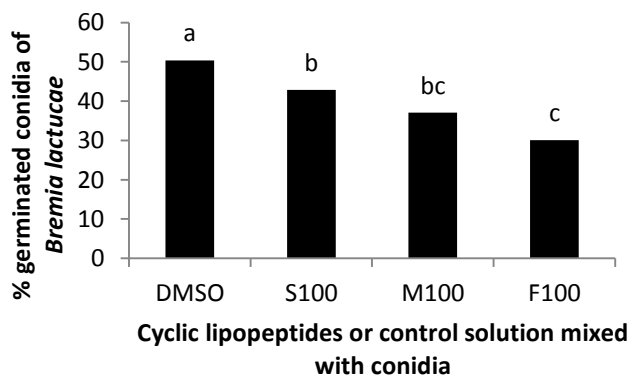


Figure 4-2: Effect of cyclic lipopeptides on *Bremia lactucae* conidia germination, assessed at 24 hours post inoculation. Conidia were mixed with 100 mg/L surfactin (S100), mycosubtilin (M100), fengycin (F100) or 0.1% DMSO (dimethyl sulfoxide), which was the control treatment because the cyclic lipopeptides were solved in DMSO with a final concentration of 0.1%. Approximate 100 spores were assessed per treatment. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

4.3.2 Effect of foliar application of the cyclic lipopeptides to the leaves

4.3.2.1 Application 1 day before inoculation

A similar experimental setup was chosen as was performed by Deravel et al. (2014) in which purified CLPs were applied as foliar spray on seven-day old seedlings. Foliar sprays in our experiments were applied one day before inoculation.

From all performed experiments, we could conclude that in experiments with about 40% disease incidence in the control plants, the observed effects were strongest (Figure 4-3A). Applications of 100 mg/L mycosubtilin (M100) or surfactin (S100) reduced the disease incidence in comparison with the disease incidence on control plants treated with 0.1% DMSO with 25% and 37%, respectively. Remarkably, 100 mg/L fengycin (F100) could not reduce the disease incidence, although a direct effect on the germination ability of the spores was observed (4.3.1) and the disease incidence was significantly reduced in experiments with higher disease pressure (Figure 4-3B). A synergistic effect of mycosubtilin and fengycin was observed. The combination of 100 mg/L mycosubtilin and fengycin resulted in the lowest number of infected plants with 94.8% reduction of the disease incidence in comparison with the disease incidence of the control plants. This was a significantly stronger reduction than the number of plants treated with 100 mg/L mycosubtilin or fengycin alone. The combination of 100 mg/L surfactin and fengycin significantly reduced the disease incidence of the treated plants in comparison with the disease incidence of the control plants, however this difference was not significantly lower than the reductions observed on plants treated with 100 mg/L surfactin alone.

In experiments with a higher disease pressure, the effect of foliar application of 50 mg/L mycosubtilin (M50), surfactin (S50) and/or fengycin (F50) was tested. The combination of 50 mg/L mycosubtilin and surfactin or mycosubtilin and fengycin reduced the disease incidence significantly in comparison with the disease incidence of the control plants. This effect was not significantly different from the disease incidence observed on plants treated with 50 mg/L mycosubtilin alone, yet mycosubtilin alone had no significant effect on the disease incidence (Figure 4-3C).

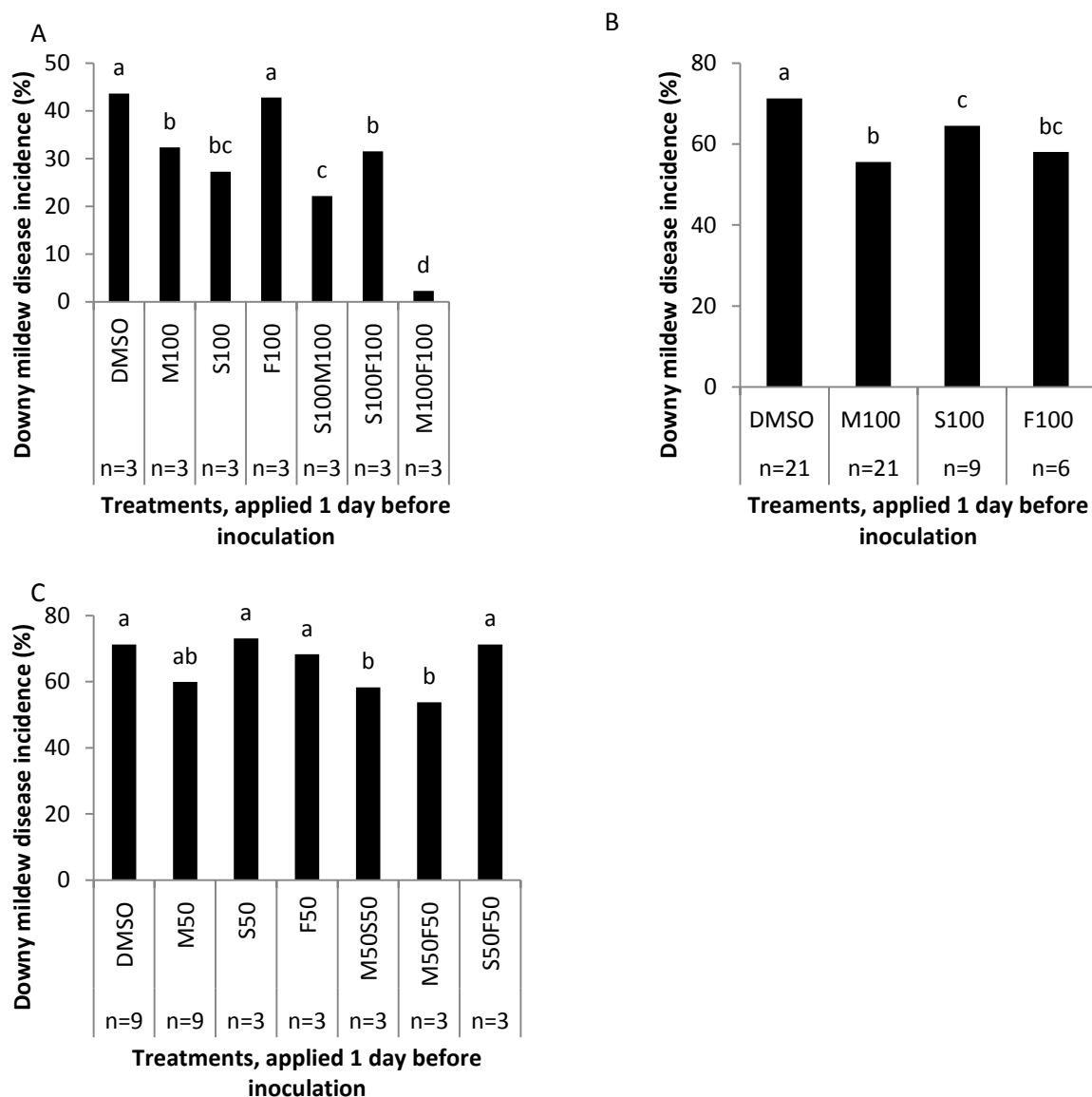


Figure 4-3: Downy mildew disease incidence determined at 10 days post inoculation on plants treated with a foliar spray of CLPs. Mycosubtilin, surfactin and fengycin were applied at 100 mg/L (M100, S100, F100) or 50 mg/L (M50, S50, F50) at 1 day before inoculation. Control plants were treated with 0.1% DMSO (dimethyl sulfoxide). The number of biological repetitions “n” varies between 3 and 21. Experiments could not all be pooled, and are therefore represented in three distinct graphs. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

4.3.2.2 Application 4 days before inoculation

Experiments were also performed with foliar sprays applied four days before inoculation. From all performed experiments, we could conclude that in experiments with disease incidence of the control plants of approximately 40%, the observed effects were strongest (Figure 4-4A). Foliar applications 4 days before inoculation of 100 mg/L mycosubtilin, surfactin or fengycin did not result in a significant effect on the disease incidence. The combination of 100 mg/L surfactin and 100 mg/L mycosubtilin reduced the disease incidence significantly in comparison with the control plants treated with 0.1% DMSO. The combination of 100 mg/L mycosubtilin with 100 mg/L fengycin had no effect on the

disease incidence. Foliar application of 10 mg/L mycosubtilin or surfactin reduced the disease incidence significantly, while no effect was observed from treatment with 10 mg/L fengycin.

In experiments with higher disease pressure, the CLPs were tested in concentrations of 50 mg/L and 10 mg/L (Figure 4-4B and Figure 4-4C). Foliar applications of 10 mg/l or 50 mg/L mycosubtilin or fengycin had no effect on the disease incidence. Also applying 50 mg/L surfactin could not reduce the disease incidence, yet application of 10 mg/L resulted in a significant reduction.

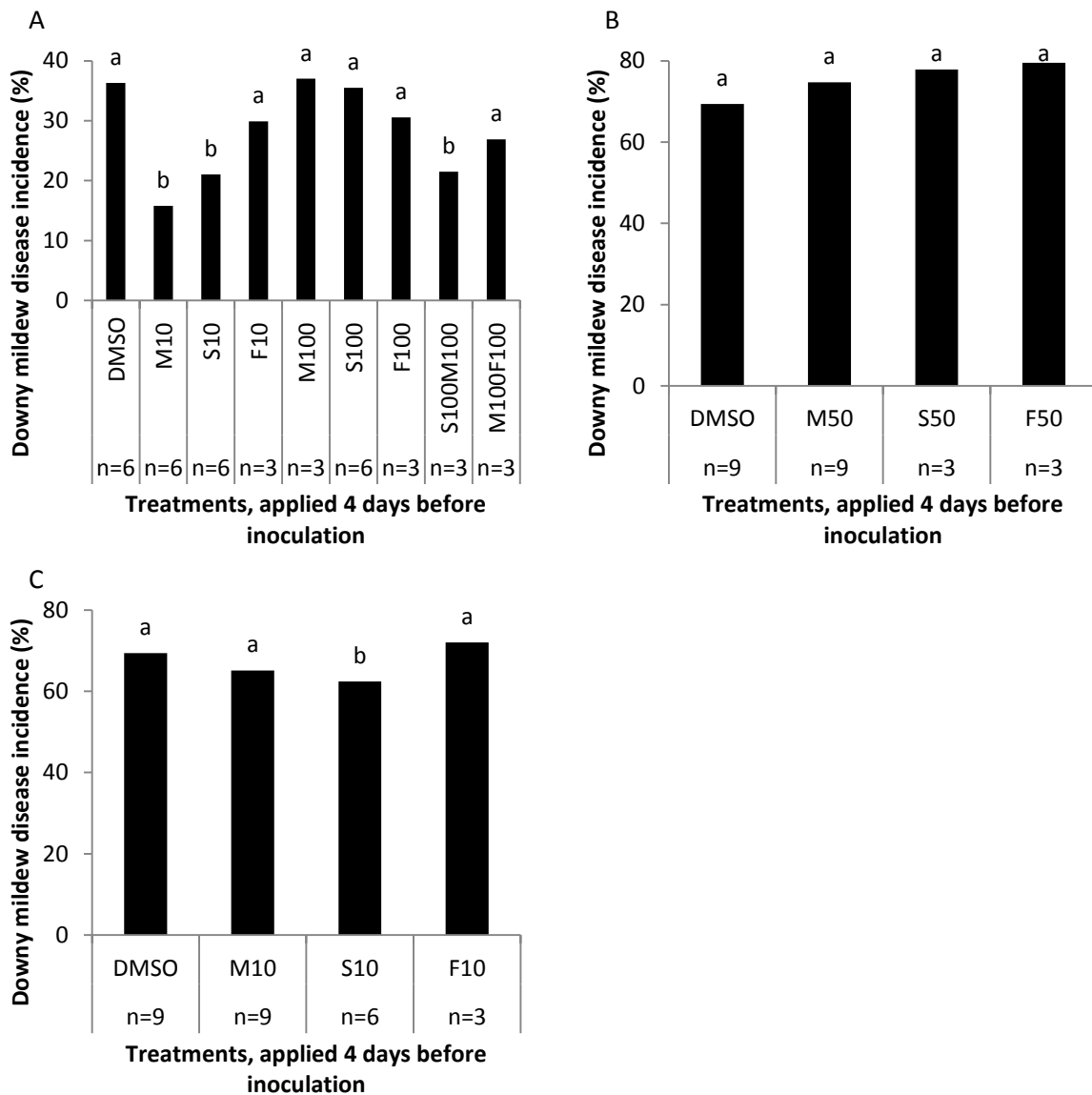


Figure 4-4: Downy mildew disease incidence determined 10 days after inoculation on plants treated 4 days before inoculation with a foliar spray of 100, 50 or 10 mg/L mycosubtilin (M100, M50, M10), surfactin (S100, S50, S10) or fengycin (F100, F50, F10). Control plants were treated with 0.1% DMSO (dimethyl sulfoxide). The number of biological repetitions is represented by “n”. The experiments could not all be pooled, therefore they are represented in three distinct graphs. Different letters indicate statistically significant differences (binary logistic regression analysis, p = 0.05). Note that no error bars are shown as data were not normally distributed.

4.3.3 Role of plant hormones in *B. lactucae* infection and defence

To assess which defence signalling pathways are involved in resistance of lettuce plants against *Bremia lactucae*, lettuce plants were treated with ethephon (Eth), a salicylic acid-analogue (BTH) and methyl jasmonate (MeJA). Eth and BTH were solved in water and MeJA was solved in DMSO with a final concentration of 1% DMSO in treatments containing MeJA.

At 10 days post inoculation plants treated with BTH or MeJA showed a significantly lower disease incidence compared with the control plants treated with water or 1% DMSO (Figure 4-5). Foliar application of the combination of BTH and MeJA resulted in plants with a disease incidence significantly lower than the disease incidence of plants treated with BTH or MeJA alone: 0.9% disease incidence versus 8.3% and 34.4% respectively. Although Eth alone did not have an effect on the *B. lactucae* disease incidence, combined with BTH or MeJA it amplified the prophylactic effect of these two components: the disease incidence of plants treated with MeJA combined with Eth was 17.5% and the combination of Eth with BTH resulted in plants with a disease incidence of 3.2%.

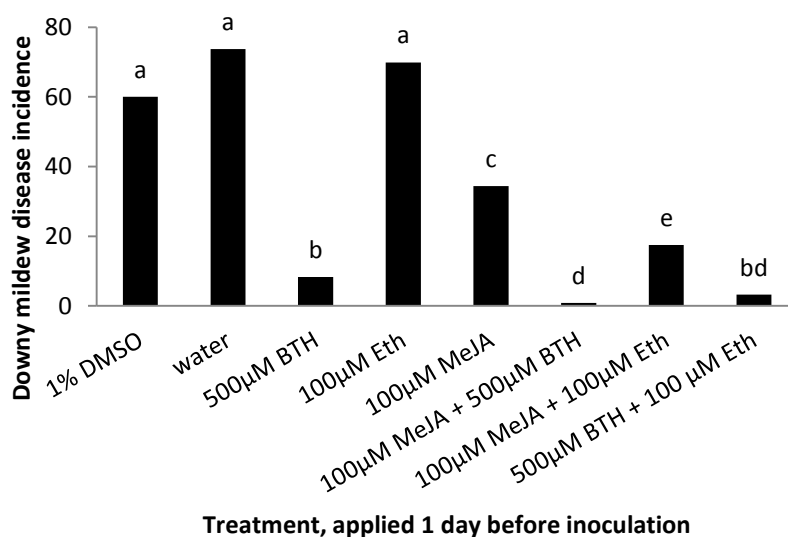


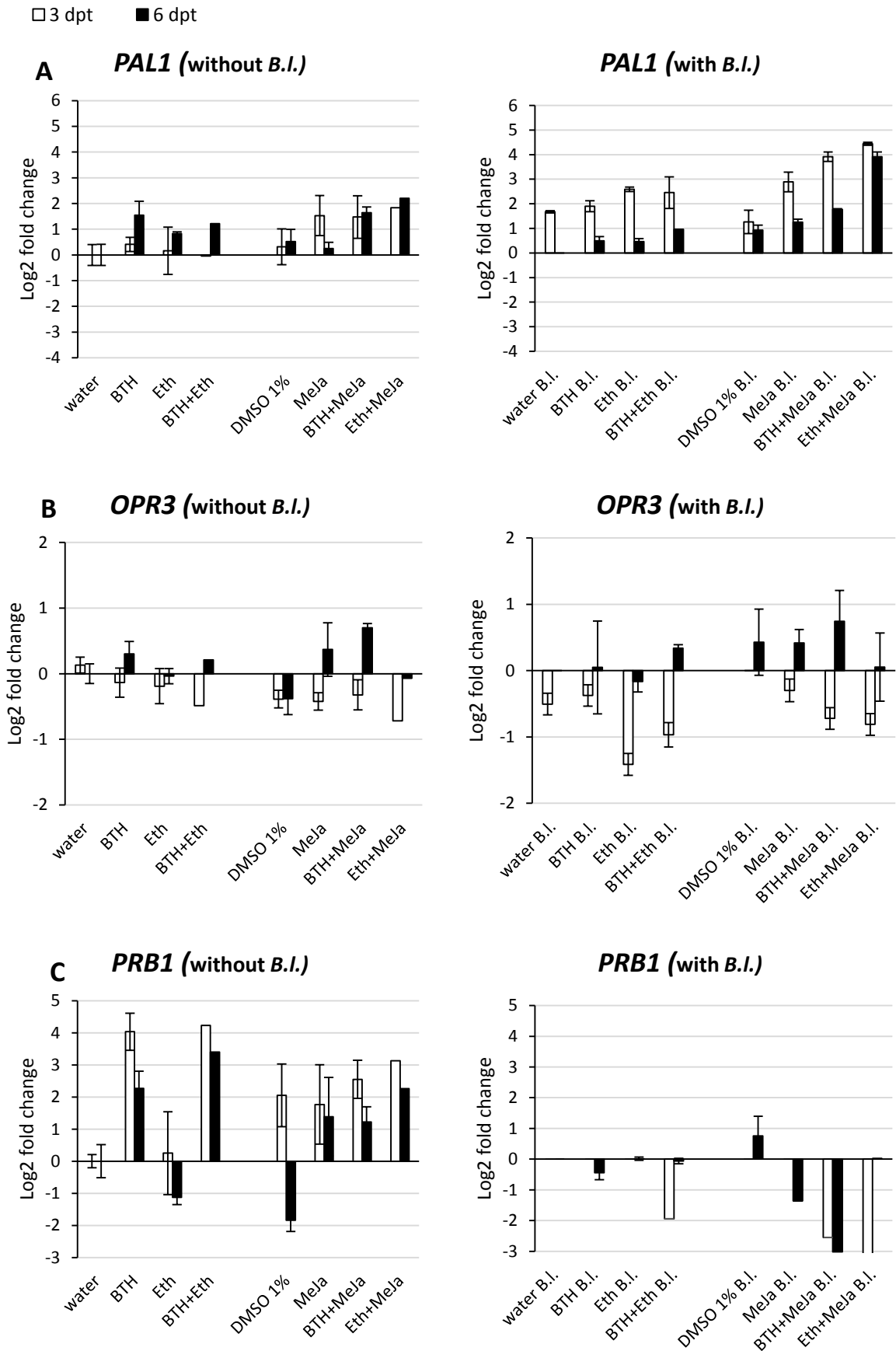
Figure 4-5: Effect on the downy mildew disease incidence of foliar application 1 day before disease incidence of BTH, ethephon and/or methyl jasmonate. Control plants were treated with water or 1% DMSO (dimethyl sulfoxide). Methyl-jasmonate (MeJA) is solved in DMSO with a final concentration of 1% in the applications containing MeJA. Ethephon (Eth) and benzo-thiadiazole-S-methyl ester (BTH) are solved in water. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

The effect of lettuce treatment with BTH, ethephon and/or methyl jasmonate on the expression on five selected stress related genes is shown in Figure 4-6. Treatment with BTH induced the expression of *PRB1* at 3 days post treatment (dpt) and the expression of *PAL1* at 6 dpt, with 4 and 1.55 log₂ fold up-regulation compared to expression in the plants treated with water. Ethephon only had a small stimulating effect on the expression of *PAL1* at 6 dpt. The effect of treatment with methyl jasmonate (MeJA) was observed on the expression of *PAL1*, *PRB1* and *LOX* at 6 dpt, all with log₂ fold changes between 1.5 and 2, compared to expression in the control plants. At 6 dpt the expression of *ERF1* in

plants treated with MeJA was up-regulated, with a log₂ fold up-regulation of 3 in comparison with the expression in control plants.

The combined treatment with BTH and ethephon, induced the expression of *PAL1* at 6 dpt, but less than in plants treated with BTH alone. The expression of *PRB1* was up-regulated to a similar level as in plants treated with BTH alone, but at 6 dpt, expression level was higher in plants treated with the combination of BTH and ethephon. In plants treated with the combination of BTH and methyl jasmonate, *PAL1*-expression was higher than in plants treated with BTH or methyl jasmonate alone, at 3 dpt, at 6 dpt the expression was similar as in plants treated with BTH alone. In plants treated with the combination of ethephon and methyl jasmonate, the expression of *PAL1* was up-regulated, with the strongest effect at 6 dpt. Also the expression of *PRB1* was enhanced in these plants both at 3 dpt and 6 dpt. The strongest effect of this treatment was observed in the up-regulation of the expression of *ERF1*.

Treatment with BTH, ethephon and/or methyl jasmonate had little or no effect on the expression of *OPR3*, which could be expected because *OPR3* encodes for an enzyme involved in the biosynthesis of jasmonic acid. Based on these results it seems that the gene coding for basic pathogenesis related protein 1, *PRB1* is mainly induced by BTH, the salicylic acid analogue. The gene encoding for the ethylene responsive factor, *ERF1* is fully induced by the combined treatment of ethephon and methyl jasmonate. *LOX* and *PAL1* are described to be ethylene and jasmonic acid-responsive. In lettuce it seems *PAL1* is responsive to salicylic acid and to a combined application of ethephon and methyl jasmonate. *LOX*-expression seems to be only responsive to methyl jasmonate in lettuce although also the combined treatment of ethephon and BTH induced *LOX*.



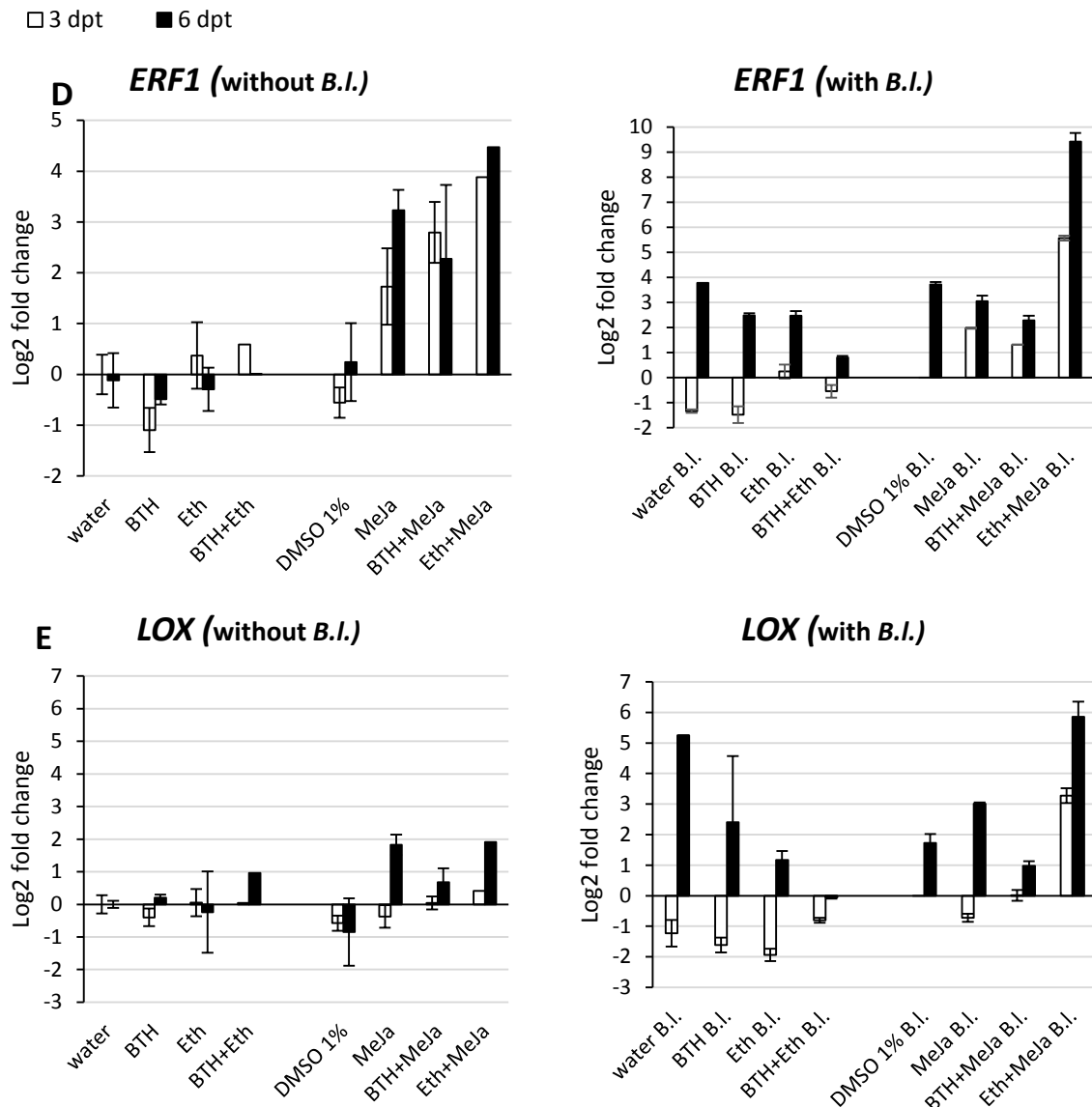


Figure 4-6: Defence gene expression in response to treatment of lettuce plants with BTH, ethephon and/or methyl jasmonate (MeJa, solved in DMSO, (dimethyl sulfoxide)). Transcript accumulation of OPR3, PAL1, PRB1, ERF1 and LOX were monitored 3 days post treatment (dpt) and 6 dpt in plants with or without inoculation with *Bremia lactucae* (B. l.). Analyses were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The transcript level was calculated using the $\Delta\Delta C_t$ -method, with β -tubulin as housekeeping gene for internal control. Results are expressed as log2 fold changes compared to the water control.

Bremia lactucae infection induces an up-regulation of 1.7 log2 fold change of *PAL1* expression in comparison with non-inoculated plants treated with water in inoculated plants treated with water. Also in inoculated plants treated with 1% DMSO the expression of *PAL1* was up-regulated in comparison with non-inoculated plants treated with 1% DMSO (Figure 4-6A). The infection had no effect on the expression of *OPR3* (Figure 4-6B). The influence of the infection on *PRB1* expression was a small up-regulation at 6 days post treatment (dpt), visible by comparing the non-inoculated plants and inoculated plants treated with 1% DMSO (Figure 4-6C) The expression of *ERF1* was down-regulated by *Bremia lactucae* infection at 2 dpt, followed by an up-regulation at 6 dpt (Figure 4-6D). A similar effect on the expression of *LOX* was observed (Figure 4-6 E). Comparison of non-inoculated and inoculated plants treated with BTH, it is clear that in inoculated plants the expression of *PAL1* at

3 dpt is increased in the inoculated plants while the up-regulation visible in non-inoculated plants is suppressed in inoculated plants. Furthermore the induced expression of *PRB1* in non-inoculated plants is suppressed in inoculated plants, while an up-regulation of *ERF1* is noticed in inoculated plants. These effects are observed for all treatments: early up-regulation of *PAL1* at 3 dpt, followed by a down-regulation in the inoculated plants, reduction of the expression of *PRB1* and the down-regulation of *ERF1* and *LOX* at 3 dpt, followed by up-regulation. The only exceptions are inoculated plants treated with ethephon and methyl jasmonate, in which the up-regulation of *PAL1* at 3 dpt is not followed by a down-regulation at 6 dpt. Another exception is the treatment with BTH and ethephon in which the strong up-regulations of *ERF1* and *LOX* are reduced in comparison with other inoculated plants.

4.3.4 Effect on the expression of stress related genes of lettuce upon treatment with mycosubtilin or surfactin

To assess which pathways were involved in the effects observed after treatment with CLPs, an experiment was set up with foliar application of 10 mg/L mycosubtilin or surfactin at 4 days before inoculation and qRT-PCRs were performed on plant samples taken at 2 dpi, 4 dpi and 6 dpi of one experiment with two biological repetitions with approximate 18 plantlets per repetition.

The foliar applications of mycosubtilin and surfactin reduced the disease incidence with more than and almost 50% respectively, in comparison with the disease incidence of the control plants (Figure 4-7).

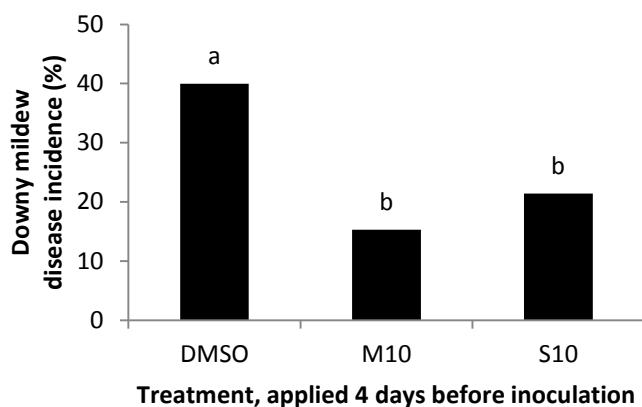


Figure 4-7: Effect of foliar spray with mycosubtilin 10mg/L (M10) or surfactin 10mg/L (S10) 4 days before inoculation on the downy mildew disease incidence (%) in comparison with control plants, treated with 0.1% DMSO (dimethyl sulfoxide). Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

The application of 10 mg/L mycosubtilin reduces the expression of *PAL1* at 10 days post treatment (6 days post inoculation, 6 dpi), although the expression was at first up-regulated (Figure 4-8). Furthermore expression of *LOX*, *PRB1*, *ERF1* and *EDS1* was reduced at 10 days post treatment (dpt). At 6 dpt (2 dpi) the expression of *ERF1* was down-regulated. Upon treatment with 10 mg/L surfactin, the expression of *PAL1* was up-regulated at 6 dpt (2 dpi). Also the expression of *EDS1* was up-regulated, at 10 dpt (6 dpi). Furthermore the expression of *ERF1* was first down-regulated at 6 dpt (2 dpi), followed by an up-regulation at 10 dpt (6 dpi). The strongest effect of surfactin was observed on

the expression of *PRB1*, which was down-regulated at 10 dpt (6 dpi). The application of these CLPs had little or no effect on the expression of *OPR3*.

When the application of 0.1% DMSO was followed by inoculation with *B. lactucae* four days later, the expression of *PAL1* was first up-regulated, followed by a down-regulation at 10 dpt (6 dpi). Also a strong up-regulation of *ERF1* and *LOX* was observed at 10 dpt. Furthermore the expression of *PRB1* was slightly up-regulated.

In plants inoculated with *B. lactucae* 4 dpt with 10 mg/L mycosubtilin, the down-regulation of *PAL1*, *ERF1*, *LOX* and *EDS1* was replaced by an up-regulation, while the expression of *PRB1* was even stronger down-regulated in comparison with non-inoculated plants treated with mycosubtilin. In inoculated plants treated with 10 mg/L surfactin the expression of *PAL1* was stronger down-regulated than in non-inoculated plants treated with surfactin, but less strong than in inoculated plants which were treated with 0.1% DMSO at 6 dpi. The up-regulation of *EDS1* and *ERF1* observed in these inoculated plants treated with 0.1% DMSO was reduced in inoculated plants treated with 10 mg/L surfactin. The down-regulated expression of *PRB1* which was observed in non-inoculated plants treated with surfactin, was similar to the expression level in inoculated plants treated with surfactin.

