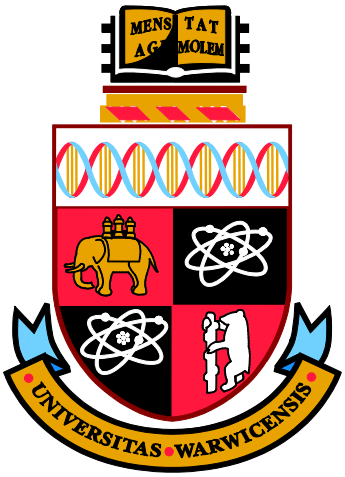


# Development of molecular approaches in the study of lettuce downy mildew (*Bremia lactucae*) population biology

by

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**A thesis submitted to the University of Warwick for the degree of**
**Doctor of Philosophy in Plant and Environmental Science**

**University of Warwick**
**Department of Life Science**

**December 2011**

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**ACKNOWLEDGEMENTS**

I would like to thank my supervisor Professor Roy Kennedy of Worcester University for offering me the opportunity to do this PhD project as well as his support and help throughout this four year study. I would also like to thank my University of Warwick supervisor Professor Eric Holub for his encouragement and support to finish the project.

I am very grateful to work with very nice and friendly people in our group. I would like to thank Dr. Alison Tör who has helped me a lot when I had difficulties in the project. I would like to also thank the help and support from Dr. Volkan Cevik for microsatellite markers development and other experiments in this Project. I also have to thank Mr. Andrew Mead for his advice on statistical experiment design and Dr. Julie Jones for helping to finish the statistical analysis.

In addition I would like to thank Dr. Michelmore (UC Davis, USA) for supplying the the *B. lactucae* isolate Cp82p24 genomic DNA and *B. lactucae* RxLR primers.

I also would like to thank Dr. Geoffery Petch, Dr. Mary Lewis, John Lynch, Rafal Gutaker, Ziyue Huang and Peijun Zhang for their great help and encouragement. And finally I have to thank my family and in particular my parents for always supporting and believing in me.

**DECLARATION**

The work contained in this thesis is the author's own work except where otherwise acknowledged. This thesis has not been submitted for a degree at another university, and has not formed a part of any previous publication unless stated.

**SUMMARY**

Downy mildew of lettuce caused by *Bremia lactucae* is a serious disease resulting in yield loss. The population structure of the pathogen in the UK is poorly understood. This PhD project concentrated on developing molecular markers to differentiate the genotypic variation of *B. lactucae* populations, with the aim of improving methods to investigate lettuce - *Bremia* interactions. Thirty-seven *B. lactucae* isolates (including single-spore and new field isolates) were collected and characterized for virulence using the conventional International *Bremia* Evaluation Board (IBEB) differential set. Microsatellite markers (SSR, ISSR) were investigated for *Bremia* race specific marker development. Three isolates of *B. lactucae* were characterized by ISSR (inter simple sequence repeat) primers, although the polymorphic DNA could not be cloned in this project due to the highly variable results of the ISSR process. Some microsatellite repeats were found in *B. lactucae* isolates sequences that amplified by *Plasmopara viticola* (grape downy mildew) SSR markers. The development of Simple Sequence Repeat (SSR) markers from *Bremia* genomic DNA was not successful, which might result from the primers used being unsuitable for *Bremia* microsatellite enrichment. *Bremia* specific ITS primers were used for quantitative PCR. RxLR primers obtained from UC Davis (USA) were tested using the collection of *B. lactucae* isolates. RxLR1 primers distinguished between isolates BL801 and BL806. Eight SNPs were identified in three isolates amplified by RxLR5. No polymorphism was observed on the gel for the remaining RxLR primers on single spore races. Unrefined field isolates showed more polymorphisms on the gel than single spore isolates. The phenotypic differences between these two isolates have been identified by the IBEB differential set. Microscopy and qPCR quantification were used to investigate the compatible and incompatible interactions. The results suggest that BL801 is more virulent than BL806, as more infection structures were observed in IBEB resistant cultivars. Results of qPCR and spore count/unit weight of cotyledons showed that BL801 and BL806 were significantly different. The qPCR quantification results from 4 and 5 dpi were correlated with the spore count/unit weight of cotyledons. Although further work is required to develop race specific markers, the methods used in this project demonstrate the potential use of molecular markers to investigate lettuce - *Bremia* interactions.

# CHAPTER 1 GENERAL INTRODUCTION

## 1.1 PLANTS AND PLANT PATHOGENS

### 1.1.1 Crops

Plants evolve oxygen and assimilate nutrients through sunlight via photosynthesis. Crop plants have always been important as they are the main nutrient source for humans and many animals. Thousands of years ago, the emergence of agriculture resulted in development of the great civilizations. The ancient Chinese civilization relied on rice, and the ancient Egyptian civilization was reliant on barley (Agrios, 2005). Crop plants not only supplied food but also have many uses including the recent development of bio-fuels.

#### 1.1.1.1 Lettuce

Lettuce (*Lactuca sativa*) is a temperate annual or biennial leafy vegetable of the family *Asteraceae*. It is a crop which grows best in cool weather and sandy, fertile, humus-rich moist soil. Therefore, lettuce is often grown in the spring or autumn in the Northern Hemisphere or often be sown in light shade if grown in summer (Lebeda *et al.*, 2002). Lettuce is grown commercially worldwide as a leaf vegetable and it probably started being used as crop plant 4500 years ago (Davis *et al*., 1997). Many different lettuce varieties were depicted in ancient Greek relics and Egyptian tomb paintings (Figure 1.1). Nowadays, there are seven commonly recognized edible lettuce cultivated groups: Butterhead, Cos, Latin, Crisphead, Cutting, Stalk (Rodenburg, 1960) and oilseed lettuce (Rulkens, 1987).

Lettuce belongs to the genus *Lactuca* which comprises about 100 mostly wild species distributed in Europe, East Africa, Asia and North America (deVries, 1997; Lindqvist, 1960a). These species are mostly diploid with a haploid chromosome

number 8, 9 or 17, and they have been divided into several sections (Lindqvist, 1960a). The classifications of some *Lactuca* species differ amongst taxonomists due to the broad diversity within the genus (Lebeda *et al*., 2007). There are three geographic groups (Asian, African and American species) within the lettuce genus. The *serriola* group that consists of lettuce and four other 9-chromosome species: *L. sativa*, *L. serriola*, *L. saligna*, *L. virosa* and *L. altaica* Fisch. and C. A. Mey (Koopam *et al*., 1998; Lindqvist, 1960b). *L. serriola*, *L. saligna*, and *L. virosa* represent the primary, secondary and tertiary gene pools of the group, respectively (McGuire *et al*., 1993; Lebeda *et al*., 2007). *L. sativa* is typically inter-fertile with the other wild species from the primary gene pool, partly inter-fertile with species from secondary gene pool and rarely inter-fertile with species from tertiary gene pool (Zohary 1991; Lindqvist 1960b). According to the study of Zohary (1991), other wild species in the primary gene pool include *L. aculeata*, *L. altaica*, *L. azerbaijanica*, *L. geprica*, *L. scariolodies* and *L. dregeana*, which potentially provides additional genetic variation that could be useful, for example for downy mildew resistance, in lettuce breeding programmes.

*L. serriola* (prickly lettuce) is regarded as the direct ancestor of *L. sativa* (Kesseli *et al*., 1991; Zohary, 1991; de Vries, 1997), and is generally resistant to the lettuce downy mildew pathogen *B. lactucae* and lettuce mosaic virus (LMV). More sources of resistance to diseases and insects have also been found in other wild species to downy mildew (*B. lactucae*) and leaf aphid (*Nasonovia ribis-nigri*) and *L. saligna* to downy mildew (*B. lactucae*) (Bonnier *et al*., 1992; deVries, 1997). Recent lettuce breeding strategies have been focusing on different aspects for the improvement of

the cultivars; one of the major focuses is introducing the *Dm* resistance genes from wild species to cultivated lettuce (deVries, 1997; Lebeda *et al*., 2009).

The first genetic linkage map for lettuce was based on the $F_2$ of two *L. sativa* cultivars (cv. Calmar x cv. Kordaat) and consisted of >13 linkage groups, using mainly RFLP and RADP markers (Kesseli *et al*., 1994). Another genetic map with nine linkage group was made on the $F_2$ of *L. saligna* crossed *L. sativa* (cv. Olof), which consisted of 476 markers (mainly AFLP) (Juenken *et al.*, 2001). In 2007, a consensus map was generated from seven individual lettuce maps, consisting of over 2700 markers (Truco *et al*., 2007).

Lettuce is a host to *B. lactucae,* but many varieties exhibit resistance to specific variants of the pathogen. This resistance is based on the presence of matching of downy mildew resistance (*Dm*) genes which correspond with matching avirulence (*Avr*) genes in the pathogen in a gene-for-gene manner (see 1.2.2). The *Dm* genes were first described in lettuce cultivars by Crute and Johnson (1976), and confer race-specific resistance both in cultivated and wild *Lactuca* species (Jeuken and Lindhout, 2002). More than 45 *Dm* genes have been predicted in the lettuce-*Bremia* pathosystem (Michelmore, 2003). To date the most effective way to control *B. lactucae* is to grow resistant cultivars in combination with fungicide (active ingredient metalaxyl) treatment.

**Figure 1. 1** A Cos-like lettuce (circled) on a relief in the tomb of the sacrificial chapel of Choe at Abydus (ca. 1800 B.C. Photographed by E. Oost, 1983, Museum of Antiquities, Leiden (deVries, 1997).

### 1.1.2 The pathogen

Plant pathogens are entities that can incite disease and there are tens of thousands of diseases that affect plants (Agrios, 2005), and they have been grouped by different criteria such as symptoms (root rots, blights, rusts). Agrios (2005) classified diseases into two groups infectious (biotic plant disease) and non-infectious (abiotic plant disease). The infectious group has been classified into eleven types of pathogens, which are: parasitic angiosperms, fungi, nematodes, algae, oomycetes, plamodiophoromoycetes, trypanosomatids, bacteria, phytoplasmas, virus and viroids (Strange, 2003).

Agrios (2005) estimated conservatively that about 31-42% of crop production is affected by disease, insects or weeds. Approximately 14.1% of this 36.5% average was assigned to the impact of plant diseases which was estimated to cause losses of approximately 220 billion US dollars in crops worldwide. In the last 100 years, pesticides have been used intensively to control crop diseases. It has been shown that pesticides are toxic to plant pathogens but also may be toxic to humans and animals and other living organisms. The residue of these chemicals can end up in soil, air and water and to potentially cause longer term contamination to the environment. Seeking environmental friendly and non-toxic diseases control regimes is, therefore, an aim for many plant pathologists.

#### 1.1.2.1 Oomycete Phylogeny

Oomycetes, also called water moulds, are a fungus-like group of microorganisms in the Kingdom Chromista (*Stramenopila*), and Phylum *Oomycota* (Cavalier-Smith, 1986; Hawksworth *et al*., 1995). Oomycetes were at one time classified as fungi based on their morphology (mycelium) and pathogenic lifestyle. However, oomycetes do not have chitinous cell walls, they are predominantly diploid karyotype and produce zoospores, which means they differ from the true fungi. Molecular phylogenetic data also confirm that they are not closely related to true fungi (Dick 2001, 2002; Kirk *et al.*, 2001).

There are eight Orders of oomycete, and the order Peronosporales includes several important plant pathogens. For example, *Pythium* sp. cause seed rots, seedling damping off and root rot. *Phytophthora* sp. can cause blight and rot (*e.g*. Late blight of potato caused by *Phytophthora infestans*) and root blight, fruit and stem rots. Many genera cause downy mildew of dicotyledonous hosts such as lettuce, tobacco, grape and cucurbits, including *Bremia*, *Peronospora*, *Plasmopara* and *Pseudoperonospora*, respectively. *Peronoslerospora*, *Sclerophthora* and *Sclerospora* are oomycetes that cause downy mildew in monocot hosts including corn, sorghum and sugarcane (Agrios, 2005). Molecular phylogenetic investigations have led to the shift from a morphological to a phylogenetic species concept (Voglmayr, 2008). The ITS (Internal Transcribed Spacer) rDNA region have been widely used in investigations for the species concept in downy mildews and white blister rusts. Nine repetitive elements have been identified from *Bremia* ITS regions showing high sequence heterogeneity between the accessions from different hosts, representing they may from distinc species (Choi *et al*., 2007).

#### 1.1.2.2 Downy mildews on crop plants

Downy mildew is a foliage blight that is caused by several genera of oomycetes. It has caused catastrophic epidemics on crops in history. In the mid to late 1800s, grape production was almost destroyed by epidemics in France and rest of the Europe. Downy mildew oomycetes are biotropic in that obtain nutrient from living host plants by producing a haustorium. They are disseminated above ground by spores called conidiospore or conidia, produced on the tips of conidiophores. The conidia and branching of conidiophores provide a means of differentiating species amongst genera of downy mildews. They generally grow best in cool and humid environments. Some genera have retained an ability to release motile spores (zoospores) from sporangia, which can swim to stomata and infect via an infection hypha (germ tube) through the stomatal opening. In contrast, non-zoosporic downy mildews penetrate via germ tubes between epidermal cells.

Downy mildews can cause rapid and serious losses in immature crops. Although the use of systemic fungicides has reduced the damage caused by this disease, it is still very difficult to control. The most common and serious downy mildew diseases are: *Bremia lactucae* causing lettuce downy mildew; *Hyaloperonospora parasitica*, causing downy mildew in brassicas; several *Peronospora spp*. causing disease in onion soybean and tobacco; *Peronosclerospora*, causing sorghum sugarcane and corn downy mildew; *Plasmopara*, causing grape and sunflower downy mildew; *Pseudoperonospora*, causing cucurbit and hops downy mildew; *Sclerophthora* causing cereal and grass downy mildew and *Sclerospora* causing grass and millet downy mildew (Agrios, 2005).

#### 1.1.2.3 Lettuce downy mildew - *Bremia lactucae*

Downy mildew of lettuce, caused by *B. lactucae*, occurs worldwide and is a serious disease of glasshouse-protected and field-grown crops (Crute, 1992b). *B. lactucae* causes foliar blights and leaf distortion. Cool temperatures and wetted leaves are necessary for germination of sporangia of *B. lactucae*. It can infect lettuce and some other *Lactucae* species such as *L. serriola* and *L. virosa* from seedlings to mature plant stages (Lebeda and Petrzelová, 2004; Lebeda and Schwinn, 1994). The symptoms comprise speckled, yellowed lesions, often angular, bounded by leaf veins on the upper surface of the leaf and with white cotton like sporulation on the lower surface (Figure 1.2).

**Life cycle of *B. lactucae***

Oospores, conidiosporangia as well as the endophytic mycelium are important elements of the downy mildew infection cycle (Clark and Spencer-Philips, 2004). The germinative spore penetrates into the leaf epidermal cell wall or stomata and commences infection. The spore produces a germ tube then forms an appressorium to penetrate the epidermal cell and form primary vesicle and secondary vesicle. It starts to colonize and obtain nutrient by the growth of hyphae and haustorium. Extensive colonization shows the conidiophores bearing conidiosporangia emerging from the stomata. Figure 1.3 shows the life cycle of *B. lactucae*.

There are two mating types of *B. lactucae* referred to as B1 and B2 (Michelmore and Ingram, 1980), which are required for sexual reproduction, to produce long-lived resting inoculum called oospores which are released into soil when the host plant dies (Crute, 1992a). There are multiple races (pathotypes) in the population of *B.*

*lactucae*. To date the 28 races of *B. lactucae* found on *L. sativa* have been denominated in Europe from 1998 to 2011 (van Ettekoven *et al*., 1999; van der Arend *et al*., 2006; Plantum NL, 2011).

**Figure 1. 2** Lettuce leaf lesion from infection by *B. lactucae*. (Source from http://www.pv.fagro.edu.uy/fitopato/FOTO%20GALERIA/Lechuga bremia/index.html).

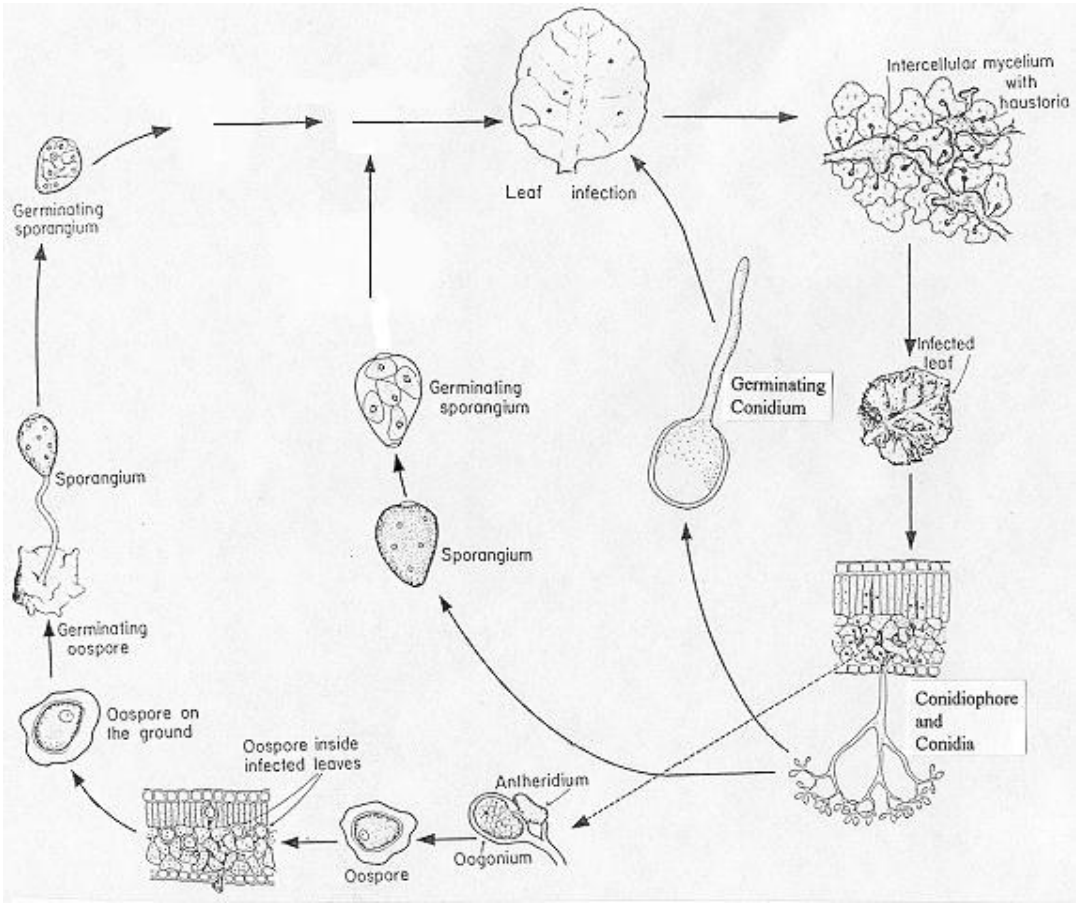



**Figure 1. 3** Disease cycle of lettuce downy mildew caused by *B. lactucae* (Agrios, 1988). (Photo has been modified from an original photo that was obtained from http://ucce.ucdavis.edu/files/filelibrary/2028/23067.pdf**).**

**Control of downy mildew in lettuce**

There are several ways for lettuce growers to manage the disease caused by *B. lactucae*. The most effective way is to grow resistant cultivars in combination with fungicide usage. However, not all lettuce cultivars are suited for all areas and seasons as the pathogen is highly variable and dynamic. Downy mildew resistant cultivars have been used to control the occurrence of the pathogen; however, these cultivars are often overcome by the emergence of new virulent isolates of *B. lactucae*. Much of the variability in *B. lactucae* isolates is in response to metalaxyl (fungicides) usage. To date there has been considerable variability in *B. lactucae* pathotypes in response to metalaxyl application. Recent breeding strategies are focusing on the improvement of the cultivars by introducing the resistance genes of wild species to the cultivated lettuces (deVries, 1997; Lebeda *et al*., 2009).

Physical control of *B. lactucae* is also used in lettuce downy mildew disease management. Cool and damp conditions on the leaf surface are good for pathogen development. In addition wind can help transmission of the pathogen. Irrigation systems can control leaf wetness duration but cannot prevent epidemics when weather conditions are conducive. Lettuce is grown as a transplanted crop and is produced in glasshouses where many cultivars are produced together. There is considerable opportunity for variability in *B. lactucae* to arise under these conditions.

## 1.2 PLANT AND PLANT PATHOGEN INTERACTIONS

The interaction between plants and pathogens is a very dynamic and complex system. It comprises of how the plants fight the parasites and how the pathogens overcome these defences to induce disease. If a pathogen can induce disease on the plant is susceptible to this pathogen. If no disease occurs then the plant is resistant. The immunity (or resistance) is a result of a successful plant defence.

Host-specific interactions means the pathogen can only cause diseases on a small group of plant species because of the specialized gene and molecular signaling required in the host-pathogen interaction (Freeman and Beattie, 2008). Most biotrophic and hemibiotrophic pathogens affect specific plants. For example, Brome mosaic virus (BMV) infects grasses such as barley but not legumes. It is crucial to understand the plants resistance for crop protection. Many model systems have been developed for the interactions studies between plants and pathogens (*e.g.* bacterial, fungal, oomycete, viral and nematode). *Arabidopsis thaliana* has been successfully implemented in the study of the interaction between plants and disease-causing pathogens. Recently, Lamour *et al*., (2007) compared the five oomycete species from which whole genome sequences are publically available (*Phytophthora sojae, P. ramorum*, *P. infestans*, *P. capsici* and *Hyaloperonospora arabidopsidis*) and indicated that these species and their hosts interactions are likely to be useful and potential as model systems.

### 1.2.1 Plant resistance mechanism

Quantitative resistance has been associated with others descriptive terms such as field, multigenic, incomplete, intermediate, partial and horizontal resistance (Mackay, 2001). This resistance is controlled by the contribution of several genes encoded for different minor effects. Environmental conditions and plant development stages can influence the expression of this resistance.

In contrast, qualitative resistance typically refers to race-specific and complete resistance. This resistance is usually monogenic and stable over a wide range of environmental conditions, and is expected to involve a mechanism of induced defence as a result of a recognition or R-protein detecting a matching avirulence protein in a gene-for-gene specific manner (Flor, 1942; Reignault and Sancholle, 2005).

### 1.2.2 Gene-for-gene theory

The gene-for-gene hypothesis of race-specific disease resistance was proposed by Harold H. Flor (1942; 1956; 1972) in his genetic analyses of interactions between flax (*Linum ultissimum*) and the rust fungus *Melampsora lini*. He proposed that an incompatible host-pathogen interaction occurs when an avirulence protein (encoded by an *Avr* gene) from the pathogen corresponds with a matching resistance protein (encoded by an *R* gene) in the host in a gene-for-gene specific manner. The absence of any matching pair of *R* and *Avr* genes will result in plants being susceptible to infection (Figure 1.4).

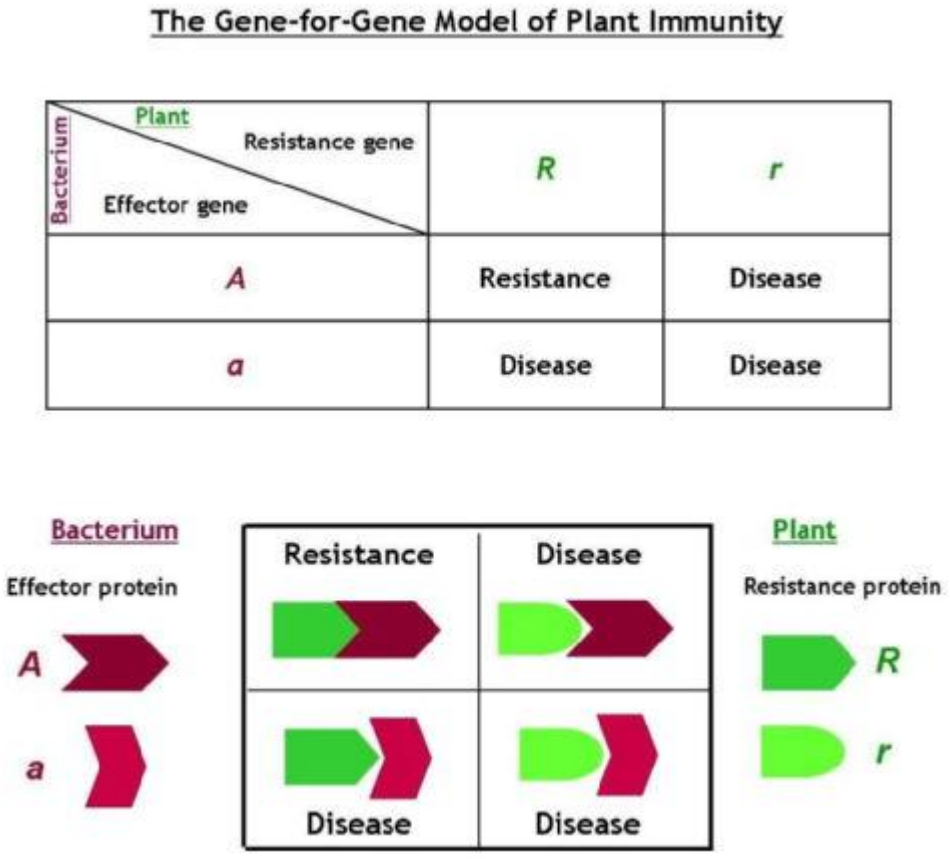



**Figure 1. 4** Gene-for-gene plant immune model.
A: avirulence gene or protein present, a: avirulence gene or protein absent, R: resistance gene or protein present, r: resistance gene or protein absent. (Source from http://www.pseudomonas-syringae.org/)

#### 1.2.2.1 Molecular characterization of resistance (*R*) genes

Since the mid-1990s, numerous *R* genes have been molecularly characterized, and have been grouped into a several classes based on distinctive features (Agrios, 2005). The largest group is represented by *R*-genes encod a nucleotidebindingsite and leucine-rich repeat (NB-LRR) class of proteins (Dangl and Jones, 2001). The first *R*-gene that was cloned from *Arabidopsis thaliana*, named *RPS2* (resistance to *Pseudomonas syringae*) that encodes a cytoplasmic receptor-like protein and confers recognition of the avirulence protein avrRpt2 (Kunkel *et al*., 1993). The bacterial protein avrRpt2 is delivered into *A. thaliana* via the Type III secretion system

(Mackey *et al*., 2003). The RPS2 protein contains a nucleotide binding site (NBS) domain and a carboxyl terminus region of leucine rich repeats (LRRs). Recognition of a pathogen effector leads to a rapid immune response often involving a hypersensitive response in which the infected plant cells undergo cell death and prevent further spread of the pathogen (Axtell and Staskawicz, 2003). The LRR domain is highly polymorphic and thought to be the most important domain for specifying recognition of matching AVR proteins (Sicard *et al*., 2003).

To date, NBS-LRR proteins represent the most common class of R-proteins found in plants (DeYoung and Innes, 2006). In the lettuce downy mildew pathosystem, for example, the *R* genes are referred to as *Dm* (downy mildew) genes and so far include only examples that encode NBS-LRR proteins (Sicard *et al.*, 2003). The *Dm* 3 gene in lettuce was cloned and characterized with a NBS-LRR region (Meyers *et al.*, 1998).

#### 1.2.2.2 Molecular characterization of avirulence (*Avr*) genes

Different pathovars of the bacterial pathogen *Pseudomonas syringae* provided the earliest examples of avirulence proteins that matched known R proteins, such as the AvrD protein from the bacterial spot of tomato pathogen *P. syringae* pv. *tomato* which elicits recognition by the *PTO* protein in tomato (Pedley and Martin, 2003).

Many examples of avirulence proteins have been characterised in the past decade from fungal and oomycete pathogens. For example, three avirulence proteins (Avr2, Avr4 and Avr9) have been cloned from the fungus *Cladosporium fulvum* which are avirulent to tomato varieties that carry the resistance genes *Cf2*, *Cf4* and *Cf9*, respectively (van Kan *et al*., 1991; Joosten *et al.*, 1994; Luderer *et al*., 2002). These

avirulence genes encode small cysteine-rich peptides that are secreted into the apoplast of a tomato host. Similarly, *ATR1* and *ATR13* are avirulence genes that were recently cloned from the *Arabidopsis* downy mildew pathogen *H. arabidopsidis* (Allen *et al*., 2004; Rehmany *et al*., 2003).

A common feature of avirulence proteins is that they appear to play an important role in pathogenicity, either for suppressing host defence or altering host metabolism in a susceptible host (lacking matching *R*-genes), and are therefore often referred to now as 'effector' proteins. Consequently, the defense response associated with *R*-genes is often described as effector-triggered immunity (ETI), and typically results in hypersensitive cell death at the site of infection (Jones and Dangl, 2006). The hypersensitive response is typically pathogen-specific resistance (Shirasu and Schulze-Lefert, 2000) and is often triggered when gene products in the plant cell recognize the presence of specific pathogen effector molecules introduced into the host by the pathogen.

## 1.3 THE SIGNALLING OF PLANT AND PATHOGEN INTERACTION

Plants do not have a circulatory system with mobile defender cells and a somatic adaptive immune system like animals, but instead rely on an innate immune system that can function in most cell types (Ausubel, 2005; Chisholm *et al*., 2006; Jones and Dangl, 2006). Plants have a basal immune system that includes a small number of membrane-bound receptor-like proteins that enable recognition of non-specific elicitors or pathogen-associated molecular patterns (PAMPs), such as flagellin protein of motile bacteria or fungal chitin. Non-pathogens as well as pathogens are capable of inducing 'PAMP-triggered immunity' (PTI) in plants due to the highly conserved nature of these molecular patterns. It has been proposed that many effectors (described above) may have evolved to enable suppression of PTI, resulting effector-triggered susceptibility.

### 1.3.1 Effectors

During the infection process, the pathogens produce a range of molecules to enable the infection, such as degrading the host cell wall, invading the host organism or changing the host metabolism (Strange, 2003). In a resistant host, these effectors are thought to trigger the host to produce enzymes like proteases, hydrolases, glucanases, and chitinases that can degrade the cell wall of an invading pathogen in the apoplast to prevent the infection (Bent and Mackey, 2007).

Bacterial pathogens utilize various secretion systems including Type II Type III, Type IV and Type VI to deliver proteins such as pathogenicity determinants (often referred to as effector proteins) into a host. The term of Type III secretion system (often abbreviated as TTSS or T3SS) was defined in 1993 (Salmond and Reeves) in

gram-negative bacteria. The cytoplasmic effectors of bacteria are generally delivered into a host cell via a Type III secretion system (Cunnac *et al*., 2009; Lindeberg *et al*., 2006), while apoplastic effectors are secreted by a Type II secretion. (Figure 1.5.c). Studies on bacterial avirulence protein have increased the understanding of the trafficking of effector proteins secreted by the Type III secretion system (Tör, 2008). In the host cytoplasm they are specifically recognized by a host resistant protein containing NBS-LRR structure. Some bacterial effectors (*e.g.* Yopj from *Yersinis* sp. and XopD, AvrBST and AvrXv4 from *Xanthomonas* sp.) have been found which show similarity to small ubiquitin-like modifier (SUMO) (Hotson and Mudgett, 2004). The finding that the fungus *Trichoderma viride* xylanase (EXI) interacts with SUMO with tomato will lead to a hypersensitive reaction (Hanania *et al*., 1999) suggested that the SUMO-like molecules from the pathogen have a function that can mimick the host's regulatory molecules involved in resistance responses (possibly to suppress the HR).

#### 1.3.1.1 Oomycetes effectors

Oomycetes form a distinct group of eukaryotic microorganisms and in recent years progress has been made on describing oomycete effectors (Kamoun, 2006). Oomycetes are thought to secrete hundreds of effector proteins that target two distinct sites (cytoplasm and apoplast) in the host plants. Apoplastic effectors are secreted into the plant extracellular space whereas cytoplasmic effectors are translocated into the plant cell, where they target different subcellular compartments (Birch *et al.*, 2006; Kamoun, 2006) (Figure 1.5). Cytoplasmic effectors consisting of an N-terminal region involved in secretion and translocation inside the host cell and a C-terminal carrying the effector activity are characterized by the conserved motifs

(RxLR) in the N-terminal region following the signal peptide (Kamoun, 2006, 2007; Morgan and Kamoun, 2007). The mechanism which oomycetes use to translocate these effectors into a host cell is currently unknown.

Four oomycete *Avr* genes have been identified; *Avr 1b-1* from the soybean pathogen *Phytophthora sojae* (Shan *et al*., 2004), *ATR13* (Allen *et al*., 2004) and *ATR1*$^{NdWsB}$ from the *Arabidopsis* pathogen *Hyaloperonospora parasitica*, and *AVR3a* from *P. infestans* (Armstrong *et al*., 2005). Most oomycete *Avr* genes share a short sequence motif downstream of the signal peptide which consists of an RxLR (Arg-X-Leu-Arg, x equals any amino acid) amino acid sequence, and a dEER (Asp-Glu-Leu-Arg) motif followsing RxLR after 5 -21 amino acid (Dodds *et al*., 2009). The RxLR motif is similar to the host cell target signal RxLR (E/Q) which promoted the hypothesis that RxLR function as a signal that mediates trafficking into host cells (Marti *et al*., 2004; Rehmany *et al.*, 2005). The oomycete effectors are modular and all known oomycete effectors with avirulence activities are cytoplasmic effectors of RxLR class (Schornack *et al.*, 2009). Figure 1.6 illustrates four oomycete AVR proteins containing within the N-terminal 60 amino acids a secretory signal peptide and a conserved domain with RxLR motif. The secreted effectors are transported across a host-derived membrane, most probably the haustorium via the RxLR leader (Tör, 2008). Recent studies indicate that the RxLR effectors show virulent activity (Bos *et al*., 2006; Dou *et al*., 2008; Whisson *et al*., 2007) to suppress the cell death. It has been discovered that AVR3a$^{KI}$ suppresses the hyposensitive cell death induced by the major *P. infestans* elicitin INF1 in *Nicotiana benthamiana* but did not suppress the cell death by other *P. infestans* (Bos *et al*., 2006) which indicates that virulent activity shows some level of specificity (Kamoun, 2006).

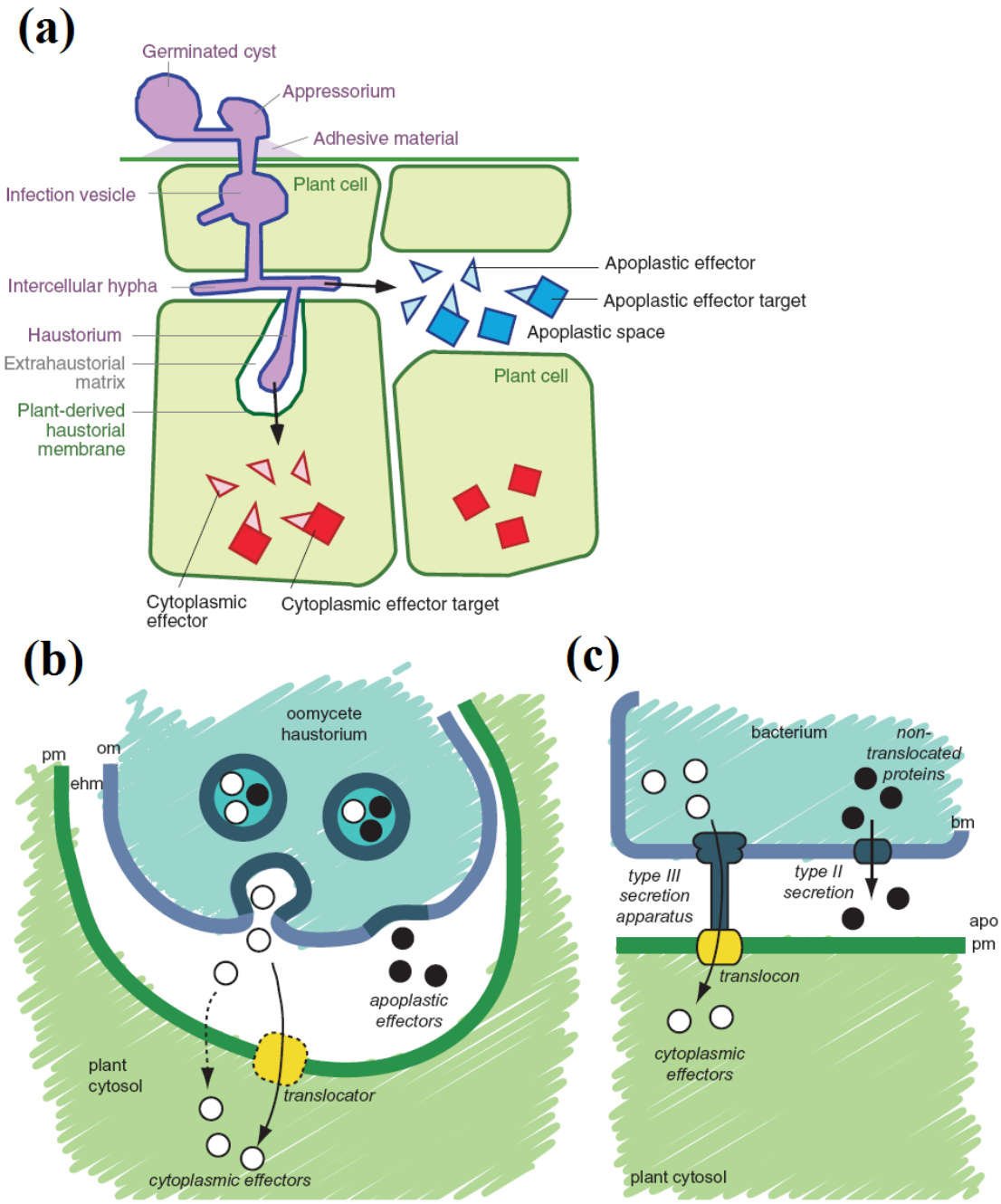



**Figure 1. 5** The secretion of oomycete and bacterial pathogen effectors. (a) Schematic view of early stage of infection by *Phytophthora infestans* illustrating the sites where apoplastic and cytoplasmic effectors are delivered to (Kamoun, 2006). (b) Sectional view of the oomycete effectors delivery into plant cell; (c) bacterial effectors are secreted by Type III secretion system (Schornack, 2009). apo: apoplast, bm: bacterial membrane, ehm: extrahuastorial matrix, om: oomycete membrane; pm: plasma membrane.