In this experiment the pathogen seems to down-regulate the expression of *PAL1*, but induce the expression of *ERF1* and *LOX*. Also an up-regulation of *PRB1* was observed.

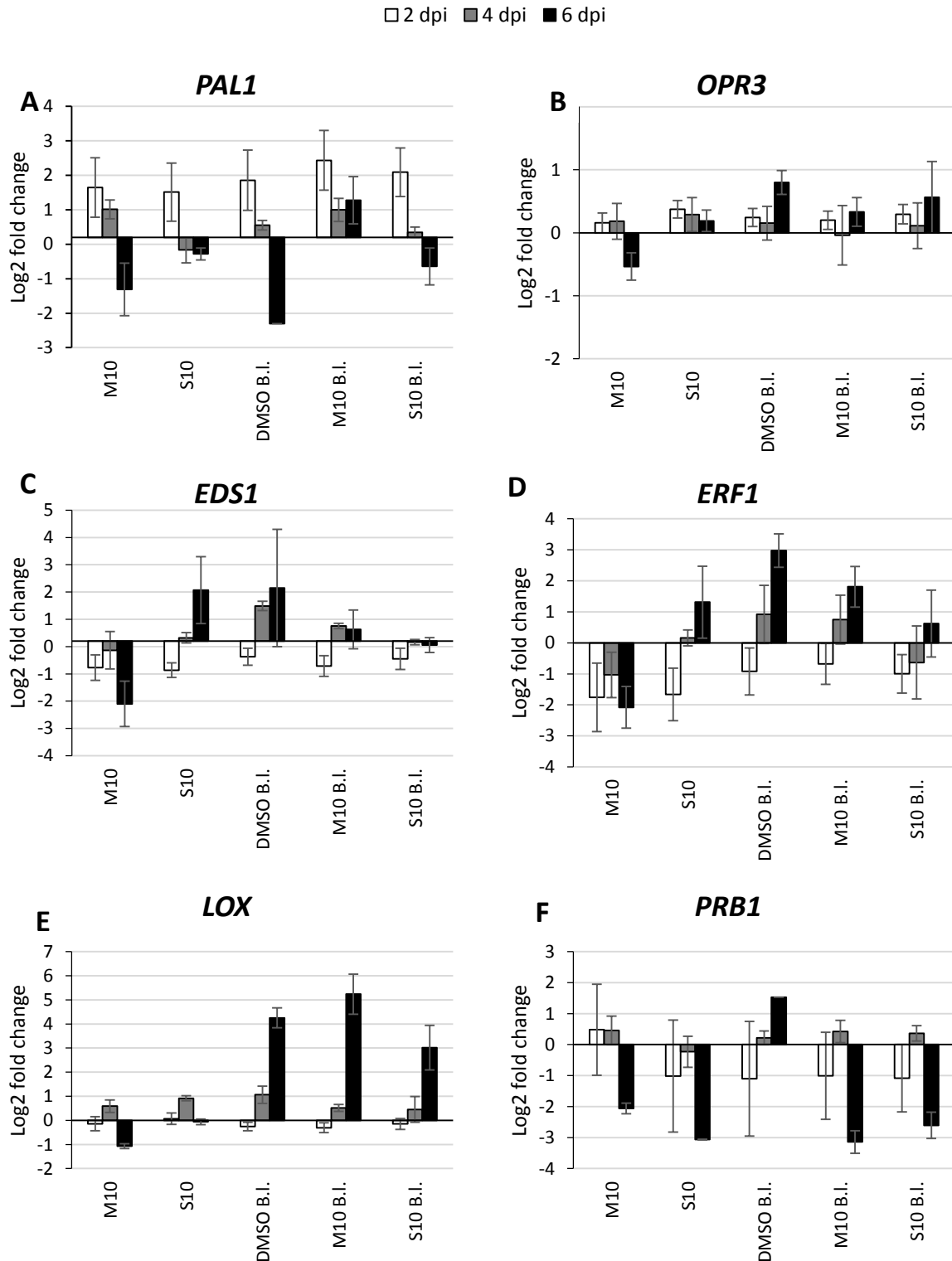


Figure 4-8: Defence gene expression in response to treatment with 10 mg/L mycosubtilin (M10) or surfactin (S10) with or without inoculation with *B. lactucae*. Transcript accumulation of *PAL1* SA-regulated marker *PRB1*, JA-regulated markers *OPR3* and *LOX* and ET-regulated marker *ERF1* were monitored 2 days post inoculation (2 dpi), 4 dpi and 6 dpi. Analyses were performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The transcript level was calculated using the $\Delta\Delta C_t$ -method, with β -*tubulin* as housekeeping gene for internal control. Results are expressed as relative transcript accumulation (fold induction) over the dimethyl sulfoxide (DMSO) 0.1% control. Data represented are means \pm SD of two replicates from a representative experiment.

4.4 Discussion and conclusions

4.4.1 *Effect of mycosubtilin, surfactin and fengycin on Bremia lactucae and downy mildew infection*

4.4.1.1 *Effect on conidia germination and of foliar application one day before inoculation*

The purpose of this study was to unravel the role that CLPs of *Bacillus subtilis* can play in the control of lettuce downy mildew. Evidence was found that the described prophylactic effect of 100 mg/L mycosubtilin and/or surfactin (Deravel et al. 2014) is a direct antagonism against *Bremia lactucae* because germination of conidia was significantly reduced by mixing the conidia with these CLPs. Furthermore it was observed that foliar application of 100 mg/L mycosubtilin or surfactin one day before inoculation reduced the disease incidence significantly, while no effect was observed for applications at 50 mg/L. This dose dependent effect might point to a direct antagonistic effect. The effect of fengycin on downy mildew has not been described before. By mixing *B. lactucae* conidia with 100 mg/L fengycin, also a significant reduction in the number of germinating conidia was noticed. Based on this result a reduction of the disease incidence was expected when 100 mg/L fengycin was applied one day before inoculation. Remarkably, in experiments with a relatively low disease pressure no effect was observed, whereas a significant reduction of the disease incidence in comparison with the control plants treated with 0.1% DMSO was observed in experiments with a higher disease pressure. Until now, we cannot explain these results.

Because the observed reduction of the disease incidence was rather small (58% disease incidence in plants treated with 100 mg/L fengycin versus 71% disease incidence in the control plants treated with 0.1% DMSO), the effect of combinations of fengycin with mycosubtilin is more interesting. The lower disease incidence of plants treated with the mixture of 100 mg/L mycosubtilin with 100 mg/L fengycin in comparison with the disease incidence of the plants treated with one of the CLPs alone, is an indication of synergism between fengycin and mycosubtilin. Interaction between iturins and fengycin was shown to play a major role in the antagonism of *Bacillus subtilis* towards *Podosphaera fusca* (Romero et al. 2007). Also in the ability of *B. subtilis* FZB42 to cope with competing organisms, iturins and fengycin played a role and act in a synergistic manner (Koumoutsi et al. 2004). In the study of Deravel and colleagues synergism between mycosubtilin and surfactin was observed (Deravel et al. 2014). In the results shown in this chapter, the combination of 100 mg/L surfactin and 100 mg/L mycosubtilin applied one day before inoculation, could reduce the disease incidence significantly in comparison with the disease incidence of control plants and plants treated with mycosubtilin alone. Yet the effect was not significantly different from the effect obtained with application of 100 mg/L surfactin alone. However, synergism of iturins, the family of mycosubtilin, and surfactin was described before as it was shown that iturins form aggregates with surfactin (Etchegaray et al. 2008; Falardeau et al. 2013) and adding surfactin in concentrations at which it is inactive, increases the haemolysis induced by iturin (Maget-Dana et al. 1992). Furthermore interaction of fengycin and surfactin was mentioned (Ongena et al. 2007; Huang et al. 2008; Ongena and Jacques 2008) e.g. in a study where it was shown that the presence of surfactin reduced the antifungal effect of fengycin against *Rhizopus stolonifer* (Tao et al. 2011). In the experiments

described in this chapter, the effect of combined application of surfactin and fengycin did not result in an effect significantly different from the effect obtained with surfactin alone.

4.4.1.2 Effect of foliar application four days before inoculation

When the CLPs were applied four days before the inoculation, only the lowest tested concentrations of surfactin and mycosubtilin (10 mg/L) could protect the plants against downy mildew. When 50 or 100 mg/L mycosubtilin or surfactin were applied four days before inoculation, no effect on the disease incidence was observed. This suggests an indirect effect of these CLPs via the plant defence mechanism. Fengycin applied in 10, 50 or 100 mg/L four days before inoculation did not have an effect on the disease incidence. When the combination of 100 mg/L mycosubtilin and fengycin was applied four days before inoculation, no effect was observed. Yet, treatment with a mixture of 100 mg/L mycosubtilin and surfactin four days before inoculation, resulted in plants with lower disease incidence than untreated plants, but the effect was less strong compared to the effect obtained by application one day before inoculation.

From these results it seems that 100 mg/L surfactin, mycosubtilin and fengycin have a direct antimicrobial effect against *Bremia lactucae*. The combination of 50 mg/L or 100 mg/L mycosubtilin and fengycin shows a strong synergism, which is lost if the mixture is applied four days before inoculation. The combination of mycosubtilin and surfactin also resulted in a strong reducing effect of the disease incidence, an effect which was maintained when the mixture was applied four days before inoculation although the observed effect of 100 mg/L mycosubtilin or surfactin alone was lost when applied four days before inoculation. Based on this results, it is possible that surfactin and mycosubtilin form aggregates with higher persistence than the lipopeptides separately.

Our results are largely in accordance with the conclusions of Deravel *et al.* as that study revealed that mycosubtilin applied one day before inoculation at 100 mg/L reduced the disease incidence of lettuce downy mildew significantly, while 100 mg/L surfactin did not have an effect in that study. This might be due to the higher concentration of the inoculum and thus the relatively high disease pressure in the study of Deravel and colleagues (Deravel *et al.* 2014). It was observed in our study as well that in experiments with higher disease pressure the effect to reduce the disease incidence mediated by surfactin was lower than the effect of mycosubtilin.

The mechanism of the CLPs to directly inhibit pathogen development is not clear yet, but might be caused by membrane-disruptive activities, which are known to be dependent on sterol composition in the membranes (Ongena and Jacques 2008).

A recent study showed in confrontation tests on PDA that the oomycete *Pythium aphanidermatum* mycelium growth was only slightly inhibited by *B. subtilis* and *B. amyloliquefaciens* and no clear correlations could be established with CLPs concentrations in the medium surrounding the bacterial colonies (Cawoy *et al.* 2015). Before it was reported that supernatants of a *B. subtilis* mutant overproducing mycosubtilin, induced growth inhibition zones significantly larger than those observed for the wild-type supernatant when tested on plates with *P. aphanidermatum* (Leclère *et al.* 2005). Therefore it might be interesting to analyse the supernatants of this bacteria strain to determine if another component is responsible for the observed effect against the tested oomycete and if it has an effect against *B. lactucae* too.

A stronger effect for a lower concentration of CLPs on induced resistance was reported before. Surfactin has shown to be able to induce resistance when applied in low concentrations and the effect is lower or gone when higher concentrations are used. This dose-dependent effect is probably due to the supposed working mechanism in which surfactin can insert into the plant cell plasma membrane which does not cause permanent damage if the concentration is low, but which may cause leakage and rupture when the concentration is higher (Heerklotz and Seelig 2007; Liu et al. 2010; Henry et al. 2011; Falardeau et al. 2013). The small disruptions caused by low concentrations of surfactin, may be sufficient to activate defence responses in the plant (Henry et al. et al. 2011). As induced systemic resistance or local induced defence upon mycosubtilin treatment is poorly described, future mechanistic studies are necessary to provide insight into the interaction mechanism with plants cells. In our study, fengycin-treatment 4 dbi did not result in enhanced resistance of lettuce plants against downy mildew. Although, fengycins were shown to be involved in triggering defence in tomato and potato, but not in bean or grapevine (Ongena et al. 2005; Ongena et al. 2007; Farace et al. 2015). This indicates the plant species-dependent response induced by CLPs.

4.4.2 Effect on expression of stress related lettuce genes

4.4.2.1 Effect of *Bremia lactucae* infection on gene expression

It is known that starting from 13 hours post inoculation formation of haustoria starts (Sargent et al. 1973) and that effectors can be secreted via the haustoria. Some effectors can interfere with the defence signalling of the plant (Stassen and Van den Ackerveken 2011). Furthermore it is reported that within 48 hours there are considerable differences in pathogen development distinguishable between compatible and incompatible interactions (Lebeda and Sedlářová 2003). To analyse the effect of the effectors and haustoria, samples should be taken on an earlier time point than done in the experiment described in this chapter.

Upon infection with *Bremia lactucae*, no effect on the expression of *OPR3* was observed, indicating that the pathogen does not influence the biosynthesis of jasmonic acid. At 2 days post inoculation an increase in the expression level of *PAL1* and a decrease in the expression level of *LOX*, *ERF1* and *PRB1* was observed. At 6 dpi the expression of *PAL1* is down-regulated, while the expression of *LOX*, *ERF1* and *PRB1* is up-regulated. The effect on the expression of *PAL1*, *LOX* and *ERF1* is very similar to the effect observed in non-inoculated plants treated with methyl jasmonate. Therefore it might be possible that the pathogen activates the jasmonate pathway. Because the up-regulation of *PRB1* induced by BTH, ethephon and/or methyl jasmonate in non-inoculated plants is suppressed in inoculated plants, it might be possible that the pathogen suppresses the salicylic acid pathway. Also in the results of De Cremer and colleagues, an induced expression of *ERF1* was reported (De Cremer et al. 2013).

4.4.2.2 Effect of BTH, methyl jasmonate and/or ethephon treatment on disease incidence and expression of stress related genes

The salicylic acid-analogue BTH is often used to induce resistance via salicylic acid-mediated pathways in plants. Treatment of lettuce plants with BTH resulted in our experiments in significant

less diseased plants. Methyl jasmonate (MeJA), an ester of jasmonic acid, is known to serve as signal molecule in plant defence systems. Treatment of lettuce plants with MeJA reduced the disease incidence significantly in comparison with the control plants. In combination with BTH, very effective protection of the plants was obtained. Cooperative effects via induction of salicylic acid and jasmonate dependent pathways were described before, for instance against *Xanthomonas campestris* in *Arabidopsis thaliana* (Rojo et al. 2003). Ethephon treatment did not have an effect on the disease incidence, yet treatments with combinations of ethephon with MeJA or BTH resulted in lower disease incidence in comparison with the disease incidence treated with MeJA or BTH alone. Synergism between the ethylene- and jasmonate or salicylic acid pathway have also been reported many times before (Rojo et al. 2003). These results show a complex interaction of the salicylic acid, jasmonic acid and ethylene pathway. Induction of the jasmonic acid or salicylic acid pathway alone, can provide resistance to the plant, however synergism between these two pathways can offer almost perfect protection. The ethylene pathway enhances the effect of the jasmonic acid pathway or salicylic acid pathway, while induction of the ethylene pathway alone cannot protect the plants. In general SA-pathway is considered to be involved in defence against (hemi)biotrophic pathogens, while JA and or ET-pathways are rather involved in defence against necrotrophic pathogens, and often these pathways are antagonistic with the SA-pathway. However the interaction between plant hormones is very complex and determines the outcome of a plant-pathogen interaction (Glazebrook 2005; Robert-Seilaniantz et al. 2011). How these pathways interact in mediating downy mildew resistance in lettuce plants needs to be further unravelled. A previous study reported no effect of foliar application of BTH (Cohen et al. 2009), however a different lettuce cultivar and a different *B. lactucae* isolate was used, which resulted in a higher disease incidence of the control plants in that study than in our experiments. Furthermore in their study BTH was applied two days before inoculation, while we applied BTH 1 day before inoculation. It is thus likely that BTH induces a defence response shortly after application, able to strongly suppress downy mildew development only if the foliar application and pathogen inoculation occur in shortly after each other.

Treatment of plants with BTH resulted in an increased expression of *PRB1* at 3 days post treatment and 6 days post treatment with a lower expression level at 6 days post treatment than at 3 days post treatment. *PRB1* codes for an pathogenesis related protein and it is often reported that salicylic acid (SA) enhances production or accumulation of PR1 proteins (Loake and Grant 2007). Yet in plants inoculated after treatment with BTH, no up-regulation of *PRB1* was observed. Probably BTH induces resistance in lettuce plants via another mechanism and expression of *PRB1* is not involved in the defence against downy mildew.

Methyl jasmonate (MeJA) treatment induces expression of *PAL1* and *ERF1* at 2 days post inoculation (dpi). Treatment with ethephon did not result in changed expression of any of the selected stress related genes, except for a down-regulation of the expression of *PRB1*. This might indicate an antagonistic reaction between the ethylene-signalling pathway and the SA-signalling pathway. Because *ERF1* encodes for an ethylene responsive factor, it could be expected that the expression of this gene would increase upon ethephon treatment because ethephon is an ethylene-releasing compound. Yet, no effect of ethephon treatment on *ERF1* was observed but in combination with MeJA, expression of *ERF1* was 6 log₂ fold higher than in control plants treated with water. Also treatment with MeJA alone induced the expression of *ERF1*. It was described before for *Arabidopsis*

thaliana that full induction of *ERF1* requires both ET and JA signalling (Pré et al. 2008; Robert-Seilaniantz et al. 2011). Furthermore upon MeJA treatment, expression of *PAL* was increased at 2 dpi, in comparison with inoculated control plants. *PAL* and *LOX* are described to be responsive to ethylene and jasmonic acid (Camm and Towers 1973; Maleck and Lawton 1998; Pieterse et al. 2000; Porta and Rocha-Sosa 2002). Resistance mediated by the combined treatment of ethephon and MeJA, might be regulated via the stronger up-regulation of *PAL1*, *ERF1* and *LOX* at 2 dpi in comparison with inoculated control plants. Also in plants treated with the combination of BTH and MeJA, expressions of *PAL1* and *ERF1* were stronger induced than in the inoculated control plants.

4.4.2.3 Effect of mycosubtilin or surfactin treatment on expression of stress related lettuce genes

Comparing the effect of foliar application of 10 mg/L mycosubtilin or surfactin on the expression of the stress related lettuce genes with the expression in inoculated control plants, might give an indication of the mode of action of the CLPs to enhance defence of lettuce against downy mildew.

The most striking difference is that the down-regulation of *PAL1* at 6 days post inoculation (dpi) in inoculated control plants is suppressed in plant treated with the CLPs. In plants treated with surfactin, the down-regulation is less strong than in the inoculated control plants. In plants treated with mycosubtilin, the expression of *PAL1* is more than 1 log₂ fold up-regulated instead of more than 2 log₂ fold down regulated in the inoculated control plants. The expression of *EDS1*, a gene described to be involved in the biosynthesis of salicylic acid (Kunkel and Brooks 2002), is up-regulated in inoculated control plants at 4 and 6 dpi. In inoculated plants treated with mycosubtilin or surfactin, almost no up-regulation is observed. The expression of *PRB1*, which is firstly down-regulated in inoculated control plants, followed by an up-regulation at 6 dpi, is strongly down-regulated at 6 dpi in inoculated plants treated with mycosubtilin or surfactin.

Because nor mycosubtilin, nor surfactin treatment has an effect on the expression of *OPR3*, these two CLPs probably do not induce production of jasmonic acid and thus defence of lettuce plants induced by these CLPs is probably not mediated via the jasmonic acid pathway.

Induction of the phenylpropanoid pathway upon treatment with surfactins, mycosubtilins or fengycins has been described several times before. Upon treatment of tobacco cells with surfactins and fengycins, major changes in defence-associated early events and in the phenolic pattern were observed. Modifications of the phenylpropanoid signature were concomitant with a significant accumulation of mRNAs coding for PAL (Ongena et al. 2007; Ongena and Jacques 2008; Jourdan et al. 2009). In tomato, root rhizosphere colonization with a *B. subtilis* mutant overproducing surfactin and fengycin also stimulates two key enzyme activities of the oxylipin pathway, LOX and lipid hydroperoxidase, in comparison with control plants and with plants treated with the wild *B. subtilis* strain, unable to produce these CLPs (Ongena et al. 2007). Treatment of potato tuber cells with fengycins resulted in the accumulation of plant phenolics involved in or derived from the phenylpropanoid pathway, while surfactins and mycosubtilins did not influence this pathway (Ongena et al. 2005). Incubating rice cell cultures with supernatant of a *B. subtilis* isolate producing fengycin and surfactin, induced a strong activation of the expression of a gene coding for PAL (Chandler et al. 2015).

The purified components surfactin, mycosubtilin and fengycin triggered a transient activation of auxin signalling upon application to rice cells. Therefore it might be interesting to analyse expression pattern of genes involved in the auxin signalling pathway in lettuce upon treatment with the CLPs.

Mycosubtilin and fengycin could switch on the rice JA and ET signal transduction pathways (Chandler et al. 2015). Treatment of grapevine cells with surfactin or mycosubtilin, induces up-regulation of genes involved in the SA-pathway or in both SA- and JA-pathway, respectively (Farace et al. 2015).

In cotton plants, iturins can induce the expression of several defence related genes and these genes were induced more rapidly and strongly in cotton seedlings upon inoculation with iturin-treated *Verticillium dahliae* compared with untreated *V. dahliae* infection. It was suggested that these defence responses may be involved in the induction of a SA- and JA-regulated basal defence and a repression of ET-regulated basal defence (Han et al. 2015).

4.4.3 Prospects and conclusions

It might be interesting to repeat the experiments with BTH, MeJA, ethephon, mycosubtilin and surfactin and take samples at an earlier time point to complete the data and give information about the expression in the plant, closer to the moment of application and before haustoria formation.

Because BTH, ethephon and MeJA were applied one day before inoculation and in our experiments effect on gene expression was determined two days after inoculation for some genes and 5 days after inoculation for others, this means the difference in expression level follows within 3 to 6 days. The *B. subtilis* CLPs, however, were applied four days before inoculation and effect of the CLP treatment on the gene expression was observed from 2 days post inoculation, which is 6 days after application.

A former study had shown potential of MeJA and SA to diminish lettuce mortality due to pill-bug and suggested further research for the use of SA and MeJA as priming components in lettuce production because no significant influence of foliar application of these components on dry and fresh lettuce head weight was observed (Tierranegra-García et al. 2011). As from our results a strong potential of BTH and MeJA to control lettuce downy mildew was concluded, we support this suggestion for further analysis. Yet attention should be paid to the age of the lettuce plants in relation with the concentration of the hormones because some of our plantlets showed leaf damage (Figure 4-9). Another interesting future experiment would consist in the inoculation of an untreated leaf of a with CLPs treated plant to assess if the induced resistance is systemic.



Figure 4-9: Leaf damage observed 10 days after inoculation upon foliar application of BTH and MeJA.

Applied at 100 mg/L mycosubtilin, surfactin and fengycin had a direct antimicrobial effect against *Bremia lactucae*. At 10 mg/L mycosubtilin and surfactin applied few days before inoculation, provide enhanced defence of lettuce plants against downy mildew. Because no effect was seen upon treatment with mycosubtilin or surfactin on the expression of *OPR3*, it is likely that the jasmonic acid pathway is not involved in the defence mechanism enhanced by these CLP. Furthermore it was observed that the effect of *Bremia lactucae* on stress related genes *PAL1*, *EDS1*, and *PRB1* was counteracted if the plants were treated four days before inoculation with these CLPs.

Chapter 5. Effect of *Bacillus subtilis* cyclic lipopeptides on *Botrytis cinerea* and *Rhizoctonia solani*

Besides downy mildew, also basal rot is a frequently observed disease of lettuce. Because a prophylactic effect of the cyclic lipopeptides of *Bacillus subtilis* against downy mildew was noticed, testing the effect of these cyclic lipopeptides against *Botrytis cinerea* and *Rhizoctonia solani*, two major causal agents of basal rot in Belgian lettuce, seemed interesting. In this chapter the effect of mycosubtilin, fengycin and surfactin on the mycelium growth of both pathogens, on germination of *B. cinerea* conidia and on the viability of *R. solani* sclerotia was tested. Furthermore the effect on basal rot development upon inoculation with *B. cinerea* or *R. solani* was evaluated on plants treated with the cyclic lipopeptides. Surfactin delayed mycelium growth of *R. solani*, yet no strong significant effect on sclerotia viability was observed. Furthermore surfactin could not permanently reduce the disease incidence of basal rot caused by *R. solani*, yet the disease index was significantly lower on leaves treated with surfactin. Surfactin had no effect against *B. cinerea*. Fengycin could reduce the development rate of basal rot caused by *R. solani*, yet no (permanent) effect on the mycelium growth or sclerotia viability was observed. Against *B. cinerea* a delay in the growth of mycelium was observed and the percentage of germinated conidia was reduced. Furthermore in a lower concentration the disease index of plants treated with fengycin was reduced. Mycosubtilin showed the potential to significantly reduce mycelium growth of both pathogens, to decrease the germination potential of *B. cinerea* conidia and moreover application 4 days before inoculation resulted in reduced disease incidence and/or index of basal rot, caused by *B. cinerea* or *R. solani*.

5.1 Introduction

Besides downy mildew, basal rot is a frequently observed disease in Belgian lettuce (Van Beneden et al. 2009). A three year study in Belgium determined that the most frequently observed causal agent of basal rot in winter was *Botrytis cinerea* and in summer *Rhizoctonia solani* (Van Beneden et al. 2009). A number of fungicides used against *Bremia lactucae* are also effective against causal agents of basal rot: Amistar, Mirador and Ortiva are effective against *Rhizoctonia solani* and/or *Botrytis cinerea* and fungicides based on mancozeb are also used to control *B. cinerea* (<http://www.fytoweb.fgov.be/indexNL.asp>). Because the cyclic lipopeptides (CLPs) produced by *Bacillus subtilis*, mycosubtilin, fengycin and surfactin showed to have potential to control downy mildew (Chapter 4) it seemed interesting to analyse their effect against these two causal agents of basal rot.

In Chapter 4 it is shown that surfactin, fengycin and mycosubtilin can reduce the ability of *B. lactucae* conidia to germinate. *B. lactucae* is an oomycete and *B. cinerea* and *R. solani* are fungi and it is known that the composition of the cell wall of these two eukaryotic groups is different. Therefore it was interesting to analyse if the CLPs also have an effect on germination of conidia of *B. cinerea* or sclerotia of *R. solani*, the main sources of primary inoculum of these two pathogens (Davis et al. 1997). It was reported before that biosurfactants can play a role in reduction of the viability of sclerotia, as Debode and colleagues demonstrated that biosurfactants played a role in biocontrol of microsclerotia of *Verticillium* (Debode et al. 2007). Sclerotia and mycelium of *R. solani* (Van Beneden et al. 2010) and mycelium of *B. cinerea* have melanins in their cell walls (Gessler et al. 2014). Melanins are darkly pigmented polymers, protecting organisms against environmental stress (Henson et al. Day 1999), various antifungal drugs (Gómez and Nosanchuk 2003) and the action of reactive oxygen species (ROS) in host cells (Gessler et al. 2014). It might be interesting to analyse if the CLPs can have an effect on the viability of sclerotia, conidia or mycelium despite the presence of melanin in the cell walls.

Biocontrol activity of isolates of the *Bacillus subtilis* group against *Botrytis cinerea* and *Rhizoctonia solani* on lettuce has been described before (Fiddaman, O'Neill, and Rossall 2000; Maronne 2002; Bardin et al. 2013). Effectiveness of *B. subtilis* against *B. cinerea* was reported on apple (Touré et al. 2004), grapevine (Farace et al. 2015), tomato (Bardin et al. 2013) and tobacco (Henry et al. 2011). Also a suppressing effect of *Bacillus* was mentioned against *Rhizoctonia solani*: an inhibition of the mycelium growth on plates due to a lipid fraction composed of lipopeptides of the iturin and surfactin type (Etcheagaray et al. 2008). *B. megaterium* had shown to possess biocontrol activities against *R. solani* (AG1-IA) on soybean (Zheng et al. 2013) and *B. subtilis* RB14 was able to reduce damping-off of tomato by *R. solani* (AG4) in which iturin A and surfactin have shown to be involved (Asaka and Shoda 1996; Mizumoto et al. 2007). Also *B. subtilis* GB03, which was registered by Uniroyal agricultural Chemical company (United states) in 1985 has shown to be highly antagonistic against *R. solani* on cotton and groundnut crops (Maksimov et al. 2011). Furthermore, a direct inhibitory effect of iturin A and surfactin, produced by *B. subtilis* strains OG, 18G and G2-6, was observed against *R. solani* on agar plates (Etcheagaray et al. 2008). Moreover, iturin A produced by *B. amyloliquefaciens* was shown to be responsible for *R. solani* 2B-12 growth inhibition *in vitro* (Yu et al.

2002). Another test indicated that *B. subtilis* showed biocontrol activity against *R. solani* both *in vivo* and *in vitro* on potato. Based on time of release, also in this study it was suggested that the antifungal effect would be the result of iturin compounds, however no analytical tests were performed to confirm this hypothesis (Elkahoui et al. 2012; Falardeau et al. 2013).

The aim of the study described in this chapter was to observe if mycosubtilin, surfactin or fengycin could have a direct effect on *B. cinerea* and on *R. solani* AG1-1B.

In Chapter 4 it was observed that treatment of lettuce plants with mycosubtilin or surfactin had an influence on the expression of stress related genes. This expression pattern coincided with enhanced defence of the plants against downy mildew. Often there is an antagonism between defence reactions of a plant responding on attack of biotrophs (such as *Bremia lactucae*) and attack of necrotrophs (such as *R. solani* and *B. cinerea*). This crosstalk and trade-off results in an optimal defence against a specific pathogen (Thomma et al. 2001; Kunkel and Brooks 2002; Rojo et al. 2003; Glazebrook 2005; Spoel et al. 2007; Koornneef and Pieterse 2008; Robert-Seilaniantz et al. 2011). Yet many exceptions and examples of synergism between the pathways are known (van Wees et al. 2000; Bostock 2005; Spoel et al. 2007; Truman et al. 2007). In the study of De Cremer and colleagues, was shown that there was a high correlation between gene expression changes induced by *B. cinerea* and during a compatible interaction with *B. lactucae*. Interestingly, for instance a gene involved in the salicylic pathway is up-regulated upon *B. lactucae* infection, while no effect was observed after inoculation with *B. cinerea* and the expression of a lipoxygenase-encoding gene was lower after inoculation with *B. lactucae* in comparison with after inoculation with *B. cinerea*. Because not much information is available of the signalling pathways involved in defence reactions of lettuce plants against downy mildew, it was interesting to analyse the effect of CLP treatment of the plants on development of basal rot caused by *R. solani* or *B. cinerea*.

5.2 Material and methods

5.2.1 Plant material

Lettuce plants of the susceptible variety Green Towers, of which seeds were kindly provided by Diederik Smilde from Naktuinbouw (the Netherlands, <http://www.naktuinbouw.com/>) were used for all experiments. Approximate 20 surface-sterilized seeds were sown on Murashige and Skoog medium with vitamins (MS-medium, 4.43 g/L with 8 g/L agar and pH 5.8). Seeds were sterilized in 1% NaOCl for 45 s and subsequently washed 6 times in sterile water. The plants were grown in a growth chamber at 17.5 °C and day/night regime of 16 h light, 8 h dark.

5.2.2 Pathogen maintenance

Botrytis cinerea strain R16 (Faretra and Pollastro 1991) and *Rhizoctonia solani* strain S001-2 belonging to AG1-1B (Van Beneden et al. 2009) were cultured on potato dextrose agar (PDA, 39 g/L) at room temperature. Plates (90 mm) with *B. cinerea* were incubated with light/darkness regime of 14 h light, 8 h darkness, plates with *R. solani* were kept in the dark.