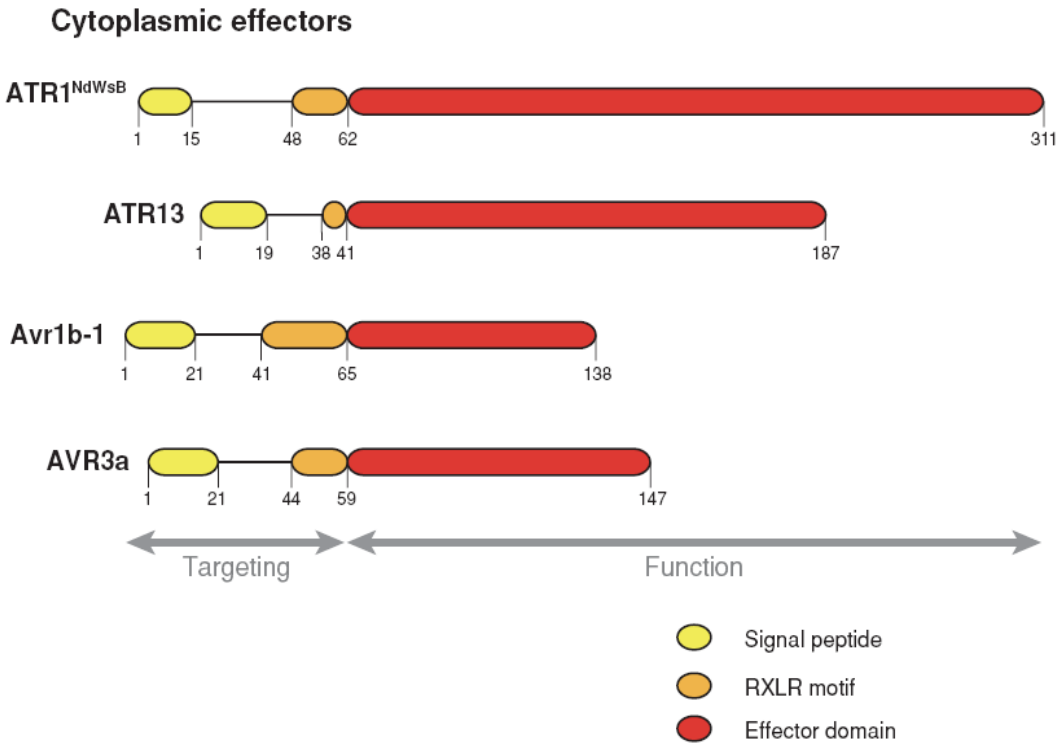



**Figure 1. 6** Four oomycete Cytoplasmic RxLR effectors.
The numbers under the sequences indicate amino-acid positions (Kamoun, 2006).

## 1.4 GENETICS OF LETTUCE - *B. LACTUCAE* INTERACTIONS

### 1.4.1 Race specific interactions between lettuce and *B. lactucae*

The interaction between *L. sativa* and *B. lactucae* is one of the most extensively characterized gene-for-gene plant pathogen relationships (Michelmore and Wong, 2008). This resistance is based on single dominant *Dm* (downy mildew) gene or *R* (resistance) factor, which means it is monogenic race-specific resistance.

More than 27 major *Dm* genes or R factors have been discovered (Farrara *et al*., 1987; Crute, 1992b; Meyers *et al*., 1998; Lebeda and Zinkernagel, 2003; Michelmore and Wong, 2008). Pathogen genetic studies have found that the host population of lettuce contained twelve well-defined race-specific resistance genes (*Dm1*, *Dm2*, *Dm3*, *Dm4*, *Dm5/8*, *Dm6*, *Dm7*, *Dm11*, *R12*, *Dm13*, *Dm16* and *R18*). These known *Dm* genes are mainly located in clusters on at least five different chromosomes in the lettuce genome (Bonnier *et al*., 1994; Hulbert and Michelmore, 1985; Kesseli *et al*., 1994). However only some of these genes (*Dm3*, *Dm7*, *Dm11*, *Dm16* and *R18*) are important in resistance management in the host crop (Lebeda and Zinkernagel, 2003). The *Dm*3 gene has been cloned (Meyers *et al*., 1998) and it was characterized to a class of resistance genes encoding a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) region similar to the gene cloned from other species giving resistance to downy mildew and other pathogens (McHale *et al*., 2006). However, resistance based on *Dm* genes is not durable since the resistance is usually overcome by rapid adaptation of *B. lactucae* races (Lebeda and Zinkernagel, 1999).

Many of *Dm* genes identified are used for phenotypic screening of *B. lactucae* isolates and to predict the characterization of their virulence (Lebeda *et al*., 2008a). Similary, genetics of avirulence genes in *B. lactucae* has been investigated (Michelmore and Wong, 2008). Lebeda *et al.* (2008b) summarised that the number of host resistance genes is expected to increase further with continuation of extensive phenotypic characterization of *Lactuca* germplasm and molecular investigation.

### 1.4.2 Phenotypic identification using reaction profile on differential sets of host cultivars

The naming methods for physiological races of plant pathogens have been developed based on the characterization of pathogenicity on a set of differential host cultivars (Herrmann *et al*., 1999). A binary/decanary classification system (Habgood, 1970) was introduced by assigning '0' as avirluence and '1' for virulence of each isolate on the given host differential set being arranged in a fixed linear order. These series of '0'and '1' are regarded as a binary number, the value of which can be converted into a decimal notation. These numbers derived in the decimal conversion are regarded as a race identity code of particular pathotype. This system was widely applied to several fungal pathogens such as rusts, mildews and potato blight (Herrmann *et al*., 1999; Limpert and Muller, 1994). Habgood's method was improved by the proposed use of octal number (Gilmour, 1973), which breaking down the series of '0' and '1' into three groups (in a triplet of differential cultivars) to generate a triplet code to name a physiological race. A laborious method for determining the virulence phenotype of *B. lactucae* had been introduced by Michelmore and Crute (1982) by developing a lettuce differential set with 24 resistance lines which have been widely used for *B. lactucae* race identification (Michelmore *et al*., 2009). A standardized

classification and nomination system has been established by the International *Bremia* Evaluation Board (IBEB) using a binary coding system which assigns a sextet code for the description of reaction patterns (van Ettekoven and van der Arend, 1999) in a joint effort of European lettuce breeders and authorities since 1999. IBEB meets annually to review the pathotypes of *B. lactucae* and to denominate new races with a Bl: prefix, until 2011 twenty-eight races have been denominated. The IBEB differential system is the physiological method used for isolate identification in this PhD project.

### 1.4.3 Genotypic identification using molecular methods

The IBEB differential system provides a macroscopic way to investigate the *B. lactucae* and lettuce interaction phenotypically. For further understanding the mechanism, molecular methods are essential tools to investigate these interactions genotypically. There are several kinds of markers showing the potential for development of *B. lactucae* race genotypic identification.

#### 1.4.3.1 RFLP, AFLP and RADP markers

RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) and microsatellite marker RAPD (randomly amplified polymorphic DNA) have been used in molecular studies and are invaluable for generating fingerprints of pathogen isolates (Dickinson, 2003). These markers have been used for genetic mapping of *B. lactucae* (Hulbert *et al*., 1988; Sicard *et al*., 2003).

Faba bean genetic diversity studies using RADP and AFLP indicated some of the technical limitation of these techniques. RAPD markers have been used to study the

genetic diversity within European and Mediterranean Faba bean germplasm (Zeid *et al*., 2003). AFLP markers have been used to assess genetic diversity among elite Faba bean inbred lines (Zeid *et al*., 2003). However, these two molecular marker systems have some technical limitations. The RAPD technique has low laboratory-to-laboratory reproducibility, and the AFLP technology has a high cost. However ISSR techniques can overcome the above limitations and yield more polymorphisms than other molecular techniques (Terzopoulos and Bebeli, 2008).

#### 1.4.3.2 Ribosomal Internal Transcribed Spacer (ITS) markers

Peagy *et al*. (2008) indicated that the internal transcribed spacer region has been widely sequenced for DNA regions in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (*e.g.* to identify geographic races). Variation among individuals rDNA repeats can sometimes be observed within both the ITS and IGS regions, due to the higher degree of variation of ITS regions than other regions of rDNA. ITS1+ITS4 primers have been frequently used and several taxon-specific primers have been described that allow selective amplification of fungal sequences specifically describing amplification of basidiomycete ITS sequences from mycorrhiza samples (Gardes and Bruns, 1993).

#### 1.4.3.3 Inter Simple Sequence Repeat markers (ISSR)

ISSR (inter-simple sequence repeat) (Zietkiewicz *et al*., 1994) is a general term for a genome region between microsatellite loci. The complementary sequences of two neighbouring microsatellites are used as PCR primers which are generated from a single-primer PCR reaction where the primer is designed from di- or trinucleotide repeat motifs with a 5' or 3' anchoring sequence of one to three nucleotides (Wolfe *et*

*al*., 1998); the variable region between them gets amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary much in length. So far, ISSR-PCR has been demonstrated to provide highly reproducible results and generate abundant polymorphisms in a number of host pathogen interactions (Ajibade *et al*., 2000). ISSR markers have been developed for identification of *B. lactucae* races (Wagner and Idczak, 2004).

#### 1.4.3.4 Simple Sequence Repeats (SSR) markers

Microsatellites or simple sequence repeats (SSRs) are tandem repeats of 2-8 base pairs (bp) that can vary extensively in the number of repeats. They are valuable as genetic markers because they are co-dominant, detect high levels of allelic diversity, and are easily and economically assayed by PCR (McCouch *et al*., 1997).

Microsatellite DNA loci have become important sources of genetic information for a variety of purposes. Specific microsatellite repeats primers must be developed to amplify microsatellite loci by PCR (Glenn and Schable, 2005). These microsatellite regions of DNA are among the most variable in the genome, thus primer-binding sites are not well conserved among distantly related species (Primmer *et al*., 1996). Although microsatellite loci have now been developed for hundreds of species these loci have not been isolated from many additional species (Glenn and Schable, 2005).

Many different strategies for obtaining microsatellite DNA loci have been described. Cloning small genomic fragments and using radiolabelled oligonucleotide probes of

microsatellite repeats to identify clones with microsatellites works well in organisms with abundant microsatellite loci (Weber *et al*., 1991). It does not work well when microsatellite repeats are less abundant. Therefore another two enrichment strategies have been developed: (1) uracil-DNA selection (Ostrander *et al*., 1992) and (2) hybridization capture (Armour *et al*., 1994; Kandpal *et al*., 1994; Kijas *et al*., 1994). Hybridization capture allows selection prior to cloning, and therefore is faster and easier to achieve with multiple samples than uracil-DNA selection (Glenn and Schable, 2005).

#### 1.4.3.5 Effector based markers

Oomycete cytoplasmic effectors have been discovered through their avirulence (*Avr*) function, where they have the ability to trigger hypersensitive cell death in host genotypes with corresponding disease resistance (*R*) genes (Morgan and Kamoun, 2007; Allen *et al*., 2004; Armstrong *et al*., 2005). Recent studies indicated that RxLR effectors possess virulence function (Bos *et al*., 2006; Sohn *et al*., 2007; Dou *et al*., 2008). Multiple candidate effectors from the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) have recently been studied to ascertain if they can suppress the host plant immunity (Fabro *et al*., 2011), which showed that Single Nucleotide Polymorphisms (SNPs) in effector candidates between seven *Hpa* isolates (Cala2, Emco5, Emoy2, Hind2, Maks9, Noco2, Waco9) were detected. These studies show a possibility that effectors candidates could be developed as markers to identify the variation between the oomycete isolates.

## HYPOTHESIS AND OBJECTIVE OF THE PROJECT

The overall aim of the project is to develop and use molecular methods to study changes in population structure of *B. lactucae* on lettuce within agricultural ecosystems. An important aspect was to develop molecular markers for epidemiological studies and compare these with existing methods for differentiating *B. lactucae* races. Physiological methods were applied for phenotypic *B. lactucae* races identification as correlated studies to molecular methods.

# CHAPTER 2 MATERIALS AND METHODS

## 2.1 EXPERIMENTAL MATERIAL

### 2.1.1 Collection of *Bremia lactucae* isolates

The lettuce cultivar of *Lactuca sativa* 'Cobham Green' supplied by (Tozer Seeds Ltd., UK) is a universal susceptible host (no known *Dm* gene) of *B. lactucae*, and has been in this study as the standard host to generate sporangia for DNA extraction (Sicard *et al.*, 2003). Seven existing isolates (BL801, BL802, BL803, BL804, BL805, BL806, and BL807) had been collected and cultured on Cobham Green. Amongst these isolates, BL801 and BL802 were used in previous studies (Prof. Eric Holub, University of Warwick, UK)，BL803 was isolated in Charlecote Garden Centre, and BL804 was isolated from Lincolnshire (Jepco, Holbeach Hurn, UK). Three other isolates (BL805, BL806, and BL807) were supplied by Langmead Ltd from Spain (Table 2.1).

**Table 2. 1** Previously collected isolates of *B. lactucae*

| Isolates | Host | Location | Date |
|---|---|---|---|
| BL801 | Cobham Green | Warwick HRI | Dec, 2007 |
| BL802 | Cobham Green | Warwick HRI | Dec, 2007 |
| BL803 | Mixed cultivars | Charlecote, UK | Jun, 2008 |
| BL804 | Iceberg | Lincolnshire | Jun, 2008 |
| BL805 | - | Spain | 2008 |
| BL806 | - | Spain | 2008 |
| BL807 | - | Spain | 2008 |

Field samples were collected from diseased crops in 2009 and 2010 to generate new isolates for population studies. Thirty-six lettuce leaf samples had been collected from Lincolnshire in August 2009, some of the leaves already had sporulation in the field whilst some showed lettuce downy mildew symptom without sporulation. Table 2.2 gives the information on the isolate collection used in subsequent studies. Twenty-five field samples had been collected from Lincolnshire (J.E. Piccaver & Co.

Norfolk House Farm, UK) and Chichester (Langmead Ltd. Ham Farm, UK) in May 2010.

After the sampling from fields, isolates were kept in a cold box before any treatment. Isolates were cultured in humid and cool conditions (15°C, 12 hours photoperiod) in a controlled environment PEL room (Plant Environment Laboratory) for more spores release before inoculation on lettuce cultivar Cobham Green.

**Table 2. 2** New isolates of *B. lactucae* collected from Lincolnshire and Chichester, England.

| Year | Location | Lettuce cultivar (host) | Number of sample collected |
|---|---|---|---|
| 2009 | Lincolnshire | Escade excel | 3 |
| | | UK COS-255 | 3 |
| | | UK MUL 1-35 | 3 |
| | | UK MUK 1-25 | 3 |
| | | UK MUL1-85 | 3 |
| | | Mirsal | 3 |
| | | CNEN4 | 3 |
| | | Caugin | 4 |
| | | Escarole Nnance | 3 |
| | | 02-354975 | 3 |
| | | L. Rossa Bastill | 3 |
| | | Iceburg Diamond | 1* |
| | | L/BIOND/LIVIGNOS | 1* |
| 2010 | Lincolnshire Southcoast | Iceberg | 16 |
| | | Mir | 1 |
| | | 7RMUS | 5 |
| | | 16LGATT | 3 |

* Denotes whole lettuce.

## 2.2 METHODS

### 2.2.1 Culture of the *B. lactucae* isolates

Cultures of the *B. lactucae* isolates were maintained on a universal susceptible cultivar of lettuce cv. Cobham Green. In this project there were two main types of inoculation techniques applied to culture the *B. lactucae* isolates. The first type of technique was non-quantitative inoculation referred to hence as the swabbing method. The other method was for quantitative inoculation (*i.e*, the amount of spores had been quantified before inoculation) which was used in experiments for droplet and spraying inoculation of seedlings.

Before the inoculation, seeds of cv. Cobham Green were sown in 90 mm crystallizing dishes filled with vermiculite moistened by Hewitt's solution (Hewitt and Smith, 1975). The lettuce seeds were placed on double layers of moist filter paper on top of the vermiculite, sealed in sandwich boxes which were placed in a growth room at 20ºC for seven days(Kapooria and Tjallingii, 1969).

#### 2.2.1.1 Swabbing inoculation

Infected leaf seedlings were used to transfer inoculum on to new seedlings by swabbing the cotyledons with sporulation. After inoculation, the infected plants were moved to a pathogen culture room (15ºC). Sporulation was observed 4-5 days later, and after 7 days, the final density of *B. lactucae* conidiophores per unit of leaf surface was sufficient for DNA extraction and in re-inoculation on to uninfected plants.

#### 2.2.1.2 Droplet and spraying inoculation

Droplet and spraying of inoculums were based on the methods described by Wood *et al*. (1988b) and Bennettand *et al*. (1994). The concentration of spores in aqueous suspension was measured and then adjusted to a standard concentration for use in experiments. Several seedlings with heavy sporulation were cut seven days post inoculation and transferred into a 50 ml Fisher centrifuge tube (Fisher Scientific, Mexico) with 20 ml of chilled, distilled water, and subsequently kept cool in an ice water bath. The tube was shaken vigorously to release the spores in water to make a suspension. The number of spores was counted with the aid of a haemocytometer (Spencer Neubauer $1^{-1}$ mm deep, UK).

The final spore concentration for inoculation was adjusted by diluting the spore concentrate. If there is Y ml spore concentrate (X spores $ml^{-1}$), and the final spore concentration was Z spores/ml then the volume of water required to make the dilution can be calculated by this formula: $((X \div Z) \times Y) - Y$. For example, 20 ml spore suspension concentration with a $9 \times 10^5$ spores $ml^{-1}$ spore count and the target concentration was $4.5 \times 10^5$ spores $ml^{-1}$. According to the formula $(9 \times 10^5 \div 4.5 \times 10^5) \times 20\text{-}20=20$ml, therefore 20 ml water has to be added to the concentrate to make the target suspension.

### 2.2.2 Phenotypic identification of *B. lactucae* races

Tests were carried out using seedlings of a set of twenty lettuce cultivars (IBEB EU-A set cultivars Table 2.3, seeds supplied by Naktuinbouw, Ltd., The Netherlands) to differentiate pathotype variants of the pathogen. The reactions of collected isolates on the cultivars (Table 2.3) can be used to identify the *B. lactucae* races according to

the IBEB nomination system. Lettuce seedlings were maintained in a propagation box.

**Table 2. 3** Twenty lettuce cultivars for *B. lactucae* race identification.

| Accession number of variety | Variety |
|---|---|
| 0 | Cobham Green |
| 1 | Lednicky |
| 2 | UC DM2 |
| 3 | Dandie |
| 4 | R4T57D |
| 5 | Valmaine |
| 6 | Sabine |
| 7 | LSE57/15 |
| 8 | UC DM10 |
| 9 | Capitan |
| 10 | Hilde II |
| 11 | Pennlake |
| 12 | UC DM14 |
| 13 | PIVT1309 |
| 14 | CGDM-16 |
| 16 | Colorado |
| 17 | Ninja |
| 18 | Discovery |
| 19 | Argeles |

The differential lettuce cultivars test sets were infected by spreading the spores on the surfaces of cotyledons of the seedling. Seedlings were cultured in a walk-in controlled environment room (15°C, 12 hours photoperiod) for seven days. Seedling infection was scored as + if sporulation was present or - if there was no sporulation, following the IBEB system. The infection phenotype was also scored using a six class scale from 0 to 5 depending on the percentage of the cotyledon that had been infected and the degree of sporulation. This scoring was useful to evaluate the susceptibility of the lettuce cultivar (see 3.2.3 for further detail).

### 2.2.3 Molecular methods for genotypic identification development

*B. lactucae* DNA was extracted by different methods in this project depending on the requirements of the experiments. The beans beating (electronic grinding) DNA extraction method was based on what has been described by Griffin *et al*. (2002). The methods for DNA extraction, PCR, cloning, sequencing were modified after the published studies (Barrett, 1987; Barbian, 1997; Clewes *et al*., 2007; Kim *et al*., 1992; Minnick and Shen *et al*., 2006; Zhang *et al*., 2007).

#### 2.2.3.1 Sample harvest

For DNA extractions, *B. lactucae* spores were usually washed prior to extraction. Spores were harvested by cutting the infected cotyledons of the Cobham Green seedlings. Infected cotyledons were placed in a beaker with 100 ml ice cold distilled water, and shaken gently to allow the spores to be released into water. Suspensions were filtered (Miracloth, CALBIOCHEM, USA) into a 50 ml universal tube. The tube was spun at 3000 rpm (1610 x g) for 20 minutes to form a pellet. The supernatant was discarded. The pellet was re-suspended in a 1.5 ml tube and was centrifugated under 14000 rpm (20,000 x g) for 1 minute to form pellet, supernatant (plant tissue) was removed by pipetting. The spores pellet was kept at -80°C.

#### 2.2.3.2 Leaf sample disruption for DNA extraction

Freeze-drying (lyophilisation) is a process of dehydration to preserve the biological material. It is suitable for drying both fresh and frozen material. In this project, samples were freeze-dried using an Edwards freeze-dry machine (Crawley, UK). Samples were placed into the freeze drying machine. Lids and covers were removed to make sure the water in the tissue evaporated. The tubes were sealed with

ventilated tissue paper. Dry samples were sealed and kept at room temperature.

Before extracting, samples were lysed for the most efficient extraction. In this project, two methods were applied to disrupt the cell; by grinding manually with liquid nitrogen or by electronic grinding. These methods were suitable to disrupt the cells of a pure spore pellet (from 2.3.1) in a 1.5ml tube. The tubes with spores were kept in liquid nitrogen for 1 minute and were ground quickly before immediate extraction.

A FastPrep-24 Tissue and Cell Homogenizer (MP, UK) and Btissuemill (Retsch, Germany) were used to disrupt the tissue. The FastPrep-24 Tissue and Cell homogenizer is suitable for dry samples, whereas the Btissue mill is a mixer that is suitable for dry, fresh or frozen samples. Before grinding, tungsten carbide beads (Qiagen UK cat. no. 69997) were placed into a 1.5 ml safe lock tube (Starlab, UK). For the FastPrep-24 homogenizer, the samples were loaded in the machine and run for 30 s with the speed set at 5.5. After grinding, the samples were removed and kept on ice for 5 min (Griffin *et al*., 2002). The above steps were repeated three times to fully disrupt the tissue. In the Btissue mill, samples were kept in liquid nitrogen. The samples were placed in the middle side of the adaptors to prevent variation in sample homogenization as the outside rack of the adaptor moves more quickly than the inside. Samples were disrupted at a high speed of 25 Hz for 30 s. The samples were removed and kept in liquid nitrogen.

#### 2.2.3.3 DNA extraction for PCR, cloning and sequencing

Different extraction methods were used in this project, depending on the condition of the samples and requirements of the test. Qiagen DNeasy Plant Mini Kit (Qiagen, UK, Cat. 69104) and Invitrogen ChargeSwithch® gDNA Plant Kit (Invitrogen, UK) are two commercial kits that were used for genomic DNA extraction from either pure spore samples or infected plant tissue. The Sigma-Aldrich REDExtract-N-Amp Plant PCR Kit was used for quick tests with small samples. It was used to extract *B. lactucae* DNA from one or two infected cotyledons with or without sporulation.

Qiagen DNeasy Plant Mini Kit (Qiagen, UK, Cat. 69104) was used in most of the DNA extraction in this project. This kit is designed to extract and purify DNA from plants and fungi (up to 100 mg) in a quick and easy way. It was used to extract the genomic DNA of *B. lactucae* from pure spores, and also used to extract the DNA of *B. lactucae* in plant tissue for both PCR and qPCR assay.

Invitrogen ChargeSwitch® gDNA Plant Kit (Invitrogen, UK) was used to extract the genomic DNA of *B. lactucae* from pure spores. However, it is not suitable where large number of samples have to be extracted at the same time.

Sigma-Aldrich REDExtract-N-Amp plant PCR kits were also used for DNA extraction. This kit contains all the reagents necessary to rapidly extract genomic DNA of *B. lactucae* in plant tissue and amplify targets of interest by PCR. The REDExtract-N-Amp PCR ReadyMix contains a tracking dye that allows direct loading of PCR reactions onto agarose gels for analysis. As one cotyledon is sufficient for the extraction it is suitable for small quantity sample (for example only

one cotyledon shows sporulation) extraction.

The cotyledons with sporulation were cut in a 1.5 ml tube. 100 µl Extraction Solution was pipetted into the tube, the tissue in the buffer was incubated at 95°C for 10 minutes; an equal volume of Dilution Solution was added to the extract to neutralize inhibitory substances prior to PCR. The extract was votexed to mix and kept in 4°C.

The PCR was carried out in 20 µl reaction containing 10 µl REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, USA), 1 µl each of 10 µM primer (forward/reverse), 4 µl DNA extract and 4 µl distilled water. All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). An initial amplification was performed for 5 cycles after denaturation at 94°C for 2 min. Each cycle consisted of 94°C for 30 s and annealing for 30 s and 72°C for 30 s followed by another 30 cycles consisted of 94°C for 30 s and annealing at 65°C for 30 s and 72°C for 30 s then 18°C infinity.

A boiling method was also used for DNA extraction. *B. lactucae* spores were harvested by washing the infected cotyledons before boiling at 95°C for 5 min to break the spore and release the DNA. This method had been used before the Sigma-Aldrich REDExtract-N-Amp Plant PCR Kit had been applied in the project to extract the DNA from small amounts of spores (spores from less than 5 sporulated cotyledons) for PCR assay.

**DNA quantification:** The DNA concentration was quantified by UV® spectrophotometry (NanoDrop, USA) (Mathimaran *et al*., 2008). 1.5 µl millipore

filtered water was loaded on the detector to do calibration. Then 1.5 µl elution buffer (Qiagen, UK) was loaded to make blank. A 1.5 µl DNA sample was loaded for measuring the concentration. 70% ethanol was used to clean the detector before and after of each loading. Alternatively, a 5 µl DNA sample (including 1 µl 5x loading dye load from Bioline, UK) was loaded on a 1% Agrose gel, and electrophoresed in 1x TBE (Tris-Borate-EDTA) buffer at 120 V for 1 hour. DNA quality can be shown on the gel photo by the brightness of the band.

**PCR amplification:** The PCR amplification systems varied in this project. Generally it was in a 25 µl reaction, containing 2.5 µl 10x buffer, 0.75 µl 25mM $Mgcl_{2,}$ 0.13 µl 25 mM dNTPs, 0.2 U Taq polymerse. The above can be used as ready mixed product such as BiomixRed (Bioline, UK). 1 µl each of 10 µM primer (forward/reverse), DNA template and PCR grade water was included. The parameters of the thermal cycles were different depending on the test. For general amplification, PCR was performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). After an initial denaturation (94°C for 2 min) 35 cycles were performed, each cycle consisted of 95°C for 30 s and annealing step (the appropriate annealing temperature for the primer pair) for 30 s and 72°C for 30 s followed by a extension step at 72°C for 10 min.

**Gel Electrophoresis:** PCR products were separated by using 1.5% Agarose Gel stained with 0.5x Gel Red, Spreadex gel EL 800 (Elchrom Scientific, Switzerland) and 6% Poly(NAT)$^{TM}$ gel (Elchrom Scientific) stained with Sybr Gold. For electrophoresis, 4 µl PCR product was loaded with 1µl loading buffer. Samples were electrophoresed alongside a DNA mass ladder (HypeLadder IV, Bioline) for Agarose

gel or 100 bp ladder (Invitrogen) for Spreadex and Poly(NAT)™ gels. Agarose gels (1.5%) were electrophoresed in 1x TBE (Tris-Borate-EDTA) buffer at 120 V for 1 hour, alongside a HyperLadder IV. Spreadex gel EL 800 and 6% Poly (NAT) Gel, were electrophoresed in 0.33x TAE (Tris-acetate-EDTA) buffer at 55°C in SEA 2000® gel tank (Elchrom Scientific) at 10 V $cm^{-1}$.

**Cloning and sequencing analysis*:*** Purified PCR products were cloned into pSTBlue-1 using AccepTorTM vector Kit (Novagen, USA). Cells positive for the insert were grown overnight, plasmid DNA was isolated using Qiaprep Spin Miniprep Kit (Qiagen, UK) and the insert was sequenced directly using BigDye Terminator® version 3.1 (Applied Biosystery, USA) (Ammiraju *et al*., 2005)and ABIPRISM 3100 Genetic Analyzer (Applied Biosystery, USA). Sequences were edited and aligned using DNAstar where sequences were cloned for re-sequencing and further analysis.

**TA Cloning and sequencing:** TA cloning is a sub-cloning technique without any use of restriction enzymes. This was quicker than traditional sub-cloning. The ability of adenine (A) and thymine (T) (complementary base pairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together is what this technique relies upon. PCR products are usually amplified using Taq DNA polymerase which preferentially adds adenine to the 3' end of the product. The target PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, UK), and quantified by UV® spectrophotometry (NanoDrop, USA) (Mathimaran *et al.*, 2008). Purified DNA was cloned into pCR®4®TOPO® Vector and the TOP10®competent *Escherichia coli* system using TOPO® TA Cloning Kit for

Sequencing (Invitrogen, U.S.), then two different concentrations of the transformation product (20 TA and 50 an) were spread on the pre-warmed selective LB plate with Kanamycin. After culture overnight at 37°C, positive colonies were picked from each plate. Plasmid culture was in 10 ml selective LB (Luria Broth) broth (Miller, USA) with Kanamycin at 37°C in shaking culture overnight (Sambrook *et al*., 1989). Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, UK). Purified plasmid DNA was sequenced using M13 forward and M13 reverse primers (Figure 2.1) separately (Mathimaran *et al*., 2008), at the Genome Centre (University of Warwick, UK).

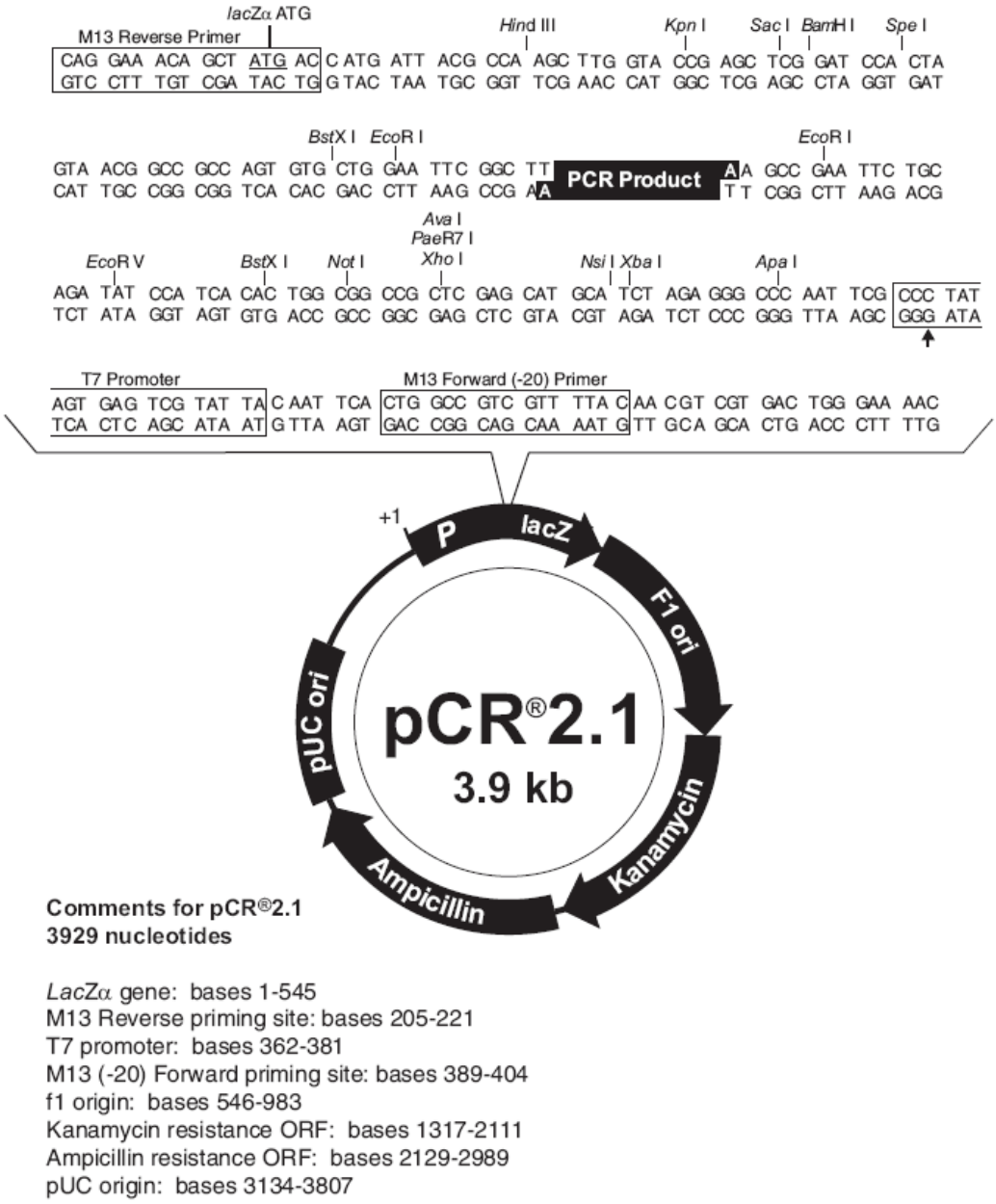



**Figure 2. 1** Map of pCR®2.1 (from the Invitrogen TA cloning Kit manual protocol) the arrow indicates the start of transcription for the T7 RNA polymerase.

### 2.2.3.4 Quantitative PCR

PCR products were quantified used a LightCycler® 480 Real-Time PCR thermocycler (Roche, Switzerland). The method was modified according to the LightCycler® Real-Time PCR Systems Application Manual and protocol. A calibration standard curve was required to enable use of this machine. The standard curve is used to quantify the relative Ct values of the samples. Ten-fold dilutions were prepared from neat BL801 including pure genomic DNA ($1.2 \times 10^6$ spore DNA). One-fold dilution ($1.2 \times 10^5$ spore DNA) labeled as Standard 1; a two-fold dilution ($1.2 \times 10^4$ spore DNA) from Standard 1 and labeled Standard 2; and further dilutions labeled Standard 3 ($1.2 \times 10^3$ spore DNA), Standard 4 ($1.2 \times 10^2$ spore DNA), Standard 5 ($1.2 \times 10^1$ spore DNA) and Standard 6 ($1.2 \times 10^0$ spore DNA).

**Sample preparation:** Samples were run in 10 µl reaction volume, which contained 5 µl Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, UK); 0.2 µl each of 10 µM concentrate forward and reverse primers (Invitrogen, UK); 0.75 µl DNA and 3.85 µl PCR grade water. The master mix was made up to 9.25 µl and was pipetted into the plate (LightCycler 480® Multiwell Plate 384, Roche), and then 0.75 µl DNA was pipetted into the same plate. The plate was sealed and the samples were mixed by centrifugating the plate in a 5810R Eppendorf Centrifuge (Hamburg, Germany) for 30 s in 1000 rpm (180 x g). The samples were run immediately avoid primer dimers.

**qPCR amplification:** Samples were run in three programme cycles. The first programme was pre-amplification, 95°C for 15 min (4.8°C/s Ramp rate), then processed to amplification: denatured at 94°C for 15 s (2.5°C/s Ramp rate) and

annealing temperature was 57°C for 30 s (2.5°C/s Ramp rate) then extension at 72°C for 30 s (4.8°C/s Ramp rate) single acquisition was detected during extension. The melting programme used was denaturing at 94°C for 15 s (2.5°C/s Ramp rate) and annealing temperature was 40°C for 15 s (2.5°C/s Ramp rate) then extended at 70°C for 15 s (4.8°C/s Ramp rate) and selected continuous for acquisition mode at 95°C (acquisitions per 25°C).