5.2.3 Cyclic lipopeptides of *Bacillus subtilis*

The experiments performed in this chapter were all done with CLPs obtained from Lipofabrik (<http://www.lipofabrik.com/>) except for the experiments with the conidia of *Botrytis cinerea*. For these experiments, CLPs were produced and purified by the ProBioGEM team from different strains of *B. subtilis* using an integrated bioprocess as recently described (Coutte et al. 2013; Farace et al. 2015). The CLPs obtained via the ProBioGEM team were delivered as powder and were only perfectly soluble in 0.1% dimethyl sulfoxide (DMSO). CLPs obtained via Lipofabrik were aqueous solutions.

5.2.4 Effect on mycelium growth

To assess the direct effect of the CLPs, concentrations of 100 mg/L were used in Chapter 4. Preliminary experiments with *B. cinerea* and *R. solani* showed no difference between treatments with concentrations of 50 mg/L or 100 mg/L on the mycelium growth of both pathogens. Therefore experiments to assess the direct effect of the CLPs (both on mycelium growth, conidia germination and sclerotia viability) were performed with the CLPs in a concentration of 50 mg/L.

PDA (39 g/L) was mixed with CLPs with a final concentrations of CLPs of 50 mg/L before pouring the plates. Positive control plates only contained PDA and negative control plates consisted of PDA mixed with fungicide Signum (3 g/L), which is effective against both *R. solani* and *B. cinerea*. The active ingredients of Signum are boscalid and pyraclostrobin (<http://www.agro.basf.be>). The CLPs for this experiments were bought from Lipofabrik (www.lipofabrik.com) as aqueous solutions.

Experiments were conducted on 55 mm plates and in the middle a 3 mm fungal plug was placed, taken from a one week-old culture of *R. solani* or a 10-days old culture of *B. cinerea*. Each treatment was conducted on ten plates. Mycelium growth was measured daily during five days starting the first day after setup as the diameter of the mycelium. Statistical analyses to assess the effect of the treatments on mycelium growth were performed using One Way Anova and Tukey analysis ($p=0.05$).

5.2.5 Effect on *B. cinerea* conidia germination

A PDA plate fully covered with *B. cinerea* mycelium was incubated for one week under continuous near-UV light to encourage sporulation (Sowley et al. 2009). One 90 mm plate covered with sporulating mycelium was cut into pieces and PDA, mycelium and conidia were mixed with 15 mL bidest water and subsequently filtered (250 μm gaze). The conidial suspension was mixed with potato dextrose broth (PDB, 6 g/L) and with the (CLPs, 50 mg/L) or DMSO or fungicide Signum (3 g/L). DMSO was used a control in the same concentrations as it was present in the CLP solutions because CLPs were obtained from the ProBioGEM team as powder which needed to be solved in 0.1% DMSO. Concentration of the conidial suspension was adapted to 10^5 spores/mL.

Lettuce leaves of fully grown purchased lettuce heads with the end of the midrib covered with moist tissue, were inoculated with a drop of the conidial suspension and inoculated leaves were placed in plastic trays. At least 5 drops per leaf and minimum 3 leaves per repetition were foreseen. The experiment was repeated with leaves of younger lettuce plants in 8-leaf stage with identical results. These younger plants were obtained from the research centre PSKW. In this experiment with the younger leaves, two drops per leaf and three leaves per treatment per tray were used and there

were three trays with all treatments. Each tray contained all treatments and there were three trays per experiment. This means 45 drops per treatment for which disease incidence and index were determined.

The small trays were put in larger trays on moist tissue and covered to create a dark environment with high relative humidity. Trays were incubated at room temperature for three days.

Disease incidence was calculated with following formula:

$$\text{disease incidence (\%)} = \frac{\# \text{ of leaves with symptoms}}{\text{total \# of inoculated leaves}} \times 100$$

Disease index was determined to assess disease severity, with following formula. Therefore the disease was scored from 0 (no symptoms) to 3 (most severe symptoms) (Figure 5-1).

$$\text{disease index (\%)} = 100 \times \frac{\# \text{ of leaves with score } 1 \times 1 + \# \text{ leaves with score } 2 \times 2 + \# \text{ of leaves with scores } 3 \times 3}{\text{total \# of inoculated leaves} \times 3}$$

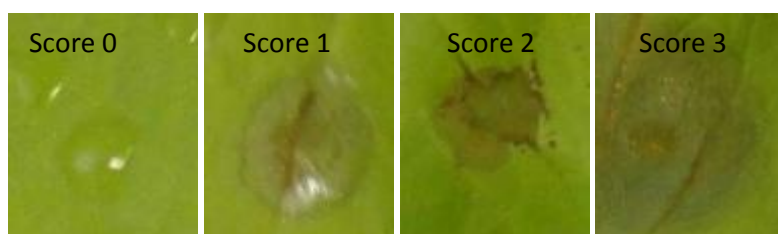


Figure 5-1: Disease score of basal rot caused by *Botrytis cinerea*. Score 0: no symptoms, score 1: brown spots within the boundaries of the droplet, score 2: spread of the necrosis outside the boundaries of the droplet, score 3: symptoms widely spread on the leaf.

Statistical analyses to assess the effect of the treatments on disease index were performed using Kruskal-Wallis and Mann-Whitney U analyses ($p=0.05$). To assess the effect of the treatments on disease incidence binary logistic regression analysis ($p=0.05$) was used.

5.2.6 Effect on *R. solani* sclerotia germination

A 90 mm PDA plate with seven-day-old *R. solani* mycelium was grinded with 25 mL sterile bidest water. This suspension was applied as a thin layer on fresh PDA plates, which were incubated for 35 days in darkness at room temperature (Manning et al. 1970). Next, sclerotia were collected from these plates by lightly scraping them from the agar surface (Bloomfield and Alexander 1967).

For each treatment, 12 sclerotia were incubated in 1 mL aqueous CLPs solution 50 mg/L for a period from 6 to 72 h, followed by washing the sclerotia in 1% NaOCl during 2 min and two additional washing steps in sterile water. Dried sclerotia were placed on a fresh PDA plate and incubated at room temperature in darkness. Diameter of mycelium growth was measured daily. Statistical analyses to assess the effect of the treatments on mycelium growth were performed using One Way Anova and Tukey analysis ($p=0.05$).

5.2.7 Effects of cyclic lipopeptides on basal rot caused by *Rhizoctonia solani* or *Botrytis cinerea* infection

Lettuce seeds were sown on MS medium and six weeks later the entire plants were dipped in a (CLP) solution (50 mg/L or 10 mg/L) for 30 seconds. Both concentrations were used to give a first indication if the observed effect would be a direct antifungal effect or an induction of the defence mechanism in the plant, because it is reported that lower concentrations are more effective to induce resistance responses in plants (Ongena and Jacques 2008). After dipping, the leaves were incubated in a growth chamber at 17.5 °C and day/night regime of 16 h light, 8 h dark for 4 days. After this incubation, the leaves were inoculated with *R. solani* or *B. cinerea*. Each leaf had 2 inoculum drops or plugs, four leaves per treatment were used in one tray and there were three trays with all treatments. This means 24 repetitions for each treatment for which disease incidence and/or index were determined.

Inoculation with *R. solani*

On each leaf two 5-days old *R. solani* mycelium plugs were placed in the centre of the leaf of which the end of the midrib was covered with moist tissue. Inoculated leaves were placed in plastic trays. These small trays were put in larger trays on moist tissue and covered to create a high relative humidity. The leaves were incubated in a growth chamber with 16 h light, 8 h darkness and a temperature of 24 °C. Disease incidence and index were evaluated daily, based on necrosis:

Score 0: no symptoms

Score 1:]0, 25%[of the leaf surface shows necrosis

Score 2: [25, 50%[of the leaf surface shows necrosis

Score 3: [50, 75%[of the leaf surface shows necrosis

Score 4: [75, 100%[of the leaf surface shows necrosis

Score 5: 100% of the leaf surface shows necrosis

Inoculation with *B. cinerea*

Lettuce leaves with the end of the midrib covered with moist tissue, were inoculated with a 2 drops of *B. cinerea* conidial suspension (10^5 spores/mL) and inoculated leaves were placed in plastic trays. These small trays were put in larger trays on moist tissue and covered to create a dark environment with high relative humidity. Trays were incubated at room temperature for three days. Next disease incidence and index were determined as described in section 5.2.5.

5.3 Results

5.3.1 Effect of *Bacillus subtilis* lipopeptides on *Botrytis cinerea*

Effect on mycelium growth

The effect of the CLPs on mycelium growth was tested *in vitro* on PDA plates. From the first day after setting up the experiment, a significant reduction in the diameter of the *B. cinerea* mycelium between the control PDA plates and the PDA plates with surfactin, fengycin or mycosubtilin was observed (Figure 5-2). Yet, the reduction of the mycelium growth was smaller on the plates with CLPs than on the PDA plates containing Signum. At the fifth day of measuring the mycelium diameter on plates with mycosubtilin or fengycin was approximate 50% larger than on plates with Signum. The mycelium diameter on plates with surfactin was no longer significantly different from the diameter on control PDA plates.

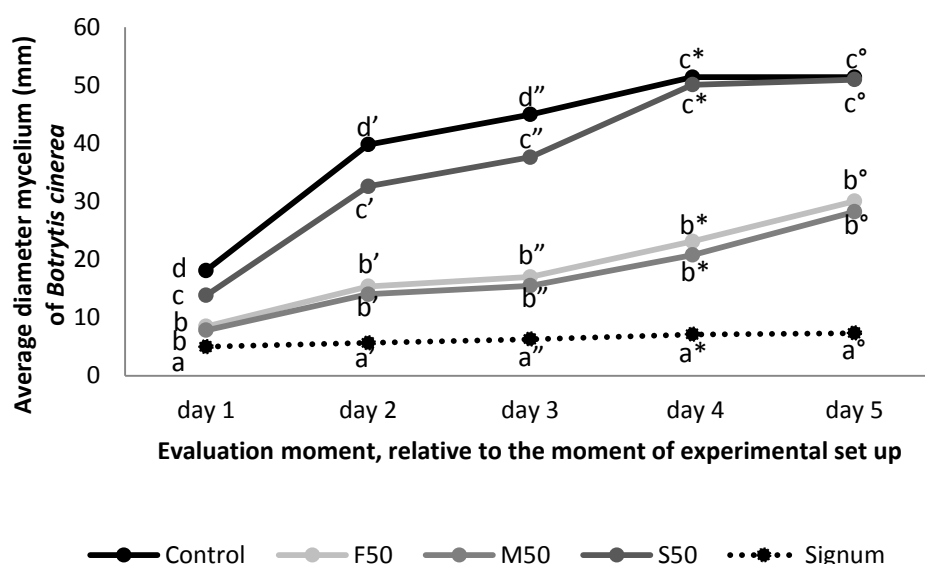


Figure 5-2: Average diameter of *Botrytis cinerea* mycelium grown on control PDA plates or PDA plates with 50 mg/L fengycin, 50 mg/L mycosubtilin, 50 mg/L surfactin (F50, M50 and S50, respectively, solved in water) or 3g/L Signum. Ten plates per treatment were evaluated and diameter was determined daily during 5 days after set-up of the experiment. Different letters indicate statistically significant differences (One Way Anova and Tukey analysis, $p = 0.05$). Error bars represent standard errors of ten repetitions but are too small to be visible.

Effect on conidia germination, disease incidence and index

Although *B. cinerea* also produces sclerotia, airborne spores are generally considered to play a prominent role in the dispersal of this pathogen (Holz et al. 2004). Therefore the direct effect of CLPs on *B. cinerea* conidia germination was studied. Conidia were mixed with CLPs and used for the inoculation of lettuce leaves. Part of the conidia suspension was not applied on lettuce leaves but kept in test tubes and the number of germinated conidia was counted after six hours of incubation at room temperature. After three days of incubation, disease incidence and disease index of the lettuce leaves were determined. The effect of the CLPs on the basal rot disease incidence and index and on the germination of the conidia is shown in Figure 5-3. Only mycosubtilin could significantly reduce the disease incidence, disease index and percentage of germinated conidia in comparison with the control treatment of 0.1% DMSO. Germination of the conidia was not significantly reduced by 0.1%

DMSO. Although fengycin did not have an effect on the disease incidence, the disease index and the percentage of germinated conidia were significantly lower in comparison with the control with 0.1% DMSO. Surfactin had no effect on the disease incidence, disease index or number of germinated conidia.

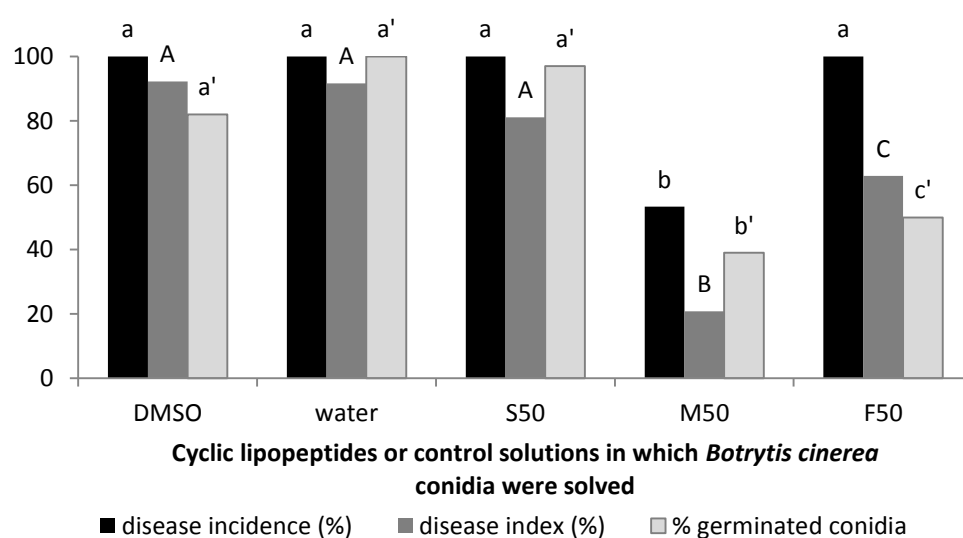


Figure 5-3: Disease incidence (%) and disease index (%) of lettuce leaves inoculated with *Botrytis cinerea* conidia solution mixed with surfactin, mycosubtilin or fengycin (50 mg/L, solved in 0.1% DMSO, S50, M50, F50) or with 0.1% DMSO or water (two control treatments). Disease incidence and index were determined 3 days after inoculation. Also the percentage germinated conidia at 6 hours after mixing the conidia with the cyclic lipopeptides, is represented in this figure. Different letters indicate statistically significant differences (binary logistic regression analysis for the disease incidence and percentage germinated conidia and Kruskal-Wallis and Mann-Whitney U analyses for disease indices, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Effect on basal rot caused by *Botrytis cinerea*

Disease incidence and disease index of plants treated with CLPs four days before inoculation were evaluated three days after inoculation (Figure 5-4).

Foliar application of mycosubtilin four days before inoculation, reduced the disease incidence and disease index significantly. Disease incidence of plants treated with water was 96% and for plants treated with 50 mg/L or 10 mg/L mycosubtilin 19% and 23%, respectively. The disease indices were 92% for plants treated with water and 12% and 16% for plants treated with 50 mg/L or 10 mg/L mycosubtilin. The effect of 50 mg/L mycosubtilin was not significantly different from the effect of 10 mg/L mycosubtilin. Treatments with surfactin did not affect the disease incidence or disease index significantly in comparison with the incidence or index of control plants treated with water. Application of fengycin at 50 mg/L did not affect the disease incidence or index, yet application of 10 mg/L reduced the disease index to 71%.

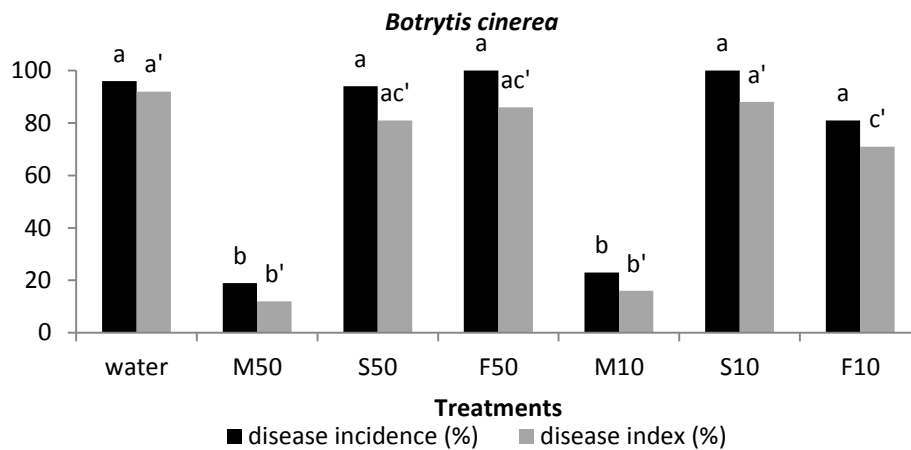


Figure 5-4: Basal rot disease incidence (%) and disease index (%) of lettuce plants treated with *B. subtilis* cyclic lipopeptides 50 mg/L or 10 mg/L mycosubtilin (M50 and M10), surfactin (S50 and S10), fengycin (F50 and F10), 4 days before inoculation with *Botrytis cinerea*. Cyclic lipopeptides were solved in water, which was the control treatment. Disease incidence and disease index were evaluated 3 days after inoculation. Different letters indicate statistically significant differences (binary logistic regression analysis for the disease incidence and percentage germinated conidia and Kruskal-Wallis and Mann-Whitney U analyses for disease indices, $p = 0.05$). Note that no error bars are shown because data were not normally distributed.

5.3.2 Effect of *Bacillus subtilis* cyclic lipopeptides on *Rhizoctonia solani*

Effect on mycelium growth

The *R. solani* mycelium diameter was not significantly different on the different plates at the first day of measuring (Figure 5-5). From the second day, the mycelium diameter on plates containing fengycin or surfactin in the medium was significantly larger than on plates with Signum or mycosubtilin in the medium. Mycelium diameters on plates with fengycin or surfactin were significantly different. On both media, growth was reduced in comparison with growth on PDA plates without additives. At four days after set up of the experiment, there was no significant different difference in the diameter of the plates containing surfactin or fengycin in the medium in comparison with the plates without additives in the PDA. Between plates with Signum and mycosubtilin in the medium, there was a significant difference in the mycelium diameter from the fourth observation day.

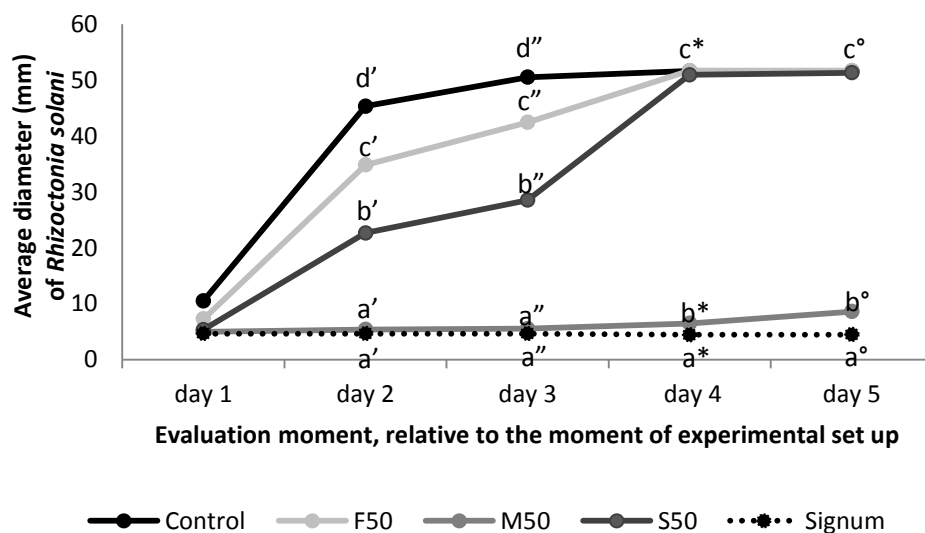


Figure 5-5: Average diameter of *Rhizoctonia solani* mycelium grown on control PDA plates or PDA plates with 50 mg/L fengycin, 50 mg/L mycosubtilin, 50 mg/L surfactin (F50, M50 and S50, respectively, solved in water) or 3 g/L Signum. Ten plates per treatment were evaluated and diameter was determined daily during 5 days after set-up of the experiment. Different letters indicate statistically significant differences (One Way Anova and Tukey analysis, $p = 0.05$). Error bars represent standard errors of ten repetitions but are too small to be visible.

Effect on sclerotia viability

Because sclerotia of *Rhizoctonia solani* are considered as a major source of primary inoculum and difficult to control with standard fungicides (Van Beneden et al. 2010), the effect of *B. subtilis* CLPs on the viability of the sclerotia was tested. Therefore the sclerotia were incubated in CLP solutions during periods with variable length, from 6 hours to 72 hours. Only sclerotia incubated for 55 hours in mycosubtilin or fengycin or 24 hours in fengycin had a significantly reduced mycelium growth (Figure 5-6). Remarkably a longer incubation period did not result in a stronger reduction of the mycelium diameter, in contrast growth of mycelium from sclerotia incubated for 60 or 72 hours was not significantly different from the growth observed for sclerotia incubated in water. The observed significant reductions of the mycelium diameter of sclerotia that had been incubated in mycosubtilin or fengycin during 24 or 55 hours were small reductions (between 6 and 16% in comparison with sclerotia incubated in water) and the growth of the mycelium from these sclerotia was delayed, but one week after the setup of the experiment the entire PDA plates were covered with mycelium. Furthermore the mycelium growth from sclerotia incubated for 55 hours in water was lower than from sclerotia that were incubated for a shorter or longer period in water. Therefore the observed reduced growth might be due to the germination rate of the sclerotia used for this time point.

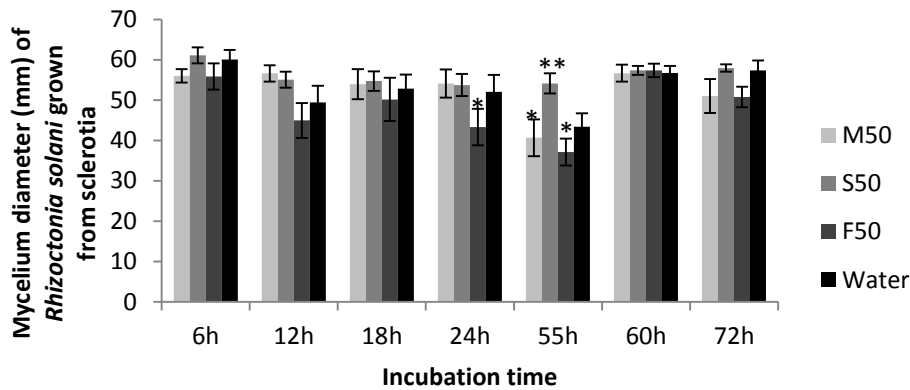


Figure 5-6: Mycelium diameter growing from sclerotia which had been incubated in *Bacillus subtilis* cyclic lipopeptides, 50 mg/L fengycin, 50 mg/L mycosubtilin, 50 mg/L surfactin (F50, M50 and S50, respectively), or water as a control. The incubation period varied between 6 hours and 72 hours. Significant differences in mycelium diameter compared to the sclerotia incubated in water, are indicated with * or ** (One Way Anova and Tukey analysis, $p = 0.05$) and standard bars represent standard errors.

Effect on basal rot caused by *Rhizoctonia solani*

Disease incidence and index were determined on plants treated four days before inoculation on a daily base, starting one day after inoculation until 6 days post inoculation (dpi) (Figure 5-7) because effect on the disease indices did not change anymore after six days post inoculation.

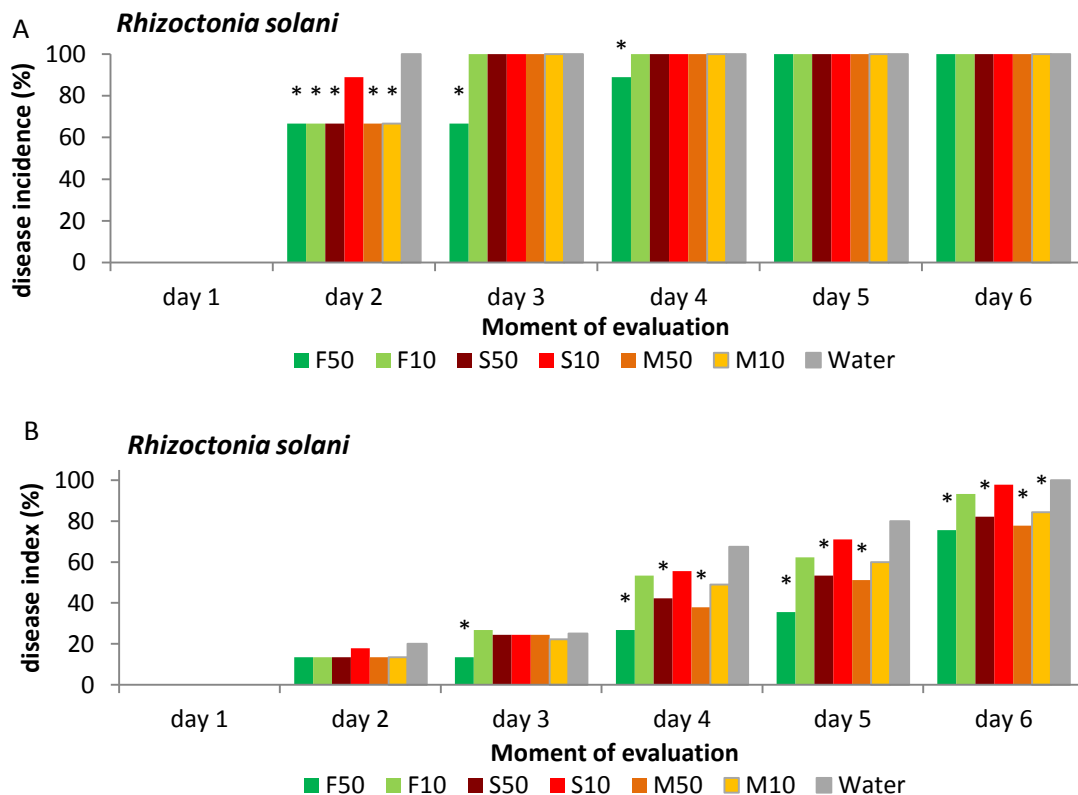


Figure 5-7: Disease incidence (A) and disease index (B) of basal rot caused by *Rhizoctonia solani* upon treatment with *Bacillus subtilis* cyclic lipopeptides (fengycin, surfactin or mycosubtilin at 10 mg/L or 50 mg/L, respectively F10, S10, M10, F50, S50 and M50) or water (control) four days before inoculation. Both disease incidence and disease index were monitored daily starting 1 day post inoculation (1 dpi) until 6 dpi. Significant differences in the disease indices of plants treated with the cyclic lipopeptide in comparison with the water treatment are indicated with *.

At 2 dpi the disease incidence was significantly different for plants treated with fengycin or mycosubtilin in both tested combinations and for plants treated with 10 mg/L surfactin. At 3 dpi and 4 dpi only plants treated with 50 mg/L fengycin had still a reduced disease incidence (Figure 5-7A). No significant effect in disease index upon CLP treatment was observed until the third day after inoculation. Starting from 3 dpi plants treated with 50 mg/L fengycin had a significantly reduced disease index. At 4 dpi, also plants treated with 50 mg/L surfactin or mycosubtilin had a significantly lower disease index in comparison with the control plants. At 6 dpi also for plants treated with 10 mg/L surfactin a reduced disease index was observed (Figure 5-7B).

5.4 Discussion and conclusions

Often it is reported that microorganisms showing biocontrol activities against *Botrytis cinerea* have no biocontrol activities against *Rhizoctonia solani*, and *vice versa*, with some exceptions amongst which some *Bacillus subtilis* strains (Fiddaman et al. 2000). In this chapter it was evaluated if mycosubtilin, surfactin or fengycin could suppress *B. cinerea* and/or *R. solani*.

5.4.1 Effect on *Botrytis cinerea*

Mycelium growth of *B. cinerea* in the *in vitro* experiments was reduced by fengycin or mycosubtilin solved in the growth medium.

Mixed with conidia, both mycosubtilin and fengycin (50 mg/L) were able to reduce the percentage of germinated conidia *in vitro*. Also in *in planta* experiments a direct effect of mycosubtilin was observed: mixing conidia with 50 mg/L mycosubtilin could reduce the disease incidence and disease index significantly in comparison with the disease observed on control plants.

Mixing conidia with fengycin before inoculation, reduced the disease index, yet not the disease incidence. A possible explanation is that fengycin delays germination of the conidia yet the dominant antifungal effect is targeted against the mycelium of *B. cinerea*. Percentage of germinated conidia was determined six hours after mixing the fresh conidia with fengycin and the effect on the disease incidence and index was evaluated three days after inoculation. Furthermore a strong reduction of mycelium growth was observed on plates with fengycin mixed in the growth medium. From the study of Farace *et al.* is concluded that fengycin can inhibit the germination of conidia. In that study the CLPs were spotted on CCM (Catalyst Coated Membrane) in a concentration of 50 mg/L and when the CLPs had dried, conidia mixed with Peptone/Tween agar medium were poured on the membrane. Fungal growth was observed 4 days later. In this experiment (almost) no mycelium growth was observed on spots with plipastin (Farace et al. 2015). Probably in this experiment not only the effect on conidia germination but also on mycelium growth was observed. Therefore these results are not necessarily contradictory with the experiments observed in the study described in this chapter. Probably the effect of mycosubtilin on the disease incidence and index was stronger compared to the effect of fengycin because mycosubtilin had a stronger and/or more permanent antifungal effect on germination of the *B. cinerea* conidia.

It was reported before for *B. cinerea* infection on apple that fengycins played a role in the biocontrol activity of *B. subtilis* as it was shown that fengycins were produced *in situ*. In this study was

suggested that the observed activity would rather be a direct antifungal effect rather than host-resistance induction because the effect was dependent on the bacterial population size and host-resistance induction is independent of the population size above a certain threshold level (Van Loon et al. 1998; Touré et al. 2004). In the experiments described in this chapter, treatment of plants with fengycin at 4 days before inoculation, resulted in a reduced disease index, only in the concentration of 10 mg/L, while no significant effect was observed for a concentration of 50 mg/L. Yet 50 mg/L fengycin solution reduced the number of germinated conidia and reduced the mycelium growth. Therefore it seems 10 mg/L fengycin triggers responses in the plant which contribute to enhanced disease resistance against basal rot caused by *B. cinerea*. It might be interesting to repeat the experiments with treatment of the leaves 4 days before and just before inoculation and inoculate leaves besides with a conidial suspension also with mycelium plugs. With this experiment could be tested if the direct effect of fengycin is predominantly against mycelium growth and not suppressing conidia germination. Furthermore could be seen if the observed reduction of the disease index observed upon treatment 4 days before inoculation is due to enhancement of the defence mechanism of the plant or a direct antifungal effect against conidia germination or mycelium growth. Treatment of lettuce leaves 4 days before inoculation with mycosubtilin, could suppress disease incidence and disease index of basal rot caused by *B. cinerea*. Both concentrations of mycosubtilin of 10 mg/L and 50 mg/L were effective to reduce the disease incidence and index. To determine if the observed reduction in disease incidence and index is due to a direct antifungal effect against *B. cinerea*, the effect of 10 mg/L mycosubtilin on conidia germination and mycelium growth should be evaluated. The findings of Farace and colleagues for the effect on *B. cinerea* on grapevine are that mycosubtilin activated expression of defence related genes involved in the salicylic acid and jasmonic acid, contributing to resistance against grey mould development in grapevine, but also that mycosubtilin inhibited germination of *Botrytis cinerea* spores (Farace et al. 2015).