### 2.2.4 Microscopy observation

Cotyledons were cut from lettuce cultivar seedlings and dipped in 100% methanol (Fisher, Germany) overnight to de-colorize, after then they were cleared in chloral hydrate (1g $ml^{-1}$ distilled water) before mounted in 50% glycerol on to glass microscope slides, a cover slip was placed on top of the tissue to produce semi-permanent slide (Wood *et al*., 1988b). These slides were examined using a Carl Zeiss light microscope (Germany). Low magnification (10x) was used to count the spores on the tissue. Higher magnification was used to identify the structure and development of the disease.

# CHAPTER 3 *BREMIA LACTUCAE* PHENOTYPIC RACE IDENTIFICATION

## 3.1 INTRODUCTION

*Bremia lactucae* is a highly variable pathogen (Petrželova and Lebeda, 2004). Natural variation within the species was first classified in terms of physiological races (Crute and Dixon, 1981), and later virulence factors (v-factors), virulence phenotypes according to the interpretation of the host-pathogen interaction in a gene-for-gene relationship (Crute, 1987; Farrara *et al*., 1987). It provided a basis for population studies in this pathogen (Lebeda, 1982; Petrželova and Lebeda, 2004). Population genetics have been widely used in studies of pathogen variation occurring on lettuce. A total of 132 isolates of *B. lactucae* collected from infected and naturally growing plants of *Lactuca serriola* during 1998-2000 were used for v-factor analysis (Lebeda and Petrželova, 2003). Thirty-seven isolates were used for determination of v-factors on the basic differential set of *Lactuca* spp. Genotype (van Ettekoven and van der Arend, 1999) and 21 v-factors (v1to v18, v36 to v38) were identified.

### 3.1.1 IBEB identification

The International *Bremia* Evaluation Board is a joint initiative of lettuce breeding companies and research institutes. IBEB established a differential test set of lettuce cultivars to provide lettuce breeders and growers with a standard means for classifying pathogenic variation within *B. lactucae* (van der Arend *et al*., 2006). This differential host set can be used for comparable tests to assign isolates a unique value per isolate/race. The denomination and identification of *B. lactucae* isolates has been a joint effort with European lettuce breeders and authorities since 1998. IBEB has identified and named new races of *B. lactucae* (Bl:) since 1998 (van der Arend *et al*., 2006). A total of 12 new races have been identified and nominated from 1998 to 2011.

There are two sets of differential host varieties referred to as EU-A and EU-B. The interaction phenotypes have been assessed using the following classification system: - resistant, (-) resistant with necrosis that appears to limit sporulation, + susceptiable, (+) susceptible with necrosis, (m) moderate/mixed level of sporulation. In the EU-A set, nineteen differential varieties (excluding the Cobham Green control) consist of three groups of six varieties and one group of one variety. The sextet code of an isolate is the sum of the sextet values of the susceptible variety in each group in the table. For example, the first sextet code of Bl: 16 is 63 because all differentials are susceptible (the sum of the sextet value is 1+2+4+8+16+32=63).

EU-B set (Table 3.1b) was defined in 2010. Three varieties have been replaced from the EU-A set because of better seed quality and five varieties have been added for breeding procedure (http://hallogijs.110mb.com/content.html). Table 3.2 shows PIVT1309, LSE18 and LS102 have been redefined to NunDm15, CG Dm16; and NunDm17. Five varieties have been added to the fourth category of sextet including: RYZ910457, RYZ2164, Bedford, Balesta and Bellissimo. Bl: 1 to Bl: 16 are new names for NL1 -NL16. Bl: 3, 11 and 19 are not available or present in the population and therefore were removed from the list (van der Arend *et al*., 2006). Three new races have recently been described by Plantum NL: Bl: 26 (EU-A 63-31-58-01) (2008); Bl: 27 (EU-A l63/63/13/19) (2009) and Bl: 28 (EU-A l59/31/10/05) (2011). The EU-B set was published in 2010 therefore during this project phenotypic comparisons were based on EU-A set. Races are defined because they may be stable in the population.

**OBJECTIVE OF STUDY**

The objective of this study was to differentiate and identify the *B. lactucae* isolates and to investigate the v-factors of the field samples. These phenotypic variations between isolates can be used to compare with the variations in genotypic level for markers development to investigate the *B. lactucae* populations in field.

**Table 3. 1** (a) The EU-A set denomination of *B. lactucae* isolates commonly used in breeding before 2010 (van der Arend *et al*., 2006); (b) The IBEB EU-B set denomination of *B. lactucae* isolates, - resistance, (-) resistance with necrosis, + susceptible (+) susceptible with necrosis, (m) moderate/mixed

**(a)**

| Variety | Cobham Gr | Lednicky | UC DM2 | Dandie | R4T57D | Valmaine | Sabine | LSE 57/15 | UC DM10 | Capitan | Hilde II | Pennlake | UC DM14 | PIVT 1309 | LSE /18 | LS-102 | Colorado | Ninja | Discovery | Argeles | Sextet code | Source |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DM nr/R nr | 0 | 1 | 2 | 3 | 4 | 5/8 | 6 | 7 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 36 | 37 | 38 | | |
| Sextet nr | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | | |
| Sextet value | | 1 | 2 | 4 | 8 | 16 | 32 | 1 | 2 | 4 | 8 | 16 | 32 | 1 | 2 | 4 | 8 | 16 | 32 | 1 | | |
| NL races | | | | | | | | | | | | | | | | | | | | | | |
| Bl:1 =NL1 | + | + | + | - | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | EU-A 11-58-00-00 | |
| Bl:2 =NL2 | + | + | + | + | + | + | + | - | + | (-) | + | + | + | - | - | - | (-) | - | - | + | EU-A 63-58-00-01 | |
| Bl:4 =NL4 | + | + | + | - | + | + | (-) | + | + | - | + | + | + | - | (-) | - | (-) | - | - | - | EU-A 27-59-00-00 | |
| Bl:5 =NL5 | + | + | - | + | - | - | - | + | + | - | + | + | - | + | (-) | - | - | - | (m) | - | EU-A 05-27-01-00 | |
| Bl:6 =NL6 | + | + | + | - | + | + | (-) | - | + | + | + | + | + | - | (-) | - | - | - | - | - | EU-A 27-62-00-00 | |
| Bl:7 =NL7 | + | + | + | + | + | - | + | + | + | - | + | + | + | - | - | - | - | - | - | - | EU-A 47-59-00-00 | |
| Bl:10 =NL10 | + | + | + | + | + | + | + | + | + | (-) | + | + | (+) | (-) | - | - | - | - | - | - | EU-A 63-59-00-00 | |
| Bl:12 =NL12 | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | EU-A 57-63-03-00 | |
| Bl:13 =NL13 | + | + | - | + | - | + | (-) | + | + | + | + | + | + | - | - | - | - | - | - | - | EU-A 21-63-00-00 | |
| Bl:14 =NL14 | + | + | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | - | (-) | EU-A 63-62-00-00 | |
| Bl:15 =NL15 | + | + | + | + | + | + | - | + | + | + | + | + | (-) | - | - | - | - | - | - | - | EU-A 31-31-00-00 | |
| Bl:16 =NL16 | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | - | - | - | EU-A 63-31-02-00 | |
| IBEB | | | | | | | | | | | | | | | | | | | | | | |
| Bl:17 | + | - | + | (+ | - | + | - | + | + | - | + | + | + | + | - | - | + | - | (+) | - | EU-A 22-59-41-00 | S |
| Bl:18 | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | - | EU-A 59-31-10-00 | UK |
| Bl:20 | + | + | + | + | + | + | + | + | + | + | + | + | (m) | - | + | - | + | - | - | - | EU-A 63-31-10-00 | D |
| Bl:21 | + | + | + | + | + | + | + | + | + | + | + | + | (-) | + | + | - | - | + | + | - | EU-A 63-31-51-00 | IT |
| Bl:22 | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | - | EU-A 59-63-09-00 | NL |
| Bl:23 | + | + | + | + | + | + | + | + | + | + | + | + | (m) | - | + | (-) | - | - | - | + | EU-A 63-31-02-01 | F |
| Bl:24 | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | + | EU-A 59-31-10-01 | NL |
| Bl:25 | + | + | + | - | + | + | + | + | + | + | + | + | (-) | - | + | - | + | - | + | - | EU-A 59-31-42-00 | NL |

**(b)**

| Isolates / Differentials | GreenTowers | Lednicky | UC DM2 | Dandie | R4T57D | Valmaine | Sabine | LSE 57/15 | UC DM10 | Capitan | Hilde II | Pennlake | UC DM14 | NunDm15 | CGDm 16 | NunDm17 | Colorado | Ninja | Discovery | Argeles | RYZ 2164 | RYZ 910457 | Bedford | Balesta | Bellissimo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BI: 1 | + | + | + | - | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 2 | + | + | + | + | + | + | + | - | + | (-) | + | + | + | - | - | - | (-) | - | - | + | - | - | - | + | + |
| BI: 3 | + | - | - | - | + | + | + | + | + | - | + | + | (+) | + | - | - | - | - | + | - | - | (-) | (-) | - | + |
| BI: 4 | + | + | + | - | + | + | (-) | + | + | (-) | + | + | + | - | (-) | - | (-) | - | - | - | - | - | - | (-) | - |
| BI: 5 | + | + | - | + | - | - | - | + | + | - | + | + | - | + | - | (-) | - | - | - | - | - | - | - | - | - |
| BI: 6 | + | + | + | - | + | + | (-) | - | + | + | + | + | + | - | (-) | - | - | - | - | - | - | - | (-) | - | - | - |
| BI: 7 | + | + | + | + | + | - | + | + | + | - | + | + | + | (-) | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 10 | + | + | + | + | + | + | + | + | + | - | + | + | + | (-) | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 11 | + | + | - | - | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | | | | | | - |
| BI: 12 | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| BI: 13 | + | + | - | + | - | + | (-) | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 14 | + | + | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 15 | + | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| BI: 16 | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| BI: 17 | + | - | + | + | - | + | - | + | + | - | + | + | + | + | - | - | + | - | + | - | - | - | (+) | (-) | - | - |
| BI: 18 | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | - | - | - | - | - | - | - |
| BI: 19 | + | + | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | + | - | - | - | (-) | (-) | - | - |
| BI: 20 | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | - | - | - | - | - | - | (-) |
| BI: 21 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | - | - | + | + | - | - | - | (-) | (-) | - | - |
| BI: 22 | + | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | (-) | - | - | (-) | + | - | - |
| BI: 23 | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | - | - | + | - | - | - | - | - | - |
| BI: 24 | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | + | - | - | + | - | - | - | - | - | - |
| BI: 25 | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | + | - | + | (-) | - | - | - | - | - | - |
| BI: 26 | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | + | + | + | + | - | - | - | - | - | - |
| BI: 27 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | - | - | + | + | - | - | + | - | - |

**Table 3. 2** The difference between EU-A set and EU-B set.

| Accession number of the variety* | R-gene | EU-A Varieties | EU-B Varieties |
|---|---|---|---|
| 0 | NONE | Cobham Green | Green Towers |
| 13 | Dm15 | PIVT 1309 | NunDm15 |
| 14 | Dm16 | LSE/18 | CG Dm16 |
| 15 | Dm17 | LS-102 | NunDm17 |
| 20 | monogenic | NONE | RYZ2164 |
| 21 | Murai | NONE | RYZ910457 |
| 22 | monogenic | NONE | Bedford |
| 23 | monogenic | NONE | Balesta |
| 24 | monogenic | NONE | Bellissimo |

*: In the EU-B set, the accession numbers 13 and 15 are replacements, and accession numbers 20 to 24 are additions.

## 3.2 MATERIAL AND METHODS

### 3.2.1 Single spore isolation

Single spore isolation was based on the protocol described by Choi *et al*. (1999). It has been used to culture single spore isolates from the mixed spore samples to ensure of the purity of the genotype. Isolates BL801, BL802, BL803 and BL804 (Table 2.1) are single spore isolates.

**Making the glass needle:** This procedure requires a glass needle to pick up the spore. A sterilised Pasteur pipette was heated in the flame of an alcohol-burner. The end of the glass pipette was melted to form a needle shape (Goh, 1999).

**Isolation of single spores:** The glass needle was assembled on to a syringe attached to a height adjustable platform. 5 ml distilled water was poured on the agar plate before the isolation. A cotyledon with sporulation was dipped gently on top of the water agar (10%) plate to allow the spores to be released in the water. The plate was

placed under the microscope, moved until a spore was in view, then the needle was slowly moved near the spore. As soon as the needle dipped in the water, the capillary flow was used to pull the spore into the needle. The spore was then ejected on to a cotyledon of cv. Cobham Green seedling. The inoculated seedling was placed in a covered sandwich box, and was incubated in a controlled environment room set at 17°C with a 12 hour photoperiod.

### 3.2.2 Mixed spore isolates collection

Thirty-six lettuce leaf samples were collected from Lincolnshire (J.E. Piccaver & Co., UK) in August 2009 (Table 3.3), and another 25 samples were collected from both Lincolnshire (J.E. Piccaver & Co., UK) and the south coast (Langmead Farms Ltd., Chichester, UK) in May 2010 (Table 3.4). Some of the leaves already had sporulation in the field. After the collection, isolates were cultured in humid conditions (17°C, 12 hours photoperiod).

**Table 3. 3** Isolates from Lincolnshire (J.E. Piccaver & Co., UK) in 2009.

| Lettuce cultivar (host) | Number of samples collected |
|---|---|
| Escade excel | 3 |
| UK COS-255 | 3 |
| UK MUL 1-35 | 3 |
| UK MUK 1-25 | 3 |
| UK MUL1-85 | 3 |
| MIRSAL | 3 |
| CNEN4 | 3 |
| CAUGIN | 4 |
| ESCAROLE NNANCE | 3 |
| 02-354975 | 3 |
| L.ROSSA BASTILL | 3 |
| ICEBERG DIAMOND | 1* |
| L/BIOND/LIVIGNOS | 1* |

* Denotes whole lettuce.

**Table 3. 4** Isolates from Lincolnshire (J.E. Piccaver & Co., UK) and Chichester (Langmead Farms Ltd., UK) in 2010.

| Lettuce cultivars (host) | Number of sample collected | Location |
|---|---|---|
| Iceberg | 16 | Lincolnshire |
| MIR | 1 | South Coast |
| 7RMUS | 5 | South Coast |
| 16LGATT | 3 | South Coast |

The successfully cultured isolations from field samples were inoculated on to Lettuce cv. Cobham Green seedlings for culture maintenance. After inoculation, the infected plants were moved to a PEL (Plant Environment Laboratory) room (18ºC). Sporulation was observed on days 4 and 5 post inoculation, and after 7 days the final density of *B. lactucae* spores per unit of cotyledon surface was sufficient for DNA extraction and re-inoculation.

### 3.2.3 Phenotypic identification of *B. lactucae* races

Race specific resistance of lettuce to *B. lactucae* has been studied for many years, based on the gene-for-gene theory. Tests were carried out using seedlings of an EU-A set of twenty lettuce cultivars (seeds were supplied by Naktuinbouw, Ltd., the Netherlands). The reactions of collected isolates on the culitivars (Table 3.5) can be used to identify the *B. lactucae* races according to the IBEB EU-A identification system (Figure 3.1). Twenty lettuce cultivar seedlings were grown in a box with divisions for *B. lactucae* race identification as shown in Figure 3.1.

**Table 3. 5** Information for *B. lactucae* race identification

| Accession number of the variety | Variety | The arrangement in Figure 3.1 |
|---|---|---|
| 0 | Cobham Green | 1A |
| 1 | Lednicky | 2A |
| 2 | UC DM2 | 3A |
| 3 | Dandie | 4A |
| 4 | R4T57D | 5A |
| 5 | Valmaine | 6A |
| 6 | Sabine | 1B |
| 7 | LSE57/15 | 2B |
| 8 | UC DM10 | 3B |
| 9 | Capitan | 4B |
| 10 | Hilde II | 5B |
| 11 | Pennlake | 6B |
| 12 | UC DM14 | 1C |
| 13 | PIVT1309 | 2C |
| 14 | CGDM-16 | 3C |
| 15 | LS102 | 4C |
| 16 | Colorado | 5C |
| 17 | Ninja | 6C |
| 18 | Discovery | 1D |
| 19 | Argeles | 2D |


**Figure 3. 1** The lettuce seedlings of EU-A differential test set for *B. lactucae* races identification. The seeds of the lettuce varieties were sown following the arrangement in Table 2.4 above (from A1=Cobham Green to D2=Argeles).

The differential lettuce cultivars test sets were infected by spreading the spores on the surfaces of fresh cotyledons of the seedling. Seedlings were cultured in a P.E.L. room (17°C, 12 hours photoperiod) for 7 days. Seedling infection was scored as + if sporulation was present or - if there was no sporulation following the IBEB system.

**Scoring systems**

The 0-3 scale scoring protocol was described by Dickinson and Crute (1974). In that protocol 0 is no sporulation on seedlings; 1 is limited sporulation; 2 is < 50 % cotyledon area covered with sporophores and 3 is > 50 % cotyledon area covered with sporophores. In my study, as to obtain more detail of the sporulation, a scoring protocol over 6 grades from 0 to 5 was applied according to the protocol of Dickinson and Crute (1974). A score of 0 was recorded if no sporulation observed; 1 if sporulation occurred on less than 20% of the surface; 2 if 20% of the surface

showed sporulation; 3 if 50% of the surface showed the sporulation; 4 if 70% of the surface showed sporulation; and 5 if 100% of the surface showed sporulation.

## 3.3 RESULTS

After the inoculation of *B. lactucae* on lettuce seedlings, the spores were ready for harvest on day 7 post inoculation (dpi). Figure 3.2 shows the sporulation of *B. lactucae on* cv. Cobham Green cotyledons (7 dpi).

**Figure 3. 2** *B. lactucae* sporulated on a cv. Cobham Green cotyledon.

### 3.3.1 Results of single spore isolation

Single-spore isolations of BL801, BL806, BL805 and NL5 from the existing collection were successful. Another two isolates (S1and S2) were single-spore isolated from field samples Bl815 (Caugin 4, 2009 field sample, J.E. Piccaver & Co., UK).

### 3.3.2 Results of characterization of isolates using differential sets

The existing isolates have been identified by the IBEB differential set. Table 3.6 shows that these exisiting isolates might belong to different races as each of them has been classified as a different sextet code. The data of Bl804 was missing as it was difficult to culture.

**Table 3. 6** Classification of the previously collected *B. lactucae* isolates

| Isolates | Sextet Code | Possible IBEB Race |
|---|---|---|
| BL801 | EU-A 63-31-02-00 | Bl:16 |
| BL802 | EU-A 59-31-10-00 | Bl:18 |
| BL803 | EU-A 59-63-09-00 | Bl:22 |
| BL804 | Missing data | Missing data |
| BL805 | EU-A 63-63-01-01 | Bl:2 or BL:10 |
| BL806 | EU-A 55-62-00-01 | Bl:10/12/15 |
| BL807 | EU-A 63-63-23-00 | Bl:21 |

Fourteen new *B. lactucae* isolates were collected from Lincolnshire (J.E. Piccaver & Co., UK) in 2009 (Table 3.7), and were tested using the IBEB differential set. Each isolate has been classified as a different sextet code (Table 3.7), but it could not be assumed that they are all different isolates, as they were possible mixed spore samples. S1 and S2 were single spore isolated from BL815 (Caugin 4), the sextet codes show they are possibly two different isolates. It is interesting to note that most of the isolates collected here sporulated on cultivar *Argeles* while only few of the known IBEB isolates sporulated on this cultivar.

**Table 3. 7** Classification of new *B. lactucae* isolates collected in 2009 from lettuce fields in Lincolnshire

| Isolates | Host Cultivars | Sextet Code | Possible IBEB Race |
|---|---|---|---|
| BL808 | Iceberg Diamond 1 | EU-A 59-31-43-01 | Bl:25, Bl:2 |
| BL809 | Iceberg Diamond 2 | EU-A 59-63-47-01 | Bl:25, Bl:2 |
| BL810 | L biond ligind | EU-A 57-63-08-00 | Bl:12, B:l22 |
| BL812 | L Rossa Bastille | EU-A 59-63-43-01 | none |
| BL813 | Caugin 1 | EU-A 59-59-59-01 | none |
| BL814 | Caugin 2 | EU-A 63-35-02-01 | Bl:23 |
| BL815 | Caugin 4 | EU-A 59-63-12-01 | Bl:22 |
| BL816 | UK COS 225 1 | EU-A 19-63-03-00 | Bl:12 |
| BL817 | UK COS 255 2 | EU-A 11-60-00-00 | Bl12, Bl:1 |
| BL818 | UK MUL1-15 3 | EU-A 51-51-03-00 | Bl:12 |
| BL819 | UK Mul-25 | EU-A 19-56-00-01 | Bl:2 |
| BL820 | UK Mul 1-95 | EU-A 32-45-00-00 | Bl:7 |
| BL821 | 025 35 4979 (2) | EU-A 59-31-43-01 | Bl:21 |
| BL822 | 025 35 4975 (3) | EU-A 59-63-11-01 | Bl:24 |
| S1 | Caugin 4 | EU-A 59-63-14-01 | none |
| S2 | Caugin 4 | EU-A 57-63-14-01 | none |

Twenty of the twenty-five samples had been cultured after sampling from Lincolnshire and the south coast (Chichester, UK) in 2010 (Table 3.8). The cultured isolates on the IBEB differential set and the results of the race identification are shown in Table 3.9. According to the results of the IBEB sextet code, the fourteen field samples from Lincolnshire were characterized in seven groups. Nine South Coast (Chichester, UK) field samples were characterized in eight groups. The field samples from Lincolnshire were found in one lettuce cultivar (Iceberg diamond), while the samples from the South Coast (Chichester, UK) were found in different varieties. This may explain why more samples from Lincolnshire belong to the same race group and South Coast samples were more variable. The sextet code of L11 was the same as Bl:18, and it is possible that L11 is Bl:18 if it is not a mixed spore sample.

**Table 3. 8** Information on the Lincolnshire and South coast (Chichester, UK) field samples collected in 2010.

| Host cultivar | Number of samples collected | Location | Number of isolates |
|---|---|---|---|
| Iceberg | 16 | Lincolnshire | 14 |
| MIR | 1 | South Coast | 1 |
| 7RMUS | 5 | South Coast | 3 |
| 16LGATT | 3 | South Coast | 2 |

**Table 3. 9** Results for 2010 Lincolnshire and South Coast (Chichester, UK) field samples identification

| Isolates | Original isolate Code | IBEB Sextet Code |
|---|---|---|
| L8 | LI0/LI1 | EU-A 27-63-10-01 |
| L9 | LI3/LI9/LI12/LI12a | EU-A 59-63-10-00 |
| L10 | LI4/LI10 | EU-A 59-63-11-00 |
| L11 | LI5/LI11 | EU-A 59-31-10-00 |
| L12 | LI7/LI16b | EU-A 59-63-11-01 |
| L13 | LI8 | EU-A 59-63-02-01 |
| L14 | LI16a | EU-A 27-63-02-01 |
| SC1 | 7RMUS/7RMUS9 | EU-A 27-27-10-01 |
| SC2 | 7RMUS1 | EU-A 59-63-40-01 |
| SC3 | 7RMUS2 | EU-A 57-31-42-01 |
| SC4 | 7RMUS2 | EU-A 59-63-36-01 |
| SC5 | 16LGATT1 | EU-A 27-27-10-01 |
| SC6 | 16LGATT(3) | EU-A 59-31-08-01 |
| SC7 | 16LGATT3 | EU-A 27-31-10-01 |
| SC8 | MIR1 | EU-A 27-23-10-01 |

### 3.3.3 The investigation of v- factor frequency in Lincolnshire

The results (Figure 3.3) show that the frequency of v1, v2, v4, v5/8, v7, v10, v11, v12, v13, v14, v16, v18, was increasing while v6, v15 and v38 decreased. The v-factors v17, v36 and v37 were absent in 2010 and v3 was not present in these two years. More v-factors present in 2009 and higher v-factor frequency occurred in 2010.

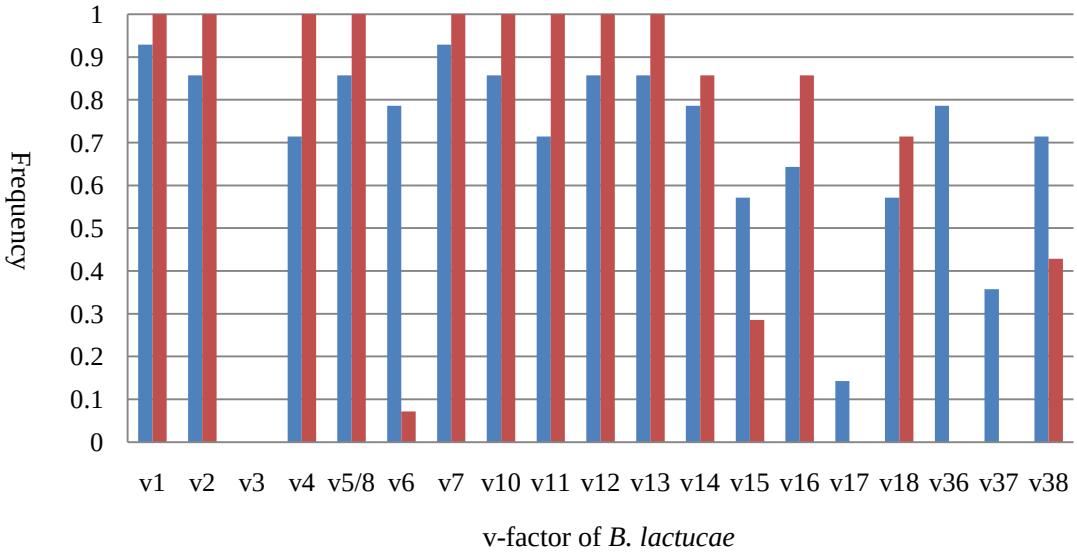



**Figure 3. 3** The frequencies of v-factors of *B. lactucae* isolates (field samples) collected from Lincolnshire in 2009 and 2010. Blue bar: 2009, red bar: 2010.

## 3.4 DISCUSSION

Field-collected and existing *B. lactucae* isolates have been identified using the IBEB system. The virulence factors of the field isolates from Lincolnshire have also been investigated by observing the results of the interaction between virulence genes in the samples and the *Dm* gene in the IBEB lettuces varieties. During the last decade, over 2000 new isolates have been found around Europe. In California isolates have been assigned up to Pathotype VIII (pathotype assignment, by University of California, Davis) (Michelmore and Wong, 2008). However, until 2011 only 28 isolates have been nominated by IBEB as Bl: 1 to Bl: 28 which is due to the decision of IBEB board based on whether the new isolates are important enough to be added to the existing Bl-set of races (Plantum NL, 2011). These new isolates were found in many different production areas and it is predicted that these new isolates will spread over Europe (Plantum NL, 2011). However, assignment of IBEB nomination is for a pragmatic utility rather than comprehensive categorization of isolates (Michelmore *et al*., 2009) and might miss important variation.

The results show some of the sextet codes of current isolate collections have the same or similar sextet code as the IBEB denominated isolates, which indicates the possibility that the collection contains the existing European Bl-set races and some of the collected isolates are new races. The IBEB sextet code results will be used to compare with the molecular marker differential results which will be described in the following chapters.

The results of v-factor frequency comparison between 2009 and 2010 might only show part of the variations of *B. lactucae* virulence in fields in Lincolnshire as the collection were not from exactly the same lines of lettuce varieties in these two years. But as an investigation it might be useful to keep records for the presence and absent of v-factors, as it is very possible that these field collections are heterogeneous which means an isolate is a mixture of races. Therefore single spore races in this sample could not be identified by the given IBEB sextet code. Therefore single spore isolation is necessary in future work. Furthermore, the identifications were based on the EU-A system, while the new EU-B system has been introduced recently, therefore it would be helpful to re-identify and re-code the collections using the EU-B set in future work.

The results of IBEB characterization of field samples show that there are 29 different isolates in the collection. However these field samples had not been single spore isolated so it is very possible that these samples are mixed spore isolates. This means they are likely to be virulent on more cultivars than the single spore line by which the IBEB set was defined (Michelmore and Crute, 1982). The problem is that the IBEB differential set is designed for samples that are single spore isolates and shows the interaction of avirulence and resistance as the presence or absence of symptom. It cannot identify how many isolates are in the sample using the IBEB differential set. If the field sample is comprised of three different isolates, for example isolates BL801, BL806 and NL5 as Figure 3.4 shows below, then the IBEB sextet code for NL5 is EU-A 05-27-01-00 (v-factors:1, 3, 7, 10, 12, 13 and 15) BL801 is EU-A 63-31-02-00 (v-factors: 1, 2, 3, 4, 5/8, 6, 7, 10, 11, 12, 13, and 16) and BL806 is EU-A 55-62-00-01 (v-factors: 1, 2, 3, 5/8, 6, 10, 11, 12, 13 and 19). But the result of

IBEB classification of this field sample would be EU-A 63-63-06-01 which comprised the v-factors of BL801 BL806 and NL5. This result could neither tell the actual race within the sample nor which race is dominant.

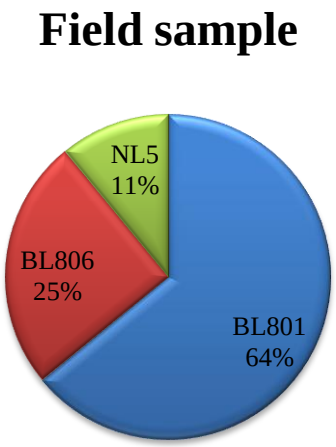



**Figure 3. 4** An example of a field collected sample that consists of three races.

The results of IBEB differentiation only show the + for compatible and - for incompatible but ignore the degree of compatibility/incompatibility. For two different races, the cultivars would show a higher degree of compatibility for the more aggressive one, but as + and - IBEB scoring, they might share the same IBEB sextet code and be considered as one race. Although there are twenty four different *Dm* genes in IBEB EU-B set, two isolates sharing one sextet code might be differentiated if more lettuce varieties carrying different *Dm* genes are introduced in the differential set. Therefore more varieties used in the differential set would make the results more accurate. However that would require more time and labour to use.

Genotypic identification using molecular markers is a promising method to identify the races. Some researchers found that avirulance to specific *Dm* genes were inherited as single dominant unlinked Loci (Norwood *et al.*, 1983; Norwood and

Crute, 1984; Michelmore *et al*., 1984; Ilott *et al.*, 1987). Avirulence is determined by dominant alleles at the unlinked loci and might be modified depending on the genetics of the host and the pathogen (Michelmore and Wong, 2008). As the avirulence would vary among races, the avirulence related marker could be developed to detect the differences and identify the *B. lactucae* races.

# CHAPTER 4 MOLECULAR MARKER DEVELOPMENT FOR IDENTIFYING GENOTYPIC VARIANCE OF *BREMIA LACTUCAE*

## 4.1 INTRODUCTION

Molecular markers have been applied in the study of pathogen populations. The variability of the pathogen could be evaluated by using molecular marker to detect the specific avirulance (*Avr*) gene. Different types of markers have been tested in this project to determine whether they will be suitable for population studies in *Bremia lactucae.*

Host-pathogen genetic studies have defined more than forty-five race-specific resistance genes in lettuce (such as *Dm1*, *Dm2*, *Dm3*, *Dm4*, *Dm5/8*, *Dm6*, *Dm7*, *Dm11*, *R12*, *Dm13*, *Dm16 and R18*) (Lebeda *et al.*, 2006). Only some of these genes (*Dm3*, *Dm7*, *Dm11*, *Dm16 and R18*) are important in resistance management in the host crop (Lebeda and Zinkernagel, 2003). The presence or absence of these genes gives rise to variability in the pathogen. Molecular markers could be developed which could detect the molecular basis of this variability.

Different types of molecular markers have been used in this PhD project, including Simple Sequence Repeats (SSR) markers, Inter-Simple Sequence Repeat (ISSR) markers, ribosomal Internal Transcribed Spacer (ITS) markers and effector markers (Chapter 5).

### 4.1.1 Microsatellites Markers

Microsatellites are considered as highly polymorphic DNA markers with discrete loci and co-dominant alleles (Schlotterer, 1998), and the hybridization method has been successfully used to construct libraries and determine flanking sequences of microsatellite DNA loci in different organisms including amphibians, birds, fish,

mammals, reptiles, insects, fungi and plants (Glen and Schable, 2005).

The Microsatellite loci from *Plasmopara viticola* had been isolated and these SSR markers (Pv14, Pv16 and Pv39) can amplify sequences of three closely related plant-pathogens oomycete species (*Plasmopara halstedii, Bremia lactucae* and *Phytophthora infestans*) (Delmotte *et al*., 2006). Therefore these three Pv makers were used in this project to look for the microsatellite loci in *B. lactucae*. Many different strategies for obtaining microsatellite DNA loci have been described. Cloning small genomic fragments and using radiolabeled oligonucleotide probes of microsatellite repeats to identify clones with microsatellites, was first described and works well in organisms with abundant microsatellite loci (Weber and May, 1989). It does not work well when microsatellite repeats are less abundant. Therefore another two enrichment strategies have been developed: (1) uracil-DNA selection (Ostrander *et al*., 1992) and (2) hybridization capture (Armour *et al*., 1994; Kandpal *et al*., 1994; Kijas *et al*., 1994). Hybridization capture allows selection prior to cloning, and therefore is faster and easier to achieve with multiple samples than uracil-DNA selection. In this project, SSR markers were being developed by modifying the method from Glenn and Schable (2005).

ISSR-PCR has been demonstrated to provide highly reproducible results and generate abundant polymorphism in a number of host pathogen interactions (Ajibade *et al*., 2000). *B. lactucae* ISSR marker development showed that ISSR markers could be used to differentiate the races of *B. lactucae* (Wagner and Idczak, 2004). In this PhD project, the ISSR markers were used to look for the variation between *B. lactucae* races.

### 4.1.2 Ribosomal Internal Transcribed Spacer (ITS) markers

The specific *B. lactuca*e primers designed by Choi *et al*. (2007) from ITS2 region had been used in this project. Br 1234F and Br 1586R had been chosen to design specific primers which can amplify smaller product for qPCR quantification studies.

Although eight *B. lactucae* isolates were collected from various host plants, the ITS2 regions of all sequences showed similar sizes of 2450–2461 bp and were constantly composed of nine repeats. This indicates that the long length and the repetitions of ITS region may be a very common event in *B. lactucae* ITS2 evolution (Choi *et al.*, 2007). These repetitions are a major factor in the huge size and sequence variability of ITS among and within species. Repetitive elements themselves are subject to high rates of evolutionary change, as indicated by the observation that copies of the repeat units show considerable sequence variation between both repeats and isolates.

**Table 4. 1** Information about ITS primers.

| **Primer name** | **Sequence (5'->3')** | **Reference** |
|---|---|---|
| ITS1 | TCCGTAGGTGAACCTGCGG | White *et al.*, 1990 |
| ITS2 | GCTGCGTTCTTCATCGATGC | White *et al.*, 1990 |
| ITS3 | GCATCGATGAAGAACGCAGC | White *et al.*, 1990 |
| ITS4 | TCCTCCGCTTATTGATATGC | White *et al.*, 1990 |
| ITS5 | GGAAGTAAAAGTCGTAACAAGG | White *et al.*, 1990 |
| ITS1-F | CTTGGTCATTTAGAGGAAGTAA | Gardes & Bruns, 1993 |
| ITS4-B | CAGGAGACTTGTACACGGTCCAG | Gardes & Bruns, 1993 |
| 5.8S | CGCTGCGTTCTTCATCG | Vilgalys lab |
| 5.8SR | TCGATGAAGAACGCAGCG | Vilgalys lab |
| SR6R | AAGWAAAAGTCGTAACAAGG | Vilgalys lab |

Information was obtained from the website of Vilgalys lab (Duck University, USA) (http://www.biology.duke.edu/fungi/mycolab/primers.htm).

The aim of using ITS primers (Table 4.1) was to develop a molecular marker that is not specifically related to pathogenicity of the organism, but instead detects the overall variation of the pathogen *B. lactucae*.

**OBJECTIVE OF STUDY**

The aim of this study was to develop race specific markers that can identify the difference between isolates. ITS, ISSR and Pv primers were based on previously published work. *B. lactucae* race specific SSR markers development was undertaken using a method modified after Glenn and Schable's protocol (2005).

## 4.2 MATERIALS AND METHODS

Published Microsatellite markers and ITS markers were used to identify variation of in the *B. lactucae* isolates and new SSR markers were attempted to be developed in this project. Genomic DNA of *B. lactucae* isolate Cp82p24 (supplied by Michelmore, UC Davis, USA) was used for new SSR markers development.