In the study described in this chapter, surfactin did not have a direct nor indirect effect on basal rot of lettuce caused by *B. cinerea*. Romano *et al.*, who did similar tests with *B. cinerea* growing on PDA plates in which *Bacillus amyloliquefaciens* CLPs were added, did not observe a direct antifungal effect of surfactin either (Romano et al. 2013). Furthermore, also in the study of Farace *et al.* no direct antagonistic effect of surfactin was observed against *B. cinerea*, (Farace et al. 2015) yet, in grapevine an immune response was activated by surfactin treatment, able to protect the plants against *B. cinerea* (Farace et al. 2015). Furthermore, when surfactin was applied on roots of tobacco plants, systemic resistance was induced against *B. cinerea* (Henry et al. 2011). These findings are indications that the abilities of the CLPs of *B. subtilis* to induce systemic resistance are plant species-dependent.

5.4.2 Effect on *Rhizoctonia solani*

Mycelium growth of *Rhizoctonia solani* was only reduced in the *in vitro* experiment by solving 50 mg/L mycosubtilin in the growth medium. Surfactin or fengycin slowed down the growth yet the effect was less effective than the effect of mycosubtilin.

Incubation of the sclerotia for 24 hours or 55 hours in 50 mg/L mycosubtilin or fengycin seemed to delay the growth of the sclerotia, however seven days after placing the sclerotia on PDA plates, mycelium covered the entire surface of the plates. Therefore the effect was not permanent and the conclusion is that the tested CLPs could not inhibit or negatively affect viability of the sclerotia. The

structure of sclerotia of AG-1 are composed of three well-defined layers. In the centre, the cells have a dense contents. These cells are surrounded by an outer layer of empty cells, which were bordered by a darkly-pigmented mucilaginous surface-layer. This constructions protects the central cells for external influences, allowing the pathogen to survive long periods without hosts (Takashi and Tadao 1978). Yet, iturins were reported to inhibit germination of microsclerotia of *Verticillium dahliae* (Han et al. 2015).

Disease incidence is only reduced upon treatment of the leaves 4 days before inoculation with 50 mg/L mycosubtilin. Fengycin and surfactin treatment reduced the severity of the disease, with a dose dependent effect: treatment with 50 mg/L reduced the disease index significantly more than 10 mg/L. The disease index of leaves treated with 50 mg/L mycosubtilin was not significantly different from the disease index of plants treated with 10 mg/L mycosubtilin. No effect was observed on the disease incidence upon fengycin treatment but the disease index was significantly yet to a limit extent reduced. Maybe this is not due to a direct effect because only a limited effect on mycelium growth was observed, but maybe plant defence was activated resulting in a reduced severity of the disease.

5.4.3 Conclusions and prospects

In this chapter we have shown that *Bacillus subtilis* CLPs could reduce basal rot caused by *Rhizoctonia solani* or *Botrytis cinerea*. Signum was only incorporated as control treatment in the experiments to assess the effect of the CLPs on mycelium growth. Yet, it would have been more consistent to provide a fungicide control in each experiment.

To understand which defence pathways are involved in the enhanced resistance mediated by mycosubtilin (against *B. cinerea*) or by mycosubtilin, surfactin or fengycin (against *R. solani*), pre-treatment of plants with the hormone homologues described in Chapter 4 should be followed by inoculation with these two pathogens. Furthermore gene expression in inoculated plants pre-treated with the hormone homologues should be compared with defence gene signature in inoculated plants pre-treated with CLPs of *B. subtilis*. Both *B. cinerea* and *R. solani* are necrotrophic pathogens, thus induction of the ET/JA signalling pathway can be expected. We have seen that upon ethephon treatment none of the selected genes was up-regulated in non-inoculated plants. Therefore an ET-marker gene should be selected to create a more complete image of the effect of the CLPs on lettuce gene expression and the effect relating defence against basal rot caused by *B. cinerea* and *R. solani*. Because only mycosubtilin could induce enhanced resistance against *B. cinerea*, while also surfactin and fengycin could enhance resistance against *R. solani*, it is possible that defence against these two pathogens is not regulated (completely) via the same pathway.

Other studies mainly focused on direct antagonistic effect of biocontrol organisms and lipopeptides, often indicating that iturin A plays a major role in the biocontrol activities, however also biocontrol effect of surfactin and fengycin is mentioned (Yu et al. 2002; Mizumoto et al. 2007; Elkahoui et al. 2012; Guo et al. 2014).

Other experiments were conducted with *Bacillus* bacteria rather than purified CLPs (Asaka and Shoda 1996; Paul et al. 1998; Touré et al. 2004; Farace et al. 2015). It might be interesting to analyse the effect of bacteria application as foliar spray or as soil treatment on basal rot, in comparison with pure

CLP application, because it might be possible that other characteristics or produced components of these bacteria can play a role in control of basal rot by direct competition with soil-borne pathogens by synthesis of antimicrobial components or competition for nutrients and niche (Stephens et al. 1993; Glick and Bashan 1997; Kamilova et al. 2005; Van Loon 2007; Neeraja et al. 2010; Maksimov et al. 2011; Beneduzi et al. 2012; Farace et al. 2015). Therefore the lettuce rhizosphere colonization capacity of different CLP producing *Bacillus* strains should be tested (Pandey and Palni 1997; McSpadden Gardener 2004), because efficient colonization is important for the bacteria to exert their beneficial effects (Chin-A-Woeng et al. 2000; Lugtenberg and Kamilova 2009). Four *Pseudomonas fluorescens* strains (KF36, KS16, KS74 and KS90) displayed strong *in vitro* antagonistic activity against *R. solani* AG1-1B and could efficiently colonize the lettuce rhizosphere (Adesina et al. 2009). It is known that *Bacillus subtilis* can efficiently colonize tomato rhizosphere (Nihorimbere et al. 2010), but also that there are significant differences in rhizosphere characteristics, probably reflecting qualitative and quantitative differences, in root exudates in lettuce and tomato rhizospheres (Maloney et al. 1997). Attention should be paid to select an adequate *B. subtilis* strain. Furthermore temperature should be taken into account because this is one of the factors determining if the bacteria can grow and if/which CLPs will be produced (Fickers et al. 2008; Deravel et al. 2014). When applying the bacteria instead of the pure lipopeptides, it would be best if the bacteria could produce CLPs of all three CLP families yet often not all CLPs are produced at the same moment (Touré et al. 2004), depending on root exudates and biofilm-related structures around root hairs (Nihorimbere et al. 2012).

Another possibility is to apply CLPs as a soil treatment. However, there are indications of low persistence of iturin A in soil because iturin A concentrations in soil decreased to undetectable levels after 17 days with starting concentrations around 500 µg /g soil (Mizumoto et al. 2007). Also in the study of Asaka and Shoda it was noticed that the concentration of iturin A was reduced after 14 days in soil, while the concentration of surfactin was more maintained (Asaka and Shoda 1996). For mycosubtilin, surfactin and fengycin there is, to our knowledge, no information about persistence in soil available, yet low persistence is predicted due to their biodegradability (Hoefler et al. 2012; Deravel et al. 2014).

In future it would be useful to repeat the tests described in this chapter with different host strains of *B. cinerea*. Strains from different host plants are often able to infect other hosts (Bardin et al. 2013; Leyronas et al. 2014) and protective efficacy of resistance-inducing products can be influenced by the *B. cinerea* strain (Bardin et al. 2013). These strain dependent results should be studied more in detail, because it can probably lead to selection of more resistant *B. cinerea* strains. However, it is also claimed that due to the complex mode of action of biofungicides based on *Bacillus subtilis* bacteria such as Serenade, resistance development is less likely than for conventional fungicides (Maronne 2002).

In conclusion the results in this chapter show the potential of *Bacillus subtilis* CLPs to control basal rot caused by *B. cinerea* or *R. solani*, two of the major pathogens causing this disease in lettuce. Surfactin treatment had only a small effect on mycelium growth of *R. solani*, while fengycin had mainly a direct antifungal effect against *B. cinerea*, although also an effect on the plant defence mechanism against basal rot caused by *B. cinerea* can be expected. Fengycin did not show a strong effect on *R. solani*. Mycosubtilin was the most effective cyclic lipopeptide with a direct antifungal

effect against *B. cinerea* and *R. solani* and also resulting in a reduction of disease incidence or index of basal rot caused by one of these pathogens when leaves were treated four days before inoculation.

Chapter 6. Effect of glycolipids on lettuce downy mildew and basal rot caused by *Rhizoctonia solani* or *Botrytis cinerea*

The cyclic lipopeptides of *Bacillus subtilis* are the topic of many studies for potential biofungicides. Yet, some glycolipids have also shown to possess antimicrobial activities. In this chapter preliminary tests were performed to evaluate the effect of three glycolipids (cellobiose lipid, glucolipid and bola sophorolipid) on the lettuce pathogens *Bremia lactucae*, *Botrytis cinerea* and *Rhizoctonia solani*. All three tested glycolipids were produced by or derived from products of the yeast *Starmerella bombicola*. Cellobiose lipid and bola sophorolipid were only effective against *B. lactucae*. Glucolipid slowed down mycelium growth of both *B. cinerea* and *R. solani*. Furthermore treatment of lettuce leaves reduced downy mildew infection. Mixing *B. cinerea* conidia with glucolipid also resulted in reduced disease index of basal rot. Overall, glucolipid seems to be most promising as potential biofungicides for the studied three lettuce pathogens, yet further study is necessary to unravel the working mechanism and determine the optimal application method.

6.1 Introduction

In search for biofungicides there are abundant research reports concerning the cyclic lipopeptides of *Bacillus subtilis*. At the research department Inbio of the Ghent University, research is ongoing for the development of biosurfactants to replace synthetic surfactants which often have an eco-toxic effect on the environment. The purpose of the surfactant production is to be used in households, in laundry detergents, as applications in the medical world or in cosmetics but also in crop protection (Tran 2012).

In this work three types of glycolipids are tested for their potential to control downy mildew or basal rot caused by *Botrytis cinerea* or *Rhizoctonia solani* on lettuce: cellobiose lipids, glucolipids and bola sophorolipids. Glycolipids are compounds which contain saccharides residues and long-chain fatty acids of hydroxylaliphatic acids (Tran 2012). Glycolipids are together with glycoproteins two types of antifungal agents secreted by yeasts (Kulakovskaya et al. 2004). Some glycolipids are known to possess antimicrobial activities (Shah et al. 2007; Bölker et al. 2008; Golubev et al. 2008; Kulakovskaya et al. 2009; Mimee et al. 2009; Kulakovskaya et al. 2010; Hammami et al. 2011; Teichmann et al. 2011; Trilisenko et al. 2012) for instance ustilagic acid, a cellobiose lipid produced by *Ustilago maydis*, was shown to exert an antagonistic effect against *B. cinerea* because wild type *U. maydis* could control *B. cinerea* infection on tomato leaves, while a mutant unable to produce ustilagic acid lost this ability (Teichmann et al. 2007). Flocculosin, a cellobiose lipid secreted by *Pseudozyma flocculosa*, demonstrated antagonistic effects against *Pythium aphanidermatum*, *Phytophthora infestans*, *B. cinerea* and *Candida albicans* (Cheng et al. 2003). The cellobiose lipid used in our study is enzymatically derived from *Starmerella bombicola* glucolipid and has shown antifungal activity against the biotrophic fungus *U. maydis*. No effect was observed against *Aspergillus flavus*, *Saccharomyces cerevisiae*, *Penicillium roqueforti*, *Cryptococcus laurentii*, *Rhodotorula bogoriensis*, *Rhizopus oryzae* or *S. bombicola*. Direct antagonistic effect was tested in experiments performed on PDA or yield extract-malt extract plates (Tran 2012).

Also some sophorolipids possess antifungal, antiviral and spermicidal properties (Christie 2014). The glucolipids used in this study are intermediates formed during the production of sophorolipids by *Starmerella bombicola* (Saerens et al. 2011; Van Bogaert et al. 2013). Some sophorolipids, produced by *S. bombicola*, are claimed to have mild antimicrobial activities (Van Bogaert et al. 2007; Christie 2014).

The bola sophorolipid used in these chapter were produced by a newly at Inbio developed *S. bombicola* strain (Sophie Roelants, InBio, personal communication on 27th of June 2014).

The purpose of this chapter was to take the first steps in analysis of the potential of these glycolipids as biofungicides against downy mildew or basal rot caused by *B. cinerea* or *R. solani*. In this chapter the effect of foliar application of the glycolipids on downy mildew and basal rot development is evaluated. Furthermore also the direct antifungal effect of the glycolipids against *B. cinerea* or *R. solani* was examined.

6.2 Material and methods

6.2.1 Plant material

Lettuce plants of the susceptible variety Green Towers, of which seeds were kindly provided by Diederik Smilde from Naktuinbouw (the Netherlands, <http://www.naktuinbouw.com/>) were used for all experiments with downy mildew. Approximate 20 seeds were sown on wetted cotton wool and a layer of filter paper in a plastic transparent box. The plants were grown in a growth chamber at 16°C and day/night regime of 16h light, 8h dark.

For the experiments with *Botrytis cinerea* and *Rhizoctonia solani*, fully grown butterhead lettuce heads were purchased, of unknown cultivar.

6.2.2 Pathogen maintenance

6.2.2.1 *Bremia lactucae*

Bremia lactucae isolate BmVL144 was used for all inoculations. This isolate was collected from Romaine lettuce (*Lactuca sativa* variety *longifolia*) in Sint-Katelijne-Waver in 2012 and maintained and multiplied on seedlings of *L. sativa* cv. Green Towers. Fresh spores were washed off with sterile water from infected leaves. The concentration of the spore suspension was adapted to 5×10^4 spores/mL and applied as a foliar spray on seven-day-old lettuce seedlings in two-leaf-stage until running-off. Boxes with inoculated seedlings were incubated under growth chamber conditions (day/night regime of 16 h light/8 h dark, 16 °C and relative humidity of minimum 90%). Under these conditions new spores appeared 5 to 6 days after inoculation and sporulation was maximal at 10 days after the inoculation, the moment disease incidence was determined in all experiments. Disease incidence was calculated as the percentage of leaves with sporulation on the total number of inoculated leaves.

6.2.2.2 *Botrytis cinerea* and *Rhizoctonia solani*

Botrytis cinerea strain R16 (Faretra and Pollastro 1991) and *Rhizoctonia solani* strain S001-2 belonging to AG1-1B (Van Beneden et al. 2009) were cultured on potato dextrose agar (PDA, 39 g/L) at room temperature. PDA plates (90 mm) with *B. cinerea* were incubated with light/darkness regime of 14 h light, 8 h darkness, plates with *R. solani* were incubated in the dark.

6.2.3 Foliar application of glycolipids

All glycolipids in this study, i.e. cellobiose lipid, glycolipid and bola sophorolipid were produced and purified by InBio. Plants were treated with the glycolipids, applied as foliar spray until running-off, one day or four days before inoculation. Bola sophorolipid and glucolipid were solved in water, cellobiose lipid was solved in dimethyl sulfoxide (DMSO) in concentrations of 10, 50 and 100 mg/L with final concentrations of 0.5, 2.5 and 5% DMSO. Water was used as control for treatments with bola sophorolipids and glucolipids, DMSO-solutions were used as control for cellobiose lipid treatments. The same concentrations were applied as the concentrations used in the experiments

with the cyclic lipopeptides of *Bacillus subtilis* (Chapter 4 and 5), to allow comparison of the cyclic lipopeptides and glycolipids. Each treatment was repeated three times and each repetition was performed with on average 25 plants in experiments with treatment 4 days before inoculation and on average 26 plants in experiments with treatment 1 day before inoculation.

Statistical analyses to assess the effect of the treatments on disease incidence were performed using binary logistic regression analysis ($p=0.05$).

6.2.4 Effect on mycelium growth

Potato dextrose agar (PDA, 39 g/L) was mixed with the glycolipids with a final concentration of the glycolipids of 50 mg/L before pouring the plates. The same concentrations were applied as the concentrations used in the experiments with the cyclic lipopeptides of *Bacillus subtilis* (Chapter 5), to allow comparison of the cyclic lipopeptides and glycolipids. Positive control plates only contained PDA and negative control plates consisted of PDA mixed with the fungicide Signum (3 g/L, BASF, active ingredients boscalid and pyraclostrobin, <http://www.agro.basf.be>), which is effective against both *Rhizoctonia solani* and *Botrytis cinerea*. To observe the effect of cellobiose lipid, only 2 mg/L concentration was tested, containing 0.1% DMSO because preliminary results had shown that higher concentrations of DMSO reduced mycelium growth significantly. Experiments were conducted on 55 mm plates and in the middle a 3 mm fungal plug was placed, taken from a 7-days-old culture of *R. solani* or a 10-days old culture of *B. cinerea*. Each treatment was conducted on ten plates for each pathogen. Mycelium growth was measured daily during four days starting the first day after setup as the diameter of the mycelium.

Statistical analyses to assess the effect of the treatments on mycelium growth were performed using One Way Anova and Tukey analysis ($p=0.05$).

6.2.5 Effect on *Botrytis cinerea* infection

Once a PDA plate was fully covered with *B. cinerea* mycelium, it was incubated for one week under continuous near-UV light to stimulate sporulation (Sowley et al. 2009). One 90 mm plate covered with sporulating mycelium was cut into pieces and PDA, mycelium and conidia were mixed with 15 mL sterile bidest water and subsequently filtered (250 μm gaze). The conidial suspension was mixed with potato dextrose broth (PDB, 6 g/L) and 50 mg/L glycolipids (50 mg/L or 2 mg/L for cellobiose lipid) or with DMSO or water or fungicide Signum (3 g/L) as controls. DMSO was the control for cellobiose treatments, water for the other tested glycolipids. The same concentrations were applied as the concentrations used in the experiments with the cyclic lipopeptides of *Bacillus subtilis* (Chapter 5), to allow comparison of the cyclic lipopeptides and glycolipids. Concentration of the conidial suspension was adapted to 10^5 spores/mL. Lettuce leaves of full grown purchased lettuce heads with the end of the midrib covered with moist tissue, were inoculated with a drop of the conidial suspension (10 μL) and inoculated leaves were placed in plastic trays. These small trays were put in larger trays on moisturized tissue and covered to create a dark environment with high relative humidity. Trays were incubated at room temperature for three days. There were 2 or 3 inoculum drops per leaf and 2 leaves per tray for each treatment. In total disease incidence and index were determined for 17 drops on average for each treatment.

Disease incidence was calculated with following formula:

$$\text{disease incidence (\%)} = \frac{\# \text{ of leaves with symptoms}}{\text{total \# of inoculated leaves}} \times 100$$

To assess the disease severity, the disease was scored from 0 (no symptoms) to 3 (most severe symptoms) (Figure 6-1) and the disease index was determined with following formula.

$$\text{disease index (\%)} = 100 \times \frac{\# \text{ of leaves with score } 1 \times 1 + \# \text{ leaves with score } 2 \times 2 + \# \text{ of leaves with scores } 3 \times 3}{\text{total \# of inoculated leaves} \times 3}$$

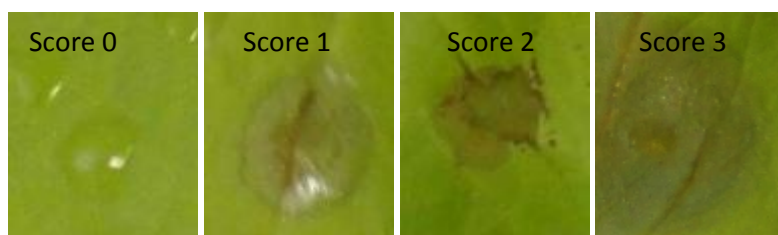


Figure 6-1: Disease score of basal rot caused by *Botrytis cinerea*. Score 0: no symptoms, score 1: brown spots within the boundaries of the droplet, score 2: spread of the necrosis outside the boundaries of the droplet, score 3: symptoms widely spread on the leaf.

6.2.6 Effect on *Rhizoctonia solani* sclerotia germination

A 90 mm PDA plate with seven-day-old *R. solani* mycelium was grinded with 25mL bidest water. This suspension was applied as a thin layer on fresh PDA plates, which were incubated for 35 days in darkness on room temperature (Manning et al. 1970). Next, sclerotia were collected.

For each treatment, 12 sclerotia were incubated in solutions of 1 mL with a concentration of 50 mg/L for all glycolipids and for cellobiose lipid also 2 mg/L was tested. The incubation period lasted 6 to 72 hours, followed by washing the sclerotia in 1% NaOCl during 2 minutes and two additional washing steps in sterile water. Dried sclerotia were placed on a fresh PDA plate and incubated at room temperature in darkness. Diameter of mycelium growth was measured daily starting after three days of incubation.

Statistical analyses to assess the effect of the treatments on mycelium growth were performed using One Way Anova and Tukey analysis ($p=0.05$).

6.3 Results

6.3.1 Effect of foliar treatment of lettuce leaves with glycolipids on downy mildew development

The effect of the glycolipids on lettuce downy mildew was assessed by applying the glycolipids as foliar spray 1 day before inoculation (dbi) or 4 dbi and evaluate the disease incidence ten days after inoculation. Concentrations of 100 mg/L were tested in a different experiment than concentrations of 50 mg/L and 10 mg/L. Results could not be pooled and are therefore shown in two figures for each application moment.

Application 1 day before inoculation

The disease incidence of plants treated 1 dbi with 100 mg/L bola sophorolipid (53%) or glucolipid (40%) was significantly reduced in comparison with the disease incidence of plants treated with water (65%). Lower concentrations of bola sophorolipid or glucolipid had no significant effect on the disease incidence (Figure 6-2b). No significant difference of disease incidence was observed for plants treated with 100 mg/L cellobiose lipid or for plants treated with the control treatment of 5% DMSO (Figure 6-2a). The DMSO solution reduced the disease incidence significantly in comparison with the plants treated with water: 6.5% versus 65%. In this concentration and at 10 days post inoculation, no negative effect was observed of the DMSO treatment on the lettuce plantlets, however experiments should be performed to determine the maximum concentration of DMSO without negative effect on full-grown plants. Lower concentrations of cellobiose lipid and DMSO were less effective in reducing the disease incidence (Figure 6-2b).

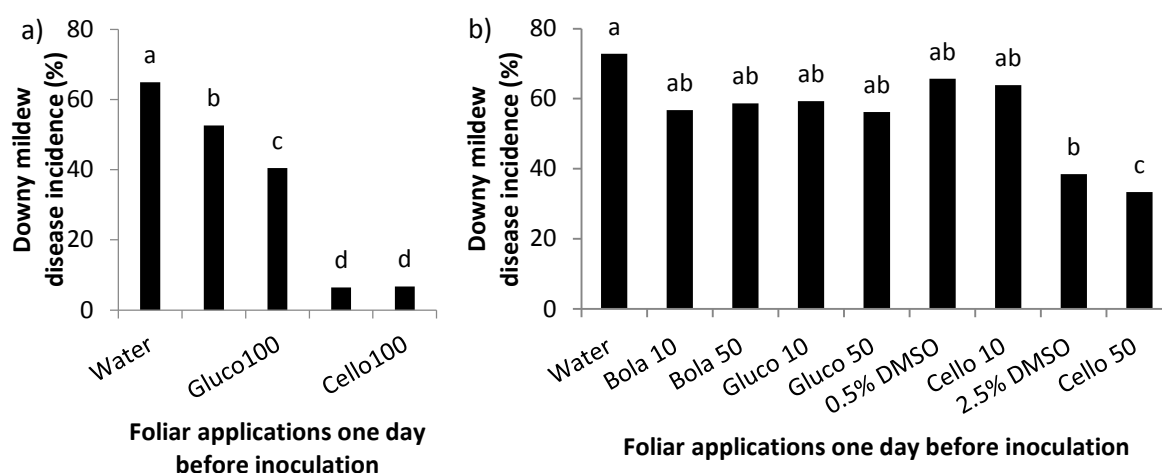


Figure 6-2: Downy mildew disease incidence (%) of plants at 10 days post inoculation. Plants were treated with glycolipids one day before inoculation. Treatments in figure a) are Bola100: 100 mg/L bola sophorolipid, Gluco100: 100 mg/L glucolipid, Cello100: 100 mg/L cellobiose lipid. Bola sophorolipid and glucolipid were solved in water, cellobiose lipid was solved in 100% DMSO, and the final concentration of DMSO in the cellobiose lipid solution was 5%. Therefore results of the cellobiose lipid were compared with a control treatment of 5% DMSO. Treatments in figure b) are bola sophorolipid (Bola), glucolipid (Gluco) and cellobiose lipid (Cello) in two concentrations: 10 mg/L and 50 mg/L. Cellobiose lipid was solved in 100% DMSO, final concentrations of DMSO in Cello10 and Cello50 were 0.5% and 2.5%, which were used as control treatments to compare the effect of cellobiose lipid with. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

Application 4 days before inoculation

When the glycolipids were applied four days before inoculation, results were very similar to the results obtained with applications one day before inoculation (Figure 6-3a). Plants treated with 100 mg/L bola sophorolipid or glucolipid had a significantly reduced disease incidence in comparison with plants treated with water: 61% and 48% respectively, versus 78%. There was a significant difference between the disease incidence of plants treated with 5% DMSO (12%) or 100 mg/L cellobiose lipid (5%). Treatments with lower concentrations of bola sophorolipid or glucolipid were not effective to reduce the disease incidence and the lower concentrations of cellobiose lipid solutions were less effective (Figure 6-3b).

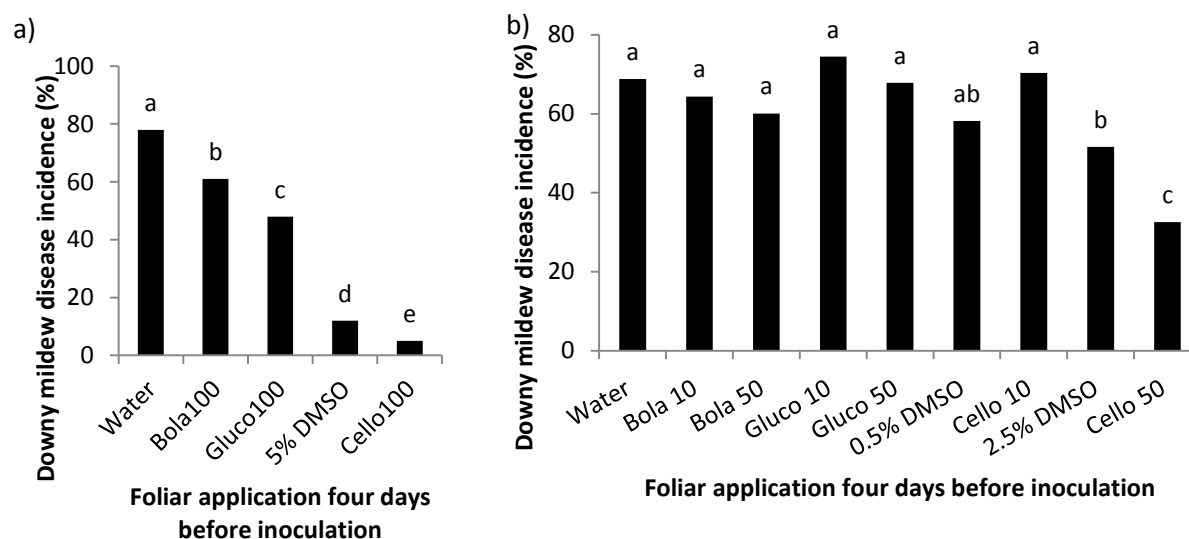


Figure 6-3: Downy mildew disease incidence (%) of plants at 10 days post inoculation. Plants were treated with glycolipids four days before inoculation. Treatments in figure a) are Bola100: bola sophorolipid, 100 mg/L; Gluco100: glucolipid, 100 mg/L; Cello100: cellobiose lipid, 100 mg/L. Bola sophorolipid and glucolipid were solved in water, cellobiose lipid was solved in 100% DMSO, and the final concentration of DMSO in the cellobiose lipid solution was 5%. Therefore results of the cellobiose lipid need to be compared with a control treatment of 5% DMSO. Treatments in figure b) are bola sophorolipid (Bola), glucolipid (Gluco) and cellobiose lipid (Cello) in two concentrations: 10 mg/L and 50 mg/L. Cellobiose lipid was solved in 100% DMSO, final concentrations of DMSO in Cello10 and Cello50 were 0.5% and 2.5%, which were used as control treatments to compare the effect of cellobiose lipid with. Different letters indicate statistically significant differences (binary logistic regression analysis, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

6.3.2 Effect of glycolipids on *Botrytis cinerea*

Effect on mycelium growth

Only on plates with 50 mg/L glucolipid in the PDA mycelium growth of *B. cinerea* was slowed down, while growth was totally inhibited on plates with Signum. Four days after experimental set up, the diameter of the mycelium on plates containing glucolipid was similar to the diameter of the fungi grown on PDA plates without supplements (Figure 6-4).

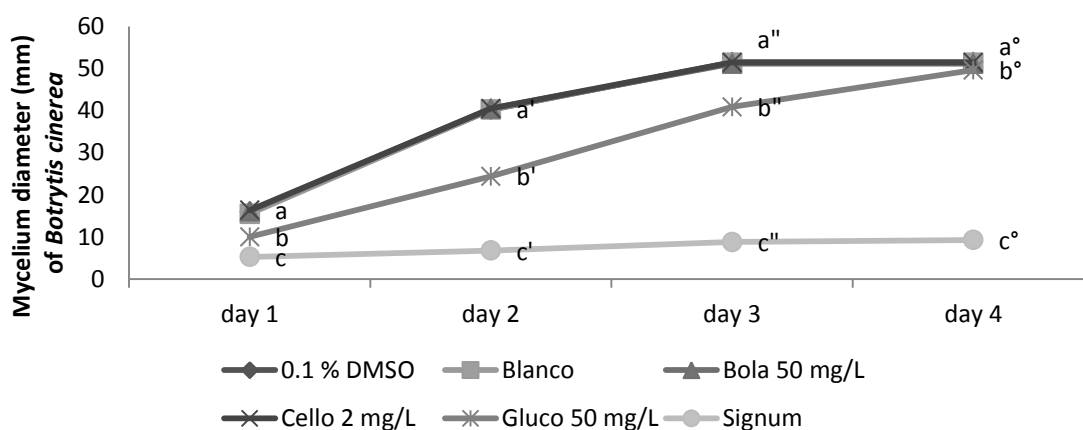


Figure 6-4: Effect of glycolipids bola sophorolipid (Bola), cellobiose lipid (Cello) and glucolipid (Gluco) solved in PDA plates on mycelium growth of *Botrytis cinerea*. Positive control plates were PDA plates without additives (Blanco) or with 0.1% DMSO added as control for 2 mg/L cellobiose lipid, which was solved in 100% DMSO with a total concentration of 0.1% DMSO in the cellobiose lipid solution. In negative control plates 3 g/L Signum was solved in the PDA. Different letters indicate statistically significant differences (One Way Anova and Tukey analysis, $p = 0.05$). Error bars represent standard errors of ten repetitions but are too small to be visible.

Effect on disease incidence and index

None of the glycolipid treatments could significantly reduce the disease incidence of basal rot caused by *B. cinerea* (Figure 6-5), however reduced disease indices were observed for 50 mg/L glucolipid, 2 mg/L cellobiose lipid and 50 mg/L cellobiose lipid. The effects on the disease index of cellobiose lipid were not significantly different from the effect obtained with DMSO controls. Remarkably DMSO reduced the disease index in these experiments in comparison with water treatment.

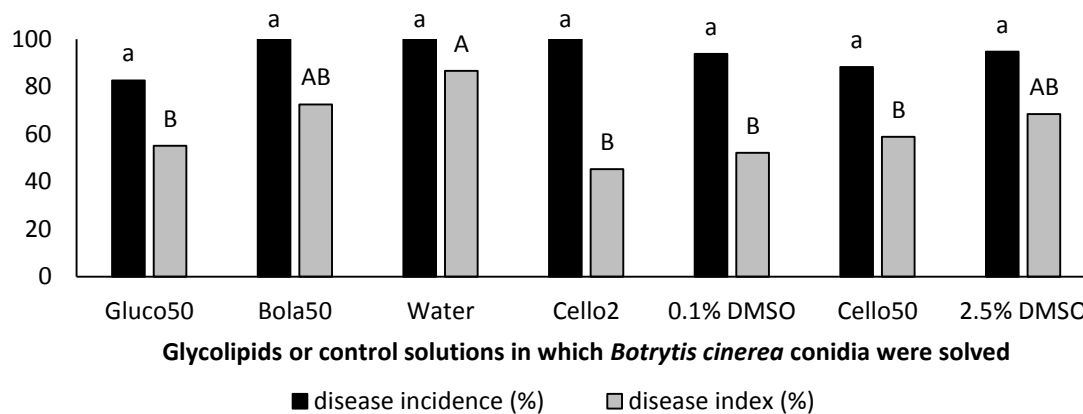


Figure 6-5: : Disease incidence (%) and disease index (%) of lettuce leaves inoculated with *Botrytis cinerea* conidia solution mixed with 50 mg/L bola sophorolipid (Bola50), glucolipid (Gluco50) or cellobiose lipid (Cello50) or 2 mg/L cellobiose lipid (Cello2). Cellobiose lipid was solved in 100% DMSO and final concentrations of Cello2 and Cello50 was 0.1% DMSO and 2.5% DMSO, respectively. Different letters indicate statistically significant differences (binary logistic regression analysis for the disease incidence and Kruskal-Wallis and Mann-Whitney U analyses for disease indices, $p = 0.05$). Note that no error bars are shown as data were not normally distributed.

6.3.3 Effect of glycolipids on *Rhizoctonia solani*

Effect on mycelium growth

Only glucolipid solved in the PDA medium, could delay the growth of *R. solani* mycelium (Figure 6-6). At 4 days post inoculation, no significant difference between mycelium growth on plates in which glycolipids were solved and on pure PDA plates was observed. The other tested glycolipids had no effect on the mycelium growth of *R. solani*.

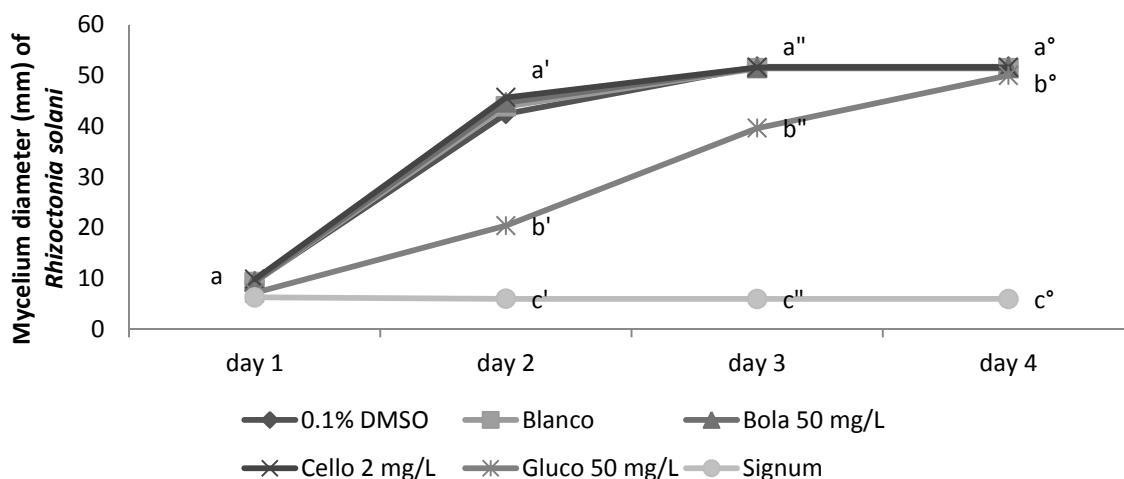


Figure 6-6: Effect of glycolipids bola sophorolipid (Bola), cellobiose lipid (Cello) and glucolipid (Gluco) solved in PDA plates on mycelium growth of *Rhizoctonia solani*. Positive control plates were PDA plates without glycolipids (Blanco) or with 0.1% DMSO added as control for 2 mg/L cellobiose lipid, which was solved in 100% DMSO with a total concentration of 0.1% DMSO in the cellobiose lipid solution. In negative control plates 3 g/L Signum was solved in the PDA. Different letters indicate statistically significant differences (One Way Anova and Tukey analysis, $p = 0.05$). Error bars represent standard errors of ten repetitions but are too small to be visible.

Effect on viability of sclerotia

None of the glycolipids could significantly reduce the viability of the sclerotia in comparison with the sclerotia incubated in water, indifferent for the incubation period, which varied from 6 hours until 72 hours (Figure 6-7).

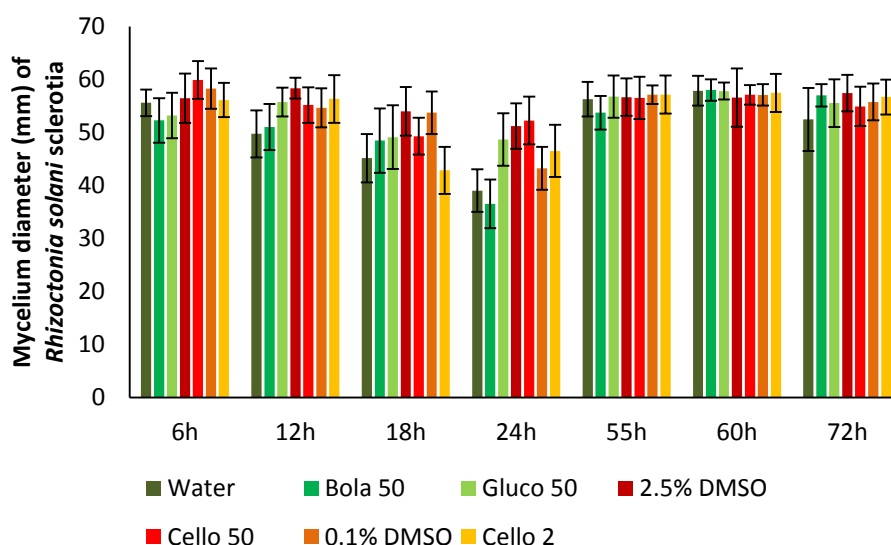


Figure 6-7: Effect of incubation in glycolipids on the viability of *Rhizoctonia solani* sclerotia. Incubation period varied between 6 hours and 72 hours. Viability was evaluated and measured as the diameter of the mycelium, measured three days after transfer of the sclerotia to PDA plates. No statistical differences were observed (One Way Anova and Tukey analysis, $p = 0.05$). Error bars represent standard errors of 12 repetitions.

6.4 Discussion and conclusions

6.4.1 Effect on downy mildew

The studied cellobiose lipid, glucolipid and bola sophorolipid were all able to reduce downy mildew disease incidence significantly when applied 1 dbi or 4 dbi. When applied 4 days before inoculation, treatments with glucolipids, cellobiose lipids and bola sophorolipids were effective to reduce the downy mildew disease incidence. Also application of glucolipid or bola sophorolipid 1 day before inoculation at 100 mg/L reduced the downy mildew disease incidence. For cellobiose lipid, only 50 mg/L concentration was effective to reduce the disease incidence, the effect of 100 mg/L was not significantly different from the control treatment with 5% DMSO. The effects observed of the glycolipids applied on the two studied application moments were thus very similar. This observation, combined with the noticed dose dependent effect of the glycolipids, suggests a direct antimicrobial effect of the glycolipids against *Bremia lactucae*. To verify this hypothesis, the effect of the glycolipids on conidia germination should be studied in future. Furthermore, another interesting experiment to test this hypothesis, would be to mix the *B. lactucae* conidia with the glycolipids just before inoculation, as was done in the experiment with *Botrytis cinerea* and compare the results of this experiment with the outcome of an experiment in which the lettuce plants were treated 4 days before inoculation with the glycolipids.

A direct antifungal effect of flocculosin, a cellobiose lipid produced by *Pseudozyma flocculosa* was observed against two oomycetes, *Pythium aphanidermatum* and *Phytophthora infestans*. This effect was only observed *in vitro* not *in planta* (Cheng et al. 2003). The mechanism of action of cellobiose lipids on yeast cells, is based on enhancement of nonspecific permeability of the cytoplasmic membrane caused by intercalation in and disturbance of the bilayer membrane structure (Puchkov et

al. 2002; Trilisenko et al. 2012). This results in rapid leakage of ATP (adenosine triphosphate) and potassium ions from the yeast cells (Kulakovskaya et al. 2005; Kulakovskaya et al. 2008; Trilisenko et al. 2012). The mycocidal activity is thought to be associated with the detergent-like, surface active properties of cellobiose lipid (Puchkov et al. 2002; Trilisenko et al. 2012).

Also some sophorolipids were proven to be effective against *Phytophthora* sp. and *Pythium* sp., by decreasing the motility of the zoospores, causing lysis of the zoospores and reducing damping-off disease incidence (Yoo et al. 2005).

6.4.2 Effect on basal rot

A relatively small, but significant inhibitory effect of glucolipid on mycelium growth of both *Botrytis cinerea* and *Rhizoctonia solani* was observed. Disease incidence of basal rot caused by *B. cinerea* was not influenced when mixing the conidia with the glycolipids, while the disease index was significantly lower on plants inoculated with conidia mixed with 50 mg/L glucolipid. This suggests that the glycolipids did not affect germination of the conidia and the observed effect is probably due to an antifungal effect on the mycelium. To test this hypothesis the direct effect of glucolipid on the germination of *B. cinerea* conidia should be tested. Furthermore the effect of leaf treatments 4 days before inoculation and 1 day before inoculation could be compared with the effect on the disease incidence and index of the described experiments in which conidia were mixed with the glycolipids just before inoculation. The glycolipids had no effect on the viability of *R. solani* sclerotia. Due to our stock solution, cellobiose lipid solutions contained relatively high concentrations of DMSO. To observe the effect of cellobiose lipid, disconnected from the effect of DMSO, we used a low concentration of cellobiose lipid. Yet, other studies report an antimicrobial effect against many species belonging to the Ascomycota and Basidiomycota (Trilisenko et al. 2012), which is concentration dependent (Kulakovskaya et al. 2007; Mimee et al. 2009). As *B. cinerea* and *R. solani* belong to the Ascomycota and Basidiomycota, respectively, an effect of cellobiose lipid could be expected when repeating the tests with a higher concentration of cellobiose lipid solutions containing a lower DMSO concentration. Another possibility is to solve the cellobiose lipid in another solvent, e.g. Tween, which has no effect on the studied pathogens.

6.4.3 Prospects and conclusions

Analysis of the expression of the stress related genes selected in Chapter 4 could be a next steps to determine if the glycolipids (also) induce enhanced defence responses in lettuce plants. To our knowledge, effect of glycolipids on stress related gene expression in plants has not been described yet.

Furthermore, to unravel the working mechanism of the glycolipids against *B. lactucae*, *B. cinerea* or *R. solani* testing the ATPase activity of the pathogen cells might be interesting. With this test it was shown that flocculosin acts by disrupting the membrane surface of sensitive micro-organism, causing leakage of the intracellular potassium and influencing plasma membrane ATPases (Mimee et al. 2009). Mixing 0.1% or 2.5% DMSO with *B. cinerea* conidia reduced the basal rot disease index, while in Chapter 5, no effect was observed for 0.1% DMSO. The reason remains unclear. A possible explanation may be that the lettuce heads used for these experiments were purchased, therefore it

is possible that the cultivar is different or the age or different treatment of the lettuce heads influences the results. The experiment should be repeated with lettuce crops grown in more controlled conditions.

Because the glycolipids studied in this chapter can be produced in relatively large volumes, future studies to evaluate their effect as biofungicides can be interesting. In the study described in this chapter, pH of the solution was not taken into account, although this can influence the activity of the glycolipids (Mimee et al. 2005) and should be considered in future experiments. Until now mostly the antimicrobial properties of cellobiose lipids were studied, yet because the glucolipid used in this study showed activity against all three studied pathogens, it might be interesting to dedicate more research on this component.

Chapter 7. General discussion, conclusions and perspectives

In this final chapter the results of this work will be summarized in a proposal for an integrated control strategy. Also how this strategy meets the requirements of integrated pest management is discussed here. Because an integrated control strategy should not cause or stimulate problems with other diseases, the side effects on basal rot are considered. The proposed control strategy describes the most effective method to use the currently available methods for downy mildew control. However, in this study also some suggestions and first steps are made to further improve the control strategy in future for instance by applying biofungicides as alternatives or additions for fungicide applications. These steps and suggestions are summarized in this chapter. Furthermore guidance needed for the implementation of the decision support tool by growers is shortly discussed. Finally, a short conclusion of this work ends the chapter.

7.1 Integrated control strategy for downy mildew

As mentioned in the problem statement, the aim of this work was to contribute to the development of an integrated, sustainable control strategy for the control of lettuce downy mildew. Based on the results described in this work the proposed control strategy starts with the choice of lettuce cultivars with optimal genetic resistance and provisions to reduce potential sources of inoculum. The next step of the strategy is to adapt the greenhouse climate during cultivation to suppress pathogen development and choose the most effective fungicides.

7.1.1 Aspects of the integrated control strategy

7.1.1.1 Potential sources of inoculum

Based on the mating type analyses in this study, observations of oospore presence and attempts to stimulate oospore germination, it was concluded that oospores are probably not the most important source of primary inoculum in Flanders. Yet, they could not be ruled out as potential source of inoculum either, because oospores were found and both mating types were detected.

L. serriola can serve as an alternative host from which conidia can spread and infect the lettuce plants. Yet this plant species is also described as source for resistance breeding (van Treuren et al. 2011; Lebeda et al. 2014). In this project we frequently found *L. serriola* plants, but never infected with *B. lactucae*. Seeds were collected and the seedlings were artificially infected with *B. lactucae* isolate Bl: 26. No difference in disease incidence between infected *L. serriola* and *L. sativa* plants was observed. This indicates it is possible the plant can harbour a source of primary inoculum for Belgian lettuce.

Although these possible sources of inoculum did not seem to be very important, in our study, it could not be proven that they were harmless either and thus cultivation should start with these precautions:

Cultivation should start with a clean greenhouse or field without lettuce plant debris in which oospores can occur, and free of the weed *Lactuca serriola*. Also the compost heap should be fenced of the greenhouse or lettuce field, to avoid conidia of old, diseased lettuce plants spreading to the new plants in the field or greenhouse (Lebeda 1984; Petzelová and Lebeda 2003).

One of the main sources of primary inoculum will be air borne conidia from infected lettuce plants cultivated. These conidia can be present when temperatures are between 0 °C and 23 °C on dry days.

7.1.1.2 Selection of lettuce cultivars

Analysis of the most effective resistance genes or factors showed that *Dm 15*, *Dm 17* and the resistance factors of lettuce cultivars Bedford, Balesta and Bellissimo provide resistance to the majority of the analysed samples. Therefore the conclusion is to cultivate lettuce with some of these resistance genes or factors. Rotation of cultivars, each possessing some of these most effective resistance genes and/or factors, can reduce the spread of isolates able to break through a resistance

gene or factor. For instance cultivation with a cultivar with resistance gene *Dm* 15 and the resistant factor of Balesta is followed by cultivation with a cultivar possessing *Dm* 17 and the resistant factor of Bellissimo. Even more effective would be to change from monoculture with one cultivar to a cultivation with different cultivars with different resistance genes or factors or with cultivars known to have field resistance.

7.1.1.3 Greenhouse climate adaptation and effective fungicide application

A third part of the suggested integrated control strategy is to adapt greenhouse climate when airborne conidia are predicted and/or apply fungicides.

Based on temperature and rainfall forecast the presence of airborne conidia is predicted. This assessment is very cautious: if it is dry and temperatures are between 0 °C and 23 °C, presence of airborne conidia is considered as an actual risk.

In this case, greenhouse climate adaptation is suggested to avoid germination and penetration. Therefore relative humidity should be below 90%. If the relative humidity was minimum 90% during 2 hours, there is an actual risk for germination and penetration. Therefore relative humidity should be adapted to below 85% during the night in the period from 5 to 32 days after potential penetration, to avoid sporulation.

If climate adaptation fails, fungicides should be applied, close to the moment of inoculation or when sporulation is expected, to be most effective. Which fungicide should be chosen, depends on the moment of application and the active ingredients which had proven to most effective at this application moment and the legal restrictions.

During this study some of the fungicides for downy mildew control were no longer legally registered in Belgium, indicating the importance of updating the decision support tool on regular basis, which might be a task for the research centres PSKW, PCG and Inagro.

7.1.2 *Link between requirements defined for integrated pest management and the proposed integrated control strategy for downy mildew*

The specifications described in the Action Plan of the Flemish Government (Vlaamse Regering 2013) and requirements for integrated pest management (IPM) are compared in Table 7-1.

Table 7-1: Comparison between IPM requirements and our results, described in this thesis.

Requirement IPM (Vlaamse Regering 2013)	Aspects of the proposed integrated control strategy for downy mildew
Prevention	<ol style="list-style-type: none"> 1. Reduce sources of primary inoculum by keeping the greenhouse free from plant waste after harvest to avoid oospore survival, remove <i>Lactuca serriola</i> plants to avert cross contamination originating from this alternative host for the pathogen, start with trustworthy plantlets and keep the compost heap fenced of the greenhouse to avoid airborne spores from old lettuce plants. 2. Use lettuce cultivars with resistance genes <i>Dm 15</i>, <i>Dm 17</i> and/or resistance factors of the lettuce cultivars Bedford, Balesta and Bellissimo in rotation or together in one cultivation cycle.
Monitoring and warning	<p>Monitoring:</p> <ul style="list-style-type: none"> • temperature and rainfall predictions lead to risk assessment for presence airborne conidia • relative humidity measurements in the greenhouse are used for prediction of inoculation and sporulation <p>Based on monitoring of temperature, rainfall and relative humidity, warnings are sent to the grower to inform about the risks</p>
<p>Intervention:</p> <ol style="list-style-type: none"> a) Only when economic damage threshold is exceeded b) Priority for biological, physical or mechanical intervention above and before chemical intervention 	<ol style="list-style-type: none"> a) Although in the current version of the forecasting model no inoculum threshold is included, suggestions are made based on results obtained with the spore sampler to determine an inoculum threshold as an improvement of the forecasting model b) Described interventions to control downy mildew <ol style="list-style-type: none"> 1) The first suggested intervention step is greenhouse climate adaptation to relative humidity conditions unfavourable for pathogen development 2) Application of biofungicides (CLPs or glycolipids) as addition to or replacement of conventional fungicides (section 7.2.4) 3) A ranking of different fungicide active ingredients was made for different application moments: relatively to the predicted moment of inoculation or the expected moment of sporulation.

7.2 Prospects

Besides studying the possibilities to improve the current control strategy of downy mildew, this work also provides some suggestions to further improve the downy mildew control strategy in future.

7.2.1 *Primary inoculum sources*

Molecular determinants for mating types are not available yet. However using molecular determinants would be a more effective approach for fast and large-scale mating type analyses. Furthermore it would give more information, for instance if *B. lactucae* is dioecious or if each mating type can produce both oogonia and antheridia, like in *Phytophthora* species (Michelmore and Ingram 1981; Michelmore and Wong 2008).

Tests with *Lactuca serriola* were now restricted to inoculations with *Bremia lactucae* isolate Bl: 26. Yet this isolate was not detected anymore after 2009. Therefore it would be useful to evaluate the current importance of *L. serriola* as alternative host by repeating the test with *B. lactucae* isolates that are nowadays regularly causing downy mildew outbreaks. Furthermore small *L. serriola* plants could be inoculated before the winter and in spring, infection of the plants should be evaluated to test if *B. lactucae* can survive during winter in this alternative host.

7.2.2 *Implementing the decision support tool in practice*

To get the model used by growers, it should be presented at information sessions at the research centres, which are in close contact with growers. As a first step to introduce the application to the growers, lettuce cultivation using the web application should be carried out at the centres to show growers how it works. In the next step, crop consultants should assist growers to install and use the application. In the research centres the web application should be adapted when legislation or gamma of fungicides changes in future to keep the application up to date. To be able to use the web application, growers should buy software for their climate box or free sensors, this is an extra cost, which does not seem to be paid off by using the application because one or two fungicide applications can be saved, but efforts should be done to adapt the greenhouse climate. Therefore it is important to convince growers that this application contributes to a more sustainable solution allowing avoidance of sudden disease outbreaks with major yield losses by reducing the chance of virulence adaptations of the pathogen, making it resistant to fungicides and able to bypass the genetic resistance of lettuce cultivars.

7.2.3 *Biofungicides*

In search for potential biofungicides to control downy mildew and basal rot, the cyclic lipopeptides (CLPs) mycosubtilin, surfactin and fengycin, produced by *Bacillus subtilis* and glycolipids produced by or derived from products of *Starmarella bombycola* were studied. Promising results were obtained, yet some remarks concerning the performed experiments should be made. To compare performance of the glycolipids and CLPs they should be tested in one experiment.

7.2.3.1 Cyclic lipopeptides of *Bacillus subtilis*

Production of *Bacillus subtilis* CLPs is dependent on environmental conditions such as temperature, pH and oxygen availability (Ohno et al. 1995; Cosby and Vollenbroich 1998; Jacques et al. 1999; Fickers et al. 2008; Guez et al. 2008; Deravel et al. 2014). Therefore it was chosen in our lettuce-CLP study (Chapter 4 and 5) to analyse the effect of the purified CLPs. Yet, analysing the effect of adequate *B. subtilis* strains colonizing the lettuce rhizosphere, as done in the rice-CLP study (Addendum I), might be interesting too because in some studies it was shown that biocontrol activity of *B. subtilis* is not (completely) dependent on CLP activities (Ongena and Jacques 2008).

Our experiments have shown that foliar application one day before inoculation of mycosubtilin or surfactin alone in a concentration of 100 mg/L could significantly reduce the disease incidence. The combination of mycosubtilin and fengycin could better protect the plants when applied together in comparison with the pure components, suggesting synergism. Mixing 100 mg/L mycosubtilin, surfactin or fengycin with *B. lactucae* conidia reduced the germination ability of the conidia (Chapter 4). Also against *R. solani* and *B. cinerea*, a direct antagonistic effect was observed from mycosubtilin or/and fengycin, respectively (Chapter 5). Also in other studies direct antagonistic effects of mycosubtilin and fengycin were reported. A study with *B. cinerea* and grapevine reported an antagonistic effect of mycosubtilin (Farace et al. 2015), while in a study with *B. cinerea* and bean besides an effect of mycosubtilin, also fengycin had antifungal activities (Ongena et al. 2005). Furthermore a direct antagonistic effect of mycosubtilin and fengycin was reported against *Pythium ultimum* (Ongena et al. 2005) and *Podosphaera fusca* (Romero et al. 2007). Other studies mention haemolytic, antiviral, antimycoplasma and antibacterial activities of surfactin (Ongena and Jacques 2008), but to our knowledge no direct effect against oomycetes was described before for surfactin. Fungitoxicity of mycosubtilin most likely relies on its membrane permeabilization properties based on formation of ion-conducting pores (Ongena and Jacques 2008). The mechanism behind fungitoxicity of fengycin is less well known, but it is reported to also be based on interaction with lipid layers, influencing the structure and permeability (Ongena and Jacques 2008). Yet, this mechanism remains focus for further studies.

The observed synergistic effects in our study from mycosubtilin and fengycin and mycosubtilin were also reported before. Interaction between iturins and fengycin was shown to play a major role in the antagonism of *Bacillus subtilis* toward *P. fusca* (Romero et al. 2007). Also in the ability of *B. subtilis* FZB42 to cope with competing organisms, iturins and fengycin played a role and act in a synergistic manner (Koumoutsi et al. 2004). Future studies should unravel whether aggregates are formed similarly as when iturins and surfactins are combined (Maget-Dana et al. 1992; Etchegaray et al. 2008; Falardeau et al. 2013).

A drawback of the experiments with the CLPs and glycolipids is that not in all experiments the effect of water and DMSO control treatments is compared. For downy mildew it was observed that treatment one day or four days before inoculation with 0.5% DMSO had no effect on the disease incidence, while a significant reduction of the disease incidence was observed for treatments with 2.5% DMSO in comparison with the disease incidence of the plants treated with water. The effect of DMSO on conidia germination was not evaluated. Furthermore, 0.1% DMSO had no effect on mycelium growth of *Botrytis cinerea* or *Rhizoctonia solani* and also viability of sclerotia was not

significantly affected by 0.1% DMSO in comparison with sclerotia incubated in water. The effect of applying DMSO on lettuce leaves 4 days before inoculation with *B. cinerea* or *R. solani* was not evaluated. Remarkably mixing *B. cinerea* conidia with 0.1% DMSO reduced the disease index and not the disease incidence while 2.5% DMSO had no effect. This might be an artefact because the difference between 0.1% DMSO and 2.5% DMSO was not significant. The results of Chapter 4 show an up-regulation of the gene coding for a basic pathogenesis related protein upon treatment with 1% DMSO at 3 days post treatment. This might indicate that DMSO can have an effect on the disease incidence or index of basal rot caused by *B. cinerea* or *R. solani*. Therefore the effect of DMSO should be studied in future. Another disadvantage is that the fungicide control is not used in all experiments to compare the performance of the glycolipids and CLPs with.

In our experiments 10 mg/L mycosubtilin or surfactin applied four days before inoculation, enhanced lettuce resistance to downy mildew. At 50 mg/L mycosubtilin treatment of lettuce leaves could suppress *B. cinerea* infection and basal rot caused by *R. solani* was reduced when leaves were pre-treated with 50 mg/L mycosubtilin, surfactin or fengycin. Pre-treatment of rice leaves with fengycin or mycosubtilin, could reduce sheath blight infection caused by *R. solani* (addendum). Also in other crops enhanced resistance against *B. cinerea* was mentioned upon treatment with CLPs of *B. subtilis*: mycosubtilin, fengycin and surfactin induced resistance responses in grapevine (Farace et al. 2015), fengycin and surfactin in bean (Ongena et al. 2005; Ongena et al. 2007), surfactin and fengycin in tomato (Ongena et al. 2007), only surfactin enhanced resistance in tobacco (Henry et al. 2011), while only fengycin was reported to support defence of apple against grey mould caused by *B. cinerea* (Ongena et al. 2005). To enhance resistance of bean against *P. ultimum* mycosubtilin or fengycin treatment were reported to be effective (Ongena et al. 2005) and upon mycosubtilin treatment tomato plants showed to be more resistant against *Pythium aphanidermatum* (Ongena et al. 2005). Defence responses against *P. fusca* were induced in melon plants treated with surfactin (Romero et al. 2007). Surfactin-triggered induced resistance, is most likely to result from small disruptions caused by insertion of small surfactin molecules into the host lipid membrane (Jourdan et al. 2009; Henry et al. 2011) and also the effectiveness of fengycin seems to be the result of binding to biomembranes (Falardeau et al. 2013). The underlying mechanism of mycosubtilin induced resistance is less well understood.

In our experiments, we did not analyse the effect of the CLPs on the signalling pathway of the pathogen. In the study concerning the effect of iturins on *Verticillium dahliae* it was reported that the iturins affect signalling pathways of this pathogen (Han et al. 2015). Therefore it might be interesting to test the effect of the CLPs on signalling pathways in *B. lactucae*, *B. cinerea* and *R. solani*, to compare with the results of the study of Han and colleagues.

Because no effect on the expression of *OPR3* is observed upon treatment with 10 mg/L mycosubtilin or surfactin, probably the jasmonate pathway was not influenced by these CLPs. A difference in the expression of *PAL1*, *EDS1* and *PRB1* in inoculated plants treated with 10 mg/L mycosubtilin or surfactin was noticed in comparison with the expression in the inoculated control plants. This might suggest that these CLPs affect the salicylic acid (SA) pathway. Upon treatment with mycosubtilin, surfactin or fengycin genes involved in the auxin-pathway were activated, while only mycosubtilin and fengycin could switch on the jasmonate (JA)- and ethylene (ET)-dependent signalling pathways in rice. JA, ET, abscisic acid (ABA) and auxin are implicated in the complex basal rice defence against *R.*

solani (Addendum). In the study with lettuce we did not analyse the expression pattern of the stress related genes upon fengycin treatment, because fengycin was not able to reduce downy mildew disease incidence when applied four days before inoculation, yet basal rot infection caused by *R. solani* was reduced in plants pre-treated with rice, making it interesting to analyse the effect of fengycin on the defence gene signature of lettuce as well. In potato tuber cell fengycin treatment resulted in accumulation of phenolic compounds (Ongena et al. 2005). From the study of De Cremer et al. (De Cremer et al. 2013) we learnt that there was a high correlation between the expression of stress related genes upon *B. lactucae* and *B. cinerea* infection. The only observed differences were the expression of *PRB1* and *EDS1*, which were more induced after *B. lactucae* inoculation in comparison with *B. cinerea* inoculation and the expression of *LOX* which was 13 times lower after inoculation with *B. lactucae* than after *B. cinerea* inoculation. This high correlation between the expressions of different stress related genes after compatible interactions with these two pathogens, might be an indication that resistance against these pathogens is also partly regulated via the same pathways and explains why mycosubtilin can induce protection against both *B. lactucae* and *B. cinerea*.

In other studies treatment of non-inoculated plants with mycosubtilin induced the expression of genes involved in the SA and JA pathway in grapevine and genes only involved in the SA pathway in *A. thaliana* (Farace et al. 2015). Surfactin treatment of non-inoculated plants was reported to switch on expression of SA dependent signalling pathway in grapevine (Farace et al. 2015), *A. thaliana* (Farace et al. 2015) and tobacco (Ongena et al. 2007). Besides genes coding for PAL and LOX showed induced expression upon treatment of tobacco with surfactin, and also an oxidative burst and accumulation of phenolics was observed (Ongena et al. 2007; Jourdan et al. 2009; Henry et al. 2011). Furthermore, reactive oxygen species (ROS) were detected in roots of tomato plantlets (Henry et al. 2011). Inoculated tomato plants pre-treated with surfactin and fengycin had an up-regulated expression of genes encoding for LOX and lipid hydroperoxidase (LHP) (Ongena et al. 2007). Table 7-2 summarizes our results concerning interaction with *B. subtilis* CLPs and lettuce, lettuce pathogens, rice sheath blight and rice in comparison with other recently reported studies of CLP interactions with plants and pathogens.

B. subtilis strain QST 713 is used in Belgian lettuce cultivation against *Botrytis cinerea* and *Sclerotinia*, known as Serenade ASO, which is applied as a foliar fungicide. This strain is known to produce iturins, plipastins and surfactins (Maronne 2002) (www.fytoweb.fgov.be). It would be interesting to test the effect of this biofungicide against lettuce downy mildew, analyse which CLPs are produced under field conditions and compare the effect of the bacterial strain with our results obtained with mycosubtilin, surfactin and fengycin. In this work Serenade was not used because it was chosen to analyse the potential of the CLPs as biocontrol and no experiments with bacteria were performed.

Due to the complex mode of action of the (combined) CLPs, the probability for pathogen resistance development is lower than for fungicides with one main single site of action (Maronne 2002). Yet, it was noticed that the effectiveness of *B. subtilis* strain QST713 against *B. cinerea* was dependent on the *B. cinerea* isolate which led to the final question in that study if the use of strain QST713 could induce selection of *B. cinerea* to more resistant isolates which may jeopardise the efficacy of the control method (Bardin et al. 2013). We only used *B. lactucae* isolate BmVL 144. The general question of this study with *B. cinerea* raises the question if this selective efficacy would also be the

case for the effect of the CLPs against *B. lactucae*. Therefore future studies should imply other *B. lactucae* isolates to compare.