### 4.2.1 Use of published SSR primers

Variable microsatellite loci have been successfully amplified in *Plasmopara viticola* using Pv14, Pv16 and Pv39 (Delmotte *et al*., 2006). According to Delmotte, these primers will also amplify DNA from *B. lactucae*. The sequences of these primers are:

Pv14 Forward: CAGAAACGCACAAGGTCTGA
Reverse: AATTGCATACTGCAGCAACG
Pv16 Forward: TAAAAATATGGTGGCGTCAG
Reverse: CCAGCAGTCTCCGTCTCATCAG
Pv39 Forward: ACGCATGGCGAACACGTAAG
Reverse: CAGACGGGAAGAAGTTGCTC

**PCR amplification:** PCR was carried out in 25 µl of reaction containing 2.5 µl (10x) buffer, 0.75 µl 25 mM $Mgcl_2$, 0.13 µl 25 mM dNTPs, 0.2 U Taq polymerase (Invitrogen, Paisley, UK). All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). After an initial denaturation (94°C for 2 min) 35 cycles were performed, each cycle consisted of 95°C for 30 s

and an annealing step (the appropriate annealing temperature for the primer pair) for 30 s and 72°C for 30 s followed by an extension step at 72°C for 10 min.

**Gel analysis and sequencing:** According to th study of Delmotte *et al*., (2006) the successful amplification of these three primers gave products of around 200 base pairs therefore the potential microsatellite region may be around 200 bp. Amplified DNA was loaded on an appropriately chosen 8.7 cm Spreadex gel selected on the basis of product sizes and resolution of the band required (Mathimaran *et al*., 2008). EL 400 was used and the running time for gels was calculated using the El-QuantTM software (all Elchrom Scientific AG, Switzerland). Bands around 200 bp were cut by BandPickTM (Elchrom Scientific AG, Switzerland) and transferred to a PCR tube before re-amplification for 20 cycles using the same conditions and primer as in the first PCR amplification (Mathimaran *et al*., 2008). The re-amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, UK), and quantified by UV® spectrophotometry (NanoDrop, USA) (Mathimaran *et al*., 2008). Purified DNA was cloned into pCR®4®TOPO® Vector and the TOP10®competent *Escherichia coli* system using TOPO® TA Cloning Kit for Sequencing (Invitrogen, UK) then spread at two different concentrations of the transformation product (20 µl and 50 µl) on two pre-warmed selective LB plates with Kanamycin. After culture overnight at 37°C, positive colonies were picked from each plate. Plasmid cultures were in 10 ml of selective LB broth with Kanamycin at 37°C in a shaking culture overnight. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, UK). Purified plasmid DNA was sequenced using M13 forward and M13 reverse primers separately (Mathimaran *et al*., 2008), at the Genomic Centre (University of Warwick, UK).

### 4.2.2 Development of SSR-based markers

The method was modified from Glenn and Schable's protocol (2005), currently there are five steps in the process which include *Rsa* I digestion, SuperSNX adaptor ligation, *Xmn* I digestion, hybridization (dynabead enrichment) and PCR recovery. There were five steps for the development from (a) to (e), and two control processes (f) and (g).

**(a) *RsaI* digestion**

In Glenn and Schable's protocol (2005) DNA was digested with *Rsa* I and *Xmn* I together, but in the following method these two digest enzymes worked separately in two different steps. The 10 µl digest reaction contained 1 µl NEB 10x Ligase Buffer, 1 µl Water, 1µl *Rsa* I (Invitrogen, UK) 7 µl DNA (60 ng $\mu l^{-1}$). Better quality and larger amounts of DNA gave better results. The solution was mixed by pipetting up and down. The mixture was incubated at 37°C for at least 2 hours or overnight. Then the digestion mixture was inactivated at 65°C for 15 min.

**(b) SuperSNX adaptor ligation**

SuperSNX24 Linker is an adapter that can be linked to digested DNA fragments. The phosphate (p) on SuperSNX24+4P Reverse primer allows ligation of the linkers to each other or the digested DNA.

SuperSNX24 Forward: 5'GTTTAAGGCCTAGCTAGCAGAATC
SuperSNX24+4P Reverse: 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA

Equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24+4p primers (100 µl of 10 µM each) were used. Salt was added to a final concentration of 100 mM (4 µL of 5M NaCl for 200 µl of primers). The mixture was heated to 95°C

in a heating block, before cooling slowly to room temperature to form the ds SuperSNX linkers. The 20 µl ligation reaction contained 10 µl digested DNA (from step a), 4.0 µl 5x Ligation Buffer, 1.0 µl T4 DNA ligase (Invitrogen), 3.0 µl ds SuperSNX linkers and 2 µl water. The mixture was incubated at 16°C overnight. To ensure ligation was successful, a PCR was performed on the linker ligation using 25 µl reaction volume which contained 2.5 µl 10x PCR buffer, 2.5 µl SuperSNX-240, 27 µl dNTP's, 0.75 µl $MgCl_2$ (25 mM -> 2.0 mM final), 0.2 µl *Taq* DNA Polymerase (5 units $\mu l^{-1}$), 2.5 µl Linker ligated DNA fragments. After an initial denaturation (95°C for 3 min) 20 cycles were performed, each cycle consisted of 95°C for 30 s, annealing at 60°C for 20 s then at 72°C for 1.5 min followed by a extension step at 72°C for 10 min. 4µl PCR product was run on a 1.0% agarose gel to observe if the reaction was successful. A successful reaction should yield a smear (approximately from 300 to 1000 bp). DNA in successful reactions was cleaned up using Qiagen PCR purification kit (Qiagen, UK).

**(c) *Xmn*I digestion**

Successfully ligated DNA was digested by *Xmn* I. 20 µl reaction volume contained 0.2 µl BSA, 2 µl NEB 10x Buffer 4, 6.8 µl water, 1 µl *Xmn* I (New England Biolab, UK), 10 µl eluted DNA (60 ng $\mu l^{-1}$) from step b.

The volume of each of the components above was multiplied by the number of DNA samples to be digested to make the master mixture. All samples were incubated at 37°C for at least 2 hours or overnight. The digestion mixture was inactivated at 65°C for 20 min. A PCR was carried in 25 µl reaction volume containing: 2.5 µl 10x PCR buffer, 5µl SuperSNX-24 ( only single primer), 0.27 µl dNTP's, 0.75 µl $MgCl_2$ (25

mM -> 2.0 mM final), 0.2 µl *Taq* DNA Polymerase (5 units $\mu l^{-1}$), 5 µl DNA fragments. After an initial denaturation (95°C for 3 min) 20 cycles were performed, each cycle consisted of 95°C for 30 s with an annealing step of 60°C for 20 s then 72°C for 1.5 min followed by a extension step at 72°C for 10 min. 4 µl of PCR product was run on a 1.0% agarose gel to check the reaction. DNA was cleaned up using a Qiagen PCR purification kit (Qiagen, UK).

**(d) Dynabead enrichment for microsatellite-containing DNA fragment**

The following procedure can be used to capture DNA fragments containing microsatellite sequences complementary to the microsatellite oligos (probes) and will wash away all other DNA fragments. Before the enrichment, 2x Hyb (hybridization) Solution (12x SSC from 20x Saline Sodium Citrate concentrate), 0.2% SDS (sodium dodecyl sulfate) and 1x Hyb Solution (6x SSC, 0.1% SDS) were produced. The biotinylated oligos (probe) mixture comprised of individual oligos all at the same concentration, and equal mixing volumes,

Mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$;

The 50 µl reaction volume contained 30 µl 2x Hyb Solution (warmed to get everything into solution), 20 µl Biotinylated microsatellite probe (mix of oligos at 2 µM total) and 10 µl Linker ligated DNA.

The thermal cycler program which denatures the DNA and the probe mixture were at 95°C for 5 minutes. Then the temperature cooled to 70°C for one minute and steps down 1°C every 1 min (*i.e.* 70°C for 1 min, 69°C for 1 min, continuing until the temperature reaches 50°C), before remaining constant at 50°C overnight. While the DNA and probe mixture was in the thermal cycler, 50µl Dynabeads (Invitrogen, UK)

were washed twice with TE (Tris EDTA buffer) and twice in 1XHyb buffer. The beads were re-suspended in 150 µl 1XHyb buffer. Beads were captured using the Magnetic Particle Concentrator (MPC) (Invitrogen, UK). The DNA and probe mixture was mixed with washed Dynabeads before incubation on an orbital shaker on slow speed at 45°C for 60 min. Beads were re-captured using the MPC. Dynabeads with DNA and probe mixture were washed twice by resuspending with 400 µl 1x Hyb buffer for 15 min at room temperature, then it was resuspended twice at 45°C with 400 µl 1x Hyb buffer for 15 min before re-suspension twice using 400 µl 6x SSC (saline-sodium citrate) for 15 min. It was resuspended twice with 400 µl 4xSSC for 10 min at room temperature. Each time the mixture was captured by MPC and supernatant was removed by pipetting. DNA was eluted with 50 µl elution buffer (Qiagen Dneasy Kit, UK) and incubated at 95°C in a heating block for 5 minutes. Beads were captured by MPC and the supernatant was transferred to a new tube

**(e) PCR recovery of enriched DNA**

A PCR was performed on the supernatant in 50 µl reaction, which contained 5 µl 10x PCR buffer, 1 µl dNTP's 5 µl, SuperSNX-24, 1.5 µl $MgCl_2$, 27 µl $dH_2O$, 0.5 µl *Taq* DNA Polymerase (5 units $\mu l^{-1}$). After initial denaturation (95°C for 3 min) 35 cycles were performed, each cycle consisted of 95°C for 30 s and annealing at 60°C for 20 s, then 72°C for 1.5 min followed by an extension step at 72°C for 10 min.

**(f) *Arabidopsis* DNA control and optimization of the DNA enrichment**

500 ng *Arabidopsis* DNA supplied by Dr. Volkan Cevik (University of Warwick, UK), was used as a control in the SSR marker development procedure following the protocol for *B. lactucae* DNA SSR marker development (as above).

**(g) Other PCR mixture control**

Without any digestion ligation and pre-amplification, the PCR mixture can be hybridized immediately. The mixture contained 30 μl 2 x hybridization buffer, 20 μl 2μM biotin primers mixture and 1 μl PCR mixture which were mixed and hybridized under 'touch down hybrid cycle' before storage at 50°C overnight. Dynabeads were used to capture the Biotinated DNA and the beads were washed and DNA eluted as in Step d (above). The eluted DNA was amplified with 6 different primers (supplied by Dr. Volkan Cevik, University of Warwick, UK) separately:

Primer 17 Nga361- GA
Primer 18 Nga32 – GA
Primer 12 Nga298 AG/TC
Primer 15 Pcs7 TA/GA
Primer 9 MHJ24 ATT
Primer 22 A+hGAPAb TTC

PCR was carried out in 25 μl of reaction containing 1 μl DNA, 2.5 μl primer, 2.5 μl 10 x buffer, 0.75 μl 25mM $Mgcl_2$, 0.5 μl 25 mM dNTPs, 0.25U Taq polymerase (all Invitrogen, Paisley, UK). All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). After initial denaturation (95°C for 3 min) 35 cycles were performed, each cycle consisted of 95°C for 30 s and an annealing step (60°C for 30 s) and 72°C for 1.5 min followed by an extension step at 72°C for 10 min. The amplified hybridization samples were run on a 1% Agarose Gel under 100 voltages for 2 hours, and the results checked under UV light.

**5'-anchored Biotinated Primers**

Another set of primers were used in SSR marker development. According to Wang *et al*. (2007), a set of 5' biotinylated primers: (TA)30, (CA)20, (GA)20, (AGA)15, (TGA)15, and (ACA)15 (Invitrogen, UK) were used in SSR marker isolation. *B.*

*lactucae* isolates CP 24 and BL806 have been pre-amplified using the method above from step a (above) to step c (above). 50 μl hybridization mixture containing 500 ng pre-amplified DNA, 6x SSC 0.1% SDS (1x hybridization buffer) in a final concentration, and 200 ng each of 5'biontinylated primers. The mixture was denatured at 95°C for 5 min and re-naturation at 60°C 3 times and re-suspended in 150 μl hybridization buffer. The hybridization mixture was added to the dynabeads and incubated at 60°C for 1 hour, with gentle agitation. A magnetic separation stand (Invitrogen, UK) was used to capture the beads before removal of the supernatant. The beads were washed twice with 300 μl 1x hybridization buffer at room temperature for 15 min, and twice in 300 μl pre-warm (60°C) 1x hybridization buffer at 60°C for 15 min. SDS was removed by washing the beads twice with 300 μl 6x SSC at room temperature for 15 min. DNA was eluted from the beads by adding 50 μl EB buffer (Qiagen, UK) and incubating at 95°C for 10 min. Beads were removed by capture using the MPC before pipetting the eluted DNA into a new 1.5 Eppendorf tube.

### 4.2.3 ISSR marker development

The ISSR primers (Wagner and Idczak, 2004) used are shown in Table 4. DNA was amplified with the primer and the PCR was carried out in 25 μl of reaction containing 2.5 μl 10x buffer, 0.75 μl 25mM $MgCl_2$, 0.13 μl 25 mM dNTPs, 0.2 U Taq polymerse (all Invitrogen, Paisley, UK). All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). After an initial denaturation (94°C for 2 min) 35 cycles were performed, each cycle consisted of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by a extension step at 72°C for 10 min.

**Table 4. 2** ISSR-primers for identification of *B. lactucae* races.
H= A, C, T; B=C, G, T; V=A, C, G; D=A, G, T (Wagner and Idczak, 2004)

| Primer type | Sequence | | |
|---|---|---|---|
| 5'-anchored tri-uncleotide | HVH-[TGT]5, DBB-[CAG]5, BVD-[CAG]5, GAG-[ACA]5 | BDB-[CAC]5, BDD-[CCA]5, GAT-[CCA]5, | BDV-[CAG]5 DBB-[CAC]5 GAC-[CGA]5 |
| unanchored tri-uncleotide | [CAG]5, [CAC]5 | [CAA]5, | [GTG]5, |

**Gel electrophoresis:** PCR products were separated using 1.5% Agarose Gel stained with 0.5x Gel Red, Spreadex gel EL 800(Elchrom) and 6% Poly(NAT)™ gel(Elchrom) stained with Sybr Gold. For electrophoresis, 4 µl PCR product was loaded with 1 µl loading buffer. Samples were electrophoresed alongside a DNA mass ladder (Hype ladder IV, Bioline; 100 bp ladder, Invitrogen) for Spreadex and Poly(NAT)™ gels. Agarose gels (1.5%) were electrophoresed in 1X TBE (Tris-Borate-EDTA) buffer at 120 V for 1hour, alongside Hyperladder IV. Spreadex gel EL 800 and 6% Poly (NAT) Gel, were electrophoresed in 0.33x TAE (Tris-acetate-EDTA) buffer at 55°C in a SEA® 2000 gel tank at 10V/cm. For each ISSR primer, bands were scored 0 (absence) or 1 (presence). The data was analysed using GenStat v9.1 (VSN International, Ltd.) using Euclidean matrices with nearest neighbour as hierarchical cluster analyses.

**Cloning and sequencing analysis**: Purified PCR products were cloned into pSTBlue-1 using AccepTorTM vector Kit (Novagen). The presence of the insert and positive clones were grown overnight, plasmid DNA was isolated using Qiaprep Spin Miniprep Kit (Qiagen, UK) and the insert sequenced using the methods described above.

### 4.2.4 ITS primers and other primers application

The published ITS 4 and 5 primers (White *et al*., 1990) and the *B. lactucae* specific primers (Choi *et al*., 2007) had been designed from the ITS regions and had been tested with the DNA samples of *B. lactucae* isolates. PCR was carried in 25 µl reaction in Biomix system.

*B. lactucae* specific primers used for this study include:

| | |
|---|---|
| BR766F | GTTTGTATTTGCAGGATTATTAGC |
| BR1234F | GCGCTGCCTTATTATTATTG |
| BR1706F | AGTTGACTTCGGTTGATG |
| BR2257F | TAGTTGATTTGCGGTATGGTT |
| BR1586R | AGCAATGTCAATCTTTACT |
| BR2120R | ATTATCATATTGCAGTAGAAACA |

All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). After an initial denaturation (93°C for 2 min) and annealing step (the appropriate annealing temperature for the primer pair for 15 s) the reaction underwent 50 cycles (93°C for 15s, the appropriate annealing temperature for the primer pair for 15 s; 72°C for 30 s) followed by a final 72°C for 10 min. In order to determine whether the DNA would amplify two primer combinations where used ITS4 forward (5'- CTT GAT TCT GCT ATA GCA AGA TGA) and ITS5 reverse (5'- GTA AAC ATT AAT TCG CAG TAT) (White *et al*., 1990), and BrlaqPCR forward (5'-GAA CTA AGA CGA TAT CGT TCT TAC) BrlaqPCR reverse (5'- ATA CTG CGA ATT AAT GTT TAC) (pers. comm. Dr. Emily Clewes).

## 4.3 RESULTS

### 4.3.1 Existing SSR markers

DNA from a North American isolate C82p24 (supplied by R. Michelmore, UC. Davis, U.S.) was optimized at three dilutions including 1:10, 1:100 and 1:1000. Dilution 1:100 gave better PCR results when used in conjunction with SSR primers (Pv14/Pv16/Pv39) (Figure 4.1). Another four isolates (BL801/BL805/BL806/BL807) have been tested with these three SSR primers. In Figure 4.2, four isolates were amplified by these three SSR primers. The bands around 200 bp as shown in Figure 4.2 were extracted by BandPick TM (Elchrom Scientific AG, Switzerland) for cloning and sequencing.

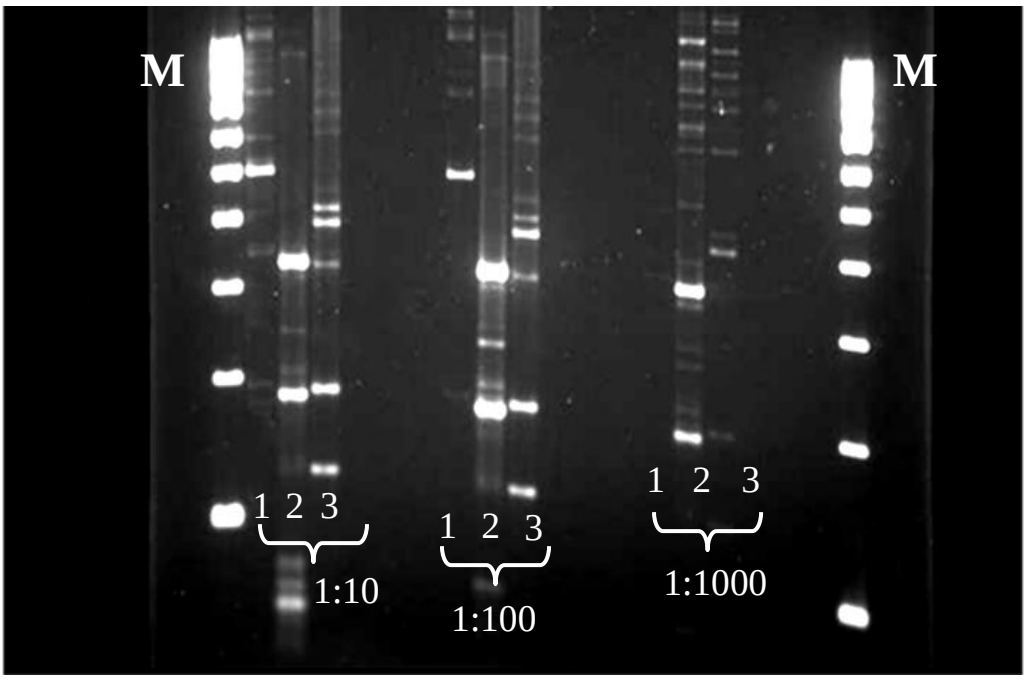



**Figure 4. 1** Different dilutions of North American *B. lactucae* isolate C82p24 amplified by Pv 14, Pv 16 and Pv 39 primers. 1: Pv14 primers, 2: Pv16 primers. 3: Pv39 primers, M: DNA marker.

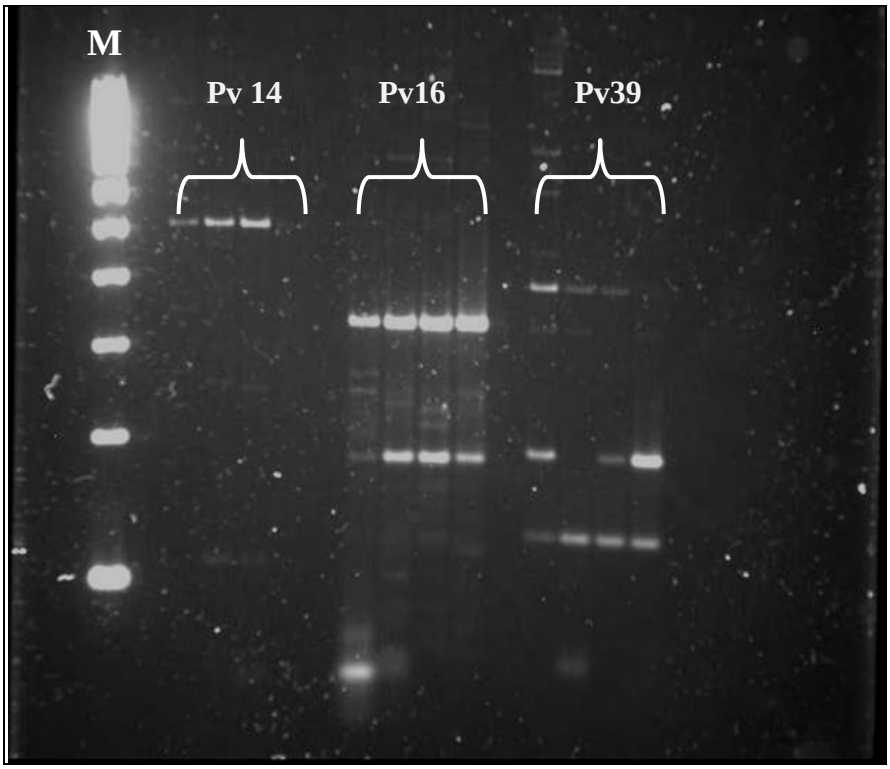



**Figure 4. 2** Four *B. lactucae* isolates (from left to right: BL801, BL805, BL806 and BL807) amplified by Pv 14, Pv 16 and Pv 39 primers. M: DNA marker.

Only BL801 amplified by Pv16 primers and BL805 amplified by Pv39 primers had positive colonies. Eight colonies were randomly picked for plasmid culture. Plasmids were extracted from the colonies and inserts sequenced using T7 primer (see Figure 2,1 for further detail), but no sequences were obtained. However, the PCR product would be inserted between the two *EcoR I* sites on the pCR®4®TOPO® Vector (Invitrogen, UK), so *EcoR I* can be used to test whether the PCR product had been inserted into the vector. Figure 4.3 shows the 1% agarose gel photo with plasmid DNA digested by *EcoR I.* The upper band represents vector DNA and the bands around 200 bp correspond to the PCR product insertions, which means cloning had been successful and the target sequence was around 200 bp.

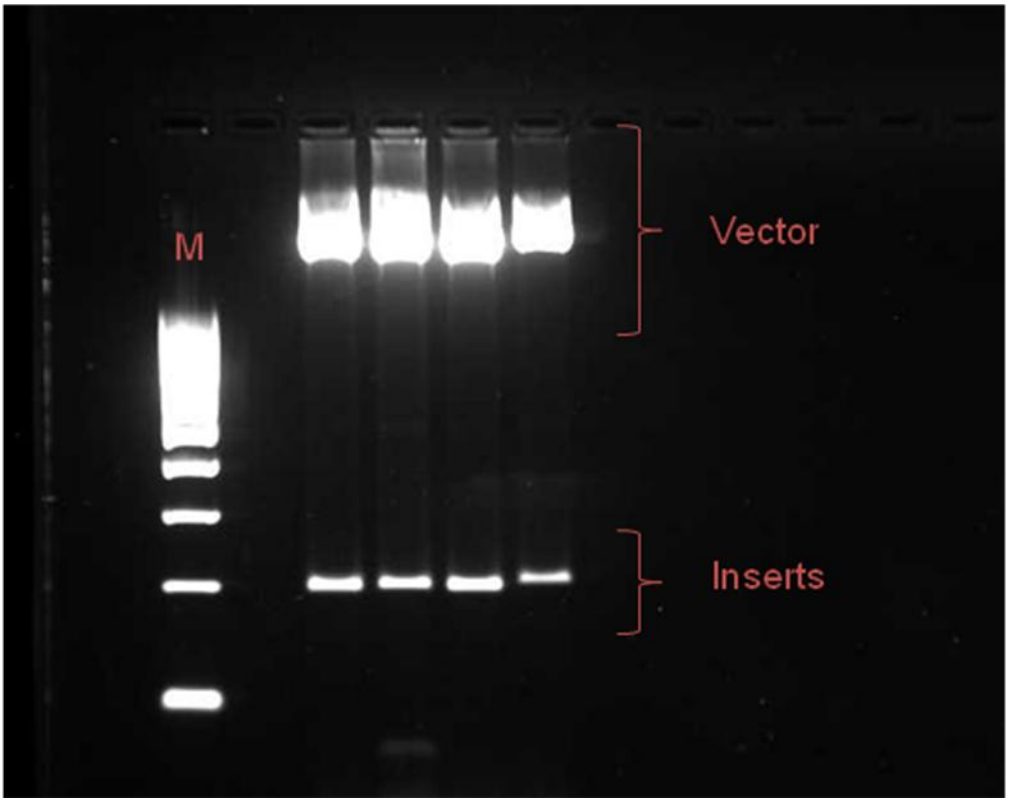



**Figure 4. 3** Plasmid DNA digested by *EcoR* I. The upper bands are vector DNA, the lower bands are insertions. M: DNA marker.

A search against NCBI Gene Bank Database using Basic Local Alignment Search Tool (BLAST) was performed for the sequences between two *EcoR* I sites. *Plasmopara viticola* microsatellite Pv39 sequence has the similarity with the PCR product. Repeated sequence can be found using web base software called Microsatellite finder:

(http://www.yourlabdata.com/index.php?option=com_bioinformatics&task=micro_sat_finder&Itemid=142). In Table 4.3 samples 1-7 (BL805) amplified by Pv39 primers and samples 8 -11 (BL801) amplified by Pv16 primers. This table shows samples 1, 5, 6, 7, 8, 10, 11 contain repeat sequences and they have a similarity to *P. viticola* microsatellite markers. The other samples show no similarity to microsatellite sequences and no microsatellite repeats.

**Table 4. 3** Microsatellite repeat finder results for *B. lactucae* samples.
Samples 1-7 (BL805) amplified by Pv39 primers; samples 8 -11 (BL801) amplified by Pv16 primers.

| Sample | Sequence | BLAST result | Microsatellite repeat |
|---|---|---|---|
| 1 | CGCCCTTTAAAAATATGGTGGCGTCAG GCTGGTTGGCTTCAGAAGAGTTTGAGT TGTTAACGAAAAGTATTTGCTCACATG TTATGGTAAGTATAAACAGACCCTGCA CAAACAGTATATATTTGAAATGCTTTG GCAATTTCTATTTTAGAATACACCGCG CTTGACTGAGCAACTTCTTCCCGTCTG AAGG | *P. viticola* sequence amplified by Pv39 primers | TATATA |
| 2 | CGCCCTTAAAAATATGGTGGCGTCAGG GGTGGANNANANNATGCCCGTGGTGA TGTCGTTCCCAATCGGCGCCCACGTGC CCGTCATCGACGTGCGTCATATCCAGT ACGCGATGCTGAAGAACAGCGATTTC AAGCGTTTTCCAACCGCTACCGCGGTC CATATTGACCAGAGCAACTTCTTCCCG TCTGAAGG | none | None |
| 3 | CGCCCTTTAAAAATATGGTGGCGTCAG GAGCAACTTCTTCCCGTCTGAAGG | none | None |
| 4 | CGCCCTTTAAAAATATGGTGGCGTCAG GAGCAACTTCTTCCCGTCTGAAGG | none | None |
| 5 | CGCCCTTCAGACGGGAAGAAGTTGCTC AGTCAAGCGCGGTGTATTCTAAAATAG AAATTGCCAAAGCATTTCAAATATATA CTGTTTGTGCAGGGTCTGTTTATACTTA CCATAACATGTGAGCAAATACTCTTCG TTAACAACTCAAACTCTTCTGAAGCCA ACCAGCCTGACGCCACCATATTTTTAA AGG | *P. viticola* sequence amplied by Pv39 primers | ATATAT |
| 6 | CGCCCTTCAGACGGGAAGAAGTTGCTC AGTCAAGCGCGGTGTATTCTAAAATAG AAATTGCCAAAGCATTTCAAATATATA CTGTTTGTGCAGGGTCTGTTTATACTTA CCATAACATGTGAGCAAATACTTTTCG TTAACAACTCAAACTCTTCTGAAGCCA ACCAGCCTGACGCCACCATATTTTTAA AGG | *P. viticola* sequence amplied by Pv39 primers | ATATAT |
| 7 | CGCCCTTCAGACGGGAAGAAGTTGCTC AGTCAAGCGCGGTGTATTCTAAAATAG AAATTGCCAAAGCATTTCAAATATATA CTGTTTGTGCAGGGTCTGTTTATACTTA CCATAACATGTGAGCAAATACTTTTCG TTAACAACTCAAACTCTTCTGAAGCCA ACCAGCCTGACGCCACCATATTTTTAA AGG | *P. viticola* sequence amplied by Pv39 primers | ATATAT |

| | | | |
|---|---|---|---|
| 8 | CGCCCTTCCAGCAGTCTCCGTCTCATC AGGCTTGACNNCNGTCACTTCGGTGGT TTCAGTGATGACCTCCGTCACCTCCGT CGTCTCATCAGGCTTGACGTCGGTCAC TTTGGTGGTTTCAGTGGTCACTTCCGTC ACCTCTGTCGTCTCGTCGTCGGTTGTTC CTGTTGTTGGCTTACGTGTTCGCCATG CGTAAGG | *P. viticola* sequence amplied by Pv16 primers | TCGTCGTC G |
| 9 | CGCCCTTCCNCAGTCTCCGTCTCATCA GGCTTGNNNNCNGNCACTTCGGTGGTT TCAGTGATGACCTCCGTCACCTCCGTC GTCTCATCAGGCTTGACGTCGGTCACT TCGGTGGTTTCAGTGGTCACTTCCGTC ACCTCTGTCGTCTCGTCATCGGTTGTTC CTGTTGTTGGCTTACGTGTTCGCCATG CGTAAGG | none | None |
| 10 | CGCCCTTCCAGCAGTCTCCGTCTCATC AGGCTTGACNNNNGTCACTTCGGTGGT TTCAGTGATGACCTCCGTCACCTCCGT CGTCTCATCAGGCTTGACGTCGGTCAC TTCAGTGGTTTCAGTGGTCACTTCCGT CACCTCTGTCGTCTCGTCGTCGGTTGTT CCTGTTGTTGGCTTACGTGTTCGCCAT GCGTAAGG | *P. viticola* sequence amplied by Pv16 primers | TCGTCGTC G |
| 11 | CGCCCTTAACGCATGGCGAACACGTAA GCCAACANCNNGAACAACCGACGACG AGACGACAGAGGTGACGGAAGTGACC ACTGAAACCACCGAAGTGACCGACGT CAAGCCTGATGAGACGACGGAGGTGA CGGAGGTCATCACTGAAACCACCGAA GTGACCGACGTCAAGCCTGATGAGAC GGAGACTGCTGGAAGG | *P. viticola* sequence amplied by Pv16 primers | CGACGAC GA |

Sat1 primers were designed from the consensus sequence of BL801 and BL806 amplified with Pv14 primers using Primer 3 software (http://frodo.wi.mit.edu/primer3/). The specificity of the primers had been checked from VBI database using the Hp Assembly 8.3 database. Sat1 primers didn't match the database of 'Hp v8.3 Assembly', the '*P. tabacina* 454 all contigs' and the 'PsojaeV4+PramorumV1+petabacina'. New Sat1 primers were generated which amplify a 300 pase pair product. The sequences if the primers were shown below.

| | |
|---|---|
| Sat1 Forward | CCGTCCAGATTGCAGCGATA |
| Sat1 Reverse | TCGTCTTCCGGCAATCCTTC |

Figure 4.4 shows the consensus sequence of BL801 and BL806 amplified with Pv14 primers and the locations of the Sat 1 primers.

Figure 4.5 shows the gel electrophoresis results of BL801 and BL806 amplified by Sat1 primers. The lower bands shown on Figure 4.5 had been cut and cloned for sequencing but no SNPs were found between these isolates.

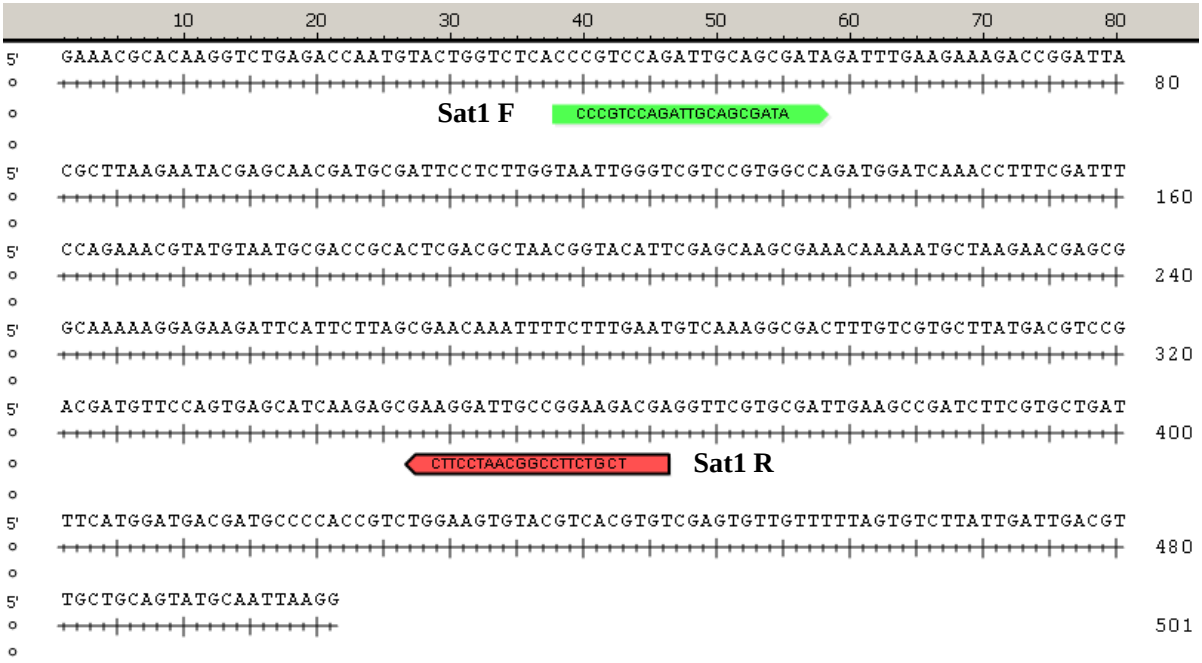



**Figure 4. 4** The consensus sequence of isolates BL801 and BL806 amplified by Pv14 primers. The locations of Sat1 primer sequences are shown in this figure. Green arrow: Sat1 forward primer, Red arrow: Sat1 reverse primer.

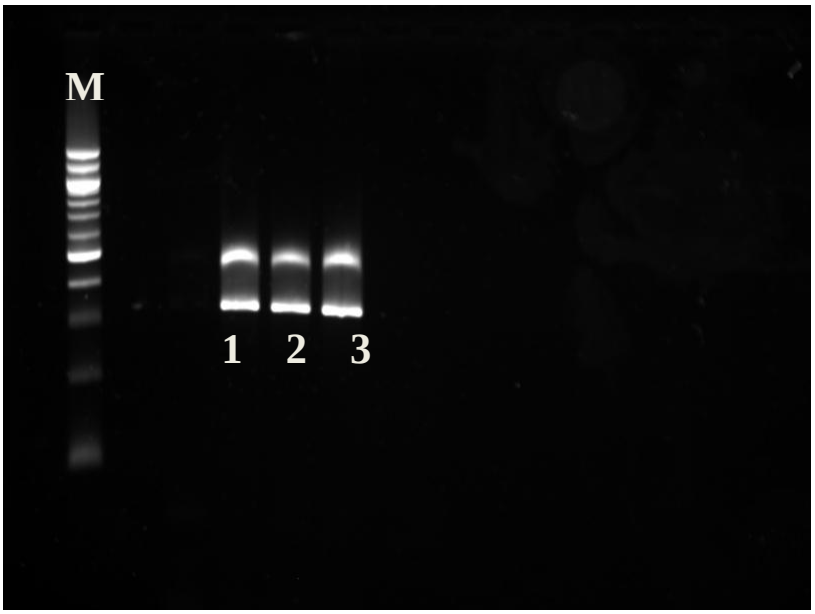



**Figure 4. 5** Results of BL801 and BL806 amplified by Sat1 primers. M: 100bp DNA marker, 1: BL801, 2 and 3: BL806.

### 4.3.2 Development of new SSR primers

During the development of SSR markers, the first few steps were successful except for the hybridization step. Primer mixtures did not appear to bind to target DNA. DNA of Cp82p24 and BL801 were digested with *Rsa*I separately first then the digested DNA fragments were ligated to two double stranded Super SNX linkers. Figure4.6 shows the successful linker ligations. Smear from 300 to 1000 bp represents the ligation product (Glenn and Schable, 2005).