7.2.3.2 Glycolipids

Due to our stock solution, cellobiose lipid solutions contained relatively high concentrations of DMSO. To observe the effect of cellobiose lipid, disconnected from the effect of DMSO, we used a low concentration of cellobiose lipid. Yet, other studies report an antimicrobial effect, which is concentration dependent (Kulakovskaya et al. 2007; Mimee et al. 2009), thus repeating our tests with cellobiose lipid solutions containing a lower DMSO concentration could be interesting. Another possibility is to solve the cellobiose lipid in another solvent, e.g. Tween, which has no effect on the studied pathogens.

In a study of Henry et al. it was shown that the several structural traits of surfactin molecules are determining the efficacy of this molecule to elicit resistance responses in tobacco cells (Henry et al. 2011). Molecules with a fatty acid chain with less than 14 carbons were ineffective to trigger resistance responses. Also linear and/or methylated derivatives lost eliciting activity. Furthermore position and amino acid substitution in the peptide could influence the eliciting capacity.

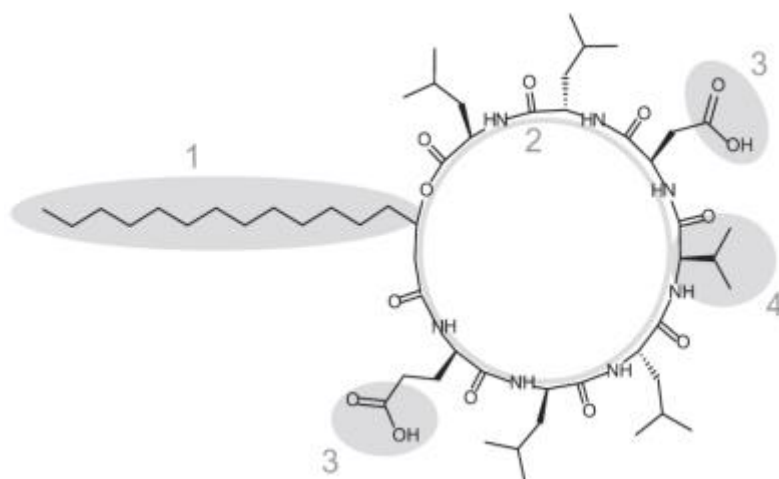


Figure 7-1: Structure of a surfactin molecule with indication of structural traits which are identified as being important for eliciting activity in tobacco cell (highlighted in grey). 1) length of the fatty acid chain 2) cyclic peptide 3) alkylated acidic amino acids 4) amino acid composition of the peptide (Henry et al. 2011)

It might be interesting to evaluate if small modifications such as fatty acid chain length in the glucolipid, cellobiose lipid or bola sophorolipid tested in Chapter 6, could also influence eliciting activities of these components.

If the observed effect of the glycolipids applied four days before inoculation with *Bremia lactucae* is due to a direct effect against the pathogen, these glycolipids degrade less fast than the CLPs. This might be an advantage of the glycolipids compared to the studied CLPs because the provided protection lasts longer.

Table 7-2: Summary of recent results concerning the interaction of *B. subtilis* CLPs mycosubtilin (M), surfactin (S) and fengycin (F) with plants and/or pathogens (fungi, oomycetes).

Study subject	Direct antagonism	Induced resistance	Induced gene expression in absence of pathogen (coding for or involved in)	Induced gene expression in presence of pathogen	Phytotoxicity
lettuce (Chapter 4 and 5, (Deravel et al. 2014))	M, S, F: <i>B. lactucae</i> M, F: <i>B. cinerea</i> M: <i>R. solani</i>	M, S: lettuce downy mildew (<i>B. lactucae</i>) M: basal rot (<i>B. cinerea</i>) M, S, F: basal rot (<i>R. solani</i>)	M: <i>PAL1</i> S: <i>PAL1</i>	M: <i>PAL1</i>	No significant effect on weight and length (data not shown)
rice - <i>R. solani</i> (Addendum I)		F,S: sheath blight (<i>R. solani</i>)	S: auxin pathway M, F: auxin, JA and ET-pathway		F, S: trigger cell death in rice cell culture
Apple – <i>B. cinerea</i> (Touré et al. 2004; Ongena et al. 2005)		F: grey mould disease (<i>B. cinerea</i>)			
Grapevine - <i>B. cinerea</i> (Farace et al. 2015)	M,F	M, S (,F)	M: SA- and JA-pathway S: SA-pathway		M: triggers cell death in grapevine cell culture
Bean (Ongena et al. 2005; Ongena et al. 2007)	M, F: <i>Pythium ultimum</i> , <i>B. cinerea</i> (Ongena et al. 2005)	M, F: damping off (<i>P. ultimum</i>) (Ongena et al. 2005) F: <i>B. cinerea</i> (Ongena et al. 2005; Ongena et al. 2007) S: <i>B. cinerea</i> (Ongena et al. 2007)			

Study subject	Direct antagonism	Induced resistance	Induced gene expression in absence of pathogen (coding for or involved in)	Induced gene expression in presence of pathogen	Phytotoxicity
Tomato (Leclère et al. 2005; Ongena et al. 2007; Henry et al. 2011)		M: <i>P. aphanidermatum</i> (Leclère et al. 2005) S, F: <i>B. cinerea</i> (Ongena et al. 2007)	S: in roots of plantlets: reactive oxygen species (ROS) (Henry et al. 2011)	S+F: plants inoculated with <i>B. cinerea</i> : LOX and lipid hydroperoxidase (LHP) (Ongena et al. 2007)	
Cotton – <i>Verticillium dahliae</i> (Han et al. 2015)	Iturins	Iturins	Iturins: SA, LOX (JA)	Iturins: SA, LOX (JA)	
<i>Arabidopsis thaliana</i> (Farace et al. 2015)			M, S: SA- pathway		

Study subject	Direct antagonism	Induced resistance	Induced gene expression in absence of pathogen (coding for or involved in)	Induced gene expression in presence of pathogen	Phytotoxicity
Tobacco (Ongena et al. 2007; Jourdan et al. 2009; Henry et al. 2011)		S: <i>B. cinerea</i> (Henry et al. 2011)	S: cells: phenylpropanoid (PAL, LOX, phenolics) (Ongena et al. 2007; Jourdan et al. 2009) and SA-pathway (Ongena et al. 2007), oxidative burst (Henry et al. 2011) S: in roots of plantlets: ROS (Henry et al. 2011)		S,F: no effect on cell viability of tobacco cell suspension culture (Ongena et al. 2007; Jourdan et al. 2009; Henry et al. 2011)
Melon (García-Gutiérrez et al. 2013)	M, F: <i>Podosphaera fusca</i>	S: <i>P. fusca</i>			
Potato tuber (Ongena et al. 2005)			F: accumulation phenolics		

7.2.4 Further adaptations on the decision support tool

The evaluating tests described in Chapter 3 indicate it might be interesting to determine which pathogens are causing basal rot when lettuce is cultivated according to the advice of the decision support tool. The identification can be linked to seasons and lettuce cultivars leading to predictions of the causal agent most likely to cause disease, similar to the results of Van Beneden and colleagues (Van Beneden et al. 2009). This prediction should influence the choice of fungicides advised by the decision support tool because it is known that Amistar, Mirador and Ortiva are not only effective against downy mildew but also against *Rhizoctonia solani* and/or *Botrytis cinerea* and fungicides based on mancozeb are also used to control *B. cinerea* (<http://www.fytoweb.fgov.be/indexNL.asp>). Furthermore in the web-application a trade-off should be made to determine if control should focus on basal rot or downy mildew or tipburn or if a compromise should be made. Therefore more experiments are needed to evaluate the effects of cultivation according to the advice of the decision support tool on yield, tipburn and basal rot pathogens. And in these experiments, not only the effects of the fungicide treatments prescribed by the decision support tool but also the effects of climate adaptation should be considered. The results of the extra studies on primary inoculum can influence the first steps of the decision support tool to avoid sources of primary inoculum.

Furthermore, because there is a trend of increased use of hydroponic cultivation of lettuce in Belgium, it would be interesting to evaluate the criteria for climate adaptation and predictions of inoculation and sporulation for cultivation in hydroponic systems.

If more research results in the development of biofungicides based on CLPs of *Bacillus subtilis* or the glycolipids studied in this work, greenhouse experiments should indicate how these biofungicides can be incorporated in the decision support tool as part of the intervention requirement of IPM. This requirement determines that priority should be given to biological intervention above and before chemical interventions. Based on the current results, 10 mg/L mycosubtilin or surfactin could be applied when presence of airborne conidia is predicted to enhance defence of lettuce plants against downy mildew. This is based on the hypothesis that the observed effect of applying these CLPs has an effect on the defence mechanism of the plants. When the decision support tool prescribes fungicide applications, 100 mg/L mycosubtilin combined with 100 mg/L fenproconazole or 100 mg/L glucolipid can be mixed with the fungicides or maybe replace a conventional fungicide. The combination of mycosubtilin and fenproconazole very strongly reduced the disease incidence of downy mildew and mostly mycosubtilin was effective against both *Botrytis cinerea* and *Rhizoctonia solani*. Also 100 mg/L glucolipid was effective against all three pathogens.

7.3 General conclusions

Firstly, the results of this work provide direct possibilities to improve the current downy mildew control strategy and make it more sustainable without causing risk of stimulating the development of basal rot caused by *Botrytis cinerea* or *Rhizoctonia solani*.

And secondly, results of this work provide suggestions for future improvements of the control strategy with biofungicides based on the CLPs of *Bacillus subtilis* and glycolipids produced by or derived from products of *Starmerella bombicola*.

Summary

Lettuce production and export represent an important sector in Belgium. Due to intensification of the crop management, lettuce became very susceptible for different diseases, among which downy mildew is considered as one of the most severe. Lettuce downy mildew is caused by the oomycete *Bremia lactucae*. This pathogen can attack all growth stages of the crop and heavily infected plants are not marketable. Furthermore this disease can spread very fast, resulting in considerable yield losses when control measures fail. Since many years these control measures usually consist of fungicide application on lettuce cultivars containing resistance genes or R-factors. Preventive and abundant fungicide spraying results in faster development of resistance against the active ingredients of these fungicides. Besides, the pathogen has the ability to evolve very fast and overcome the genetic resistance of the lettuce cultivars. Because of the restrictions of the current control strategy, a more sustainable control strategy for this disease is desirable.

Lettuce breeding for resistance against downy mildew should focus on resistance genes with the highest efficacy for resistance against the current pathogen population. Therefore 55 isolates were collected in Flanders from 2008 until 2013 and their virulence characteristics were analysed. From our analyses, resistance genes *Dm* 15 and 17 and the resistance genes of Bedford, Balesta and Bellissimo are most effective against *B. lactucae* in Belgium. Nine of the 55 isolates were identical to the official IBEB races and 41 had a unique virulence-phenotype; which shows the known genetic variability of this pathogen. As a possible explanation for this variability sexual reproduction is often suggested. Yet, because only in six of the 55 samples oospores were detected and of the 11 analysed isolates 10 were mating type B1 and only one B2, it was concluded that sexual reproduction might be not the major source of primary inoculum in Belgium and the observed variability might be probably due to asexual genetic variation caused by gene duplication, polyploidy or nonsense mutations.

Because only relying on the resistance offered by these resistance genes in lettuce cultivars is too risky, fungicide applications are a necessary addition of a lettuce downy mildew control strategy. To improve the effectiveness of fungicides, they should be applied as close as possible to the moment of infection. To predict this moment, forecasting models were developed. Similarly to these models, we developed our own model for greenhouse grown lettuce, in which the first intervention step to inhibit pathogen development was not to apply fungicides but to adapt the greenhouse climate. Therefore profound knowledge of the epidemiology of the pathogen was needed. Based on our epidemiological study and sampling with a spore sampler, presence of conidia was estimated to be highest when temperature is between 0 and 23°C and there is no long lasting or heavy rainfall. If there is an actual risk for conidia, germination and penetration can be inhibited by avoiding periods of two hours or longer with a relative humidity of at least 90%. If, despite the attempts to adapt the greenhouse climate, the period with relative humidity of minimum 90% exceeds two hours, fungicides should be applied. Which fungicide, depends on the moment of application relatively towards the predicted moment of penetration, the growth season, the time until harvest and which fungicides were used before. The suggestion for the grower is based on the experiments performed in the greenhouses of the three research centres in which fungicides were applied on different

moments relatively towards the moment of inoculation in different growth seasons. Starting from the sixth night and lasting until 32 days after the predicted moment of penetration of the host, relative humidity at night should be attempted to be kept beneath 85% to avoid sporulation. This period from six until 32 days, is determined by the minimum and maximum incubation period observed in our greenhouse experiments. If, despite the attempts to keep the relative humidity below 85% at night, a period of minimum five hours with a higher relative humidity was observed, a new fungicide application should be performed, if the legal time between two applications is passed. Once our model was developed we tested it several times in greenhouses of the three research centres and could usually save two or three fungicide applications with similar downy mildew control in comparison with standard fungicide applications based on a calendar schedule. Also on basal rot, tipburn and yield, our model had no negative effects, according to our evaluating experiments. Because the moments of application and choice of fungicides are more accurate, fungicide applications can be saved, which may indirectly lead to less disease outbreaks and less resistance development. Therefore the model was converted into a web application, available (after subscription to one of the research centres) at <http://lap.inagro.be>.

Extension of the current control measures can be by the development of new (bio)fungicides. Well studied components in this context are the cyclic lipopeptides of *Bacillus subtilis*: mycosubtilin, fengycin and surfactin. In our experiments as well, they showed to have potential to control downy mildew when applied as foliar spray before inoculation. Their effect seems to be a combination of direct antagonism against *B. lactucae* and an indirect effect via induced expression of stress related genes. It was observed that the effect of *Bremia lactucae* on stress related genes *PAL1*, *EDS1*, and *PRB1* was counteracted if the plants were treated four days before inoculation with these CLPs. Also against two important pathogens causing basal rot, *R. solani* and *B. cinerea*, the *B. subtilis* cyclic lipopeptides were effective, which makes them promising for lettuce cultivation.

Furthermore a cellobiose lipid, a glucolipid and a bola sophorolipid were tested and were able to reduce downy mildew. Moreover, glucolipid was also able to suppress development of *B. cinerea* or *R. solani*, making this glycolipid interesting for future research.

In conclusion, the findings of this thesis give suggestions to optimize the current available lettuce downy mildew control measures and suggest cyclic lipopeptides of *B. subtilis* and the tested glycolipids as promising biofungicides, which can complement a sustainable control strategy.

Samenvatting

Slaproductie en export vormen een belangrijke economische sector in België. Door de intensifiëring van de teelt, werd het gewas gevoeliger voor verschillende ziekten, waaronder valse meeldauw. Deze ziekte wordt beschouwd als één van de meest ernstige bedreigingen binnen de teelt. Valse meeldauw in sla wordt veroorzaakt door de oömyceet *Bremia lactucae*. Deze pathogeen kan alle groeistadia van sla infecteren en sterk geïnfecteerde planten zijn niet verkoopbaar. Verder verspreidt deze pathogeen zich zeer snel in het gewas, waardoor ernstige opbrengstverliezen kunnen voorkomen als de controle maatregelen ontoereikend zijn. Reeds lange tijd controleren telers deze ziekte met fungicide toepassingen op slarassen met resistentiegenen of –factoren tegen deze ziekte. Preventief en overvloedig spuiten van een beperkt aantal fungiciden leidde tot resistentie ontwikkeling van deze pathogeen tegen de actieve componenten van de gebruikte fungiciden. Daarnaast kan de pathogeen snel evolueren waardoor de genetische resistentie van de slarassen geen of onvoldoende bescherming biedt tegen deze pathogeen. Doordat deze bestrijdingsstrategieën vaak tekort schieten, is de ontwikkeling van een duurzamere, geïntegreerde strategie wenselijk.

Veredeling van sla voor resistentie tegen valse meeldauw, moet gefocust zijn op resistentiegenen met de grootste effectiviteit tegen de huidige isolaten in de pathogeen populatie. Daarom werden 55 isolaten verzameld in Vlaanderen tussen 2008 en 2013 en werden hun virulentie eigenschappen geanalyseerd. Uit onze resultaten bleek dat resistentiegenen *Dm 15* en *Dm 17*, naast de resistentiegenen van slacultivars Bedford, Balesta en Bellissimo het effectiefst waren tegen *B. lactucae* isolaten in Vlaanderen. Negen van de 55 isolaten waren identiek aan de officiële IBEB rassen en 41 van de 55 isolaten had een uniek virulentie-phenotype, wat de gekende genetische variabiliteit van deze pathogeen aantoont. Een mogelijke verklaring voor deze genetische variabiliteit is seksuele reproductie. Omdat slechts in zes van de 55 stalen oösporen gedetecteerd werden en omdat er van de 11 geanalyseerde isolaten 10 mating type B1 waren en slechts 1 B2, is ons besluit dat seksuele reproductie waarschijnlijk niet de voornaamste bron van primair inoculum is in België. De geobserveerde variabiliteit is mogelijk te wijten aan asexuele, genetische variatie veroorzaakt door genetische duplicatie, polyploidie of nonsense mutaties.

Enkel vertrouwen op de resistentie in de slacultivars is risicovol en daardoor worden de planten ook regelmatig behandeld met fungiciden. Om de effectiviteit van de fungiciden te verhogen is het nodig om ze zo dicht mogelijk tegen het moment van inoculatie toe te passen. Om dit moment te voorspellen, werden verschillende modellen ontwikkeld. Naar analogie met deze modellen, hebben we ook een model ontwikkeld specifiek voor sla geteeld in serres. In ons model bestaat de eerste interventiestap niet uit fungicide behandelingen, maar uit het aanpassen van het klimaat in de serre om zo de ontwikkeling van de pathogeen te vermijden. Hiervoor was een grondige kennis van de epidemiologie van de pathogeen nodig. Gebaseerd op onze epidemiologische studie en analyses met een sporenvanger, werd bepaald dat risico op aanwezigheid van conidia het hoogst was wanneer de temperatuur tussen 0 en 23°C was en er geen langdurige of hevige regen was. Wanneer de kans groot is dat er conidia aanwezig zijn, moet kieming van deze sporen vermeden worden door perioden

van 2 u of langer met een relatieve vochtigheid hoger dan 90% te vermijden. Als er ondanks pogingen om het klimaat te sturen toch zo'n periode heeft plaatsgevonden, moet er zo snel mogelijk behandeld worden met fungiciden. Het type fungicide dat aangeraden wordt, is afhankelijk van het toedieningstijdstip relatief ten opzicht van het geschatte moment van binnendringen in de plant, het groeiseizoen, de tussentijd tot de oogst en welke fungiciden voordien reeds gebruikt werden in deze teelt. De aanbeveling voor fungiciden is gebaseerd op experimenten die uitgevoerd werden op de proefcentra waarbij verschillende fungiciden op verschillende momenten t.o.v. het moment van inoculatie werden toegediend in verschillende seizoenen. In een periode van zes tot 32 dagen na de vermoedelijke inoculatie moeten de klimaatsinstellingen in de serre aangepast worden om te streven naar een relatieve vochtigheid onder 85% gedurende de nacht om sporulatie te vermijden. De periode van zes tot 32 dagen werd bepaald door onze bevindingen dat dit de minimum en maximum duur van de incubatieperiode is. Als er, ondanks de pogingen om het klimaat te sturen, toch een periode langer dan 5 u met een RV hoger dan 85% geweest is, de wettelijk vastgestelde periode tussen twee fungicide toepassingen verstreken is en de periode tot de oogst nog lang genoeg is, moet opnieuw een fungicide behandeling toegepast worden.

Zodra ons model ontwikkeld was, hebben we het verschillend keren getest in de serres op de proefcentra. Hierbij konden doorgaans twee fungicide behandelingen uitgespaard worden in vergelijking met het normale spuitschema dat in de praktijk gangbaar is. We bestudeerden ook het effect van ons model op rand, smet en opbrengst. Op geen van deze aspecten had telen met het model een negatieve invloed. Omdat het toepassingsmoment en de keuze van de fungicide accurater is, kunnen fungicide toepassingen uitgespaard worden, wat onrechtstreeks kan leiden tot minder ziekte uitbraken en minder isolaten die resistentie ontwikkelen. Omwille van deze gunstige verwachtingen, werd het model omgezet in een web applicatie die beschikbaar is voor de telers op <http://lap.inagro.be>.

Een mogelijke uitbreiding van de bestaande controle strategie tegen valse meeldauw kan het gebruik van biopesticiden zijn. Componenten die vaak bestudeerd worden in de zoektocht naar nieuwe biopesticiden, zijn de cyclische lipopeptiden van *Bacillus subtilis*: mycosubtiline, fengycine en surfactine. Ook uit onze experimenten is gebleken dat ze potentieel gebruikt kunnen worden tegen valse meeldauw. Het geobserveerde effect blijkt een combinatie te zijn van een direct antagonistisch effect van de drie bestudeerde lipopeptiden tegen *B. lactucae*, maar er werd ook een indirect effect waargenomen waarbij de toegepaste concentraties van mycosubtiline en surfactine 10 keer lager zijn, de lipopeptiden 4 dagen voor de inoculatie over de planten worden verstoven en de expressie van genen gerelateerd aan stress geactiveerd wordt met en/of zonder aanwezigheid van de pathogeen. Uit onze studie bleek dat het effect dat *B. lactucae* heeft op de expressie van de geselecteerde genen *PAL1*, *EDS1* en *PRB1* tegengewerkt wordt in planten die vier dagen voor de inoculatie behandeld werden met 10 mg/L mycosubtilin of surfactin. Om meer duidelijkheid te krijgen over welke genen betrokken zijn in de afweer van slapplanten tegen valse meeldauw, hebben we de planten behandeld met hormonen en de resultaten van die experimenten lijken erop te wijzen dat deze afweer via de SA-en JA-sigtaal pathways verloopt en geprimed wordt door ethyleen. Naast het effect van de cyclische lipopeptiden tegenover *B. lactucae*, hebben we ook het effect bestudeerd tegen twee pathogenen die smet in sla veroorzaken: *Rhizoctonia solani* en *Botrytis cinerea*. Ook tegen deze twee pathogenen waren de lipopeptiden werkzaam.

Verder hebben we een cellobiose lipide, een glucolipide en een bola sophorolipide getest en ook deze drie konden valse meeldauw infectie onderdrukken. Bovendien kon het glucolipide ook de ontwikkeling van *B. cinerea* en *R. solani* afremmen, wat dit glycolipide interessant maakt voor verder onderzoek.

Ter conclusie kan gesteld worden dat in deze thesis suggesties gegeven worden om de huidige bestrijdingsstrategie van valse meeldauw in sla te optimaliseren en cyclische lipopeptiden en bepaalde glycolipiden worden gesuggereerd als beloftevolle, mogelijke biopesticiden, die een aanvulling kunnen betekenen voor een duurzame, geïntegreerde bestrijdingsstrategie.

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Addendum I: Role of cyclic lipopeptides produced by *Bacillus subtilis* in mounting induced immunity in rice (*Oryza sativa* L.)

Because in our laboratory a group is specialized in rice studies, it was interesting to compare some tests performed with lettuce with results that could be obtained with similar experiments on rice. In this study, we demonstrate the potential of *Bacillus subtilis* BBG111 to trigger ISR in rice (*Oryza sativa* L.) against *Rhizoctonia solani*, while there was no effect against *Magnaporthe oryzae*. We show that plant recognition of BBG111 induces jasmonic acid (JA) and ethylene (ET) as well as abscisic acid (ABA) and auxin signalling. In addition, *B. subtilis* supernatants also boosted immune responses triggered by chitin, suggesting that BBG111 triggers ISR at least in part by reinforcing basal plant defence responses. Finally, our results reveal an indispensable role of the BBG111 cyclic lipopeptides, fengycin and surfactin, in the induced defence state.

Chandler, S., Van Hese, N., Coutte, F., Jacques, P., Höfte, M., De Vleeschauwer, D. (2015) Role of Cyclic Lipopeptides Produced by *Bacillus subtilis* in Mounting Induced Immunity in Rice (*Oryza sativa* L.). *Physiological and Molecular Plant Pathology* 91: 20-30.

A.1 Introduction

Rice (*Oryza sativa* L.) is the most consumed staple food grain for more than three billion people living in tropical and subtropical Asia (Khush 2005). However, rice production is severely affected by a variety of biotic and abiotic factors including pests, weeds, drought, heat and salinity. In addition, more than 70 diseases caused by bacteria, fungi, viruses or nematodes have been recorded on rice, among which rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) are the most important fungal constraints on high productivity (Ou 1980; Lee and Rush 1983).

The filamentous ascomycete *M. oryzae* (Hebert) Barr [anamorph *Pyricularia oryzae* Cavara syn. *Pyricularia grisea*] is one of the most devastating pathogens of rice worldwide due to its widespread distribution and destructiveness (Talbot 2003). A recent survey saw *M. oryzae* ranked as the world's top 1 plant-pathogenic fungus based on scientific/economic importance (Dean et al. 2012). *M. oryzae* is a hemibiotrophic pathogen as its infection cycle combines successive biotrophic and necrotrophic growth stages. Following appressorium-mediated penetration, successful infection requires an initial period of biotrophy during which the fungus forms bulbous invading hyphae within apparently healthy plant cells (Howard et al. 1991). Once established within the plant, the pathogen gradually switches to a necrotrophic lifestyle, resulting in the appearance of visual disease symptoms. *R. solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk], on the other hand, is an archetypal necrotroph which kills host cells at early stages of the infection process and feeds on the remains.

Management of fungal rice diseases usually involves the use of fungicides and cultivation of resistant varieties. However, whilst the use of hazardous chemicals is environmentally undesirable as well as economically costly, resistant blast cultivars often do not withstand more than one or two years of cultivation before succumbing to diseases, due to either breakdown or gradual erosion of the resistance in face of the high variability of the pathogen population (Mew 2004). Moreover, though partial resistance to sheath blight has been reported, no major resistance genes have been identified despite screening more than 3000 accessions of germplasm worldwide (Zheng et al. 2013). Hence, there is considerable incentive to develop new disease control strategies providing durable, environmentally sound, and broad-spectrum pathogen protection. Among such strategies, approaches capitalizing on the plant's own defensive repertoire seem especially promising (Song and Goodman 2001).

To effectively combat invasion by microbial pathogens, plants have evolved sophisticated mechanisms providing several strategic layers of coordinated defences. Pre-formed structural and physical barriers as well as inducible plant defences that are activated upon pathogen recognition constitute the first line of defence and result in a basal level of immunity (Pieterse et al. 2009). Besides these primary attacker-specific responses, plants can also mount a non-specific systemic resistance response that is effective against future pathogen attack. Depending on the organism interacting with the plant, plants are able to activate several types of this so-called induced resistance, including systemic acquired resistance (SAR). SAR is triggered by a localized infection with a necrotizing pathogen and is marked by local and systemic increases in salicylic acid (SA) and the accumulation of pathogenesis related (PRs) proteins (Grant and Lamb 2006). Colonization of the roots by selected strains of plant growth-promoting rhizobacteria (PGPR) leads to a phenotypically

similar form of induced resistance, commonly referred to as induced systemic resistance (ISR) (Van Loon et al. 1998). Seminal studies in *Arabidopsis* and rice have shown that rhizobacteria-mediated ISR often functions independently of SA but requires components of the jasmonic acid (JA) and ethylene (ET) response pathways (Pieterse et al. 1998; Verhagen et al. 2004; De Vleeschauwer et al. 2008). However, much progress has since been made and is now becoming increasingly clear that various hormone-dependent signalling conduits can govern the ISR phenotype depending on the rhizobacterium and the plant–pathogen system used (De Vleeschauwer and Höfte 2009).

Over the past few years, multiple PGPR strains have been shown to successfully control rice blast and sheath blight diseases. These include various *Pseudomonas* and *Bacillus subtilis* strains, including *Pseudomonas fluorescens* PF1 and FP7 (Nandakumar et al. 2001), *P. fluorescens* PfALR2 (Rabindran and Vidhyasekaran 1996), and *B. subtilis* MBI 600 (Kumar et al. 2012), all of which are effective against sheath blight, as well as *Pseudomonas aeruginosa* 7NSK2 and *P. fluorescens* WCS374r, which are both effective against rice blast (De Vleeschauwer, Cornelis, and Höfte 2006; De Vleeschauwer et al. 2008). However, the ability of the strains *B. subtilis* BBG111 and RFB104 to trigger ISR in rice has not been tested yet.

B. subtilis strains BBG111 and RFB104 are two strains that are known to produce different pattern of cyclic lipopeptides (CLP). Composed of a cyclized oligopeptide lactone or lactam ring coupled to a fatty acid (Raaijmakers et al. 2006; Jacques 2011), CLPs are known for their powerful activity against a wide range of organisms, including fungi, bacteria, protozoa and plants and they also have low environmental toxicity (Raaijmakers et al. 2006; Ongena and Jacques 2008; Raaijmakers et al. 2010; Deravel et al. 2014). *B. subtilis* strain BBG111 produces two CLPs, fengycin and surfactin (Coutte et al. 2010), whereas *B. subtilis* RFB104, a derivate of *B. subtilis* ATCC6633, produces surfactin and mycosubtilin (Bechet et al. 2013). Ongena et al. (Ongena et al. 2007) have already shown the importance of surfactin and fengycin in induced systemic resistance in bean, but no studies thus far have evaluated their impact on ISR in cereal crops.

In the present study, we demonstrate the ability of *B. subtilis* BBG111 to mount ISR against sheath blight, while no effect on rice blast was noticed. Furthermore, we show the involvement of CLPs in the establishment and maintenance of ISR and study their mode of action by analysing defence gene expression, hormone pathway activation and cell death responses.

A.2 Material and methods

A.2.1 Plant materials and cell cultures

Seeds of rice (*Oryza sativa* subsp. *japonica*.) cultivar Taichung 65 (abbreviated as T65), were used throughout this study. Unless stated otherwise, rice plants were grown in commercial potting soil (Structural; Snebbout) under growth chamber conditions (constant temperature of 28°C, relative humidity: 60%, 12/12 light/dark period). Cell suspension cultures of rice cultivar Kitaake (*Oryza sativa* subsp. *japonica*) were grown in the dark on a rotary shaker (120 rpm) at 28°C in liquid AA medium (Baba et al. 1986). The cells were diluted in fresh medium every week, and all experiments were performed 5 days after transfer.

A.2.2 Cultivation of rhizobacteria and pathogens

Bacterial strains used in this study are listed in Table A-1. *B. subtilis* strains BBG111 and RFB104 were routinely grown for 24 to 28h at 28°C on Luria-Bertani (LB) agar plates. Alternatively, a single bacterial colony was inoculated in LB broth and grown for 24 to 28h at 28°C under shaking conditions (150 rpm). Bacterial suspensions were adjusted to the desired concentration based on their optical density at 620 nm.

M. oryzae isolate VT5M1 (Thuan et al. 2006) was grown at 28°C on potato dextrose agar (PDA) in darkness. Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 7 days to induce sporulation. Conidia were harvested according to De Vleeschauwer et al. (De Vleeschauwer et al. 2006), and inoculum concentration was adjusted to a final density of 5×10^4 spores mL⁻¹ in 0.5% gelatine (type B from bovine skin; Sigma-Aldrich G-6650).

R. solani isolate MAN-86 (AG-1, IA) (Taheri et al. 2007), obtained from symptomatic plants (cv. IR-50) in rice fields in the state of Karnataka (India) was maintained on Potato Dextrose Agar (PDA) medium at 28°C in the dark.

Table A-1: Microorganisms used in this study.

Strains	Relevant characteristics	Reference
Bacterial strains		
<i>B. subtilis</i> BBG111	Derivate of <i>B. subtilis</i> 168 (Sfp ⁺) Produced CLPs: fengycin, surfactin	(Coutte et al. 2010)
<i>B. subtilis</i> RFB104	Derivate of <i>B. subtilis</i> ATCC 6633 (Amy ⁻ Cm ^R) Produced CLPs: mycosubtilin, surfactin	(Bechet et al. 2013)
Fungal pathogens		
<i>Magnaporthe oryzae</i> VT5M1		
<i>Rhizoctonia solani</i> MAN-86	Anastomosis group AG1- IA	(Thuan et al. 2006) (Taheri et al. 2007)

A.2.3 ISR bioassays

Induced resistance bioassays were performed essentially as described by De Vleeschauwer et al. (De Vleeschauwer et al. 2006). Briefly, plants were grown under growth chamber conditions (28°C, relative humidity: 60%, 12/12 light regimen) in commercial potting soil (Structural; Snebbout) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for 2 min, rinsed three times with sterile, demineralized water, and

incubated on wet sterile filter paper for 5 days in the dark at 28°C to germinate. The bacterial inoculum was thoroughly mixed with the potting soil to a final density of 5×10^7 cfu g⁻¹ and, 12 days later, applied a second time as a soil drench (5×10^7 cfu g⁻¹). In control treatments, soil and rice plants were treated with equal volumes of sterilized saline solution.

For chemical induction of resistance against *M. oryzae*, plants were treated with benzothiadiazole (BTH) at 3 days prior to challenge inoculation. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was dissolved in sterilized demineralized water for use and applied as a soil drench. Control plants were treated with an equal volume of water. BTH was provided by Syngenta Crop Protection.

A.2.4 Pathogen inoculation and disease rating

Five-week-old rice seedlings (five-leaf stage) were challenge inoculated with *M. oryzae* isolate VT5M1 as described before (De Vleeschauwer et al. 2006). Seven days after inoculation, disease was assessed by counting the number of elliptical to round-shaped lesions with a grey centre indicative of sporulation of the fungus, and expressed relative to non-bacterized control plants.

For inoculation with *R. solani*, fourth-stage leaves of five-week-old rice seedlings were excised, cut into 10-cm segments and placed in Petri dishes lined with moist filter paper. Next, an 8-mm-diameter mycelium disc of a 7-day-old PDA culture was carefully placed in the centre of the detached leaf segments and inoculated leaves were incubated under growth chamber conditions (28°C, relative humidity: 60%, 12/12 light regimen). Six days after inoculation, disease was evaluated by measuring the percentage of diseased leaf area using ASSESS 2.0 software (Digital Image Analysis Software for Plant Disease Quantification by Lakhdar Lamari)

A.2.5 Application of purified lipopeptides

All lipopeptides used in this study, i.e. fengycin, mycosubtilin and surfactin, were produced and purified by the ProBioGEM team from different strains of *B. subtilis* using an integrated bioprocess as recently described (Coutte et al. 2013; Farace et al. 2015). For all experiments, lipopeptides were applied at a concentration of 35 µM in 0.72% dimethyl sulfoxide (DMSO) and equivalent volumes of DMSO were added to control treatments.

ISR was induced by floating detached leaves of 5-week-old rice plants on an aqueous solution of lipopeptides or DMSO. One day after cyclic lipopeptide application, detached fourth-stage leaves were inoculated with *R. solani*.

Five-day-old rice cell suspension cultures were treated with the purified lipopeptides fengycin, mycosubtilin and surfactin at a concentration of 35 µM. Control (Ctrl) cell cultures were treated with 0.72% DMSO. At the indicated time points after inoculation, cells were harvested and stained with Evans Blue. The amount of cell death was quantified by recording A₅₉₅.

A.2.6 RNA extraction, cDNA synthesis and quantitative RT-PCR Analysis

Total RNA was isolated from frozen cell culture using TRI reagent (Sigma) and subsequently treated with Turbo DNase according to the manufacturer's instructions (Ambion/Applied Biosystems). RNA concentration was checked before and after Turbo DNase digestion with a Nanodrop ND-1000 Spectrophotometer. First-strand cDNA was synthesized from 1 µg of total RNA using GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions. Quantitative PCR amplifications were conducted on optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green Master Mix (Stratagene/Bio-Connect) to monitor double-stranded DNA synthesis. The expression of each gene was assayed in duplex in a total volume of 25 µL including a passive reference dye (ROX) according to the manufacturer's instructions (Stratagene). The thermal profile used consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s. Fluorescence data were collected during the annealing stage of amplification. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer. The amount of plant RNA in each sample was normalized using actin (*Os03g0718100*) or eukaryotic translation elongation factor 1A (*Os03g0178000*) as an internal control, and samples collected from control cell cultures were selected as a calibrator. For all amplification plots, the optimal baseline range and threshold cycle values were calculated using the Mx3005P algorithm (Stratagene). Gene expression in control, lipopeptides-treated, and bacteria-treated samples was expressed relative to the calibrator and as a ratio to actin or eukaryotic translation elongation factor 1A expression using the measured efficiency for each gene. Primer sequences are listed in Table A-2.

A.2.7 qPCR analysis and cell death assays in rice cell cultures treated with CLPs

Fengycin, mycosubtilin or surfactin were added to 5-day-old cell cultures at a concentration of 35 µM. Control cells were treated with 0.72% DMSO only. All cells were harvested 2, 6 or 24 h post treatment and subjected to RNA extraction analysis. Alternatively, cells were harvested 1, 2 or 3 days post treatment and analysed for cell viability as described by Kurusu et al. (Kurusu et al. 2010).

A.2.8 qPCR analysis of cell cultures treated with rhizobacteria supernatant

Strains BBG111 and RFB104 were cultured for 24h at 28°C on either LB plates or LB broth. Bacteria cultured on solid LB medium were scraped off the plates and suspended in 10 mL of sterile demineralized water. Next, bacterial suspensions were vortexed and centrifuged for 10 min at 10,000 x g. The supernatant was passed through a 0.22 µm filter and added to 5-day-old rice cell suspension cultures (1 mL supernatant per 3 mL of cells). Cells were harvested at 1, 2 and 6 h post treatment and subjected to RNA extraction analysis as described above. According to the bacteria growth conditions, either LB broth or sterile demineralized water was used as control.

A.2.9 qPCR analysis of cell cultures treated with chitin

Bacteria cultures were grown for 24h at 28 °C in LB broth. Five-day-old rice cell suspension cultures were treated simultaneously with supernatant of LB-grown *B. subtilis* BBG111 and RFB104 bacteria and/or insoluble chitin (100 µg / mL; chitin from crab shells, Sigma). Two h later, rice cell cultures were harvested and subjected to RNA extraction analysis. Mock-treated controls were treated with sterile demineralized water only.

Table A.2: Gene-specific primers used for quantitative real-time PCR

Pathway	Gene	Locus number	Annotation	Reference	Forward (5'-3')	Reverse (3'-5')
HK	<i>Actin</i>	Os03g0718100	Rice actin 1	(McElroy et al. 1990)	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC
	<i>eEF1a</i>	Os03g0178000	Eukaryotic elongation factor 1A	(Kidou and Ejiri 1998)	GGCTGTTGGCGTCATCAAGA	CCGTGCACAAAACCTACCATT
ABA	<i>Rab21</i>	Os05g0341600	Responsive to ABA 21	(Mundy and Chua 1988)	AAGCGCCTTGTTGTTGAAGGTGTG	AGAGCAGCGTCTGCATCAGATAG
ET	<i>EBP89</i>	Os03g0182800	Ethylene-responsive TF 89	(De Vleeschauwer et al. 2010)	TGACGATCTTGCTGAACTGAA	CAATCCCACAAACTTTACACA
	<i>ACS1</i>	Os03g0727600	ACC synthase	(Zarembinski and Theologis 1997)	TCGGCCAAGACCCTCGACG	CGAAAGGAATCTGCTACTGCTGC
IAA	<i>IAA9</i>	Os02g0805100	IAA-responsive gene 9	(Zarembinski and Theologis 1997)	CAACGACCACCAAGGCGAGAAG	CCAGGCAACCAAAACCGAGCTG
	<i>ARF1</i>	Os11g0523800	ADP ribosylation factor 1	(Yang et al. 2005)	AACGTTGAGCTGAAGCCGAGC	ATGAGTGCACCCGTGGCCTAAC
	<i>GH3-8</i>	Os07g0592600	IAA-amino acid synthetase	(Contreras et al. 2004)	AGTACAAGGTGCCACGCTGC	ACGAGGTGGGGATTTGACCGAC
JA	<i>JAMYB</i>	Os11g0684000	JA-inducible Myb TF	(Ding et al. 2008)	TGGCGAAACGATGGAGATGG	CCTCGCCGTGATCAGAGATG
	<i>JiOsPR10</i>	Os03g0300400	JA-inducible PR10 protein	(Lee et al. 2001)	CGGACGCTTACAATAAATCG	AAACAAAACCTTCTCCGACAG
	<i>PR10b</i>	Os12g0555500	Basic PR10b	(Jwa et al. 2001)	CCCTGCCGAATACGCCTAA	CTCAAACGCCACGAGAATTTG
SA	<i>NPR1</i>	Os01g0194300	protein nonexpressor of PR1	(Han et al. 2004)	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACTAG
	<i>WRKY45</i>	Os05g0322900	WRKY TF 45	(Chern et al. 2001)	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC
CS	<i>β-Glu</i>	Os05g0495900	Beta-1,3-glucanase precursor	(Shimono et al. 2007)	ATTCTCGCTTGCGGGTTCTT	CCAGCATGCCGTAGTTCACA
	<i>Mlo</i>	Os03g0129100	Receptor-like kinase MLO	(Zhu et al. 2007)	CACCCTGCAGCGACAGAAAG	CCTCTCGCTGGAGCTCGATA
	<i>PAL</i>	Os02g0627100	Phenylalanine ammonia-lyase	(Kim et al. 2002)	CCCTGCCAATCTGTGAACTA	GCCGCTATGCAACGAAGAAT
<i>Mg</i>	<i>28S rDNA</i>	none	28S ribosomal RNA	(Zhu et al. 1995) (Takaiwa et al. 1985)	TACGAGAGGAACCGCTCATTAGATAATT	TCAGCAGATCGTAACGATAAAGCTACTC

HK: housekeeping gene; ABA: abscisic acid; IAA: indole acetic acid; JA: jasmonic acid; ET: ethylene; SA: salicylic acid; CS: chitin sensitive; *Mg*: *Magnaporthe*; ACC: 1-aminocyclopropane-1-carboxylic acid; PR: pathogenesis-related; TF: transcription factor.

A.3 Results

A.3.1 Effect of *B. subtilis* BBG111 on *Rhizoctonia solani* in rice

To determine the ability of *B. subtilis* BBG111 and *B. subtilis* RFB104 to trigger ISR against the necrotrophic sheath blight pathogen *R. solani* susceptible rice plants were grown in bacteria-inoculated soil and subsequently challenged with the virulent *R. solani* strain MAN86. As shown in Figure A-1, treatment with *B. subtilis* BBG111 caused an approximately 50% reduction in disease development compared to control plants, whereas a substantial but not significant reduction was observed for *B. subtilis* RFB104. Repeat experiments also failed to show a significant reduction of disease in response to RFB104 treatment, suggesting that unlike BBG111, RFB104 is incapable of protecting rice against *R. solani*.

To exclude direct antagonism between the inducing bacterium and the challenging pathogen, possible systemic plant colonization was checked. However, none of the bacterial strains were detected in sheath or leaf extracts of root-treated rice seedlings at any time during the course of the experiments, indicating that bacterial plant colonization remained confined to the root zone (data not shown). Furthermore, no significant and/or consistent differences in appearance, size or weight of control or bacteria-treated plants were observed prior to challenge infection in any of the experiments (data not shown). Hence, under the experimental conditions used in this study, root treatment with either BBG111 or RFB104 did not lead to detectable effects on plant growth that could have affected the growth or development of the respective pathogens. Therefore, these findings suggest that the protective effect exerted by *B. subtilis* BBG111 is based on activation of host immunity, rather than being caused by microbial antagonism.

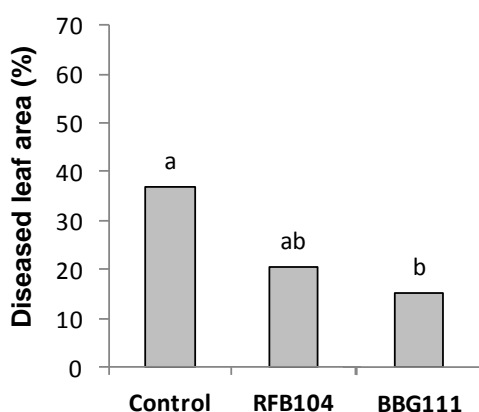


Figure A-1: Influence of soil treatment with rhizobacteria on rice sheath blight (*R. solani*) severity. ISR was induced by growing rice T65 plants in soil containing *B. subtilis* BBG111 or RFB104 bacteria. Control plants were treated with water. Five-weeks-old plants were challenged by inoculating an 8-mm disc of *R. solani* mycelium on detached fourth-stage leaves of individual plants. Six days after fungal inoculation, disease was rated by measuring the percentage of disease foliar area using ASSESS 2.0 (Image Analysis Software for Plant Disease Quantification by Lakhdar Lamari). Different letters indicate statistically significant differences between treatments (Mann-Whitney; $\alpha = 0.05$; $n = 6$). Repetition of the experiment led to results very similar to those shown. No error bars are shown because data were not normally distributed.

A.3.2 Effect of *B. subtilis* on *Magnaporthe oryzae* in rice

To test the spectrum of effectiveness of the BBG111-triggered ISR, we next assayed for induction of resistance against the hemibiotrophic leaf blast fungus *M. oryzae*. As a positive control, plants were treated with benzothiadiazole (BTH), a synthetic SA analogue and well-studied chemical inducer of blast resistance (Shimono et al. 2007). By seven days after fungal inoculation, leaves of non-treated, control plants developed large spindle-shaped lesions (diameter 3-6 mm) with a grey centre, indicative of fungal sporulation (Figure A-2). BTH-treated plants, on the other hand, exhibited a strong reduction in the number of susceptible-type lesions, displaying a resistance phenotype characterized by the occurrence of small necrotic non-sporulating lesions. In contrast, no statistical differences could be observed between control plants and plants colonized with the *B. subtilis* strains. Importantly, this impaired ISR response was not due to insufficient root colonization as bacterial counts in the rhizosphere of bacterized rice seedlings were comparable to those obtained in the *R. solani* bioassays ($1.87 \pm 0.27 \times 10^5$ CFU. g⁻¹ for BBG111; $1.66 \pm 0.42 \times 10^5$ CFU. g⁻¹ for RFB104) and similar to those reported in previous ISR studies on rice (De Vleeschauwer et al. 2006; De Vleeschauwer et al. 2008). In conjunction with the results of *R. solani* bioassays, these findings demonstrate that *B. subtilis* BBG111 acts as a positive regulator of resistance to the necrotroph *R. solani* while being ineffective against the hemibiotroph *M. oryzae*.

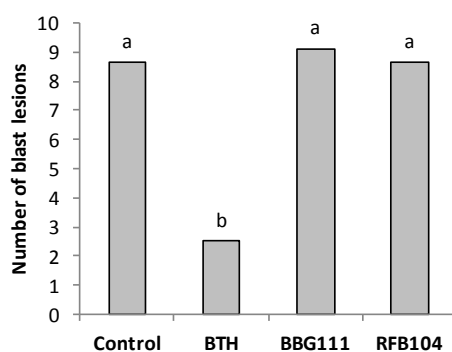


Figure A.2: Influence of soil treatment with rhizobacteria on rice blast (*M. oryzae*) severity. ISR was induced by growing rice T65 plants in soil containing *B. subtilis* BBG111 and RFB104 bacteria. For chemical induction of blast resistance, plants were soil drenched with BTH (0.05 mM) at 3 days prior to challenge. Control plants were treated with water. Plants were challenged when five weeks old (five-leaf stage) by spraying a spore suspension of virulent *M. oryzae* VT5M1 at 5×10^4 conidia ml⁻¹. Seven days after challenge inoculation, disease was rated by counting the number of susceptible-type lesions on the fourth leaves of individual plants. Different letters indicate statistically significant differences between treatments (Mann-Whitney; $\alpha = 0.05$; $n = 6$). Photographs depicting representative symptoms were taken at 7 days after inoculation. The experiment was repeated twice with similar results. No error bars are shown because data were not normally distributed.

A.3.3 Effect of *B. subtilis* BBG111 supernatant and chitin

Perception of invariant pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) by membrane-bound pattern recognition receptors (PRRs) is one of the most peculiar events in the early phase of plant-pathogen interactions and leads to a basal level of resistance, termed PAMP-triggered immunity or PTI. Although unequivocal evidence is still lacking, rhizobacteria-mediated ISR has long been thought to operate by reinforcing basal PTI responses (De Vleeschauwer and Höfte 2009). Therefore, to begin to unravel the mechanistic basis of BBG111-mediated ISR, we assessed the

effect of bacterial supernatant on defence responses induced by the archetypal fungal PAMP elicitor, chitin. To this end, rice cell suspension cultures were treated or not with chitin and/or supernatant of BBG111 and RFB104 and tested for expression of several well-established PTI marker genes. These genes included *PHENYLALANINE AMMONIA LYASE (PAL)*, a central regulator of the phenylpropanoid pathway, the receptor-like *MLO* protein gene and an antimicrobial *β-GLUCANASE (β-GLU)* encoding gene. Figures 3A and C show that supernatant of *B. subtilis* BBG111 caused a much stronger activation of *PAL* and *β-Glu* gene expression compared to chitin alone, suggesting that ISR mediated by *B. subtilis* BBG111 supernatant results in a more powerful immune response than chitin-mediated PTI. Moreover, adding chitin to cell cultures pre-treated with *B. subtilis* BBG111 supernatant strongly enhanced gene expression compared to separate treatments, with *β-GLU*, *MLO* and *PAL* gene transcript levels showing an approximate and respective 21, 22 and 27-fold increase in case of *B. subtilis* BBG111 (Figures A-3A, B and C). In contrast, pre-treatment with supernatant of the ISR non-inducing strain *B. subtilis* RFB104 had only a minor effect on chitin-induced gene expression. Together these results support the notion that chitin and BBG111 metabolites act synergistically on defence gene expression in rice and strengthen the hypothesis that BBG111-mediated ISR may function, at least in part, by amplifying pathogen-triggered PTI responses.

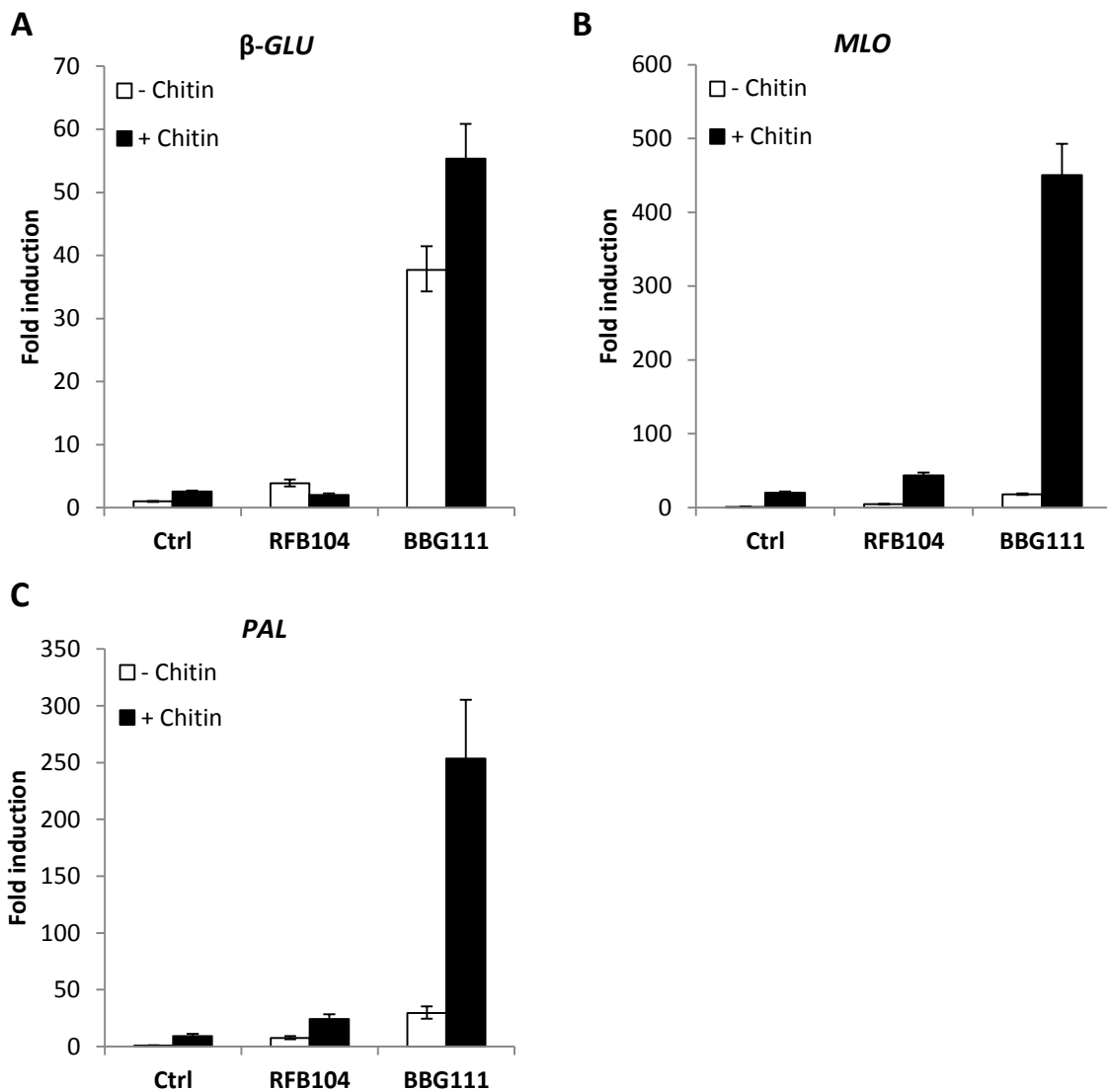


Figure A.3. Effect of *B. subtilis* BBG111 and RFB104 supernatant on chitin-induced defence gene expression in rice cell cultures. Supernatant of LB-grown *B. subtilis* BBG111 and RFB104 bacteria was applied to 5-day-old rice cell suspension cultures. One day later, control and bacteria-treated cells were incubated for 2 h in the presence of chitin (100 $\mu\text{g}/\text{mL}$). Controls were treated with sterile demineralized water. Quantitative RT-PCR analysis was performed using primers for the following transcripts: (A) β -GLUCANASE (β -Glu), (B) receptor like MLO protein (MLO), and (C) PHENYLALANINE AMMONIA LYASE (PAL). Gene expression levels were normalized using *eEF1a* (*Os03g0178000*) as an internal reference and calculated relative to the expression in mock-treated control (Ctrl) cells. Data presented are means \pm SD of two replicates from a representative experiment.

A.3.4 Effect of *B. subtilis* BBG111-secreted molecules on signalling pathways of rice

Given the importance of hormones in the plant defence signalling network (Pieterse et al. 2012; De Vleeschauwer et al. 2013; De Vleeschauwer et al. 2014), we next tried to determine the hormone signalling pathways involved in BBG111-induced resistance. To this end, the expression of various hormone marker genes was measured in rice cell cultures treated with supernatant of bacteria cultured in liquid LB medium.

Gene expression analysis revealed an accumulation of JA-associated transcripts upon treatment with *B. subtilis* supernatant (Figure A-4F). For instance, application of BBG111 supernatant entailed a fast and strong accumulation of *JiOsPR10*, with mRNA levels peaking at 1 hpi at 16 times the levels found in mock-inoculated controls (Figure A-4G). Administration of BBG111 supernatant also induced a strong up-regulation of *JAMYB*, another JA marker gene (Figure A-4E), as well as of the ET-responsive gene *EBP89* (Figure A-4B) and the auxin biosynthesis gene *ACS1* (Figure A-4C). In addition, BBG111 treatment caused a 4- and 3-fold up-regulation of the ABA- responsive genes *Rab21* (Figure A-4A) and auxin-responsive gene *IAA9* (Figure A-4D), respectively, but failed to induce any substantial (more than 3-fold) and/or consistent changes in expression of the SA marker genes *NPR1* and *WRKY45* (Figures A-4G and H). Similar yet less pronounced trends were observed in response to RFB104. These transcript changes suggest that perception of BBG111 by rice cells triggers activation of mainly JA/ET and to a lesser extent ABA and auxin signalling pathways.

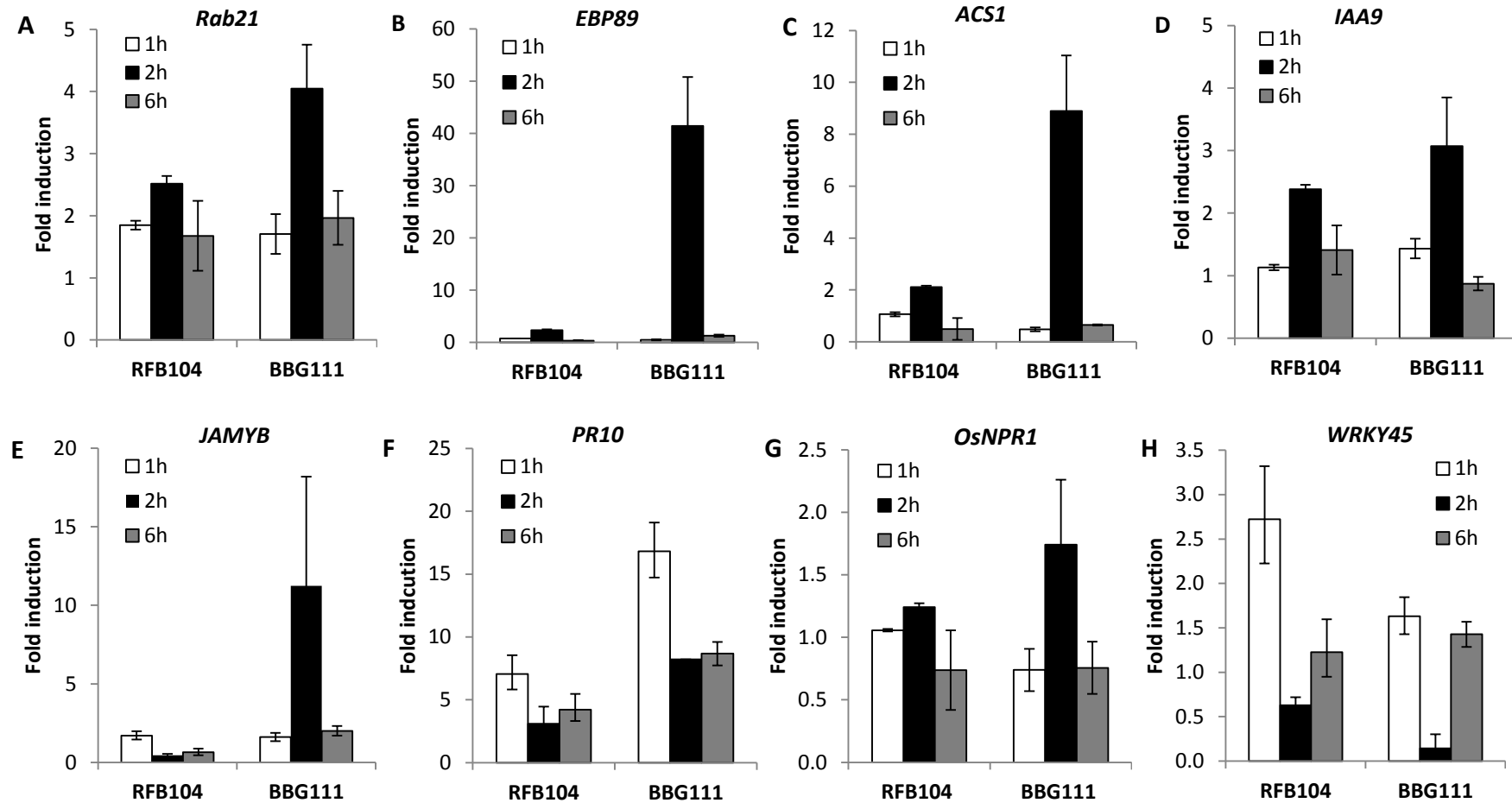


Figure A.4: Expression of hormone marker genes in rice cell cultures treated with supernatant of *B. subtilis* BBG111 and RFB104. Supernatant of *B. subtilis* BBG111 and RFB104 bacteria grown in LB broth was applied to 5-days-old rice cell suspension cultures. Control samples were treated with LB only. At different time points after inoculation (1, 2 and 6 h), cell cultures were harvested and subjected to quantitative RT-PCR analysis for the following transcripts: (A) *Rab21*, (B) *EBP89*, (C) *ACS1*, (D) *IAA9*, (E) *ARF1*, (F) *JAMYB*, (G) *JiOsPR10*, (H) *NPR1* and (I) *WRKY45*. Gene expression levels were normalized using actin (*Os03g071810*) as an internal reference and calculated relative to the expression in mock-treated control (Ctrl) cells at 1 h. Data presented are means \pm SD of two replicates from a representative experiment. Gene expression changes less than 3-fold are not considered as biologically relevant.

A.3.5 Effect of *B. subtilis*-produced cyclic lipopeptides on protection in rice to *R. solani*

Cyclic lipopeptides produced by *B. subtilis* strains are well known to be involved in plant immunity as elicitors of ISR (Ongena et al. 2007). *B. subtilis* BBG111 and RFB104 produce different types of CLPs with BBG111 synthesizing surfactin and fengycin, and RFB104 producing surfactin and mycosubtilin. In order to evaluate the role of these cyclic lipopeptides in inducing resistance in rice to *R. solani*, a bioassay was performed on detached rice leaves pre-treated with purified CLPs. As shown in Figure A-5, no statistically significant differences in disease severity were observed between the DMSO controls and leaves pre-treated with mycosubtilin. In contrast, fengycin pre-treatment significantly decreased the extent of necrosis induced by *R. solani* compared to control plants. Moreover, no statistical differences were found between fengycin and surfactin treatments, suggesting the involvement of both fengycin and surfactin in mediating induced resistance in rice against *R. solani*. However, since there is no strict spatial separation between the CLPs and the pathogen in these experiments, it cannot be ruled out that direct antimicrobial effects may also have contributed to the observed reduction in disease severity.

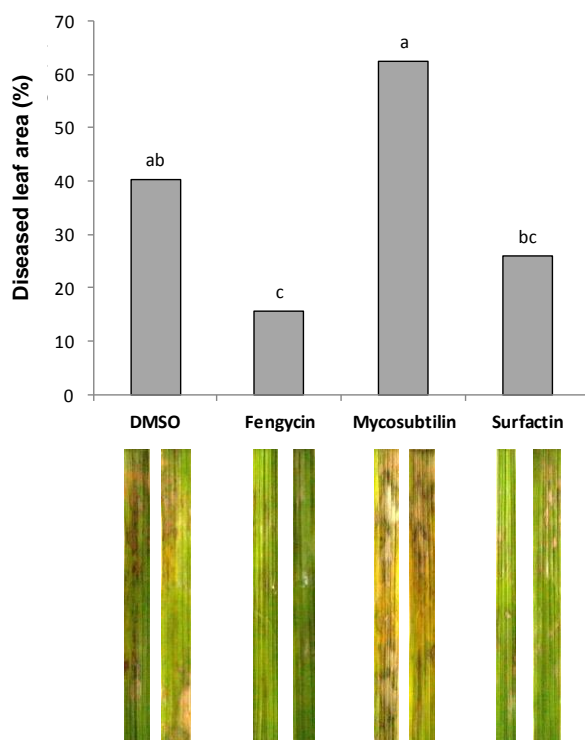


Figure A.5: Effect of pre-treatment with cyclic lipopeptides on rice sheath blight (*R. solani*) severity. ISR was induced by floating detached leaves of 5-week-old rice plants on an aqueous solution of lipopeptides (35 μ M). Control detached leaves were treated with 0.72% DMSO (dimethyl sulfoxide). One day after cyclic lipopeptide application, detached fourth-stage leaves were inoculated with *R. solani* as described in Figure 1. Six days after challenge inoculation, disease was rated by measuring the percentage of disease foliar area using ASSESS 2.0 (Image Analysis Software for Plant Disease Quantification by Lakhdar Lamari). Different letters indicate statistically significant differences between treatments (Mann-Whitney; $\alpha = 0.05$; $n = 10$). Photographs depicting representative symptoms were taken at 6 days after inoculation. The experiment was repeated once with similar results. No error bars are shown because data were not normally distributed.