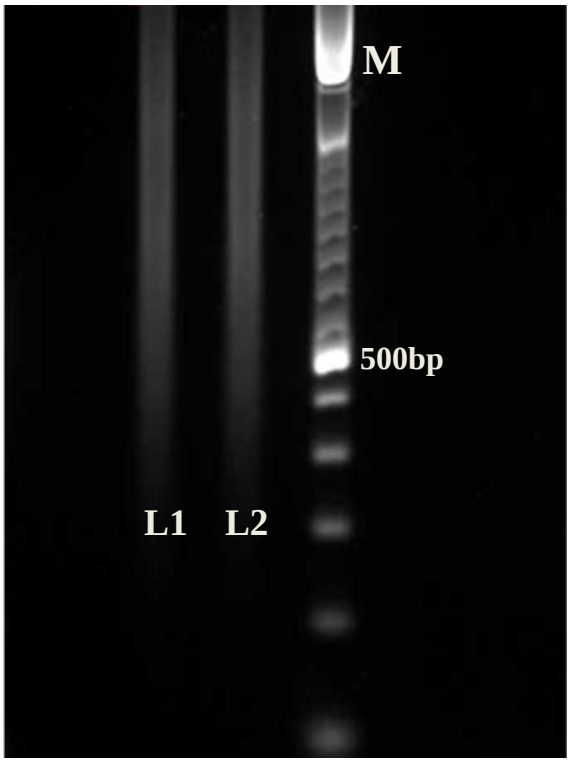



**Figure 4. 6** Products of linker ligation. L1: BL801, L2: Cp82p24, M=100 bp Marker.

The ligation products were digested again by *Xmn* I before enrichment. Figure 4.7 shows the result of PCR recovery of hybridization DNA (Dynabead enrichment product). A distinct band was present around 100 bp size range, it was possible that this could comprise of the target DNA, however after gel extraction and pre-amplification no DNA could be identified which means this band was not the target DNA and may have been a primer dimer.

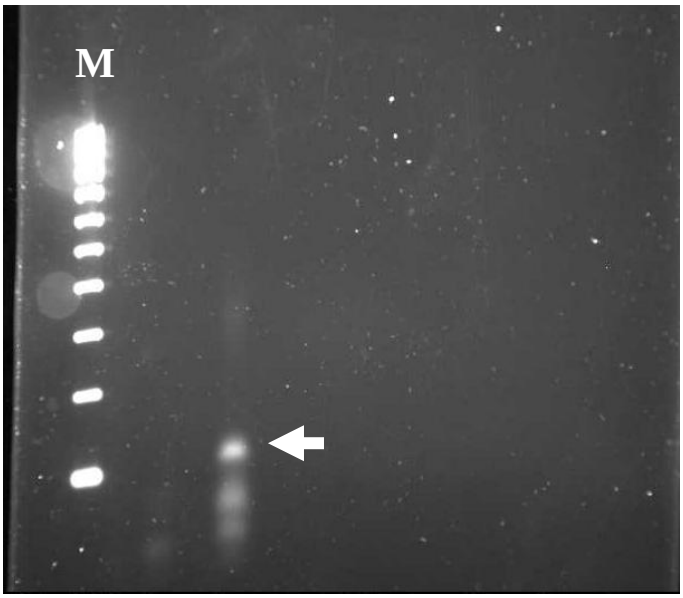



**Figure 4. 7** PCR recovery of hybridization DNA. The hybridization product is indicated by the arrow between 100 to 200 bp, M=DNA marker.

### *Arabidopsis* DNA positive control

Six microsatellite regions from the *Arabidopsis* genome were tested to develop positive controls to evaluate this method. In Figure 4.8 no band was observed indicating that the primers did not hybridize products from *Arabidopsis* DNA.

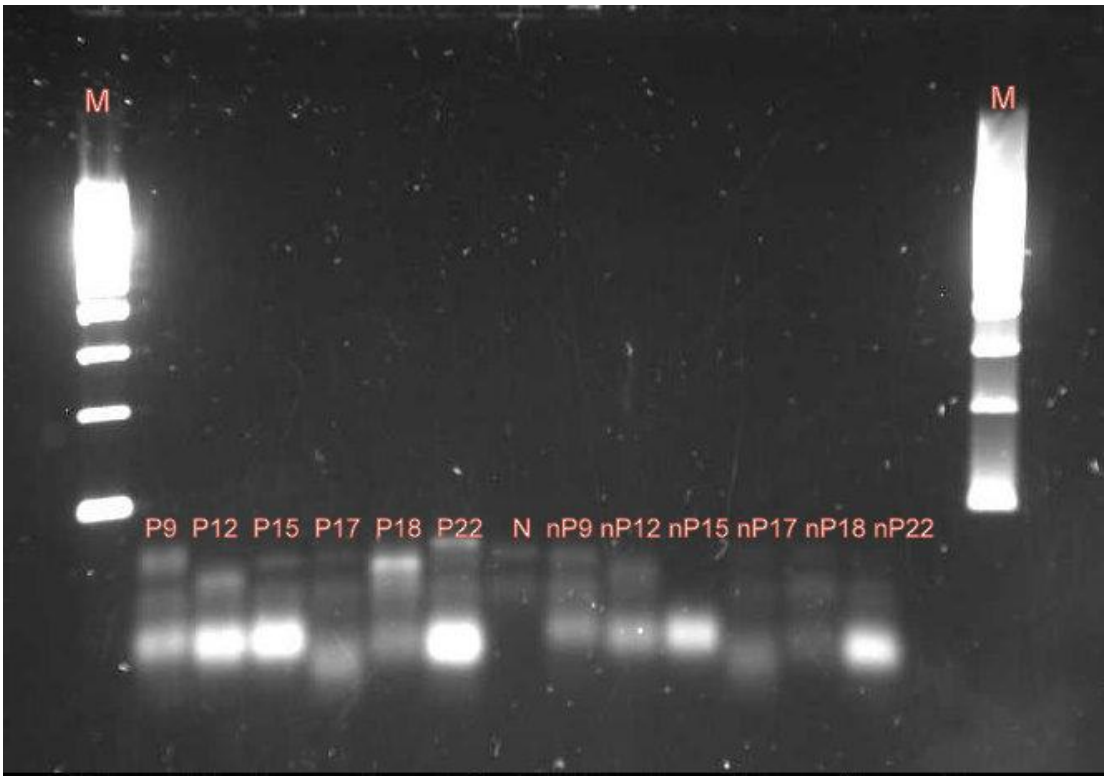



**Figure 4. 8** *Arabidopsis* positive control. Six lanes on the left (P9 to P22) are regions of *Arabidopsis* hybridized using the six microsatellite primers; six lanes on the right (nP9 to nP22) are just primers DNA. N is *Arabidopsis* DNA hybridized without primers. M: DNA marker.

Optimization of the SSR protocol was required to overcome problems encountered in this study. The concentration of the biotinylated oligo mixture was increased from 2 μM to 3μM and a more gentle washing procedure employed. A new 5' - anchored biotinylated primers mixture has been used to optimize the hybridization results of the new protocol which is shown in Figure 4.9. Although there are two clear smears of Cp82p24 and BL 806, the hybridization negative control also shows a clear smear and the PCR was negative, which means the smear could be background.


**Figure 4. 9** Result of the application of new 5'-anchored biotinylated primers. Cp82P24 and BL806 are two *B. lactucae* isolates. PCRN: PCR negative control, HybN: hybridization negative control, M: marker.

### 4.3.3 Use of existing ISSR primers application

In this project, agarose gels were initially used but did not give clear resolution of DNA banding patterns. Using ISSR primers (Table 4.2) produced DNA banding patterns after electrophoresis. However agarose gels could not separate the banding sufficiently. Elchrom Spreadex® and Poly(NAT)® ready-to-use gels in the size range of 20 bp to 1000 bp, were used to improve the resolution. The electrophoresis results have shown that 6% Poly (NAT) gel improves the separation of banding and gives better resolution. Fourteen ISSR primers (Table 4.2) produced good PCR amplicons; however these were poorly separated when electrophoresed on a 1.5% agarose gel (Figures 4.10 and 4.11). To resolve the banding pattern, amplicons were electrophoresed on a Spreadex EL800 gel. However, using this system most bands were greater than the exclusion limit of the gel.


**Figure 4. 10** Gel eletrophoresis of BL801 amplified with 16 different ISSR primers. 1=[CAG]5, 2=[CAA]5, 3=BVD-[CAG]5, 4=[CAC]5, 6=BDD-[CCA]5, 7=BDV-[CAG]5, 8=GAC-[CGA]5, 9=DBB-[CAC]5, 10=DBB-[CAG]5, 11=[GTG]5, 12=GAG-[ACA]5, 13= GAT-[CCA]5, 14= HVH-[TGT]5, 15= ITS 4 and 5 primers, 16= BrlaqPCR primers.

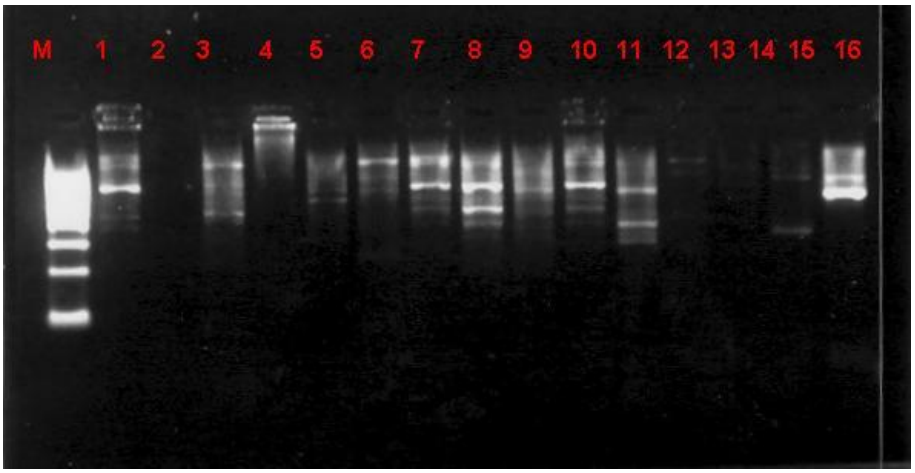



**Figure 4. 11** Gel eletrophoresis of BL802 amplified with 16 different ISSR primers. 1=[CAG]5, 2=[CAA]5, 3=BVD-[CAG]5, 4=[CAC]5, 6=BDD-[CCA]5, 7= BDV-[CAG]5, 8= GAC-[CGA]5, 9= DBB-[CAC]5, 10= DBB-[CAG]5, 11=[GTG]5, 12= GAG-[ACA]5, 13= GAT-[CCA]5, 14= HVH-[TGT]5, 15= ITS 4 and 5 primers, 16= BrlaqPCR primers.

Electrophoresis of ISSR amplicons on Elchrom 6% Poly(NAT)® Gel shows that resolution and separation was better than the agarose gel and Spreadex EL800 gel. Clear banding patterns are seen on the gel. BL801, BL802 and BL803 (IBEB identifications shown in Table 3.6) show different banding patterns on the gels which indicated that these three isolates are genetically different (Figure 4.12). Cluster analysis of banding patterns indicated that the three isolates were genetically distinct (Figure 4.13).

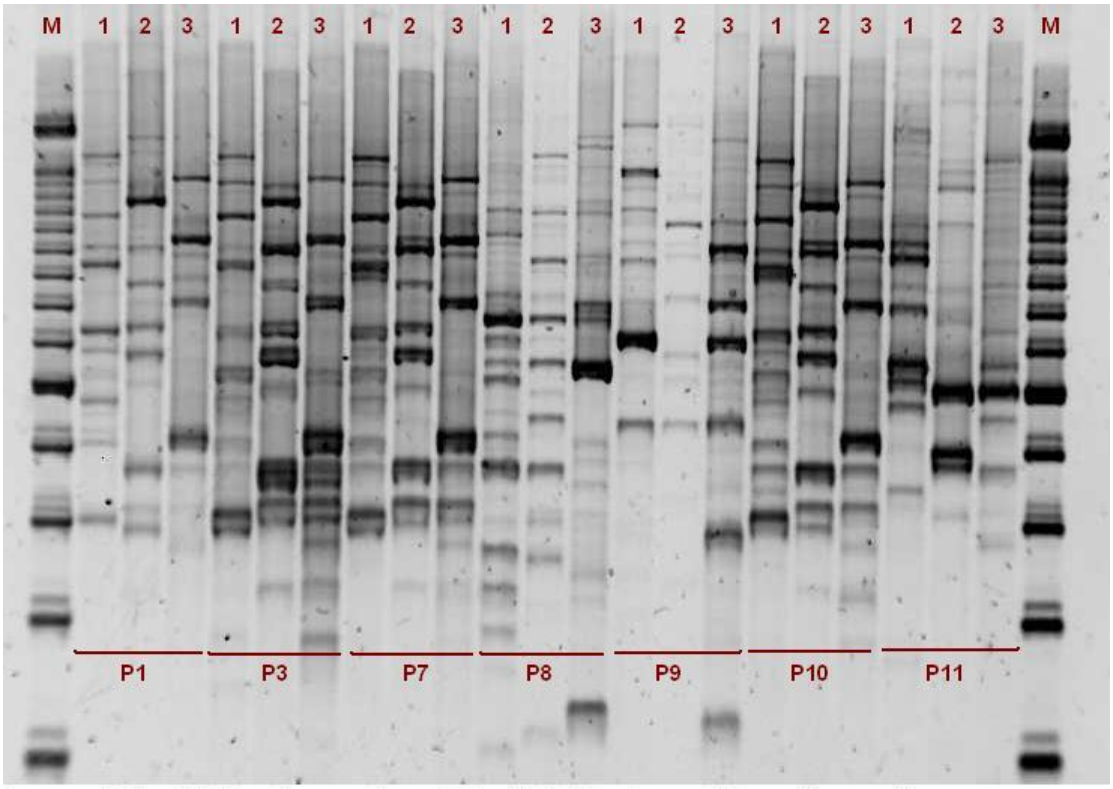



**Figure 4. 12** Banding profile of tested race of BL801, BL802 and BL803 with 7 different ISSR primers. M=Marker, 1=BL801, 2=BL802, 3=BL803, P=primer. 1=[CAG]5, 3= BVD-[CAG]5, 7=BDV-[CAG]5, 8= GAC-[CGA]5, 9= DBB-[CAC]5, 10= DBB-[CAG]5, 11=[GTG]5.

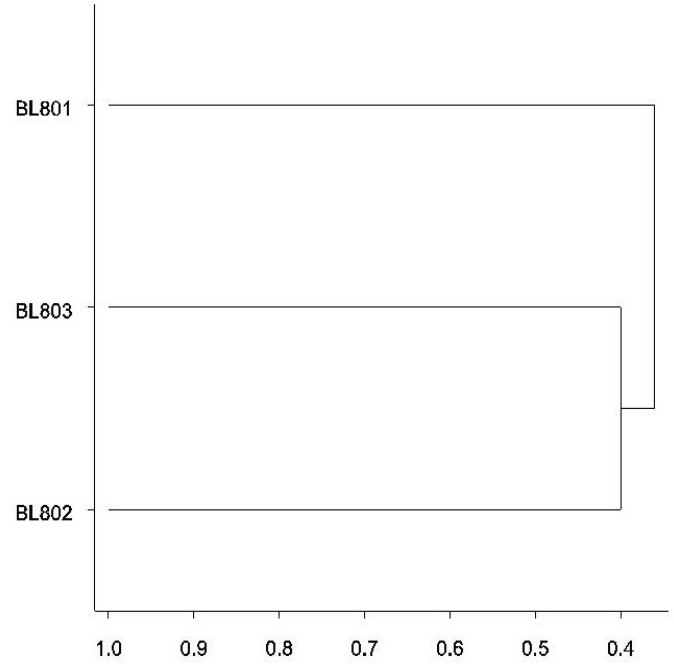



**Figure 4. 13** Dendrogram derived from ISSR data of three *B. lactucae* isolates using hierachial cluster analysis of Euclidean matrices with 'nearest neighbour' in GenStat v9.1. Bands were scored for 0 (absences) or 1 (presence).

DNA gel extraction was optimized for the development of ISSR markers. Distinct bands of each isolates around 500 bp (Figure 4.12) were cut for analysis. As Elchrom 6% Poly (NAT)$^{TM}$ Gel could not be dissolved in the normal gel extraction solution, other methods had to be applied. This was achieved by using the method described byMathimaran *et al*. (2008) to extract DNA from gels using the BandPick TM (Elchrom Scientific AG, Switzerland) method before re-amplification of the DNA by PCR. This method can extract the DNA from the Elchrom 6% Poly(NAT)$^{TM}$ Gel. After re-amplification the product was run on a gel. The original 500 bp single bands increased to 1000 bp and more than one band were shown on the gel. This might be caused by contamination. To solve this problem, the product was run on a 1.5% Agarose gel, with a single band round 500 bp cut from the gel and then the DNA re-extracted using QIAquick gel purification Kit (Qiagen, UK) before cloning and

sequencing.

DNA sequences were obtained from *B. lactucae* isolates with the plasmid DNA cultured from the target DNA which had been cloned into pSTBlue-1 using the AccepTor™ vector Kit (Novagen, USA), and T7 primer (Okura *et al*., 2005). However empty insertions were reported when searched against NCBI Gene Bank Database using Basic Local Alignment Search Tool (BLAST).

### 4.3.4 Development of ITS primers and other primers

In Figures 4.10 and 4.11 no distinct band was observed for *B. lactucae* DNA amplified by ITS 4 and ITS 5 primers. BrlaqPCR primers only amplified BL802. Figure 4.14 shows the results of the published *B. lactucae* specific primers designed from the ITS region. Each two bands are BL801 and BL806 with cv. Cobham Green. These three DNA samples were amplified by Br766F with Br1586R, Br766F with Br2120R, Br1706F with 2120R and Br1234F with Br1586R. The results show the primers are highly specific for *B. lactucae* as no DNA from the host plant was amplified. These bands were cut and cloned by Invitrogen TOPO® TA cloning Kit for sequencing. The Seqman result showed that no differences were found between isolates BL801 and BL806. However, these sets of primers are highly specific therefore they could be designed for other purposes such as the detection of the pathogen.

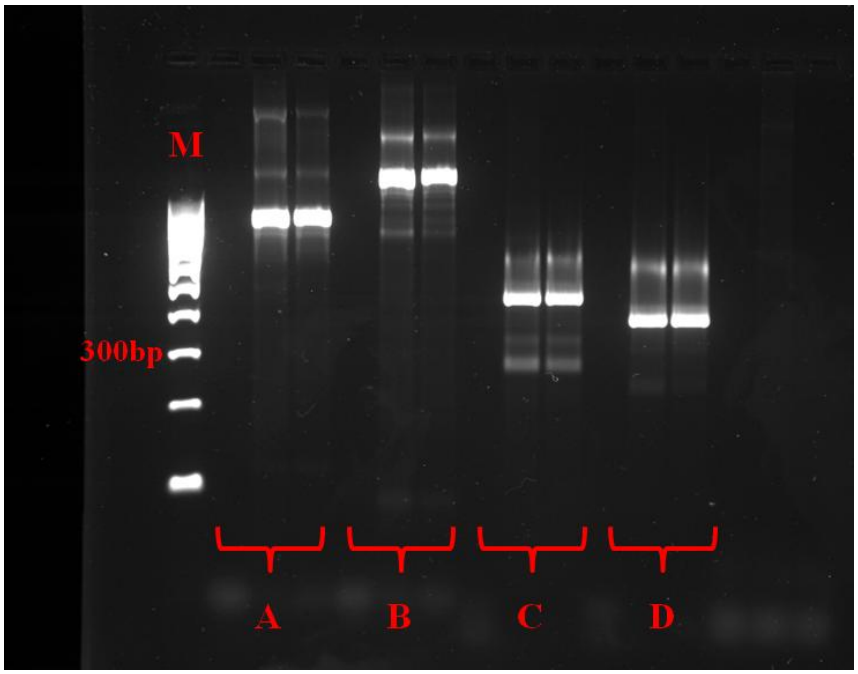



**Figure 4. 14** Results for cv. Cobham Green, BL801 and BL806 amplified by *Bremia* specific primer. Each group samples were loaded as this order (from left to right): cv. Cobham Green, BL801, BL806. M= Hyperladder marker. Primer pairs: A=Br766 and Br1586; B= Br766 and Br2120; C=Br1706 and Br2120; D=Br1234 and Br1586.

## 4.4 DISCUSSION

The results show that ISSR markers can be useful for detecting polymorphisms amongst *B. lactucae* isolates, with each isolate having distinct banding patterns. Cluster analysis of banding patterns indicates that the three isolates tested were genetically distinct using ISSR markers. Therefore ISSR markers could be developed as race identification markers. However, a contamination problem may exist in the isolates with the presence of other organisms. The gel extraction method has also been difficult. Another possibility is the quality of the DNA. Before the Invitrogen ChargeSwitch Kit was used, DNA was extracted by boiling spores which may be contaminated by plant tissues and with the non-specificity of the ISSR marker it may amplify any DNA fragment in the reaction. Before all these problems have been solved ISSR markers could not be developed as race differential markers.

### 4.4.1 Micrasatellite markers development

Microsatellite repeat 'TATATA', 'TCGTCGTCG' and 'CGACGACGA' were found in the sequences of the clones. These results show the possibility of the development of microsatellite markers which are 'neutral markers' similar to ISSR marker. Currently, the results are a trial of testing whether *B. lactucae* DNA can be amplified by these published Pv SSR markers. If the different isolates amplified by the same SSR primers show differences in sequences that mean this marker can differentiate these two isolates and specific race microsatellite marker can be designed. However, as the test with BL801 was with Pv39 and BL805 was amplified by Pv 16 their sequences could not be used for comparison. Therefore DNA from all the isolates should be amplified with the same SSR markers and sequences compared. This could provide suitable molecular marker for differentiating isolates. These markers could

be used for differentiating isolates in competition experiments. The designed SSR marker Sat 1 could not be used as a differential marker to identify BL801 and BL806. However it could be used as a *B. lactucae* specific marker to detect the pathogen.

The results show that current SSR marker development was not successful due to hybridization problems. This enrichment procedure followed Glenn and Schable (2005) protocol which can be used to developed SSR markers of *Arabidopsis* DNA successfully. Before enrichment all the steps showed positive results so at the beginning the protocol was optimized by increasing the concentration of the biotinylated primers mixture and a more gentle washing procedure was applied to capture more DNA, but this did not give any positive results. The result of the positive control (*Arabidopsis* DNA) containing microsatellite region) could not be captured. It is possible that the problem was related to the probe or there was a problem in the hybridization PCR. So new 5'-anchored biotinylated primers mixtures were used to optimize the hybridization result of the new protocol however nothing could be improved and no DNA could be captured.

Microsatellites are considered as highly polymorphic DNA markers with discrete loci and co-dominant alleles (Schlotterer, 1998), and the hybridization method has been successfully used to construct libraries and determine flanking sequences of microsatellite DNA loci in different organism amphibians, birds, fish, mammals, reptiles, insects, fungi, plants (Glen and Schable, 2005). However, in this project it was not successful during the hybridization, while the *Arabidopsis* DNA control was enriched and showed the positive result. This result could be explained if the *Bremia*

DNA had been washed away during the washing procedure. Another reason might be the 5' anchored biotinylated primers in this protocol was not suitable for *Bremia* microsatellite regions. The results of Microsatellite repeat found in the Pv SSR primers amplified sequence are repeats of TA, TCG and CGA, which were not included in the 5' anchored biotinylated primers in this protocol. In Delmotte's study (2006), a 5'-biotinylated $(CT)_{10}$ and $(GT)_{10}$ oligonucleotide were used as probes, therefore these unsuccessful results might be caused by the 5'-biotinylated primer designs which were not suitable for oomycete microsatellites regions. An optimized protocol is necessary for microsatellites markers development.

The identified SSR markers of *B. lactucae* have not been reported before; the most recent and closely related finding is the isolation of microsatellite loci from *Plasmopara viticola* (Delmotte *et al*., 2006). The cross-amplification of three closely related plant-pathogen Oomycete species (*Plasmopara halstedii*, *Bremia lactucae* and *Phytophthora infestans*) of *P. viticola* had been tested, and these species can be amplified by Pv14 Pv16 and Pv39 (Delmotte *et al*., 2006).

Delmotte *et al*., (2006) found microsatellite isolation from *P. viticola* was difficult with a density of non redundant positive clones of 1.6%, and the microsatellite sequences were often short and yielded low variable markers. That might be the reason why the designed Sat1 markers have difficulty in differentiating the BL801 and BL806.

### 4.4.2 ISSR development

In the early 1990's, ISSR began to be used as a new type of DNA marker which involves the use of microsatellite sequences directly in PCR for DNA amplification (Zietkiewicz *et al*., 1994) which enables amplification of genomic DNA and provides information about many loci simultaneously. Microsatellites are widely distributed in eukaryotic genomes and they are highly polymorphic due to variability of the number of tandem repeats motif at a specific locus (Davierwala *et al*., 2000). In Wagner and Idczak (2004) found that ISSR primers were developed to determine the genetic variability of *B. lactucae* isolates, thirty one ISSR primers were tested to distinguish 17 races of *B. lactucae*. They found primers with a repeat motif of $(CCA)_5$, $(CGA)_5$, $(CAC)_5$ or $(CAG)_5$ anchored, or unanchored are able to form polymorphic band patterns (Wagner and Idczak, 2004). In this PhD project the 5´-anchored tri-nucleotide and un-anchored tri-nucleotide ISSR primers were chosen from the Wagner and Idczak (2004) primers list. Figure 4.12 shows that the ISSR primers can differentiate three *B. lactucae* isolates. The cluster analysis of banding patterns scoring indicated that the three isolates were genetically distinct (Figure 4.13). However, due to highly variable ISSR results, they were not repeatable and it caused difficulties isolating the specific loci in the amplified DNA product. Although it was difficult to isolate the race-specific markers for *B. lactucae* in this project, ISSR markers have been developed as race differentiate markers for other oomycete species. The recent study of *Sclerospora graminicola* (pearl millet downy mildew) characterization using ISSR markers showed that 22 isolates of *S. graminicola* were assessed using 20 ISSR primers and the results of ISSR fingerprints revealed significant genetic diversity among the *S. graminicola* isolates, therefore ISSR markers could be a powerful tool for fingerprinting and diversity analysis in fungal

pathogens (Sudisha *et al.*, 2010). *S. graminicola* is closely related to *B. lactucae* and the characterization of its population as highly variable with extreme virulence leading the breakdown of resistance (Hess *et al.*, 2002) is similar to *B. lactucae*. Furthermore suitable and efficient ISSR markers have been presented for discriminating other oomycete pathogen like *Phytophthora* and *Pythium* (Cooke and Lees, 2004), therefore ISSR markers should be regarded as a promising tool for *B. lactucae* population study in future work.

### 4.4.3 ITS markers

The specific *B. lactuca*e primers designed by Choi *et al*. (2007) from ITS2 region were used in this project. Br 1234F and Br 1586 had been chosen to redesign specific primers which can amplify smaller product for qPCR quantification studies. Although eight *Bremia* isolates were collected from various host plants, the ITS2 regions of all sequences showed similar sizes of 2450–2461 bp and were constantly composed of the nine repeats. This means that the long length and the repetition of ITS region may be a very common event in *B. lactucae* ITS2 evolution (Choi *et al*., 2007). These repetitions are a major factor in the huge size and sequence variability of ITS among and within species. Repetitive elements themselves are subject to high rates of evolutionary change, as indicated by the observation that copies of the repeat units show considerable sequence variation between both repeats and isolates. The Br primers are very specific to *B. lactucae*, they could be the PCR amplified DNA sequence can be used to design more specific primers for pathogen detection. Therefore the Br primers were applied in qPCR for *B. lactucae* DNAquantification (see Chapter 6 for detail).

# CHAPTER 5 DEVELOPMENT AND USE OF RXLR EFFECTOR MARKERS FOR IDENTIFICATION OF *BREMIA LACTUCAE* ISOLATES

## 5.1. INTRODUCTION

Oomycetes are thought to secrete hundreds of effector proteins that target distinct sites in their host plants. In five examples, an oomycete effector has been identified as an avirulence (AVR) determinant, by eliciting defense in a host that contains a matching receptor-like resistance gene (encoding an *R* protein): Avr 1b-1 from soybean pathogen *Phytophthora sojae* (Shan *et al*., 2004); ATR13 (Allen *et al*., 2004), $ATR1^{NdWsB}$ and $ATR5^{LerEmoy2}$ (Bailey *et al*., 2011.) from the *Arabidopsis* downy mildew pathogen *Hyaloperonospora parasitica,* and AVR3a from *Phytophthora infestans* (Armstrong *et al*., 2005). The RxLR motif (Arg-X-Leu-Arg, x equals any amino acid) is common to four oomycete avirulence proteins and other pathogen effector proteins and it is similar to the host cell target signal RxLR (E/Q) which promoted the hypothesis that RxLR functions as a signal that mediates trafficking into host cells (Marti *et al*., 2004; Rehmany *et al.*, 2005).

RxLR proteins are typically highly variable within an oomycete species due to selection pressure for evading R protein detection in the host, therefore the adjacent sequences are likely to vary amongst pathogen isolates. This natural variation could potentially provide molecular markers to detect polymorphisms driven directly by natural selection. *Hyaloperonospora arabidopsidis* (*Hpa*) is an obligate biotroph of *Arabidopsis thaliana*. A recent study (Fabro *et al*., 2011) of *Hpa* candidate effectors (HaRxLs) showed that most of the HaRxLs enhanced plant susceptibility, which indicates that these effectors can suppress the host immunity. The aim of this chapter was to determine whether such markers could also be readily developed for use in *Bremia lactucae*, using RxLR sequences in this pathogen provided by Professor Richard Michelmore (UC Davis, USA).

Several cDNA libraries of *B. lactucae* from a variety of sources have been generated to identify avirulence effector proteins and one subtraction library was made; sequences from all libraries are being analyzed for candidate effectors using bioinformatics approaches (Michelmore and Wong, 2008). The presence of a secretion signal peptide and the RxLR amino acid motif are also being searched for, and more than fifteen candidate sequences had been identified by the beginning of this project (Michelmore and Wong, 2008).

Seventeen different pairs of RxLR primers were provided by Richard Michelmore and had been designed from sequences of RxLR effectors of *B. lactucae* isolate SF5. These were tested on all the isolates of *B. lactucae* collection used in this project to investigate the genotypic polymorphisms between isolates.

An investigation was also made of the compatible and incompatible interactions of two *B. lactucae* isolates on the IBEB differential set of cultivars, using selected RxLR markers. The aim was to discover whether using RxLR markers would give a more accurate identification of isolates than the IBEB method.

**OBJECTIVE OF STUDY**

The aim of this study was to determine whether RxLR markers could be readily developed for use in *B. lactucae*. RxLR markers were also used to investigate the compatible and incompatible interactions to discover whether using molecular markers would give a more accurate identification of *B. lactucae* isolates than the IBEB method.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Single spore isolates tested with RxLR primers

Six *B. lactucae* isolates were generated for use in experiments including: BL801, BL806, Bl805 (Table 3.6), NL5 (Table 3.1 a), S1 and S2, (Table 3.7). Each isolate was maintained on seedlings of a universally susceptible cultivar (Cobham Green) in an individual propagation box. Further details of methods for single-sporing and maintenance of isolates are provided in Chapter 3.

### 5.2.2 Optimization of RxLR-based markers

RxLR primers were tested with all of the *B. lactucae* isolates in the collection assembled for this to investigate the genotypic polymorphisms between isolates. The primers were provided by Richard Michelmore (UC Davis, USA), which were designed from DNA sequence of RxLR alleles (Table 5.1) in the *B. lactucae* isolate SF5.

**Table 5. 1** Sequences of RxLR primers from *B. lactucae* DNA.

| RxLR | Forward Primer | Reverse Primer |
|---|---|---|
| 1 | ATGCGGCTTCCTTTCC | TCAATCATGGCGTACTGA |
| 2 | ATGCGTTTGCTAAATATCG | TCATCTTCCTCTAAAACTGAAAA |
| 3 | ATGCCCAGGTGCCTTG | TTAGTCTGGTCCTCTAATCATG |
| 4 | ATGCGCGTGAAGGACT | TTACTTTAAAATGTTGTCCTTACA |
| 5 | ATGCAGCTTCCTTTCCA | CTATATACCCTCGTTAAGGCGT |
| 6 | ATGCTTGCTCTATCCAAGATT | TTACATACCATGTTGCATTCT |
| 7 | ATGGTGCGGAAAAATATTC | CTAATTAGCCCTGGGGC |
| 8 | ATGACTTTGAACGCCAAG | CTACTCTAAACCATCTGTCTGTCG |
| 9 | ATGCATGTCGATGTTTGTT | TTAAACTATGCCAGTGCTGC |
| 10 | ATGTACATGCGCGACAAT | CTATTTCGAGAAAAACGACA |
| 11 | ATGCGCTACAAGCTTATCG | TTACAAATCGAGCGTGTTT |
| 12 | ATGCAGCGCTTCCTTC | TTGAGAAGAGTAATATTAACAAG |
| 13 | ATGCGATCGCTACGG | GTCGGATGTTGTGGGTA |
| 14 | ATGAATGACTTGACAATTGC | TCATACGGGTCCCACA |
| 15 | ATGTGCGCGCGACAA | TCCGAGATTAAGAACATCGT |
| 16 | ATGCTTTCCTGCAAAAA | TTAAACGGAAAGCAAATCA |
| 17 | ATGGCGTCGTCTCGT | CTAGCAGCATCCGTTGT |

### 5.2.3 DNA extraction of single spore isolates from pure spores

**Spore harvest:** To ensure the purity and quality of the DNA the washed-spore harvest method was applied (see Chapter 2). The harvest was conducted in a BioMAT Class II laminar flow cabinet (MAT Medical Air Technology, UK) to prevent contamination from other isolates. Sporulating cotyledons were cut and placed in beaker of chilled distilled water and shaken gently to dislodge the spores. The spore suspension was then filtered through mirarocloth (CALBIOCHEM, USA) into a clean centrifuge tube and placed on ice.

**Spore purification:** The spore suspension was centrifuged at 3000 rpm (1610 x g) for 20 minutes in an Eppendorf Centrifuge 5810 R (Eppendorf, Germany). The spore pellet was re-suspended in 1 ml Millipore water vortexed and transferred to a 1.5 ml Eppendorf tube. This was centrifuged at 14000 rpm (20,000 x g) for 1 minute in a bench top centrifuge (ThermoScientific, Germany). The supernatant was removed without disturbing the pellet and the tube was stored in at -80 $^{o}$C.

**DNA extraction:** The tube containing the spore pellet and a micro pestle were cooled in liquid nitrogen and the spore pellet was then ground using the micro pestle. The DNA was then extracted using a Qiagen DNeasy Mini Plant Kit (Qiagen, UK) according to the manufacturer's protocol.

### 5.2.4 PCR amplification with RxLR primers

PCR reaction were carried out in a 25 µl reaction containing 12.5 µl Biomix red (Bioline, UK) 0.5 µl 50mM MgCl2 (Bioline, UK), 1 µl each of 10 µM primer (forward/reverse) 1 µl template DNA and 9 µl distilled water. All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). An initial amplification was performed for 5 cycles after denaturation at 94°C for 2 min. Each cycle consisted of 94°C for 30 s, annealing (temperature appropriate to the TM value of the primers) for 30 s and 72°C for 30 s. This was followed by another 30 cycles consisted of 94°C for 30 s and annealing at 65°C for 30 s and 72°C for 30 s then cooling to1 °C.

The amplification product was purified using Qiagen PCR Purification Kit (Qiagen, UK), and the purified DNA was cloned into pCR®4®TOPO® Vector and the TOP10®competent *Escherichia coli* system using TOPO® TA Cloning Kit for Sequencing (Invitrogen, UK). Positive colonies containing the insert were selected by colony PCR using M13 (forward and reverse) primers. Products were visualized by agarose gel electrophoresis.

The positive colonies were cultured in 10 ml selective LB broth with Kanamycin at 37°C in a shaking incubator overnight. The plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, UK). Sequencing PCR was in a 10 µl reaction volume containing 2 µl Big Dye, 2 µl 5x sequence buffer, 1 µl M13 primer (forward or reverse, Invitrogen, UK), 1 µl purified plasmid DNA and 4 µl distilled water. Sequencing was carried out at the Genomic Centre (University of Warwick, UK) using BigDye Terminator v3.1 chemistry (Applied Biosystem, USA) and ABIPRISM

3100 genetic Analyzer (Applied Biosystem, USA).

### 5.2.5 Field sample isolates tested with RxLR primers

Field samples were collected from lettuce farms in Lincolnshire and in the south coast of England. Samples that were collected from the field were assumed to be mixed spores of isolates. These isolates were maintained in a propagation box. A more efficient and economic method was applied for DNA extraction from this set of samples for time was not sufficient for the application of the pure spore DNA extraction method. One or two sporulating cotyledons from each isolate were collected for DNA extraction. The procedure for single spore harvests was carried out in a BioMAT Class II laminar flow cabinet (MAT Medical Air Technology, UK). One or two sporulating cotyledons were placed in a 1.5ml tube on ice, 100 µl Extraction Buffer (RedExtract-N-Amp, Sigma-Aldrich) was pipetted into the tube before incubating the tissue at 95°C for 10 min. An equal volume of Dilution Buffer (RedExtract-N-Amp, Sigma-Aldrich) was added to the extract to neutralize inhibitory substances prior to PCR. The extract was vortexed and then kept at 4°C.

The PCR was carried out in a 20 µl reaction containing 10 µl REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, USA), 1 µl each of 10 µM primer (forward/reverse, Invitrogen, UK), 4 µl extracted DNA and 4 µl distilled water. All amplifications were performed in an Applied Biosystems thermal cycler (Applied Biosystems, UK). An initial amplification was performed for 5 cycles after denaturation at 94°C for 2 min. Each cycle consisted of 94°C for 30 s and annealing at 60°C for 30 s and 72°C for 30 s followed by another 30 cycles consisted of 94°C for 30 s and annealing (temperature is variable for different primers) for 30 s and 72°C for 30 s then 18°C to

infinity.

### 5.2.6 Comparison test between RxLR marker and IBEB identification

The molecular markers RxLR1 and RxLR7 were used to investigate compatible and incompatible interactions of isolates BL801 and BL806 on the IBEB lettuce differential set of cultivars. In the first experiment, cotyledons from the entire IBEB lettuce differential set of cultivars were inoculated with BL801 and BL806 and were then tested with the primers RxLR1 and RxLR7 using the Sigma-Aldrich RedExtract-N-Amp system. In the second experiment, only cultivars that gave incompatible reactions were used to avoid spore contamination. In this experiment there were two trials: in trial A, samples were harvested on the 4$^{th}$ day post inoculation while in trial B, samples were harvested on the 7$^{th}$ day post inoculation. Table 5.2 shows which cultivars were inoculated for these two experiments.

**Table 5. 2** Information of the isolates and cultivars used in the first and second experiments.

| Isolate | IBEB cultivars | 1.Lednicky | 2. UC DM2 | 3.Dandie | 4. R4T57D | 5. Valmaine | 6. Sabine | 7. LSE 57/15 | 8. UC DM10 | 9. Capitan | 10. Hilde II | 11. Pennlake | 12. UC DM14 | 13.PIVT 1309 | 14.LSE18 | 15.LS-102 | 16Cororado | 17.-Ninja | 18Discovery | 19 Argeles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BL801 | IBEB | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - | - | - |
| | RxLR | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | - | - | - |
| | Exp. a | * | * | * | * | * | * | * | * | * | * | * | * | - | * | + | - | + | - | - |
| | Exp. b | * | * | * | * | * | * | * | * | * | * | * | * | + | * | - | + | + | - | + |
| BL806 | IBEB | + | + | - | + | + | - | + | + | + | + | + | + | - | - | - | - | - | - | + |
| | RxLR | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | + |
| | Exp. a | * | * | - | * | * | - | * | * | * | * | * | * | - | + | + | + | - | - | * |
| | Exp. b | * | * | + | * | * | - | * | * | * | * | * | * | - | - | - | - | - | - | * |

+: cultivars were inoculated in the experiment, *: cultivars were not inoculated in the experiment. Exp.: experiment, A: trial A, B: trial B.

## 5.3 RESULTS

### 5.3.1 Single spore isolates tested with RxLR primers

The products from PCR amplification of the single spore isolates with 16 pairs of RxLR primers were electrophoresed on 1.5% agarose gels. The figures below from 5.1(a) to 5.1(f) show the same gel loading order for each RxLR primer pair: 1:S1, 2:S2, 3:BL801, 4:Nl5, 5:Bl805 and 6:BL806. RxLR1 can differentiate between isolates BL801, BL806 and NL5 as Figure 5.1(a) shows below. BL801 shows a band around 300 bp, while BL806 and NL5 have a band above 1000 bp. In Figure 5.1(b), RxLR 5 showed two bands for NL5, and the band for BL806 is slightly higher than the others. No polymorphism was observed between these isolates when amplified by other RxLR primers as shown in Figure 5.1 (a) – (f). The results of gel electrophoresis of the PCR products from the amplification of the single spore isolates with each of the RxLR primer pairs are summarised in Table 5.3 (fade bands were not counted).

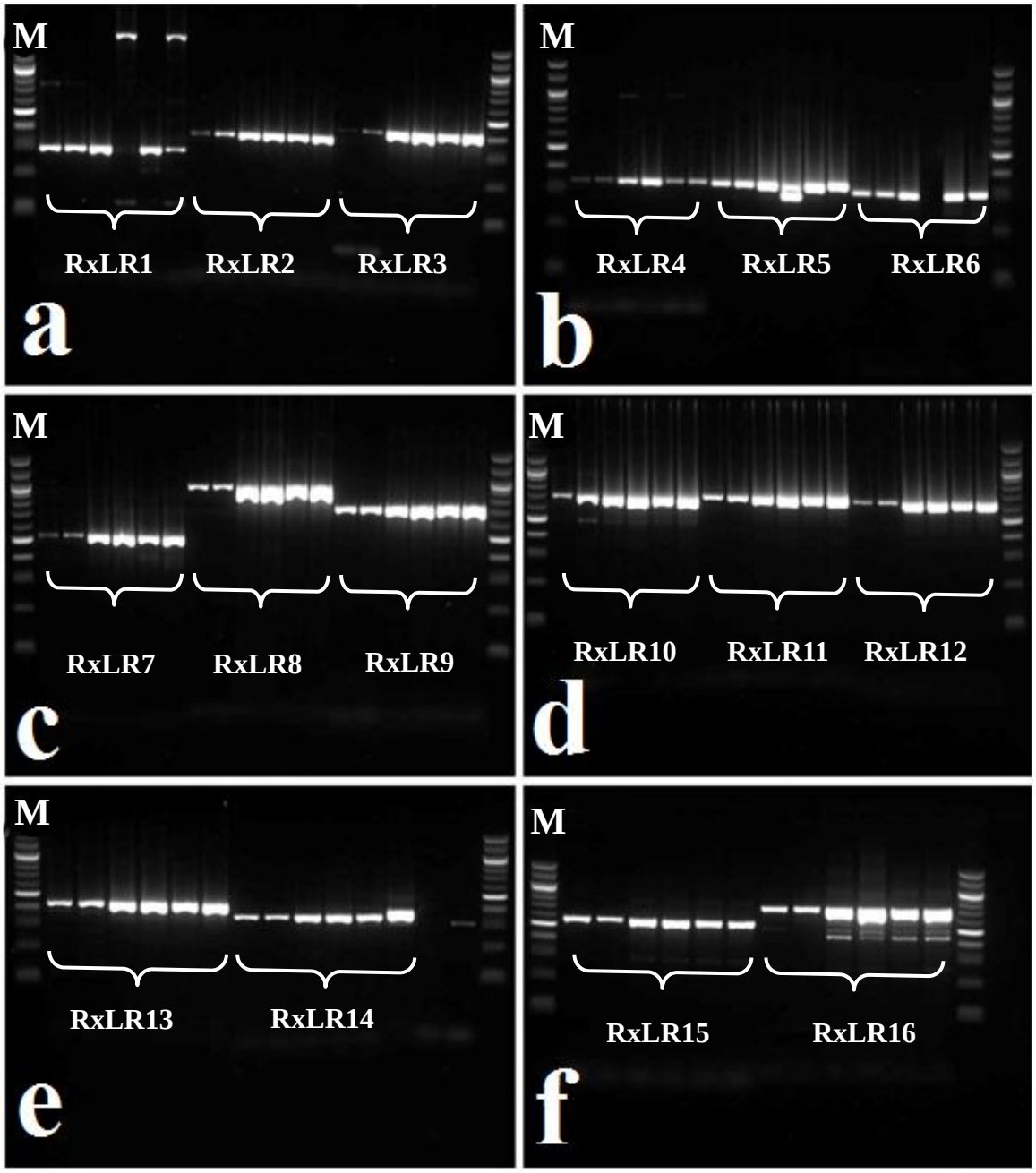



**Figure 5. 1** Gel electrophoresis photos of six single-spore isolates (from left to right, S1, S2, BL801, NL5, Bl805, BL806) amplified with 16 pairs of RxLR primers. Different RxLR amplification are shown in each photograph including (a) RxLR1, 2 and 3; (b) RxLR 4, 5 and 6; (c) RxLR7, 8 and 9; (d) RxLR10, 11 and 12; (e) RxLR13 and 14; and (f) RxLR16 and 17; DNA amplified from each isolate was loaded onto the gels in the order indicated above, M=DNA marker, faint bands were not scored.

**Table 5. 3** Summary of products from PCR amplification of single spore isolates with 16 pairs of RxLR primers. x indicates where a band was observed at a specific size of PCR product. Only bright bands were scored.

| RxLR | Size (bp) | Single Spore Isolates | | | | | |
|---|---|---|---|---|---|---|---|
| | | 801 | 806 | NL5 | 805 | S1 | S2 |
| 1 | 1500 | | x | x | | | |
| | 300 | x | x | | x | x | x |
| 2 | 350 | x | x | x | x | x | x |
| 3 | 400 | x | x | x | x | x | x |
| 4 | 320 | x | x | x | x | x | x |
| 5 | 320 | x | x | x | x | x | x |
| | 250 | | | x | | | |
| 6 | 300 | x | x | x | | x | x |
| 7 | 500 | x | x | x | x | x | x |
| 8 | 1000 | x | x | x | x | x | x |
| 9 | 750 | x | x | x | x | x | x |
| 10 | 700 | x | x | x | x | x | x |
| 11 | 700 | x | x | x | x | x | x |
| 12 | 650 | x | x | x | x | x | x |
| 13 | 420 | x | x | x | x | x | x |
| 14 | 350 | x | | x | x | x | x |
| 16 | 550 | x | x | x | x | x | x |
| 17 | 700 | x | x | x | x | x | x |

### 5.3.2 Results of field-collected isolates tested with RxLR primers

The products of PCR amplification of the field-collected isolates with 16 pairs of RxLR primers were electrophoresed on 1.5 % agarose gels. The gel loading order of the products and their IBEB codes are summarized in Table 5.4.

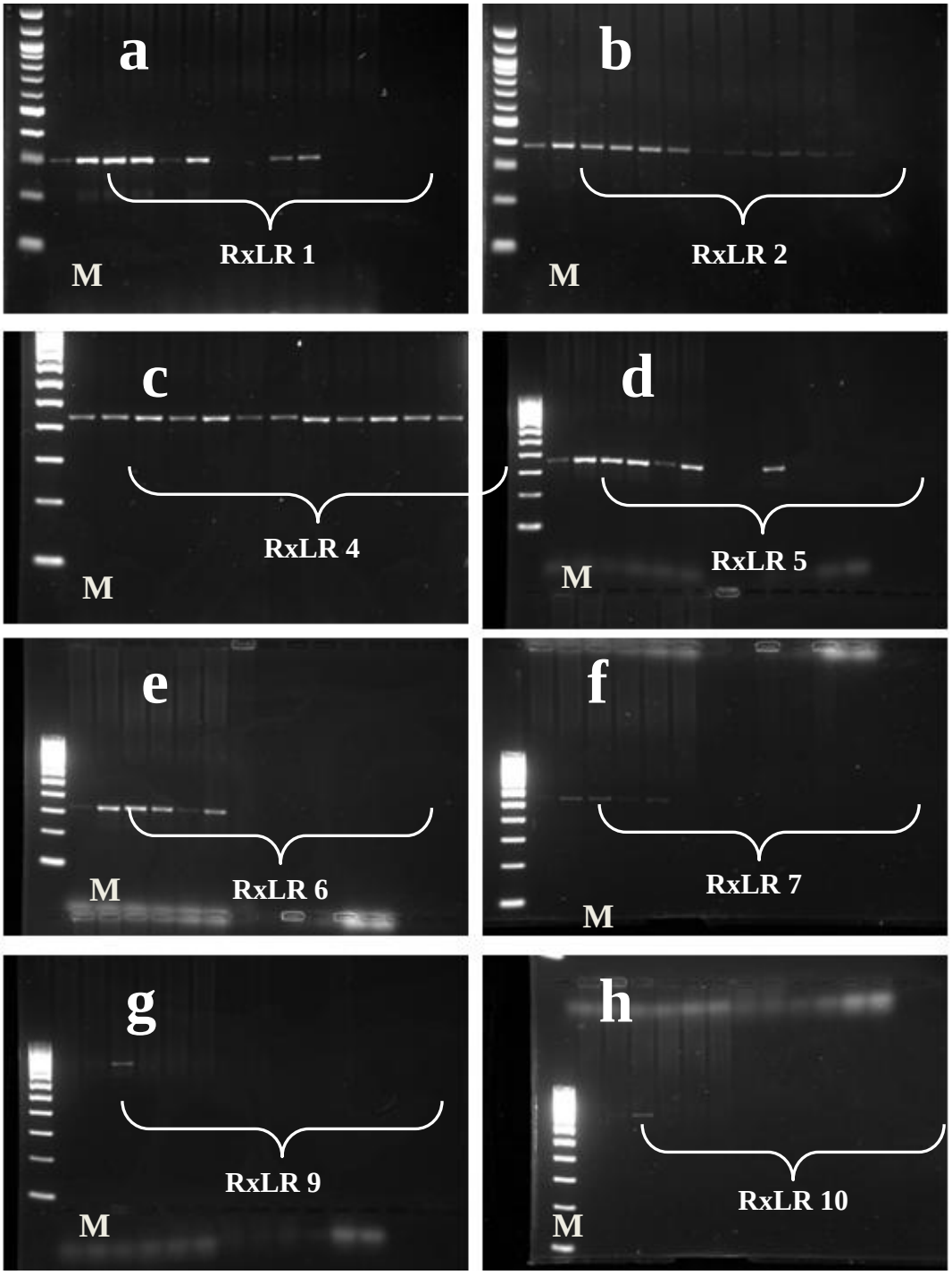
**Table 5.4** Summary of gel loading order and IBEB code of *B. lactucae* field-collected isolates.

| Sample Year | Gel loading order | Isolates | IBEB code |
|---|---|---|---|
| 2010 | 1 | L1 | EU-A 27/63/10/01 |
| | 2 | L2 | EU-A 59/63/10/00 |
| | 3 | L3 | EU-A 59/63/11/00 |
| | 4 | L4 | EU-A 59/31/10/00 |
| | 5 | L5 | EU-A 59/63/11/01 |
| | 6 | L6 | EU-A 59/63/02/01 |
| | 7 | Li 16 1st | EU-A 27/63/02/01 |
| | 8 | 7RMUS | EU-A 27/27/10/01 |
| | 9 | 7RMUS1 | EU-A 59/63/40/01 |
| | 10 | 7RMUS2 | EU-A 59/63/36/01 |
| | 11 | 16LGATT1 | EU-A 27/27/10/01 |
| | 12 | 16LGATT3 | EU-A 59/31/08/01 |
| 2009 | 1 | Site F55 Lignial | EU-A 57/63/08/00 |
| | 2 | Caugin 4 | EU-A 59/63/12/01 |
| | 3 | Iceberg diamond | EU-A 57/63/42/01 |
| | 4 | UK MUL1-15 (4)a | EU-A 19/56/00/01 |
| | 5 | UK MUL1-15 (4) | EU-A 51/51/03/00 |
| | 6 | Unknown | EU-A 59/63/10/01 |
| | 7 | 02354915 | EU-A 59/63/11/01 |

#### 5.3.2.1 Results of field samples collected in 2010

The results of gel electrophoresis of PCR amplified products of field samples with 13 pairs (RxLR 3,8,15 didn't amplify any product of these samples, therefore they are not shown in Figure 5.2) of RxLR primers are shown in Figure 5.2 (a) to (m). In Figure 5.2 (a) Li16 1st and 7RMUS could not be amplified by RxLR1. In Figure 5.2 (b), RxLR2 amplified a 350 bp product with each isolate. In Figure 5.2 (c) RxLR4 amplified a 320 bp product for each isolate. In Figure 5.2 (d) RxLR 5 did not amplify isolates Li 161$^{st}$ 7RMUS 7RMUS2 16LGATT1 and 16LGATT3. In Figure 5.2 (e)

RxLR6 primers only amplified 300 bp products of isolates L1 L2 L3 L4 L5 and L6. In Figure 5.2 (f) RxLR7 primers only amplified 500 bp product of isolates L1 L2 L3 L4 and L5, In Figure 5.2 (g) RxLR9 primers only amplified 7500 bp product of isolates L2 and L3. In Figure 5.2 (h) RxLR10 primers only amplified 700 bp products of isolates L2 and L3. In Figure 5.2 (i) RxLR11 primers did not amplify isolates L4 and L6 but amplified the others around 700 bp. In Figure 5.2 (j) RxLR12 primers did not amplify isolates L4 and L6 but amplified the others around 650 bp. In Figure 5.2 (k) RxLR13 primers amplified 420 bp products of each isolate. In Figure 5.2 (l) RxLR14 primers amplified 350 bp products of each isolate. In Figure 5.2 (m) RxLR17 only amplified five isolates: L2, L6, 7RMUS1, 7RMUS2, 16LGATT1 and 16LGATT3.


a
RxLR 1
M
b
RxLR 2
M
c
RxLR 4
M
d
RxLR 5
M
e
M
RxLR 6
f
RxLR 7
M
g
RxLR 9
M
h
RxLR 10
M

(Figure 5.2)

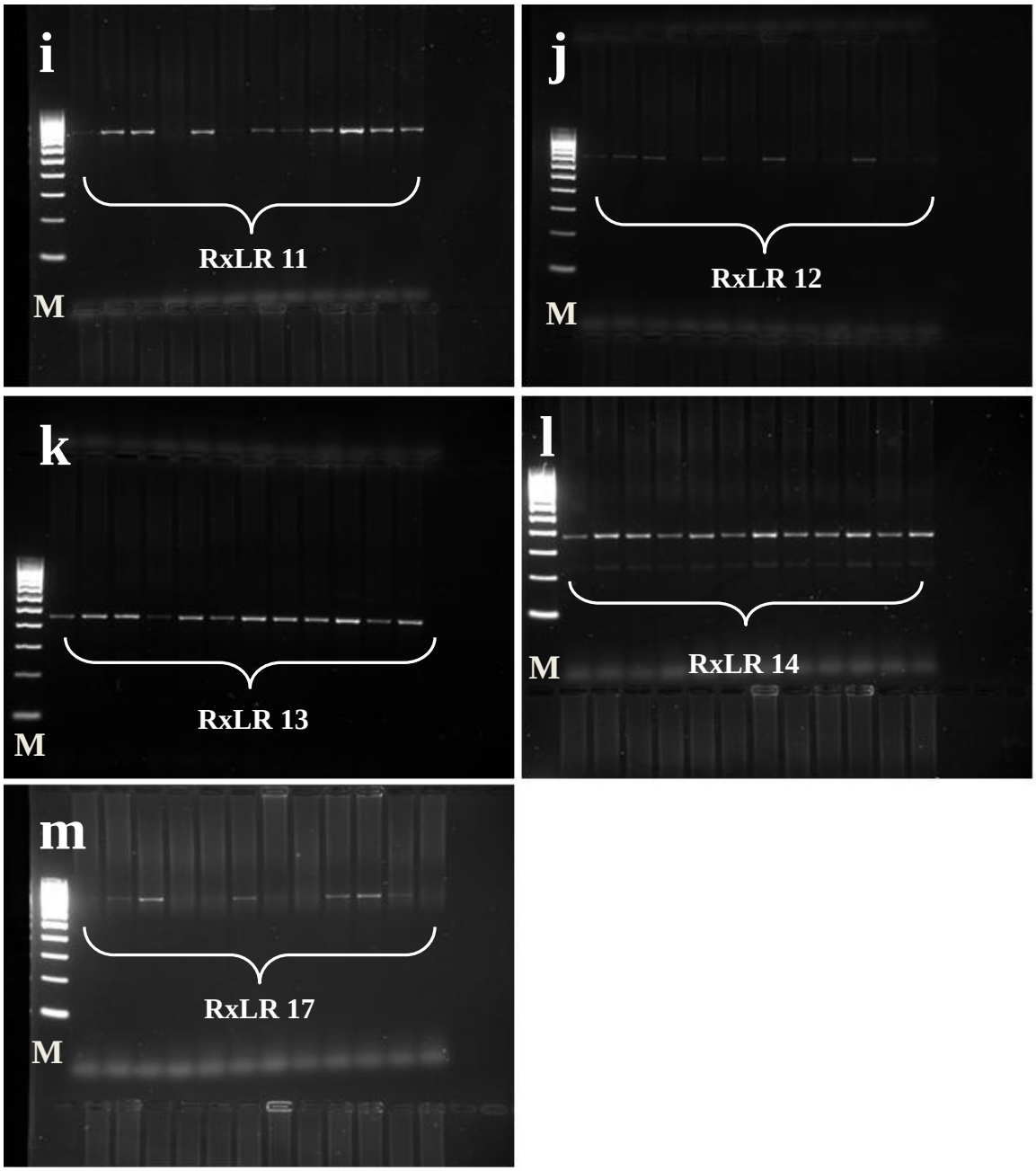
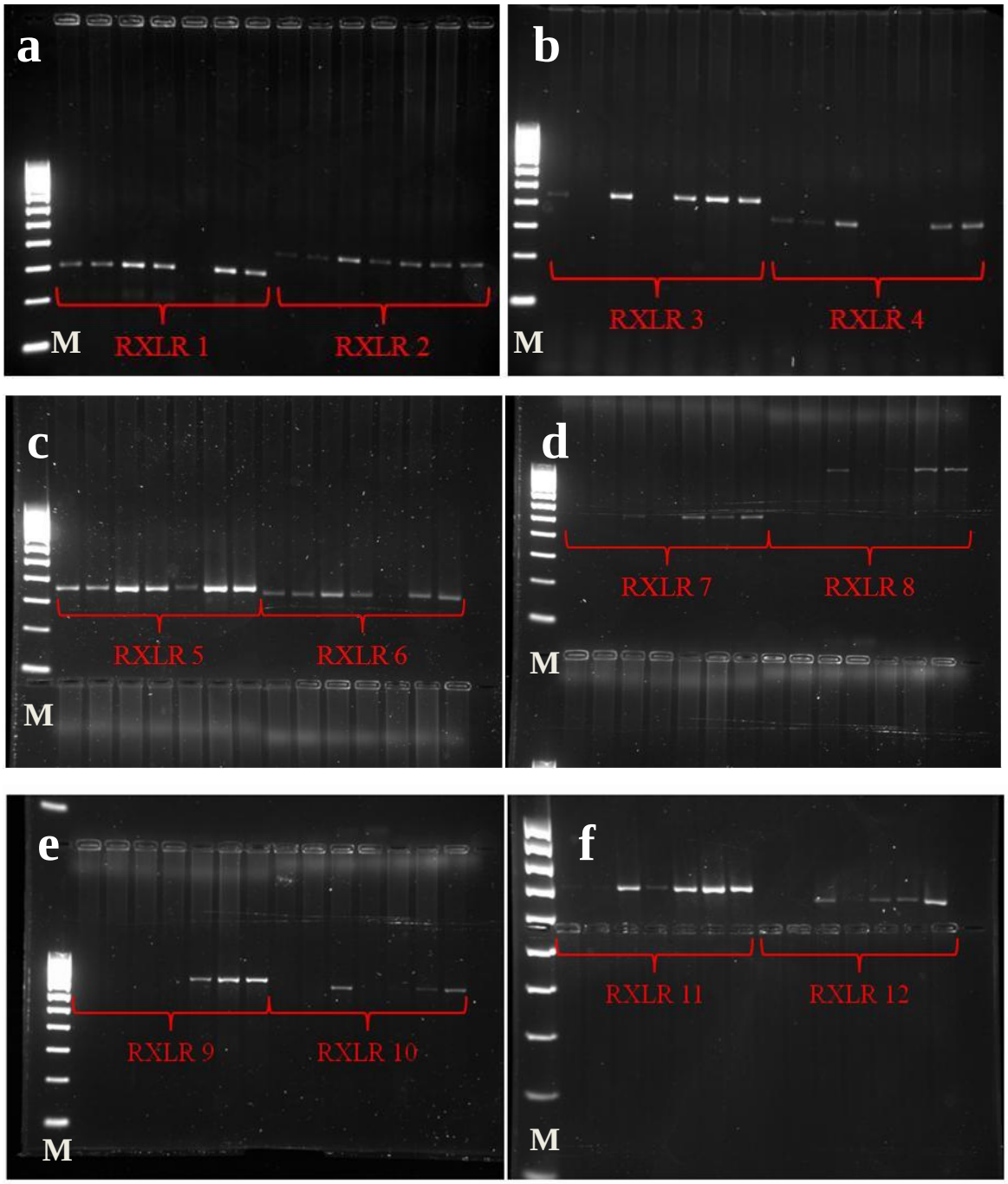



**Figure 5. 2** Gel eletrophoresis photos of ten field samples collected in 2010 amplified with RxLR primers. (a) RxLR1, product sizes are around 300 bp; (b) RxLR2, ca. 400 bp; (c) RxLR4, ca. 320 bp; (d) RxLR5, ca. 320 bp; (e) RxLR6, ca. 300 bp; (f) RxLR7, ca. 500 bp; (g) RxLR9, ca. 750 bp; (h) RxLR10, ca. 700 bp; (i) RxLR11, ca. 700 bp; (j) RxLR12, ca. 650 bp; (k) RxLR13, ca. 420 bp; (l) RxLR14, ca. 350 bp; (m) RxLR17, ca. 700 bp; DNA amplified was loaded onto the gels in the order indicated in Table 5.4, M=DNA marker. RxLR 3,8,15 didn't amplify these samples were not present.

#### 5.3.2.2 Results of field samples collected in 2009

The products of PCR amplification of the field isolates collected in 2009 with 16 pairs of RxLR primers were electrophoresed on 1.5 % agarose gels. The gel loading order of the samples can be found in Table 5.4.

The results of RxLR PCR amplification of field samples collected in 2009 are shown in Figure 5.3 (a) to (h) below. In Figure 5.3 (a) RxLR1 amplified the isolates around 300 bp except UK MUL1-15(4), RxLR 2 amplified the isolates around 350 bp. In Figure 5.3 (b) RxLR3 amplified the isolates around 400 bp except Caugin4 and UK MUL1-15(4)a, RxLR4 amplified the isolates around 320 bp except UK MUL1-15(4) and UK MUL1-15(4)a. In Figure 5.2 (c) RxLR5 amplified the isolates around 320 bp, RxLR6 amplified 300 bp product of the isolates except UK MUL1-15(4). In Figure 5.3 (d) RxLR7 amplified around 500 bp product of the isolates except SiteF55Liginal Caugin4 and UK MUL1-15(4)a, RxLR8 amplified around 1000 bp product of the isolates except SiteF55Liginal Caugin4 and UK MUL1-15(4)a. Figure 5.3 (e) shows RxLR9 only amplified isolates UK- MUL1-15(4)a, isolate6 and 02354915 around 750 bp, RxLR10 only amplified isolates Iceberg diamond isolate 6 and 02354915 around 700 bp. Figure 5.3 (f) shows RxLR11 amplified the isolates around 700 bp except Caugin4, RxLR12 did not amplify isolates SiteF55Liginal, Caugin, or UK MUL1-15(4). It amplified the other isolates around 650 bp. In Figures 5.3 (g) RxLR13 and 14 amplified the isolates around 420 bp and 320 bp respectively. In Figure 5.3 (h) RxLR16 did not amplify any isolate, RxLR17 only amplified isolate 02354915 around 700 bp.


a
M
RXLR 1
RXLR 2
b
M
RXLR 3
RXLR 4
c
M
RXLR 5
RXLR 6
d
M
RXLR 7
RXLR 8
e
M
RXLR 9
RXLR 10
f
M
RXLR 11
RXLR 12

(Figure 5.3)

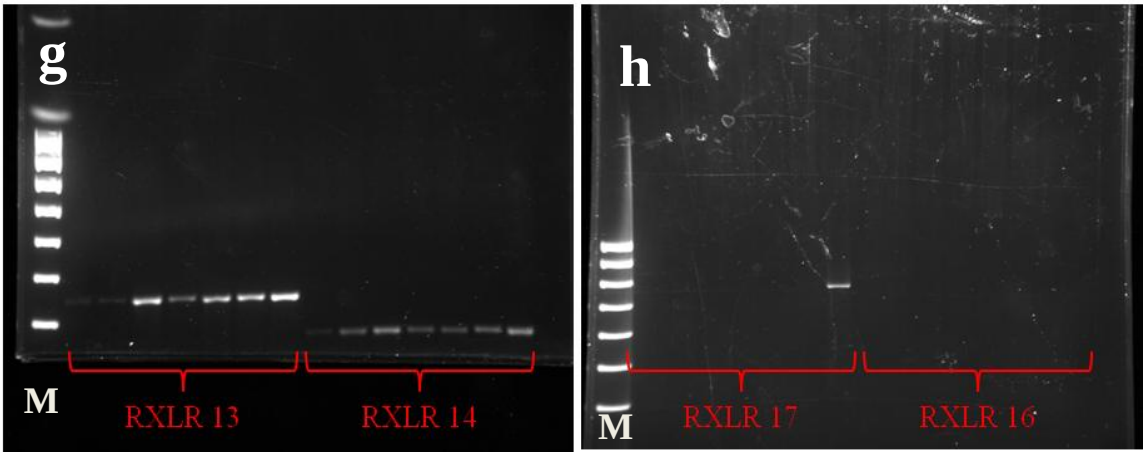



**Figure 5.3** Gel eletrophoresis photos of field samples collected in 2009 amplified with RxLR primers (Table 5.4). (a) RxLR1 and 2; (b) RxLR3 and 4; (c) RxLR5 and 6; (d) RxLR7 and 8; (e) RxLR9 and 10; (f) RxLR11 and 12; (g) RxLR13 and 14; and (h) RxLR16 and 17; DNA amplified was loaded onto the gels in the order indicated in Table 5.4, M=DNA marker.

The results of PCR amplification of both 2010 and 2009 collected field samples with RxLR primers have been summarized in Table 5.

**Table 5. 5** Summary of results of gel electrophoresis of the products from PCR amplification of the field isolates with 16 pairs of RxLR primers (1= one band present, 0 = no band present).

| | Size | Field samples collected in 2010 | | | | | | | | | | | | Field samples collected in 2009 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RxLR | (bp) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 1 | 300 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 2 | 350 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 |
| 4 | 320 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 5 | 320 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 300 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 7 | 500 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| 8 | 1000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| 9 | 750 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| 10 | 700 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 |
| 11 | 700 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 12 | 650 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| 13 | 420 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | 350 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16 | 550 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 700 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

### 5.3.3 Sequencing of selected RxLR PCR amplification products

To determine whether differences existed between PCR products from different isolates, which could not be seen by gel electrophoresis, sequencing was carried out on selected samples. Thus, the 320 bp band obtained from isolates BL801, BL806 and NL5 after amplification with RxLR5 primers were cut from the gel, cloned and sequenced. Some SNPs were found between the isolates and these are shown in Table 5.6. Isolate NL5 shows G at position 64, C at position 99 and T at position 100 while the other two isolates are A, T and C. Isolate BL801 shows A at position 217 whereas the other two isolates are G. Isolate BL806 shows C at position 51, G at position166 and C at position261 while the other are T, A, and G respectively. The base at 273 was absent in NL5 but shows C with BL801 and T with BL806.

**Table 5. 6** SNP differences between iaoltes NL5, BL801 and BL806 (sequences amplified by RxLR5).

| **Isolate/Loci** | **51** | **64** | **99** | **100** | **166** | **175** | **217** | **261** | **273** |
|---|---|---|---|---|---|---|---|---|---|
| NL5 | T | G | C | T | A | C | G | G | - |
| BL801 | T | A | T | C | A | C | A | G | C |
| BL806 | C | A | T | C | G | A | G | C | T |

### 5.3.4 Comparison of systemic detection between IBEB phenotypic method and RxLR genotypic method

BL801 DNA extracted from cultivars LS-102 and Colorado (incompatible cultivars to BL801 in IBEB system) show bands in Figure 5.4 (a). Cultivars PIVT1309, LS-102 and Ninja (incompatible cultivars to BL801 in IBEB) show bands in the second experiment (sampled on the 4$^{th}$ day after inoculation) in Figure 5.5. In Figure 5.5 (d) cv. PIVT1309, cv. Colorado, cv. Ninja and cv. Argeles show bands on the second experiment (sampled on the 7$^{th}$ day after inoculation).

BL806 DNA extracted from cultivars PIVT1309, LSE18 and Ninja (incompatible cultivars to BL806 in the IBEB system) show bands on the first experiment in Figure 5.4 (b). In Figure 5.5 (c), cv. LSE/18 and cv. LS102 and cv.Colorado show a band in the 2$^{nd}$ Experiment A. Only cultivar Dandie shows band in the second 2$^{nd}$ Experiment B in Figure 5.5 (d).

**Table 5. 7** The comparison of results between IBEB differential test set and RxLR primer. *= cultivar was not inoculated, + = symptom present (compatible reaction), —= symptom absent (incompatible reaction), Exp.=experiment. For the results of BL801 1st and 2nd experiment were tested with primer RxLR1, while BL806 were tested with primer RxLR7.

| Isolate | IBEB lettuce cultivars | 1.Lednicky | 2. UC DM2 | 3.Dandie | 4. R4T57D | 5. Valmaine | 6. Sabine | 7. LSE57/15 | 8. UC DM10 | 9. Capitan | 10. Hilde II | 11. Pennlake | 12. UC DM14 | 13.PIVT 1309 | 14.LSE18 | 15.LS102 | 16Cororado | 17 -Ninja | 18Discovery | 19 Argeles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bl801 | IBEB | + | + | + | + | + | + | + | + | + | + | + | + | — | + | — | — | — | — | — |
| | 1st Exp. | + | + | + | + | + | + | + | + | + | + | + | + | — | + | + | + | — | — | — |
| | 2nd Exp. A | * | * | * | * | * | * | * | * | * | * | * | * | — | * | + | — | + | — | — |
| | 2nd Exp. B | * | * | * | * | * | * | * | * | * | * | * | * | + | * | — | + | + | — | + |
| Bl806 | IBEB | + | + | — | + | + | — | + | + | + | + | + | + | — | — | — | — | — | — | + |
| | 1st Exp. | + | + | — | + | + | + | + | + | + | + | + | + | + | + | — | — | + | — | + |
| | 2nd Exp. A | * | * | — | * | * | — | * | * | * | * | * | * | — | + | + | + | — | — | * |
| | 2nd Exp. B | * | * | + | * | * | — | * | * | * | * | * | * | — | — | — | — | — | — | * |

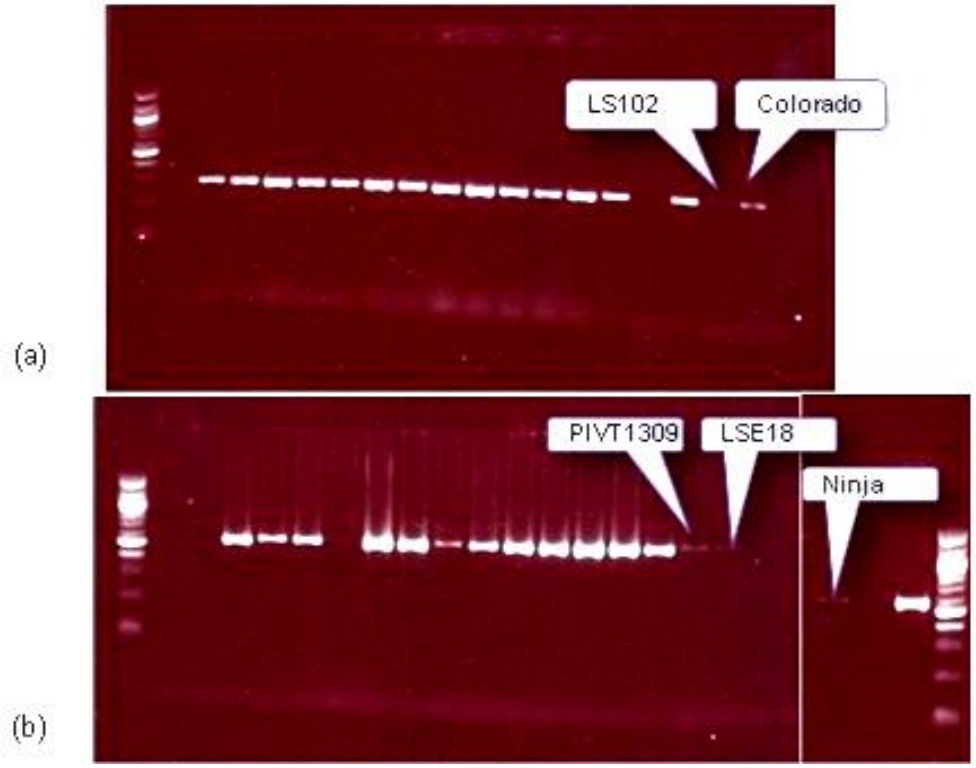



**Figure 5. 4** Gel electrophoresis results of infected IBEB both + and - cultivars amplified with RxLR primers in the first test. (a) BL801 amplified by RxLR1; (b) BL806 amplified by RxLR7.