A.3.6 Involvement of cyclic lipopeptides of *B. subtilis* strains in defence gene expression and hormone pathway activation in rice

To shed more light on the resistance-inducing capacity of *B. subtilis*-produced CLPs, we analysed defence gene expression and hormonal pathway activation in rice cell cultures treated with the various peptides. As shown in Figure 6, administration of fengycin and mycosubtilin resulted in a strong up-regulation of all tested JA marker genes (JAMYB, JiOsPR10 and PR10b; Figures A- 6E, F and G). At 6 hpi, the same treatments also resulted in a strong and fast induction of the ET biosynthesis gene ACS1 (Figure A-6C). In contrast, addition of surfactin to the cell cultures had a rather weak effect on expression of JA and ET marker genes (Figures A-6C and 6E to G). However, all three treatments triggered a fast and transient up-regulation of the auxin marker genes ACS1 and IAA9 (Figures A-6C and D) and a strong down-regulation of the SA marker gene WRKY45 at 24 hpi (Figure A-6I). Expression of NPR1, another SA marker gene, and the ABA-responsive gene Rab21, showed no strong changes upon CLPs application (Figures A-6A and H). Together these results suggest that although surfactin, mycosubtilin and fengycin all trigger a transient activation of auxin signalling at early stages of the interaction, only mycosubtilin and fengycin are able to switch on the rice JA and ET signal transduction pathways.

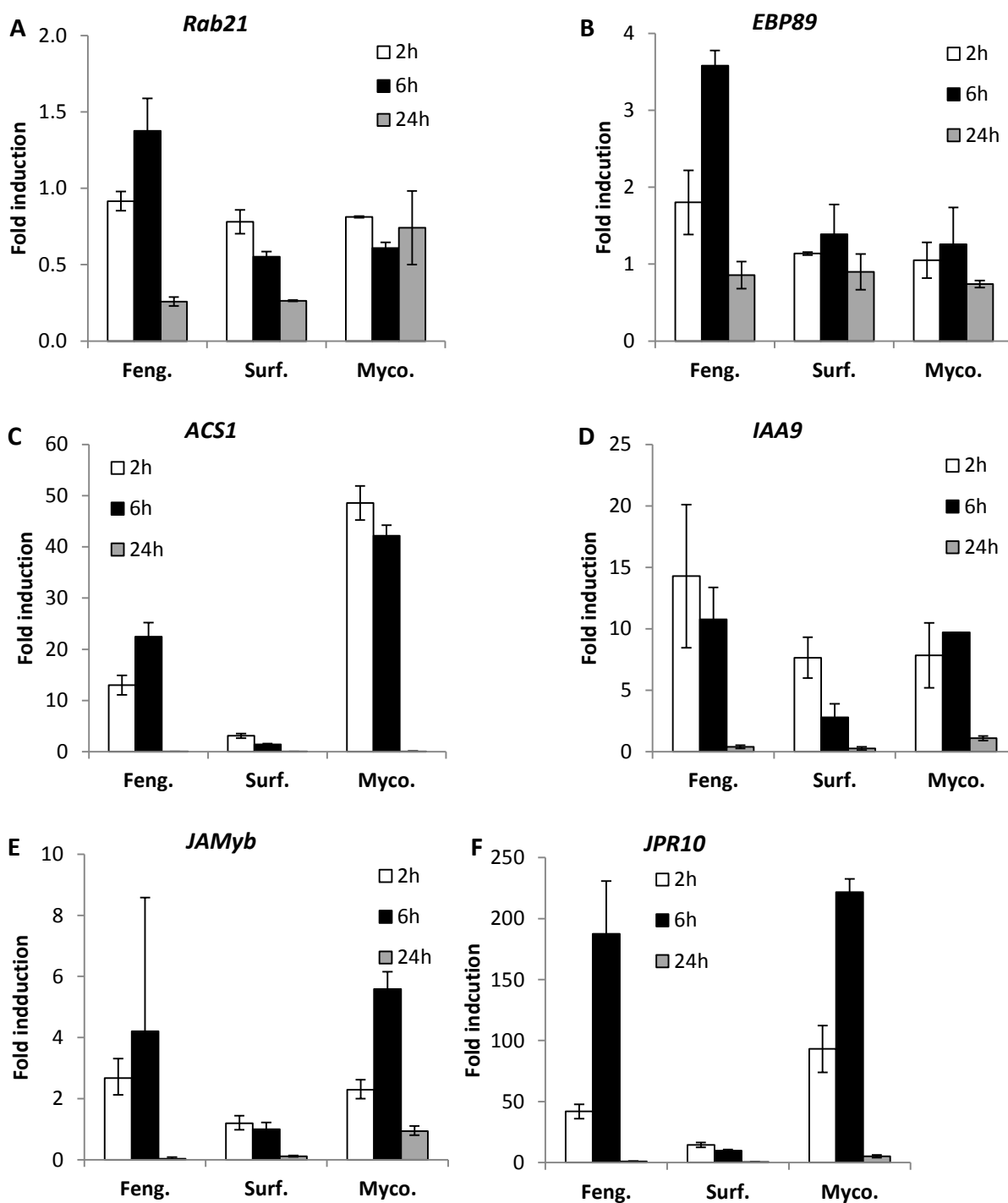


Figure A.6: Expression of hormone marker genes in rice cell cultures treated with cyclic lipopeptides. The cyclic lipopeptides fengycin (Feng.), mycosubtilin (Myco.) and surfactin (Surf.) were applied at a concentration of 35 μ M to 5-day-old rice cell suspension cultures. Control (Ctrl) cultures were treated with 0.72% DMSO. At different time points after inoculation (2, 6 and 24 h), cell cultures were harvested and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin (*Os03g0718100*) as an internal reference and calculated relative to the expression in mock-treated control (Ctrl) rice cell cultures at 2 h in DMSO. Data presented are means \pm SD of two replicates from a representative experiment. Gene expression changes less than 3-fold are not considered as biologically relevant.

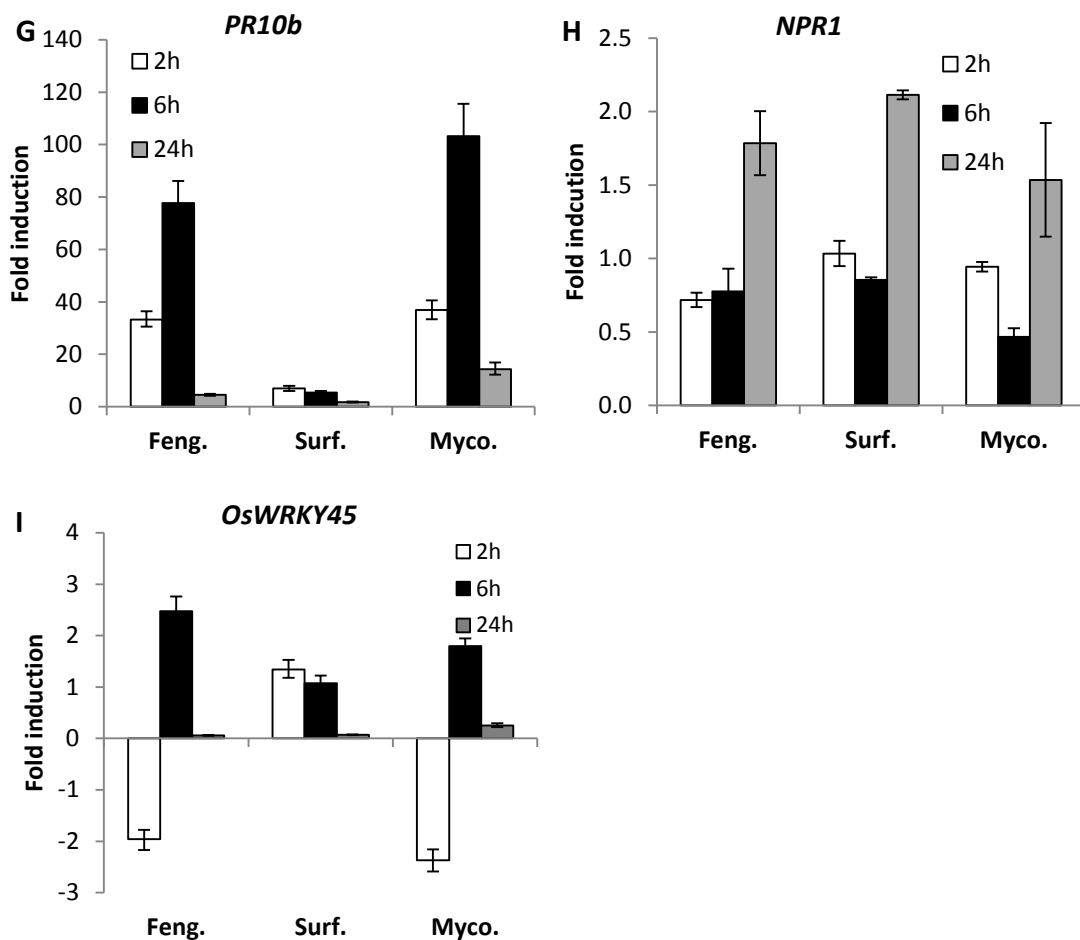


Figure A.6 continued.

7.3.1 A.3.7 Effect of *B. subtilis*-produced cyclic lipopeptides on cell death in rice cell suspension cultures

The activation of plant innate immune responses often results in a form of programmed cell death (PCD) known as the hypersensitive response (Pieterse et al. 2009). To quantify the level of cell death induced by *B. subtilis*-produced CLPs, cell viability assays using Evans blue staining were performed. As shown in Figure A-7, all treatments induced a certain level of cell death, with the exact levels reaching a plateau from 24 hpi onwards. Control and mycosubtilin treatments showed fairly similar readings, suggesting that mycosubtilin does not trigger PCD. In contrast, fengycin and surfactin treatments nearly doubled the rate of dying cells, with absorbance values ranging from 1.3 to 1.6 between 24 and 72 hpi. Thus, contrary to mycosubtilin, both fengycin and surfactin are able to trigger cell death in rice cell suspension cultures.

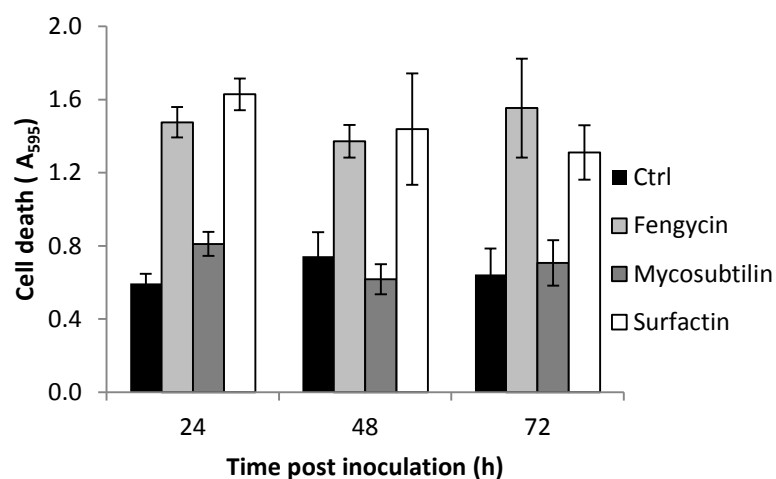


Figure A.7: Effect of cyclic lipopeptides on cell viability in rice cell cultures. Five-day-old rice cell suspension cultures were treated with the purified lipopeptides fengycin, mycosubtilin and surfactin at a concentration of 35 μ M. Control (Ctrl) cell cultures were treated with 0.72% DMSO. At the indicated time points after inoculation, cells were harvested and stained with Evans Blue. The amount of cell death was quantified by recording A595. Data represented are means \pm SD of 6 replicates from a representative experiment.

A.4 Discussion and conclusions

Induced systemic resistance is a phenomenon by which plant exhibit increased levels of resistance to a broad spectrum of pathogens in response to root colonization with selected strains of plant growth-promoting rhizobacteria (PGPRs). Contrary to the relative wealth of information in dicots, little is known about the plant signalling pathways and bacterial determinants underlying ISR in cereal crops. In this study, we have focused on PGPR strains producing CLP-type biosurfactants and their role in mounting ISR in the model monocot rice. We show that soil application of *B. subtilis* BBG111 protects foliar tissues against rice sheath blight (*R. solani*) (Figure A-1) but not against rice blast (*M. oryzae*) (Figure A-2). We also identified CLPs in *B. subtilis* BBG111 with resistance eliciting-activity (Figure A-5). In addition, our findings reveal that the *B. subtilis* CLPs fengycin and surfactin target JA, ET and/or auxin pathways, indicating the existence of multiple ISR resistance pathways in rice (Figure A-4 and Figure A-6).

Plant growth-promoting rhizobacteria (PGPR) have been reported to stimulate plant growth through a variety of mechanisms, including the reduction of deleterious effects caused by plant pathogens (Lugtenberg and Kamilova 2009). This biocontrol capacity is enabled by antibiosis, parasitism, competition for nutrients and, more indirectly, through induction of plant defence mechanisms. Given the spatial separation between the root-colonizing PGPR strains and challenging leaf pathogens, our data strongly suggest that the beneficial protective activity of *B. subtilis* BBG111 observed in this study is due to activation of ISR, rather than being caused by microbial antagonism (Figure A-1).

Besides ISR induction of *B. subtilis* to *R. solani* in intact plant assays, BBG111 secreted molecules also proved to be more effective in inducing defence gene expression in suspension cell cultures compared to the fungal PAMP elicitor, chitin (Figure A-3). Moreover, co-application of chitin and supernatant of *B. subtilis* BBG111 had a clear synergistic effect on gene transcription, suggesting that

BBG111 may trigger ISR at least in part by boosting PTI-mediated defences (Figure A-3). Further evidence supporting this concept came from analysis of the hormone signalling pathways governing the ISR phenotype. Consistent with the classic ISR signalling route in *Arabidopsis* and mirroring *Pseudomonas fluorescens* WCS374-mediated ISR in rice (De Vleeschauwer et al. 2008), supernatant of *B. subtilis* BBG111 strongly triggered activation of the JA and ET defence pathways. Interestingly however, we also found BBG111 to up-regulate ABA- and auxin- related genes (Figure A-4). Considering that JA, ET, ABA and auxin all have been implicated in activation of basal rice defences against *R. solani* (De Vleeschauwer et al. 2010; Peng et al. 2012; Helliwell et al. 2013; Van Bockhaven et al. 2013), BBG111 thus seems to switch on all hormone pathways required for basal sheath blight resistance. Although further experiments using hormone-deficient and/or –insensitive rice mutants and time-resolved hormone measurements are imperative to unequivocally delineate the signalling circuitry mediating BBG111-triggered resistance, these results not only highlight the importance of plant hormones in orchestrating and fine-tuning the plant's immune response but also reinforce the notion that the same pathway operative in ISR boosts whatever basal resistance is conditioned by that pathway.

Previous studies revealed that *B. subtilis* strains BBG111 produces both surfactin- and, fengycin-type CLPs, while RFB104 synthesizes surfactin and mycosubtilin (Coutte et al. 2010) [Fickers, unpublished results]. Detached leaf assays with the purified CLPs revealed that fengycin, and to a lesser extent, surfactin, trigger partial levels of resistance against *R. solani* (Figure A-5), suggesting the involvement of these metabolites in the BBG111-mediated ISR (Figure A-2). Moreover, our findings demonstrated a strong induction of JA and ET signalling and a transient activation of auxin signalling upon fengycin addition to rice cell cultures, while the effect of surfactin appears to be limited to activating auxin signalling (Figure A-6). Interestingly, the hormone pathways induced by fengycin not only resemble those triggered by *B. subtilis* BBG111 supernatant (Figure A-6) but also strongly overlap with the signalling circuitry activated in *R. solani*-infected rice (Peng et al. 2012; De Vleeschauwer et al. 2013; Helliwell et al. 2013; Van Bockhaven et al. 2013). Although we are aware that final proof for fengycin as an essential determinant for ISR elicitation will require bioassays with CLP-deficient mutant strains, the importance of the latter compound in the BBG111-triggered resistance to *R. solani* seems apparent. Moreover, given the often reported redundancy in bacterial traits operative in ISR (De Vleeschauwer and Höfte 2009) and considering the differences in gene expression between supernatant- and CLP-treated rice cells (Figures A-4 and A-6), it should be noted that fengycin may not be the only factor contributing to the onset and/or maintenance of BBG111-induced resistance.

Corroborating the results of our detached leaf assays, we found surfactin and fengycin, but not mycosubtilin, to trigger increased levels of cell death in rice cell suspension cultures (Figure A-7). Considering that mycosubtilin induced similar levels of JA-, ET- and auxin-responsive gene expression as did fengycin (Figure A-6), the ability to induce plant cell death seems indispensable for CLPs to trigger ISR to *R. solani*. This concept may however seem at odds with the reported necrotrophic lifestyle of *R. solani*. Contrary to biotrophs which develop a sophisticated relationship with their host and thrive on living plant tissues, necrotrophic pathogens have long been thought of as aggressive, indiscriminate pathogens that kill host cells by means of a varied arsenal of lytic enzymes and toxic molecules. Recent advances in plant immunity research have added some nuances to this view. Perhaps most conspicuously, it has been found that necrotrophic pathogens may also have a short

biotrophic phase early after penetration during which they are sensitive to cell death-based plant defences (Shlezinger et al. 2011). Most tellingly in this regard, Asselbergh et al. (Asselbergh et al. 2007) showed that timely activation of hypersensitive-response like cell death in the ABA-deficient tomato mutant *sitiens* is a key component of resistance against the archetypal necrotroph *Botrytis cinerea*. In this context, our recent observation that at least some *R. solani* strains similarly adopt a short biotrophic phase in which living rice cells are colonized before switching to necrotrophy is of particular interest [De Vleeschauwer et al., unpublished]. Moreover, recent studies by Helliwell et al. (Helliwell et al. 2013) uncovered ET as a powerful inducer of sheath blight resistance, the role of which in activating various types of cell death is well described. Taking these facts into account, it is tempting to speculate that *B. subtilis* BBG111 mounts ISR to *R. solani* at least in part by secreting fengycin and surfactin in the rhizosphere, thereby rapidly inducing localized cell death and JA, ET, ABA and auxin-steered plant immune responses that block the pathogen in its biotrophic phase. Interestingly, such a scenario may also provide a mechanistic framework for the ineffectiveness of BBG111 against *M. oryzae* which is well known to hijack the rice ABA and auxin machinery in order to cause disease (Koga et al. 2004; Domingo et al. 2009; Jiang et al. 2010; Fu et al. 2011; Yazawa et al. 2012).

In keeping with our findings, CLPs have been repeatedly implicated in ISR triggered by *B. subtilis* and *Pseudomonas* bacteria and all three families of *B. subtilis* CLPs, iturins, surfactins and fengycins, are reported to exert prophylactic effects. For instance, surfactins are known to trigger immunity in bean, melon, tomato, tobacco and grapevine, whereas fengycins elicit defence responses in potato, tomato and tobacco (Ongena et al. 2005; Ongena et al. 2007; Ongena and Jacques 2008; García-Gutiérrez et al. 2013; Farace et al. 2015). Mycosubtilin on the other hand, was recently found to trigger defence signalling mechanisms in grapevine (Farace et al. 2015), while a CLPs mixture produced by *Bacillus amyloliquefaciens* was able to reduce infection caused by the rhizomania disease vector *Polymyxa betae* in sugar beet (Desoignies et al. 2013). Finally, bacterial mutant analysis and testing of purified compounds identified massetolide A, a CLP of *P. fluorescens*, as a powerful elicitor of local and systemic resistance of tomato against *Phytophthora infestans* (Tran et al. 2007).

Despite this relative large body of evidence connecting CLPs to the onset and maintenance of ISR, local defence responses after CLP perception have been poorly documented (Tran et al. 2007; Ongena and Jacques 2008; Jourdan et al. 2009; Falardeau et al. 2013). Yet, an interesting picture emerges when comparing our results to those of Farace et al. (Farace et al. 2015) who observed a long lasting tolerance to *Botrytis cinerea* in grapevine plants upon treatment with mycosubtilin or surfactin. Contrary to the repressive effect of both CLPs on SA-inducible WRKY45 expression in rice (Figure A-5), this mycosubtilin and surfactin-induced protection to *B. cinerea* was associated with increased SA and JA signalling. Moreover, clear differences were evident in the cell death-provoking ability of the different CLPs with fengycin and surfactin inducing increased levels of cell death in rice but not in grapevine, and mycosubtilin being effective in grapevine only (Farace et al. 2015). Although it should be mentioned that slightly different concentrations were used in the different studies (50 mg/L for each CLP in the study of Farace et al. (Farace et al. 2015) as compared to 35µM or 41.9 mg/L mycosubtilin, 36.3 mg/L surfactin and 51.2 mg/L fengycin in this work), these differential effects point towards specificity in CLP-host cell interactions. Supporting this assumption,

recent findings revealed that surfactin-triggered ISR most likely results from small disruptions caused by the insertion of surfactin molecules into the host lipid membrane (Jourdan et al. 2009; Henry et al. 2011). Although not large enough to destroy membrane integrity, these membrane disruptions may be sufficient to induce plant cells to activate immune responses (Henry et al. 2011). Appreciative binding to biomembranes also seems to underpin the differential effectiveness of fengycin-type CLPs in direct antibiosis towards a variety of plant pathogens (Falardeau et al. 2013). Alternatively though less parsimoniously, CLPs may have a different mode of recognition and/or action in monocot (rice) and dicot (grapevine) plant species. In support of this assumption, Ortmann et al. (Ortmann et al. 2006) previously demonstrated that exopolysaccharides of *Pantoea agglomerans* exert different priming and eliciting activities in suspension-cultured cells of monocots and dicots. More recent work however, has suggested that PTI-associated signalling pathways are conserved across plant species, families, and even clades, and that the transferred PRRs can efficiently recruit these potentially conserved immune components (Lacombe et al. 2010; Mendes et al. 2010; Afroz et al. 2011; Fradin et al. 2011; Tripathi et al. 2014; Holton et al. 2015). Future mechanistic studies should provide additional insights into the precise mechanisms by which CLPs interact with eukaryotic cells, further explaining the differential effectiveness of CLPs across plant species and host-pathogen combinations.

In summary, this study demonstrates the potential of *B. subtilis* BBG111 to mount ISR in rice against *R. solani*, while there was no effect on rice blast. Moreover, our findings point to a role of various CLP molecules in triggering and modulating the various resistance processes leading to the induced defence state. Further elucidation of the plant defence responses and signalling pathways underlying ISR in rice mediated by CLPs will not only advance our fundamental understanding of induced resistance phenomena in cereals, but also enable the exploitation of these environmentally friendly biological metabolites for effective utilization of induced resistance in crop protection.

Addendum II: Transformation of *Bremia lactucae*

In this work, we studied interactions between pathogens and components with possible biocontrol activity. We concluded that it would be easier to study this interaction, but also the interaction between different pathogens or even different mating types of one pathogen if fluorescent strains would be available. Therefore we took the first steps to transform *B. lactucae*, with a method based on the description of Dubresson et al. (Dubresson et al. 2008). The construct for this transformation (Figure AII-1), plasmid p34GFN, was kindly supplied by Prof. Dr. Mauch (University of Fribourg, Switzerland). This construct contains the Green Fluorescent Protein (GFP) gene and the geneticin resistance (nptII) gene, flanked by *HAM34* promoters and terminators (Si-Ammour et al. 2003; Mccarren 2006). The construct was multiplied in the bacterial strain *E. coli* DH5 α . Fresh conidia were collected 10 days after the inoculation of the lettuce Green Towers seedlings and concentration of the conidial suspension was adapted to 2.4×10^6 spores/mL in distilled water, as described for *Plasmopara viticola* (Dubresson et al. 2008). Subsequently 15 μ g of p34GFN vector DNA was added to 500 μ L conidial suspension and this mixture was electroporated using the Gene Pulser Xcell (Bio-Rad) with 300V. After electroporation, immediately 500 μ L distilled water was added to the cuvette and the electroporated suspension was used to inoculate lettuce Green Tower leaves. For this inoculation we did not use a foliar spray until run off as for the other inoculations described before, but pipetted the suspension directly on seven day old lettuce leaves. From seven days after inoculation, sporulation was monitored with an Olympus BX-51 microscope with filters GFP-L (ex. 480; em. 510) and GFP-B (ex. 480; em. 535/50). The majority (76%) of the spores was transformed (Figure AII-2), yet in comparison with not-transformed *B. lactucae*, development of the transformed pathogen strain, seems to be slower. Conidia were collected and used in a concentration of 10^5 spores/mL to inoculate fresh seven-day-old seedling. Ten days later conidia were again monitored with fluorescence microscopy, yet the section fluorescent conidia was reduced very strongly. This procedure was repeated, but in a third generation, almost no fluorescent conidia could be observed. The loss of fluorescence might be due to the observation of slower development of transformed conidia in comparison with not-transformed spores, resulting in the last ones outcompeting the first ones. Selecting transformed conidia by mixing them with geneticin could be a solution and might provide a useful tool to study interactions with *B. lactucae* isolates in future.

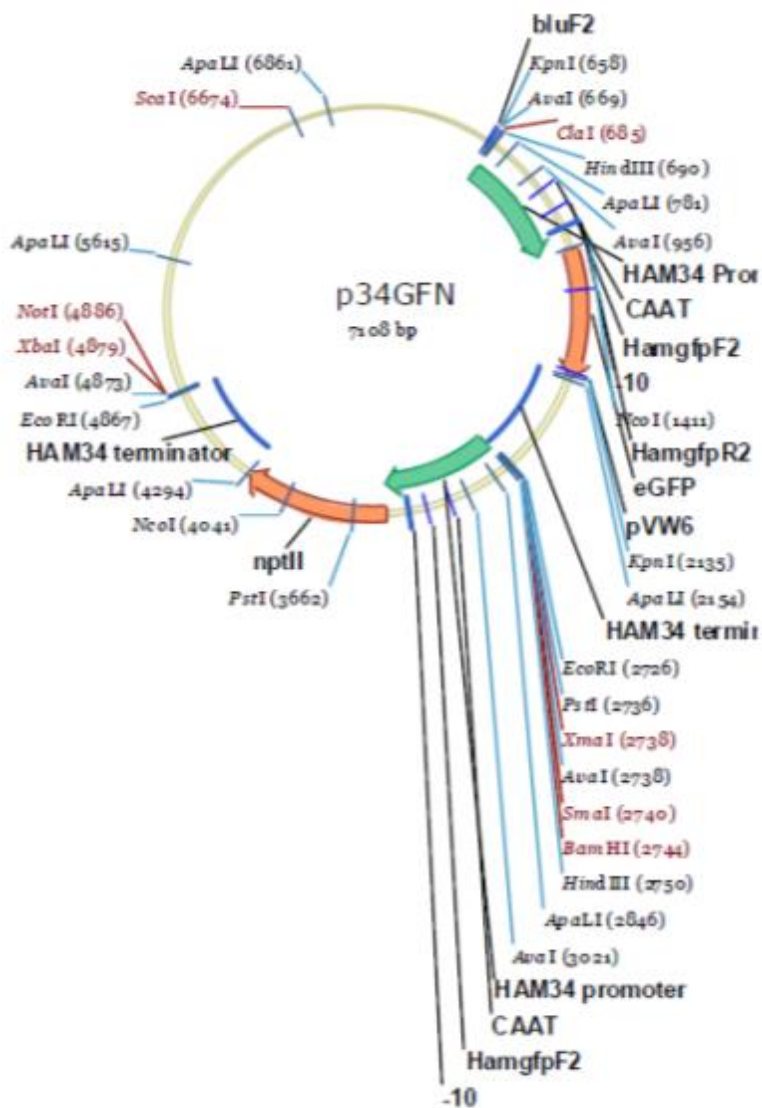


Figure AII-1: Map of plasmid p34GFN with the Green Fluorescent Protein (GFP) gene and geneticin resistance gene (*nptII*). Both are flanked by the HAM promoters and terminators (Si-Ammour et al. 2003; Mccarren 2006).

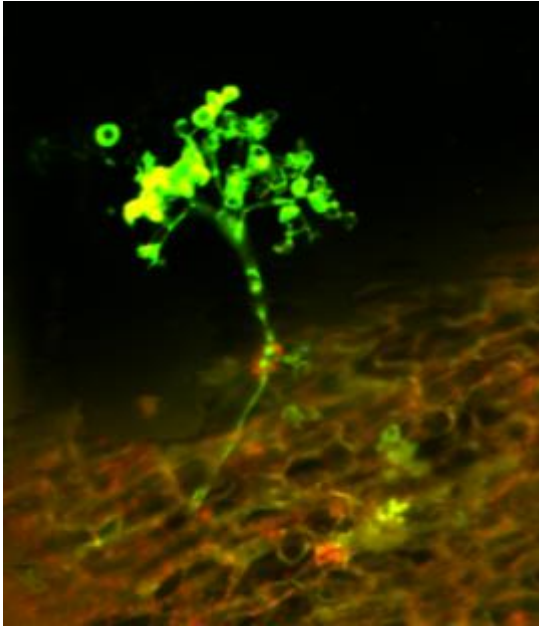


Figure All-2: Transformed *B. lactucae* with GFP construct. A sporangiophore with conidia is emerging out of the leaf surface.

Curriculum vitae

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Publications

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A sustainable approach to control lettuce downy mildew.

Poster presentation by N. Van Hese

10th Conference of the European Foundation for Plant Pathology, Wageningen, the Netherlands (2012)

Van Hese, N., Bleyaert, P., Leenknecht, I., Bogaert, A., Vergote, N., Coutte, F., Jacques, P., Höfte, M.

A sustainable approach to control downy mildew (*Bremia lactucae*) in greenhouse-grown lettuce.

Poster presentation by N. Van Hese

64th International symposium on Crop Protection, Ghent, Belgium (2012)

Van Hese, N., Bleyaert, P., Leenknecht, I., Bogaert, A., Vergote, N., Coutte, F., Jacques, P., Höfte, M.

A sustainable approach to control downy mildew (*Bremia lactucae*) in greenhouse-grown lettuce.

Poster presentation by N. Van Hese

SHE 2nd Symposium on Horticulture in Europe, Angers, France (2012)

Van Hese, N., Leenknecht, I., Bogaert, A., Bleyaert, P., Höfte, M., Vergote, N.

A sustainable approach to control downy mildew in lettuce.

Oral presentation by N. Vergote

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Van Hese, N., Höfte, M. Potential new biopesticides against downy mildew in lettuce.

Oral presentation by N. Van Hese

65th International symposium on Crop Protection, Ghent, Belgium (2013)

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An integrated control strategy for downy mildew in greenhouse-grown lettuce.

Poster presentation by N. Van Hese

10th International Congress of Plant Pathology, Beijing, China (2013)

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Sustainable control of downy mildew on greenhouse grown lettuce

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Attended conferences and symposia without participation

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