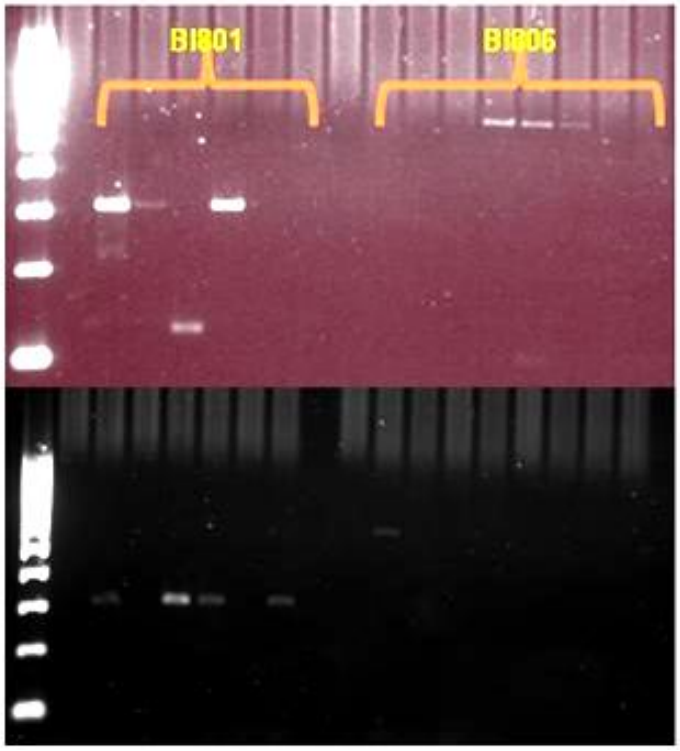



**Figure 5. 5** Gel electrophoresis results of infected IBEB resistant cultivars (scored as - in IBEB system) amplified with RxLR primers in the 2nd Experiment. (c) 2nd Experiment A sampled on the 4th day post inoculation; (d) 2nd Experiment B sampled on the 7th day post inoculation.

## 5.4 DISCUSSION

### 5.4.1 The application of RxLR effector markers to the differentiation of *B. lactucae* isolates

The results of PCR amplification of single spore isolates with RxLR primers show that this method is promising for the differentiation of *B. lactucae* isolates. For example, the RxLR1 primers amplified a 300 bp product from isolates BL801, Bl805, S1 and S2 whereas a 1500 bp product was amplified from isolates BL806 and NL5/2. Since these differences can be observed from gel electrophoresis it is easy to distinguish between these two groups of isolates. Interestingly, BL801 S1 and S2 are UK isolates, whereas the others are not. Only the non-UK isolates (except Bl805) amplify a 1000 bp product. It is possible that the polymorphisms are related to geographic difference. Further testing with isolates from a range geographic location would be required. Thus, RxLR primers may have the potential to be used as markers to identify isolate origin. But this would require further investigation.

Although no polymorphisms were observed when these isolates were amplified with the rest of the 15 pairs of RxLR primers, differences might exist which could not be seen by gel electrophoresis. The sequencing results of the PCR products amplified from isolates BL801 BL806 and NL5 with the RxLR 5 primers show that, although no polymorphism were visible by gel electrophoresis, SNPs were present between the isolates. This result suggests that further SNPs may exist between isolates which could be discovered by sequencing the products from amplification with other RxLR primers. Marker development on these SNPs could be used to differentiate between isolates and Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, USA)

could be used to test the isolates (Clewes *et al*., 2007) and design specific primer probes. These results are similar to those observed in isolates of *H. parasitica* (the brassica downy mildew pathogen). Once the primers for each SNP have been designed, infected plant samples can be analyzed by Melt Curve Analysis. The energy required to break the base-base hydrogen bonding between two strands of DNA is dependent on their length, GC content and their complementarily. Different SNPs will give different melting curves. In this way, isolates that have different SNPs can be differentiated by comparing the melting curves of amplified RxLRs. Such a method would enable many samples to be processed and analysed rapidly for both experiments and for diagnostic purposes. Thus differentiating SNPs between isolates is worth further investigation.

More polymorphisms were seen between the field isolates than the single spore isolates when RxLR PCR products were viewed by gel electrophoresis. However, field isolates are likely to be comprised of a mixture of several pure isolates. It may therefore be necessary to single spore the samples in the future work to examine whether the polymorphisms are consistent. If they were, then the bands could be sequenced and any differences could be used to design race specific molecular markers.

High polymorphism was found in SNP in effector candidates between seven *Hyaloperonospora arabidopsidis* (*Hpa*) isolates (Fabro *et al.*, 2011). The sequencing of the Hpa isolate Emoy2 genome revealed its potential to encode at least 140 RxLRs (or HaRxLs) and 56% of these showed polymorphism between 1 to 10 SNPs. From the results of this HaRxls study, RxLR sequences of *B. lactucae* might exist SNP

differences that could not be observed on gel. Therefore further study in investigating *B. lactucae* RxLR SNP polymorphism might be worthwhile.

### 5.4.2 The comparison between IBEB phenotypic cultivar identification and RxLR genotypic identification

The results of the comparison of the IBEB phenotypic and RxLR genotypic methods of identification show that the IBEB method may not be 100% reliable using the method for denominating isolates of *B. lactucae*. In the first experiments, DNA from the interaction between isolate BL801 and the IBEB cultivars had been amplified with RxLR1. Although the IBEB method showed cultivars LS102 and Colorado to be incompatible, bands indicating the presence of BL801 were observed by gel electrophoresis. Similarly, the interactions between isolate BL806 and cultivars PIVT1309, LS102 and Ninja, which are incompatible according to the IBEB test, gave bands after amplification with RxLR7. This again indicates the presence of the pathogen. Thus the molecular markers suggest that the pathogen does grow on cultivars designated 'incompatible' by the IBEB method.

To confirm this result was not affected by contamination by spores from the adjacent compatible cultivars, the second experiment was designed to exclude the compatible cultivars. The results of 2$^{nd}$ Experiment A show that isolate BL801 was detected on cultivars LS102 and Ninja. Isolate 806 was detected on cultivar LSE18. In the 2$^{nd}$ Experiment B, isolate BL801 was detected on cultivars PIVT1309, Colorado, Ninja and cv. Argeles; isolate BL806 was detected on cv. Dandie. Thus the pathogen existed in the cultivars without visible symptoms being present.

One explanation of these results might be systemic infection of cultivars by *B. lactucae*. Variation in systemic infection of brassica seedlings by *H. parasitica* has been observed. Resistance of a host to a pathogen depends on the interaction between the avirulence factor of the pathogen and the resistance factor of the host. Thus the degree of resistance, which is caused by specific *R* gene/*Avr* gene interactions may vary according to which genes are present. Such variation in resistance will not always be visible phenotypically. There may be considerable systemic development of the pathogen within the host that may be detrimental, but may not be sufficient to lead to sporulation. This may lead to inaccurate identification of an isolate by the IBEB method, since two isolates may both be scored as incompatible on a particular cultivar, but in fact, one may grow substantially whereas the other may not.

A more accurate way of identifying an isolate would be to be able to detect which avirulence genes are present. However, we do not currently have any markers derived from avirulence gene products. Nevertheless, since it has been found that avirulence factors exported from oomycete pathogens into their hosts usually contain an RxLR motif (Bhattacharjee *et al*., 2006; Rehmany *et al*., 2005; Whisson *et al*., 2007), it is likely that any marker based on such RxLRs may be closely related to the virulence/avirulence of the pathogen and so may be more reliable for isolate identification.

Since these experiments have shown that there may be systemic development of the pathogen in the host in incompatible interactions, it was decided that this should be investigated further. Therefore microscopy observations and qPCR detection of pathogens have been developed and will be described in the following chapter.

# CHAPTER 6 THE INTERACTION BETWEEN *BREMIA LACTUCAE* AND IBEB DIFFERENTIAL LETTUCE CULTIVARS

## 6.1 INTRODUCTION

The relationship between plants and oomycetes is very complex and heterogeneous, and has been studied for decades, increasing our understanding on this subject. However, more information is still needed to obtain a complete view of their interaction (Lebeda *et al*., 2008b). Crute (1992c) and Lebeda *et al*. (2008b) regarded the *Lactuca* spp. - *B. lactucae* relationship as one of the most important pathosystems. It provides a model system for studies of the recognition processes underlying race specific resistance and host-parasite specificity as well as variability of plant defence mechanisms (Crute and Lebeda, 1981, 1983; Lebeda *et al.*, 2008b).

### 6.1.1 The categories of the resistance interaction

In plant – pathogen interactions there are three most common categories: race-specific resistance, race non-specific resistance and field resistance (Lebeda *et al*., 2008b). Race-specific resistance is based on a gene-for-gene relationship between host and pathogen, which has been described in Chapter 1 and which is the principle for recent studies of the interaction between *B. lactucae* and host lettuce cultivars. Race non-specific resistance relies on passive plant defence and is a combination of different types of defence that are similar for all plant-pathogen interactions. This resistance is conferred by multiple genes that encode proteins with a diversity of functions within the plant-pathogen interaction (Agrios, 1997; Dickinson and Lucas, 1982; Lebeda *et al.*, 2008b). Field resistance is found in mature plants grown in the field, which will reduce the susceptibility of the lettuce to natural *B. lactucae* infection (Lebeda *et al*., 2008b; Grube and Ochoa, 2005). This type of resistance can be either race-specific or race non-specific.

According to the gene-for-gene theory, race-specific resistance is determined by dominant resistance genes (R genes; or referred to as *Dm* genes in lettuce) in the host and complementary dominant avirulence genes (*Avr* gene or v-factor) in the pathogen (Crute, 1992b). More than 45 host race-specific *Dm* genes and their complementary *Avr* gene or v-factors have been predicted in the lettuce downy mildew pathosystem (Lebeda *et al.*, 2006). At least 15 *Dm* genes have been characterized in lettuce species which are *Dm*1, *Dm*2, *Dm*3, *Dm*4, *Dm*5/8, *Dm*6, *Dm*7, *Dm*10, *Dm* 11, *Dm*12, *Dm*13, *Dm*14, *Dm*15, *Dm*16 and R18 (review from Lebeda *et al*., 2008b) and two other related *R* factors R36 and R37 which are found in *L. sativa* but originate from *L. saligna* (Michelmore *et al*., 2005).

The hypersensitive response is considered as a programmed cell death, triggered by the resistance reaction (Kamoun *et al*., 1999), and it is important for race-specific resistance of lettuce to *B. lactucae* (Lebeda *et al*., 2001). This plant resistance response has been reported to happen in some compatible and or non-host interactions, but only on a small scale (Lebeda *et al.*, 2002). The hypersensitive response (cell death) normally occurs within few hours after the pathogen contact with the host plant (Agrios, 1988). Not only the incompatible reaction showed hypersensitive cell death, it also has been found in compatible combination (Viranyi and Blok, 1976). The irreversible membrane damage study of *B. lactucae* and lettuce interaction indicated that the timing of the hypersensitive cell death is determined by the resistance gene (Wood *et al*., 1988a) which means the timing of hypersensitive cell death varies depending on the interaction between *R* gene and *Avr* gene combination. Figure 6.1 is the hypersensitive reaction under light microscopy, shows as yellow necrosis of the lettuce epidermal cell. Primary and secondary vesicles

produced by the pathogen caused the cell death.

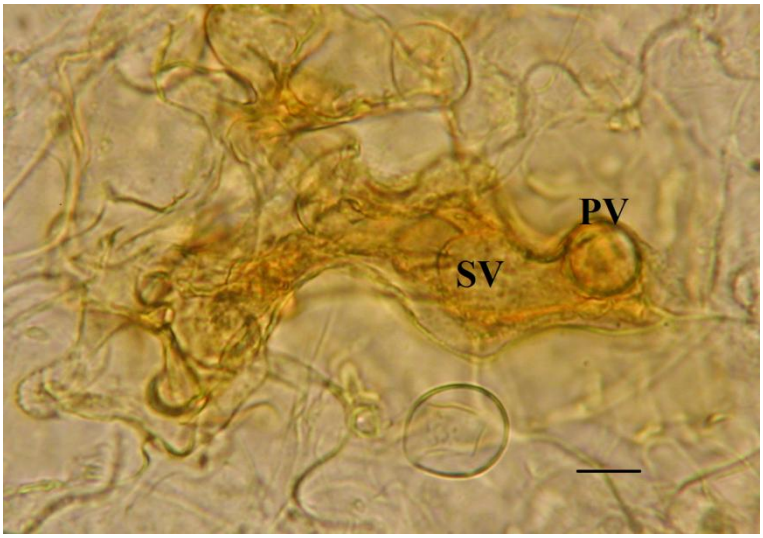



**Figure 6.1** Hypersensitive cell death response. PV: primary vesicle, SV: secondary vesicle, the bar corresponds to 15µm.

### 6.1.2 The infection structures of *B. lactucae*

The initiation of infection by an oomycete pathogen is the penetration of the plant surface. A few steps are needed prior to the penetration; adhesion of spores to the leaf surface, the formation of germ tubes and appressoria, and the penetration pegs (reviewed in Lebeda *et al.*, 2008b; Latijnhouwers *et al.*, 2003). Once the pathogen has overcome the barrier of the host cell wall, a primary vesicle is formed within the epidermal cell. This vesicle then swells to form the secondary vesicle from which hyphae develop and exit the cell. The hyphal branches elongate and penetrate more host cells, forming structures, called haustoria, which are responsible for taking nutrients from the host to enable growth and colonization by the pathogen.

In the lettuce - *Bremia* interaction the recognition of disease development is based upon the specific *Dm/Avr* gene combination (Mansfield *et al.*, 1997). Woods *et al.* (1988b) found that variation also existed between isolates in the timing of cell death,

as indicated by irreversible membrane damage, according to the *Dm/Avr* gene combination. An estimate of this timing was made by observing the degree of expansion of the primary vesicle or the initiation of the secondary vesicle. Thus isolates can be distinguished by using microscopy to identify differences between disease developments.

The first 24 to 48 hours after inoculation is the most important period for *B. lactucae* hyphal development (Lebeda *et al.*, 2008b). Therefore observation of the early stage of infection is also important for quantitative comparative studies between isolates.

### 6.1.3 The IBEB method and quantitative method to evaluate the interaction

In this project, a scoring system has been developed to differentiate the susceptible interactions (which have been described in Chapter 3), however it is not a quantitative method. Various non-genetic factors could affect the seedling susceptibility to *B. lactucae*, such as inoculum concentration, mineral nutrition, seedling vigour and age (Dickson and Crute, 1974, Michelmore and Crute, 1982; Norwood and Crute, 1984), as well as visual error.

The compatible and incompatible cultivar-isolate combinations are distinguishable by observing the presence or absence of the disease symptoms. The IBEB differential lettuce cultivar set is based on this specific interaction between host and pathogen to generate a + or - (compatible/incompatible) pattern to differentiate the isolates. However, this application solely pays attention to the appearance of the symptoms to identify the virulence; it is difficult to determine differences between the compatible interactions.

Therefore the IBEB set could not be used to investigate variation of susceptibility although this is considered important when studying resistance not readily attributed to major genes (Michelmore and Crute, 1982; Norwood *et al*., 1983b; Yuen and Lorbeer, 1983).

In Chapter 5, RxLR markers were determined that could detect *B. lactucae* DNA in IBEB resistant cultivars, indicating there was pathogen growth. In order to investigate these interactions further, a measureable and visible method might be necessary. Therefore microscopic observation was applied to quantify the variance of the infection structures between different isolate/cultivar combinations. The limitation of this traditional method of assessment, however, is that it is time consuming and prone to observer error. Therefore a more efficient and high throughput method is worthy of investigation.

Quantitative polymerase chain reaction (qPCR) is a sensitive specific and efficient method to detect pathogens *in planta*, especially in the early stages of infection when only very small amount of pathogen exists, which is difficult to quantify by traditional visual assessment (Eshraghi *et al*., 2011). qPCR quantification has been successfully used in other oomycete pathosystems such as *Phytophthora infestans*, *P. citricola* and *P. cinnamomi* (Böehm *et al.*, 1999; Schaad and Frederick, 2002; Eshraghi *et al*., 2011). In this project, qPCR quantification was developed for the study of the lettuce-*Bremia* interaction.

In this chapter in order to investigate the difference of resistance interactions with difference *Dm*/*Avr* combination (BL801 and BL806 with different cultivars), five

resistant cultivars were selected for microscopic observation. Secondary vesicle, hyphal development and cell death were recorded to evaluate the pathogen development. For the purpose of developing a rapid and sensitive molecular technique for the assessment of different *B. lactucae* - lettuce interactions, three experiments were performed on susceptible and resistant cultivars to compare the isolates BL801 and BL806. Spore per cotyledon unit weight count was a complementary physiological assessment for the qPCR experiments. ANOVA (analysis of variation) and correlation analysis were used to evaluate the results. The aim of these experiments was to look for rapid and reliable techniques to identify the variance of *B. lactucae.*

## 6.2. MATERIALS AND METHODS

### 6.2.1 Cultivars and isolates

*B. lactucae* isolates BL801 and BL806 were used to inoculate the lettuce cultivars in this test (details of the IBEB phenotypic classification of these two isolates can be found in Appendix 1, Table A1). Seeds of the cv. Cobham Green were used as the susceptible control in all experiments in this chapter and were supplied by Tozer Seeds Ltd. (Cobham, Surrey, UK).

**Microscopy experiment**: Resistant cultivars of *L. sativa* were chosen from the IBEB differential set for comparison with the susceptible control, cultivar Cobham Green (no known resistance genes). The chosen cultivars and their corresponding *Dm* genes and the set positions were as follows: PIVT 1309 (*Dm* 15), LS-102 (*Dm* 17), Colorado (*Dm* 18), Ninja (*Dm* 36) and Discovery (*Dm* 37).

**qPCR quantification experiment**: For both Experiment 1 and 2, susceptible cultivars UC DM2 (*Dm* 2), Valmaine (*Dm* 5/8), UC DM10 (*Dm* 10), Capitan (*Dm* 11), Pennlake (*Dm* 13) and UC DM14 (*Dm* 14) were used; resistant cultivars LS-102 (*Dm* 17), Colorado (*Dm* 18), Ninja (*Dm* 36) and Discovery (*Dm* 37) were used as well. In Experiment 3, the entire IBEB EU-A varieties were used for qPCR quantification for both *B. lactucae* isolates.

**Spore count per unit weight of cotyledons experiment**: It was a complementary test for qPCR quantification. In experiment 1 and 2, the same susceptible cultivars were used as qPCR experiments 1 and 2. In Experiment 3, additional cultivars were tested which are susceptible to one of the two isolates (see Appendix 1, Table A1).

### 6.2.2 Microscopy experiments

**Inoculation:** Cotyledons of lettuce cultivars were cut and the abaxial surface of the petiole were placed on moist filter paper (Whatman, England) in a petri dish (Greiner Bio-one, Hungary) (Figure 6.2). The cotyledons were inoculated with droplet of spore suspension (approximate 500 spores were inoculated on the cotyledon surface). The petri dish was sealed in a transparent bag (Figure 6.3) and placed in PEL (plant environment laboratory) room incubated at 15°C.

**Fixation and examination for light microscopy:** This method was based on what has been described by Wood *et al*. (1988b). Cotyledons were picked and stored in 100% methanol overnight to be fixed and de-colorized. They were then cleared in chloral hydrate (1g $ml^{-1}$ distilled water). After the tissue became transparent and the pathogen structures were clearly seen, cotyledons were mounted in 50% glycerol

onto glass microscope slides, a cover slip was placed on top of the tissue to have semi-permanent slides. These slides were examined using a Carl Zeiss light microscope (Germany).

**Timed observation plan and statistical analysis:** To compare the development of *B. lactucae* isolates in host tissue of different lettuce cultivars, three cotyledons of each cultivar were petiole inoculated with each isolate (BL801 or BL806) and samples were taken at seven times (24, 48, 72, 96, 120, 144 and 168 hours after inoculation) for microscopy examination. The numbers of secondary vesicles, total hyphae and hypersensitive cells that appeared dead were counted for each combination of host, isolate and time point. The numbers of short hyphae (<50 µm), medium hyphae (>50 µm, <100 µm) and long hyphae (>100 µm) were counted. Percentages of the infection structures (secondary vesicle without further development of hypha, short hypha, medium hypha and long hypha) and cell death in total infection (formation of secondary vesicle) were calculated (see Appendix 1, Table A2).

Each sample (cotyledons) of this observation experiment was classified by 'day/block /leaf' (block stands for each replicate) for statistical analysis. The value of the count was analyzed by ANOVA using general analysis of variance design in GenStar 12$^{th}$ edition (VSN International Ltd., Hemel Hempstead, UK, 2009). Data were subjected to square root transformation to stabilize the variance, leaf nested within block nested within day. The means of transformed data of numbers of secondary vesicles, hyphae and cell death were analysed to compare the differences between isolates and the isolate-cultivar interaction.

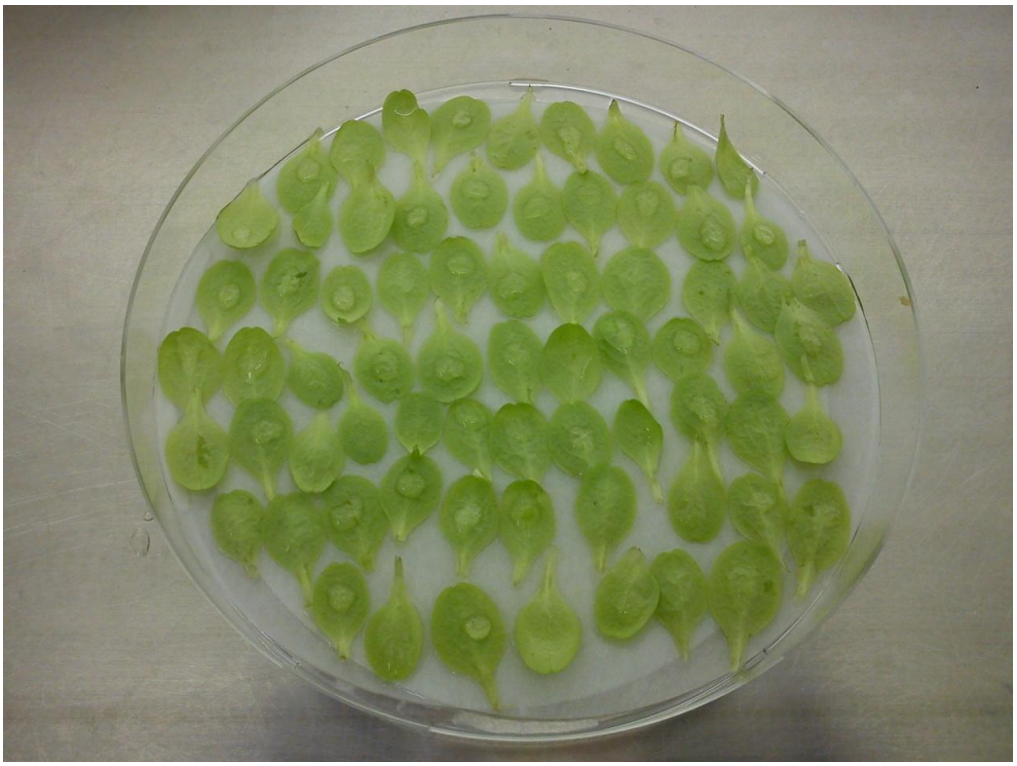


**Figure 6. 2** The droplet inoculation on lettuce cotyledons

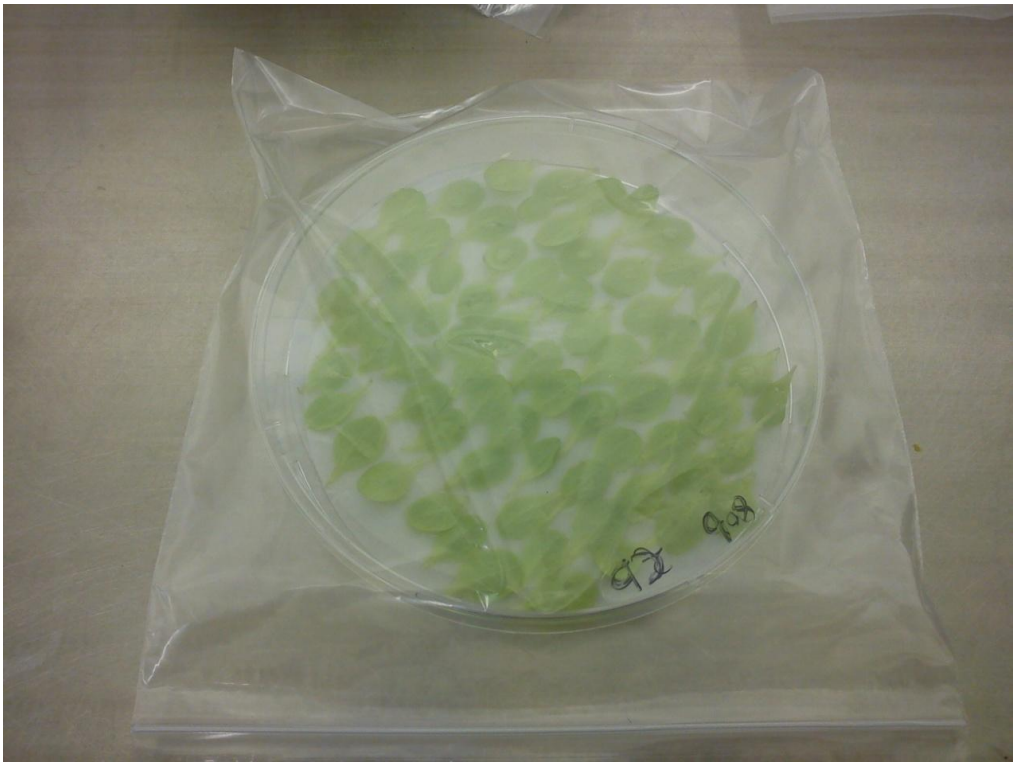


**Figure 6. 3** The inoculated cotyledons were sealed in a transparent bag.

### 6.2.3 qPCR quantification

**Inoculation:** In both susceptible and resistance tests, spray inoculation was applied (detail in Section 2.2.2.2). Cultivar Cobham Green and the chosen cultivars were sown separately in plastic pots with moistened peat/sand mix covered by vermiculite on top. Then susceptible cultivars and resistant cultivars were sealed separately in a propagation box with cultivar Cobham Green, and were cultured in a growth room at 18ºC for seven days. The spore suspension was quantified before inoculation, and sprayed evenly on lettuce cultivars, using a 50ml spray bottle (Boots, UK). The inoculated samples were cultured in a PEL room at 15ºC.

**Sampling:** For susceptible cultivars, a seven-day time course of sampling was applied. Eight or ten cotyledons were cut from each cultivar every day post inoculation until the seventh day. For resistant cultivars, eight or ten cotyledons were cut from each cultivar at the first, fifth and seventh day post inoculation. For experiment 3, the cotyledons were sampled on the fifth day post inoculation. For spore count per unit weight of cotyledons experiments, cotyledons were only sampled on the seventh day after inoculation.

**Spore count per unit weight of cotyledons experiment:** The cotyledons were weighed before spore count. The count of spores in the sample was divided by the cotyledons weight to obtain the result (spores per gram of cotyledons).

**DNA extraction:** Samples were freeze dried prior to being disrupted by using Btissue mill (see Section 2.3.2 for detail). DNA was extracted by using Qiagen Dneasy Mini Plant Kit (see Section 2.3.3 for detail). Extracted DNA was stored at

-20ºC.

**Primer design:** Several primer sets had been tested to improve the efficiency of the calibration curve. The original *Bremia* specific primer Br1234/Br1586 (Choi *et al.*, 2007) amplifies a 350 bp product which was too large for qPCR. Re-designed primers Br150 pair and Br155 pair were not specific which also amplify plant DNA. The idea of keeping one primer from the original Br1234/Br1586 and pairing with Br150/Br155 worked well. Br150 forward primer and br 1586 reverse primer produce a 200 bp amplicon and were very specific to *Bremia* DNA and did not amplify plant DNA.

**Establishing a calibration curve**: A standard calibration curve was needed to compare the Ct values of the samples. Ten fold serial dilutions were from neat BL801 pure genomic DNA ($1.2x10^6$ spores DNA) to make six standards (Table 6.1).

**Table 6. 1** The 10 fold serial DNA dilutions of standard curve.

| Standards | NEAT | Std. 1 | Std. 2 | Std. 3 | Std. 4 | Std. 5 | Std. 6 |
|---|---|---|---|---|---|---|---|
| Concentrate | $1.2x10^6$ | $1.2x10^5$ | $1.2x10^4$ | $1.2x10^3$ | $1.2x10^2$ | $1.2x10^1$ | 1.2 |
| Dilutions | 0 | 1 fold | 2 fold | 3 fold | 4 fold | 5 fold | 6 fold |

Std.: Standard.

**Sample preparation:** In this test, samples were run in 10 µl reaction volume, which contains 5 µl Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, UK); 0.2 µl each of 10 µM concentrate forward and reverse primers (Invitrogen, UK), 0.75 µl DNA and 3.85 µl PCR grade water. Before making the master mix, a layout was drawn for the loading of each sample on the plate. The master mix was made up, 9.25 µl master mix was pipetted into each well, and then 0.75 µl DNA was pipetted

into the plate. The plate was sealed with sealing foil. The samples were mixed by centrifugating the plate in a 5810R Eppendorf Centrifuge (Hammbury, Germany) for 30 s at 1000 rpm (180 x g). The samples were run immediately. The plate (LightCycler 480® Multiwell Plate 384, Roche) was kept on ice during pipetting to avoid primer dimers.

**qPCR Cycle:** Samples were run in three programmes cycle. The detail of the programme had been described in Chapter 2 (see 2.3.4 for detail).

**Statistical analysis:** The quantification data were classified by (day.block)/leaf (block stands for each replicate) for statistical analysis. The value was analyzed by ANOVA using general analysis of variance design in GenStar 12th. Data were subjected to $\log_{10}$ transformation to stabilize the variance, leaf nested within block nested within day. For experiment 3, only the 5th day post inoculation samples were tested, therefor the data were classified by (block/leaf). Spore DNA qPCR quantification results and spore count per unit weight of cotyledons results were compared using the *t* – test of a correlation coefficient (Kanji, 1999).

## 6.3 RESULTS

### 6.3.1 Microscopic observation and evaluation

Secondary vesicle, hyphae and cell death were observed and the numbers on each lettuce cultivar were counted and analyzed. Figure 6.4 shows microphotos of the infection structures of the *B. lactucae* isolate. Figures 6.5 and 6.6 show the development of *B. lactucae* in five IBEB resistant cultivars (cv. PIVT1309, cv. LS-102, cv. Colorado, cv. Ninja and cv. Discovery) and a susceptible control (cv. Cobham Green) over a 7 day period after inoculation. Cultivar Cobham Green was susceptible to both *B. lactucae* isolates BL801 and BL806, with colonization by the pathogens two days after the inoculation. Infection structures and cell death were difficult to measure under microscope. Therefore for cv. Cobham Green, the numbers of infection structures and cell death were counted for the first two days after inoculation, after 2 days it was regarded as 100% infected for analysis. Hypersensitive cell death of cv. Cobham Green could vary 2 days after inoculation. After this it was regarded as missing data and was predicted by Genstat 12$^{th}$ edition. The predicted cell death was included in the analysis and the results are presented as the analysis of variance of cell death (the predicted values are shown in the graph generated by Genstat 12$^{th}$ edition).

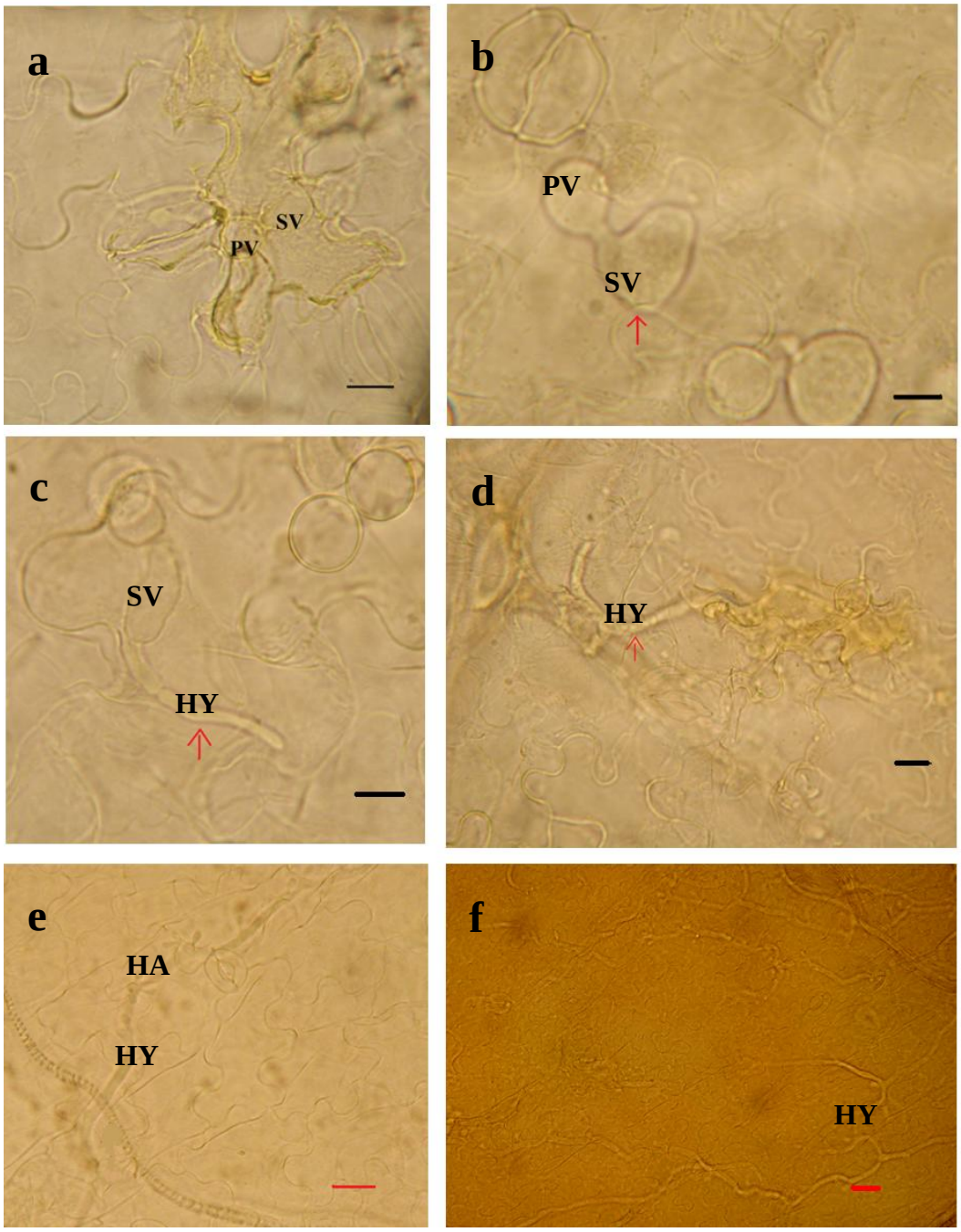



**Figure 6. 4** The development of *B. lactucae* (BL801) and lettuce hypersensitive cell death response. (a) Hypersensitive cell death, (b) secondary vesicle, (c) short hypha (<50 µm), (d) medium hypha (>50 µm, <100 µm), (e) long hypha (>100 µm), (f) too many long hyphae. Black bar: 10 µm, red bar: 30 µm. PV: primary vesicle, SV: secondary, HY: hypha, HA: haustorium.

#### 6.3.1.1 Microscopic observation of BL801

Figure 6.5 shows the development of BL801. Figure 6.5 (A) shows the percentage of secondary vesicles (without hypha) in total infection. A high percentage indicates fewer hyphae produced and *vice versa*. The results with cv. Cobham Green shows the amount of secondary vesicles was growing before the 2$^{nd}$ day and it decreased quickly to 0% after which 100% of secondary vesicles produced hyphae after. BL801 shows fewer hyphae grew after 24 hours on cv. PIVT 1309, cv. LS-102 and cv. Colorado, but more were produced on cv. Discovery and cv. Ninja. The percentages of secondary vesicles were decreasing during this 7 day period on most of the cultivars except cv. Ninja.

Figure 6.5 (B) shows the percentage of short length hyphae (<50 µm) in the total infection. The percentage of short hyphae decreased quickly from 60% to 0% from the 1$^{st}$ day to 2$^{nd}$ day on cv. Cobham Green. For cv. PIVT 1309, cv. LS-102 and cv. Colorado the percentage increased and reached approximately 20% at the 7$^{th}$ day. For cv. Discovery and cv. Ninja, the percentages were generally declining with time, on the 7$^{th}$ day 0% short hyphae grew on cv. Discovery and 20% grew on cv. Ninja.

Figure 6.5 (C) shows the percentage of medium length (>50 µm, <100 µm) hyphae in the total infection. For cv. Cobham Green, the percentage of medium length hyphae increased from 0% to 40% from the 1$^{st}$ day to 2$^{nd}$ day then decreased to 0% on the third day. Cultivars Ninja cv. LS-102 and cv. Discovery fluctuated during the 7 day period, and cv. Discovery decreased to 0% on the 7$^{th}$ day. Cultivar Colorado and cv. PIVT1309 gave a very low percentage on the 1$^{st}$ day and dropped to 0% on the 2$^{nd}$ day.

Figure 6.5 (D) shows the percentage of long length hyphae (>100 µm) in the total infection. The percentage of cv. Cobham Green started to increase from the 1st day and reached 100% on the 3rd day, while the other cultivars started to show an increase on the 2nd day but cv. Colorado stopped growing and there was no growth on cv.PIVT1309. Cultivar Discovery reached 90% on the 7th day and the other cultivars were approximately 10%.

Figure 6.5 (E) shows the percentage of cell death. Cultivar Cobham Green started to grow on the 1st day, while the others cultivars gave high percentages of cell death from the 1st day. Cultivar cv. PIVT 1309 was excluded from the analysis due to error from unrepresentative outlier values.

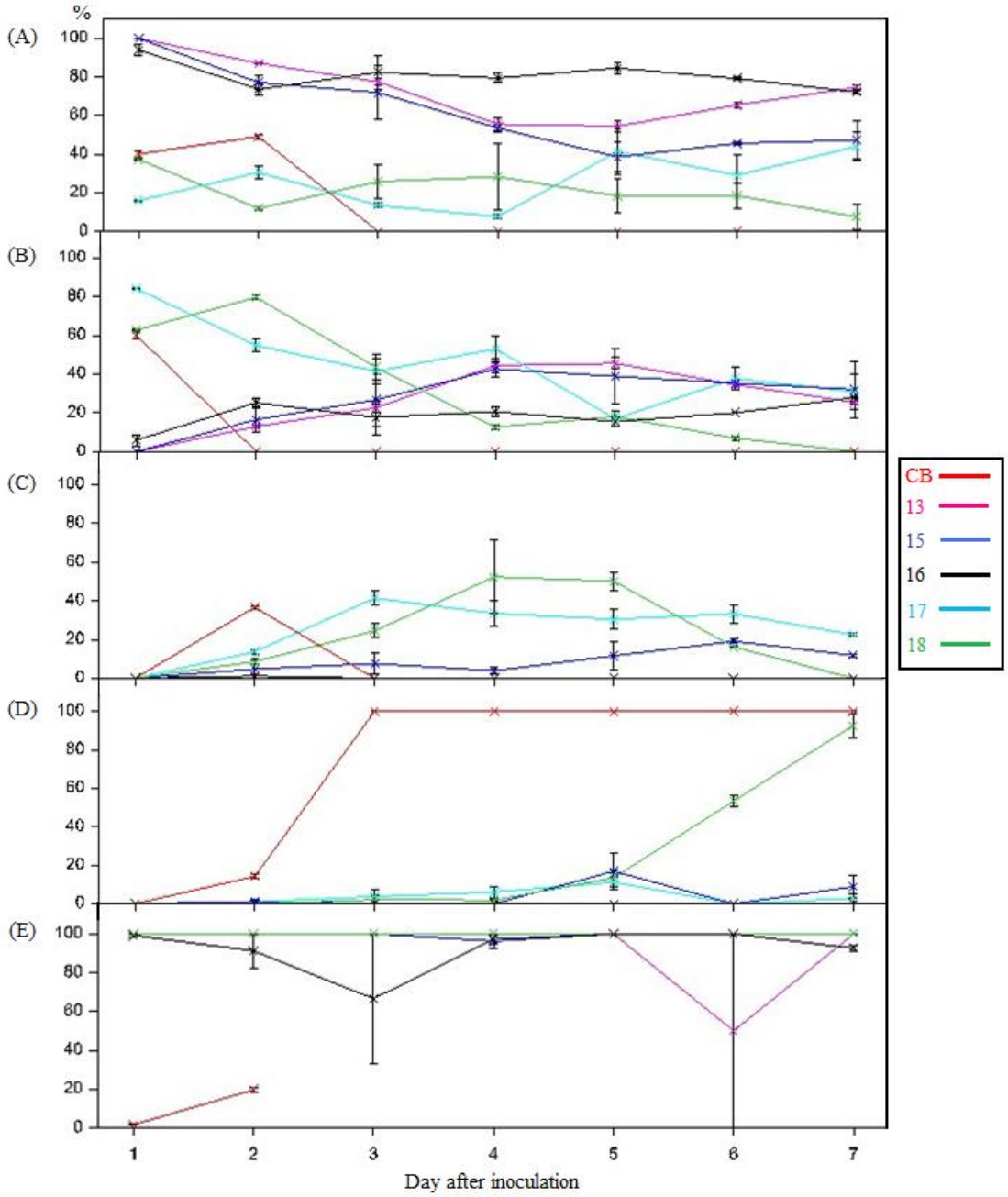



**Figure 6. 5** The development of infection structures of BL801 and host cell death expressed as percentages of the total observed in cultivars. Y-axis: percentage (%), x-axis: day after inoculation; (A) percentage of secondary vesicle without hypha producing; (B) percentage of short hyphae; (C) percentage of medium hyphae; (D) percentage of long hyphae; (E) percentage of cell death. CB=cv. Cobham Green, 13=cv. PIVT 1309, 15=cv. LS-102, 16=cv. Colorado, 17=cv. Ninja, 18=cv. Discovery.

#### 6.3.1.2 Microsopic observation of BL806

Figure 6.6 shows the development of BL806. Figure 6.6 (A) shows the percentage of secondary vesicles (without hyphae) in the total infection. The result on cv. Cobham Green shows secondary vesicles declined from 60% on the 1st day and dropped to 0% on the 3rd day, cv. Colorado started decreasing from 100% on the first day. On the 7th day, the percentage of cv. Discovery was around 70% and cv. LS-102 was approximately 60% while the other cultivars were around 90%.

Figure 6.6 (B) shows the percentage of short length hyphae (<50 µm) in the total infection. The percentage of short hypae on cv. Cobham Green started decreasing from 40% on the 1st day and reached 0% on the 2nd day, while the other cultivars showed an increase from the 1st day except cv. Colorado in which hyphae was visible on the 3rd day. The resistant cultivars generally showed an increase then decreased, on the 7th day. The percentage of short length hyphae of cv. LS-102 and cv. Discovery were around 30% and the others were around 10%.

Figure 6.6 (C) shows the percentage of medium length (>50 µm, <100 µm) hyphae in the total infection. Cultivar Cobham Green did not show medium hypae, only cv. LS-105 and cv. Discovery showed medium hyphae. The medium hyphae became visible on the 1st day on cv. Discovery, and increased on the 4th day with a slight decrease on the 7th day to approximately 10%. The medium hyphae started became visible on the 4th day on cv. LS-102, and then increased to about 10% on the 7th day.

Figure 6.6 (D) shows the percentage of long length hyphae (>100 µm) in the total infection. In cv. Cobham Green they became visible on the 1st day and reached to 100%

on the 3rd day. The other resistant cultivars did not show long hyphal development except for cv. Discovery. Long hyphae became visible on the 4th day, and decreased after the 5th day (less than 5%).

Figure 6.6 (E) shows the percentage of cell death. Cultivar Cobham Green showed an increase in cell death from 0% on the 1st day, while most of the other cultivars show 100% of cell death from the 1st day. Eighty percent cell death was recorded on cv. Ninja from the 1st day and it increased to 100% on the 2nd day.

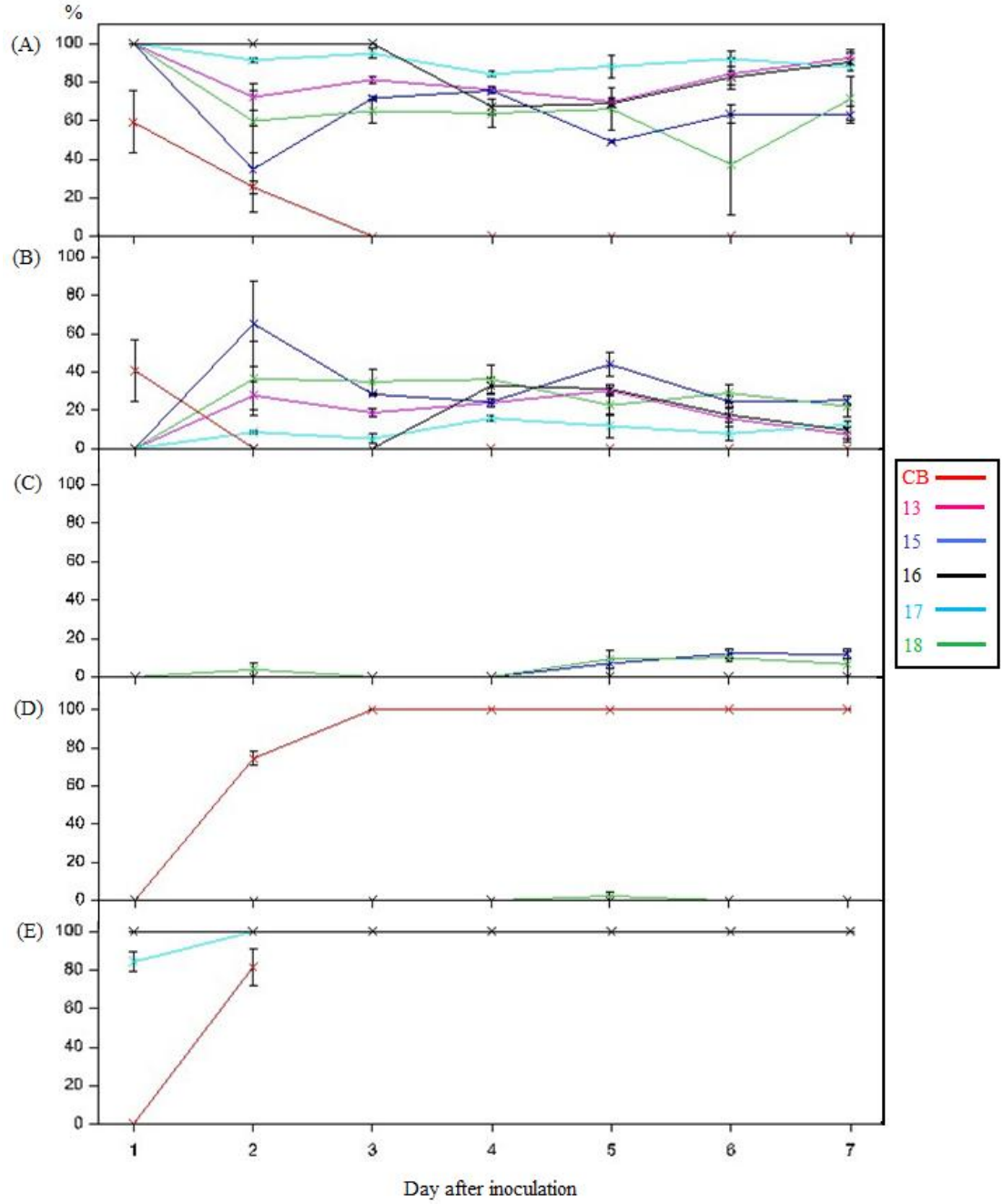



**Figure. 6** The development of infection structures of BL806 and host cell death expressed as percentages of the total observed in cultivars. Y-axis: percentage (%), x-axis: day after inoculation; (A) percentage of secondary vesicle without hypha producing; (B) percentage of short hyphae; (C) percentage of medium hyphae; (D) percentage of long hyphae; (E) percentage of cell death. CB=cv. Cobham Green, 13=cv. PIVT 1309, 15=cv. LS-102, 16=cv. Colorado, 17=cv. Ninja, 18=cv. Discovery.

#### 6.3.1.3 Results of statistical analysis

The microscopy data were subjected to a square root transformation. Analysis of variance looked at effects including: isolate, cultivar, cultivar by isolate, isolate by sample time, cultivar by sample time, cultivar by isolate, and cultivar by isolate by sample time. The main effects of isolate and cultivar were both significant at $p< 0.05$ (see Appendix 2). Isolate by sample of hyphae ($p= 0.212$) and cell death ($p= 0.233$) were not significant which means isolates BL801 and BL806 behaved similarly over time. The analysis results are shown in Tables 6.2 to 6.7. In each case, a difference between means was considered to be significant if larger than the least significant difference (LSD) of means (at the 95% probability level) (see Appendix 2 for further detail). Negative and zero results (Tables 6.5 and 6.6) were from small original data subjected by analysis and resulted in small negative or zero values.

Table 6.2 shows the mean number of secondary vesicles (without further development of a hypha), Table 6.3 shows the mean number of total hyphae, Table 6.4 shows the mean number of short hyphae, and Table 6.6 shows the mean number of long hyphae. For each of these measurements, the difference between isolates BL801 and BL806 is significant ($p<0.05$). As expected, the test cultivars are significantly different ($p<0.05$) from the susceptible control cv. Cobham Green. The isolate by cultivar interactions were significantly different ($p<0.05$), indicating that BL801 and BL806 performed differently in each cultivar.

Table 6.5 shows the mean number of medium hyphae. The difference between isolates BL801 and BL806 is significant ($p<0.05$). The cultivars are significantly different ($p<0.05$) from cv. Cobham Green. The isolate by cultivar interactions were

significantly different ($p<0.05$) between BL801 and BL806 on each cultivar. The interactions on cultivars Discovery, LS-102 and Ninja were significantly different ($p<0.05$) between isolates. Only interactions on Discovery and LS-102 were significantly different ($p<0.05$) from cv. Cobham Green of BL806.

**Table 6. 2** Table of transformed means obtained from analysis of variance of numbers of secondary vesicles observed in lettuce cotyledons at seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of secondary vesicles[1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 9.81 | | | | | | 0.18 | 140 |
| | BL806 | 5.63 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.32 | |
| | | 13.77 | 5.98 | 7.02 | 5.45 | 7.56 | 6.61 | | |
| Interaction | BL801 | 15.8 | 8.17 | 9.97 | 6.21 | 10.09 | 8.62 | 0.45 | |
| | BL806 | 11.74 | 3.71 | 3.96 | 4.73 | 5.07 | 4.51 | | |

[1] Data subjected to square root transformation.

**Table 6. 3** Table of transformed means obtained from analysis of variance of numbers of total hyphae observed in lettuce cotyledons at seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of total hyphae[1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 7.23 | | | | | | 0.2 | 140 |
| | BL806 | 3.35 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.34 | |
| | | 13.13 | 2.17 | 5.49 | 3.21 | 4.95 | 2.78 | | |
| Interaction | BL801 | 14.91 | 3.37 | 8.85 | 3.78 | 8.63 | 3.83 | 0.49 | |
| | BL806 | 11.34 | 0.96 | 2.14 | 2.63 | 1.27 | 1.73 | | |

[1] Data subjected to square root transformation.

**Table 6. 4** Table of transformed means obtained from analysis of variance of numbers of short hyphae observed in lettuce cotyledons at seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of short hyphae[1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 3.78 | | | | | | 0.19 | 140 |
| | BL806 | 1.45 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.33 | |
| | | 0.84 | 2.16 | 3.27 | 2.77 | 3.86 | 2.78 | | |
| Interaction | BL801 | 1.2 | 3.35 | 4.7 | 3.11 | 6.46 | 3.83 | 0.47 | |
| | BL806 | 0.47 | 0.96 | 1.84 | 2.43 | 1.27 | 1.73 | | |

[1] Data subjected to square root transformation.

**Table 6. 5** Table of transformed means obtained from analysis of variance of numbers of medium hyphae observed in lettuce cotyledons at seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of medium hyphae[1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 1.92 | | | | | | 0.15 | 140 |
| | BL806 | 0.21 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.25 | |
| | | 0.57 | 0.03 | 2.19 | 1.16 | 2.43 | 0.003 | | |
| Interaction | BL801 | 1.14 | 0.06 | 3.85 | 1.62 | 4.84 | 0.005 | 0.37 | |
| | BL806 | 0 | 0 | 0.53 | 0.7 | 0.01 | 0 | | |

[1] Data subjected to square root transformation.

**Table 6. 6** Table of transformed means obtained from analysis of variance of numbers of long hyphae observed in lettuce cotyledons at time points seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of long hyphae[1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 3.07 | | | | | | 0.12 | 140 |
| | BL806 | 1.82 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.21 | |
| | | 11.98 | -0.01 | 1.70 | 0.31 | 0.69 | -0.001 | | |
| Interaction | BL801 | 13.08 | -0.02 | 3.33 | 0.65 | 1.37 | 0.05 | 0.3 | |
| | BL806 | 10.87 | 0 | 0.07 | -0.03 | -0.001 | 0 | | |

[1] Data subjected to square root transformation.
Negative value subjected to small mean in the original data.

Table 6.7 shows the mean number of dead cells. The difference between isolates BL801 and BL806 is significant ($p<0.05$). The cultivars are significantly different ($p<0.05$) from cv. Cobham Green. The isolate by cultivar interactions were significantly different ($p<0.05$) between BL801 and BL806 on each cultivar except on Cobham Green. The interaction on cultivars Colorado and Discovery of BL806 are not significantly different from Cobham Green.

**Table 6. 7** Table of transformed means obtained from analysis of variance of numbers of dead cells observed in lettuce cotyledons at seven time points (1, 2, 3, 4, 5, 6 and 7 days post inoculation).

| Mean number of dead cells [1] | | | | | | | | LSD | DF |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | BL801 | 8.17 | | | | | | 0.29 | 120 |
| | BL806 | 4.47 | | | | | | | |
| Cultivar | | Cobham Green | Colorado | Discovery | LS-102 | Ninja | PIVT1309 | 0.51 | |
| | | 3.98 | 5.82 | 7.46 | 6.06 | 7.97 | 6.61 | | |
| Interaction | BL801 | 4.35 | 7.81 | 10.65 | 6.81 | 10.86 | 8.55 | 0.72 | |
| | BL806 | 3.61 | 3.84 | 4.27 | 5.31 | 5.08 | 4.68 | | |

[1] Data subjected to square root transformation.

### 6.3.2 qPCR quantification of susceptible cultivars

Figures 6.7 to 6.10 show the qPCR quantification of DNA extracted from isolate BL806 in susceptible cultivars. Figures 6.12 to 6.15 show the qPCR quantification results of isolate BL801 (DNA extracted from susceptible cultivars). Figures 6.11 and 6.16 show the results for spore count per unit weight of cotyledons of BL806 and BL801 on the susceptible cultivars, respectively.

#### 6.3.2.1 Results of BL806 on susceptible cultivars

The results of qPCR quantification of BL806 in seven selected susceptible cultivars in Experiment 1 and 2 are shown in the Figure 6.7 to Figure 6.10. The unit scale was the DNA copy of each spore (one spore was regarded as one copy of DNA). Figure 6.11 shows the results for spore count/unit weight of cotyledons sampled on the 7$^{th}$ day after inoculation.

For Experiment 1, the early stage (1 to 4 dpi) is shown in Figure 6.7 and the late stage of infection (4 to 7 dpi) is shown in Figure 6.8. Cultivar Cobham Green was a susceptible control in these experiments. The 4$^{th}$ and 7$^{th}$ dpi data of this cultivar were missing but isolate BL806 was growing on this cultivar on the 5$^{th}$ and 6$^{th}$ day suggesting Cobham Green might be less susceptible to BL806 than the others. The development of BL806 on cv. UC DM2 was increasing during this 7 day period. The development of BL806 on cv. Valmiane fluctuated but increased and reached a maximum on the 6$^{th}$ day before declining on the 7$^{th}$ dpi. The development of BL806 on cv. UC DM10 increased during the 7 days. For cv. Captain, BL806 increased before declining on the 7$^{th}$ day. For cv. Pennlake BL806 showed a similar pattern as on cv. Captain. However BL806 increased on cv. UC DM14 over the 7 day period.

Figure 6.8 shows that the DNA increased sharply after the 4th dpi, and on cv. Valmaine, cv. Pennlake and cv. UC DM14 BL806 developed to the highest level in comparison to other cultivars.

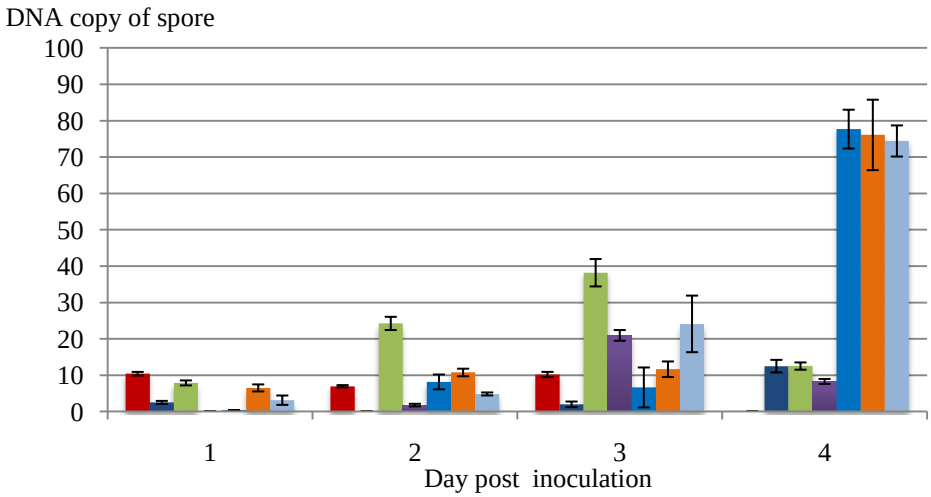



**Figure 6. 7** qPCR results from experiment 1 for BL806 DNA sampled from days 1 to 4 post inoculation. = cv. Cobham Green, =cv. UCDM2, =cv. Valmaine, = cv. UCDM10, =cv. Captain, = cv. Pennlake, = cv.UCDM14.

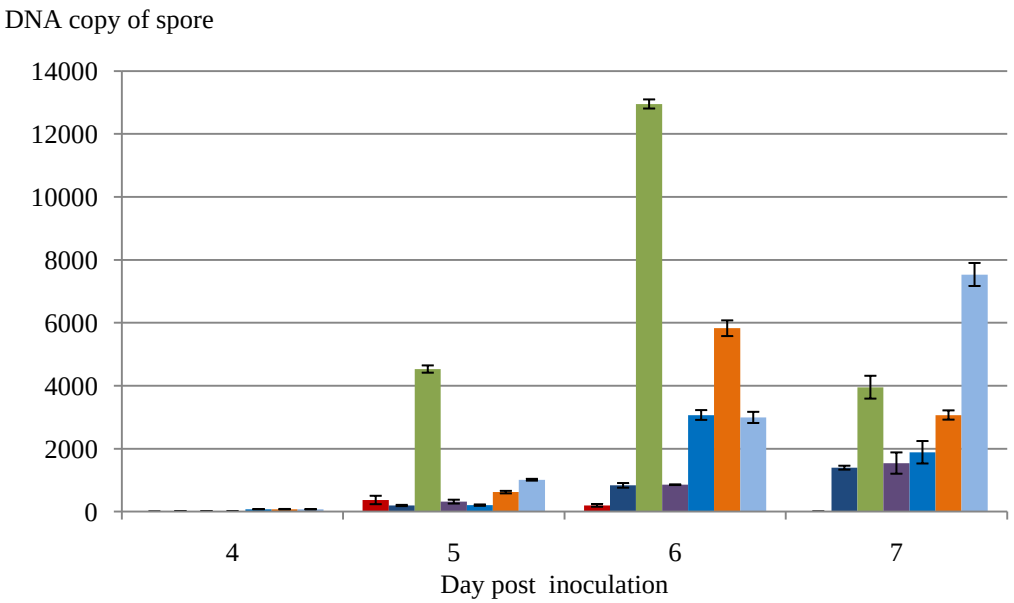



**Figure 6. 8** qPCR results from experiment 1 for BL806 DNA sampled from days 4 to 7 post inoculation. = cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, = cv. Captain, = cv. Pennlake, = cv.UCDM14.

Figure 6.9 and 6.10 show the results of Experiment 2. For cv. Cobham Green the data from 6$^{th}$ and 7$^{th}$ dpi were missing. The results of 5$^{th}$ day shows less DNA can be detected on cv. Cobham Green than on the others cultivars except cv. Pennlake. The *B. lactucae* DNA extracted from cv. UC DM2 cv. Valmaine cv. Captain and cv. UC DM14 was increasing during the 7 day period, and on cv. Valmaine and cv. UC DM14 it increased sharply on the 7$^{th}$ dpi. The *B. lactucae* DNA extracted from cv. UC DM10 was generally increasing, and the amount extracted on the 6$^{th}$ dpi was slightly higher than that which occurred on the 5$^{th}$ and 7$^{th}$ dpi. The DNA amount extracted from cv. Pennlake was increasing quickly on the first 4 days then declined slightly on the 5$^{th}$ and 6$^{th}$ dpi before increasing sharply on the 7$^{th}$ dpi. The the development of isolate BL806 in Experiment 2 was slightly different from the first experiment, as most of the cultivars showed the highest DNA level extracted on the 7$^{th}$ dpi. However, cultivars Valmaine, Pennlake and UC DM14 produced the highest amount of BL806 DNA on the 7$^{th}$ dpi which is the same as Experiment 1.

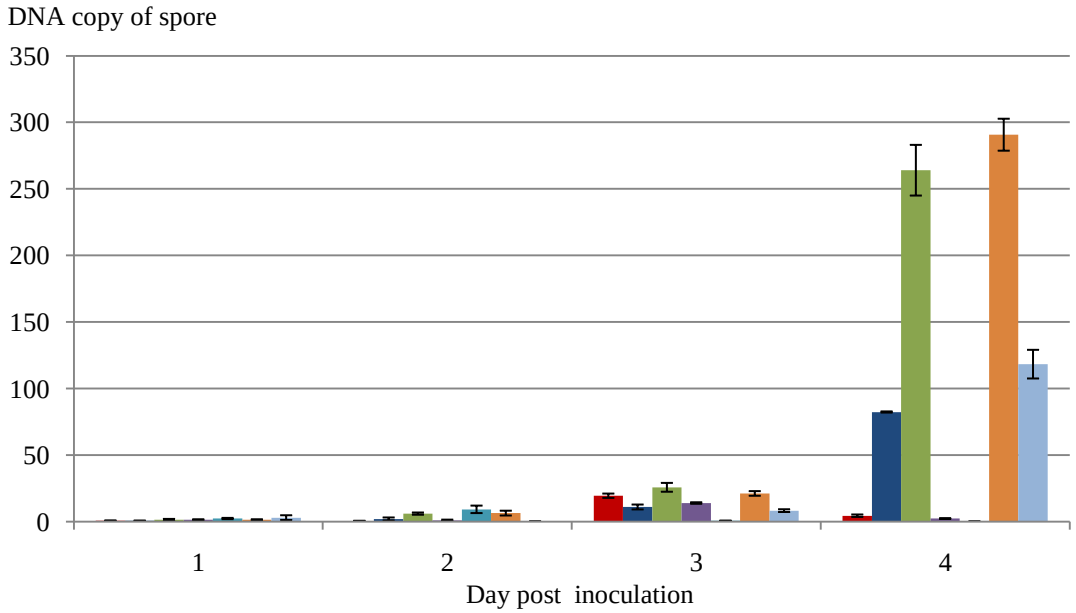



**Figure 6. 9** qPCR results from experiment 2 for BL806 DNA sampled from days 1 to 4 post inoculation. = cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, = cv. Captain, = cv. Pennlake, = cv. UCDM14.

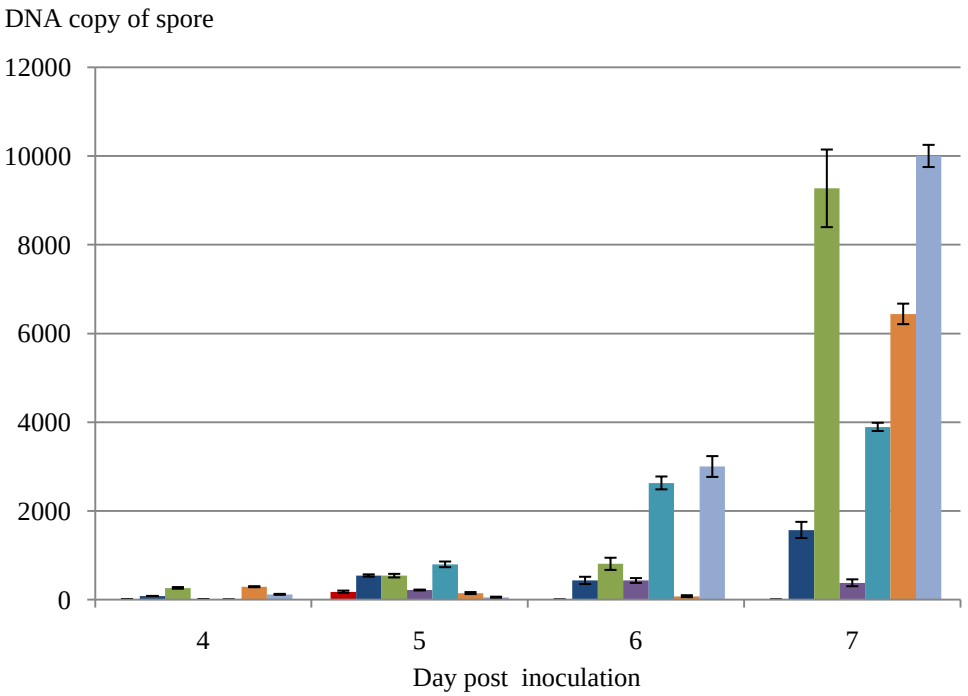



**Figure 6. 10** qPCR results from experiment 2 for BL806 DNA sampled from day 4 to 7 post inoculation. = cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, =cv. Captain, = cv. Pennlake, = cv. UCDM14.

Figure 6.11 shows the results of BL806 spore count per unit weight of cotyledons on the 7$^{th}$ dpi, which was compared to the results of qPCR. In Figure 6.11, cv. Cobham Green shows the greatest value of spore count with cv. Valmaine and cv. Pennlake showing the second highest amount. Cultivars UCDM10 and Captain show similar values and cv. UCDM2 gave the lowest value of spore count. The spore counts on other cultivars show similar levels of DNA as assessed using qPCR quantification except for cv. Cobham Green. Further analysis and description will be included in statistical analysis section (section 6.3.5).

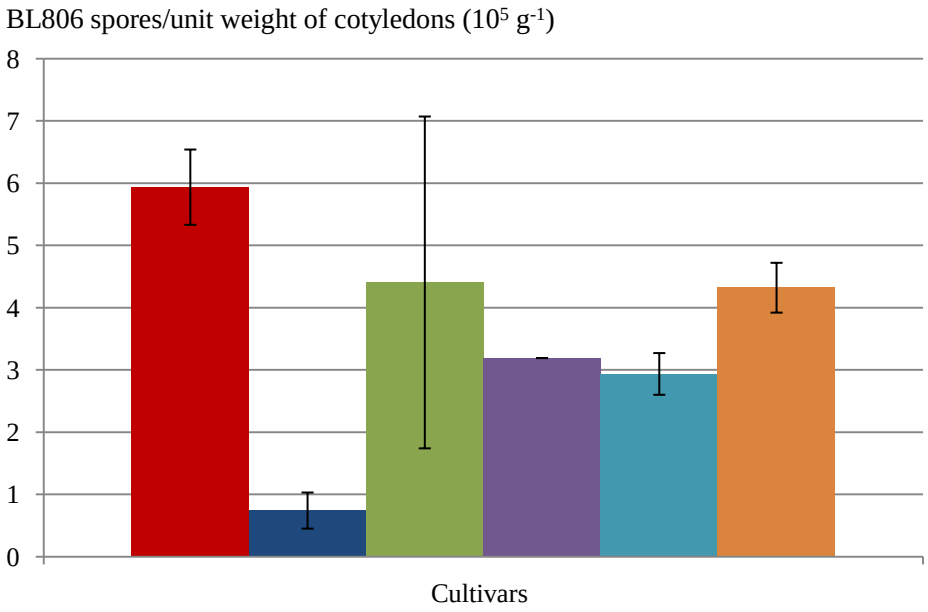



**Figure 6. 11** The results for spore count per unit weight of cotyledons of BL806 on day 7 post inoculation. = cv. Cobham Green, =cv. UCDM2, =cv. Valmaine, = cv. UCDM10, =cv. Captain, = cv. Pennlake.

#### 6.3.2.2 BL801 on susceptible cultivars

The results of qPCR quantification of BL801 on six selected susceptible cultivars and one resistant cultivar (cv. UCDM14) of Experiment 1 and 2 are shown in Figures 6.12 to Figure 6.15. The unit scale is the DNA copy of each spore (one spore was regarded as one copy of DNA). Figure 6.16 shows the results for spore count/unit weight of cotyledons sampled on the 7$^{th}$ day after inoculation.

In Experiment 1, the early stage is shown in Figure 6.12 and late stage of infection is shown in Figure 6.13. Cultivar Cobham Green was used as a control in these experiments. Figure 6.12 and Figure 6.13 show that the amounts of *B. lactucae* DNA were generally increasing on these selected cultivars and reached the highest value on the 7$^{th}$ dpi. During the early stage, the susceptible cultivars gave DNA amounts increasing on the 3rd day. The amount of DNA on cv. Cobham Green cv. Valmaine, cv. Captain and cv. Pennlake increased quickly on the 4$^{th}$ dpi. For the later stage, some data was missing on the 5$^{th}$ and 7$^{th}$ dpi, however cv. UCDM2 showed the highest amount of *B. lactucae* DNA extracted from it, and cv. Valmaine was the second highest. Cultivars Cobham Green, UC DM10 and Pennlake gave a similar amount, and cv. Captain gave the lowest amount extracted among these susceptible cultivars. Less BL801 DNA were dectected in these cultivars after the 6$^{th}$ day. Although cv. UC DM14 was regarded as a resistant cultivar, the amount of DNA was increasing in it during this 7 day period.

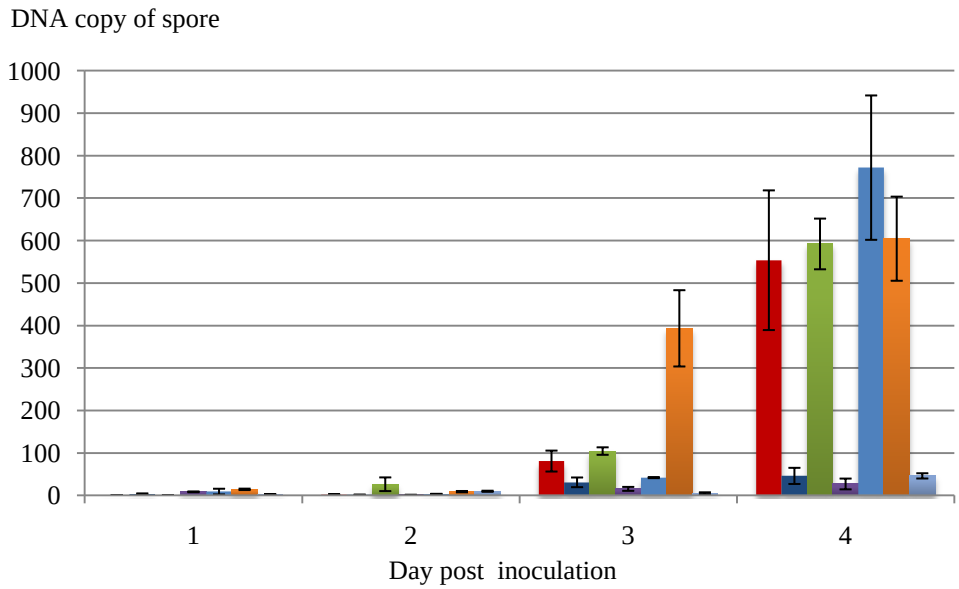



**Figure 6. 12** qPCR results from experiment 1 for BL801 DNA of sampled from the days 1 to 4 post inoculation. = cv. Cobham Green, =cv. UCDM2, =cv. Valmaine, = cv. UCDM10, = cv. Captain, = cv. Pennlake, =cv. UCDM14.

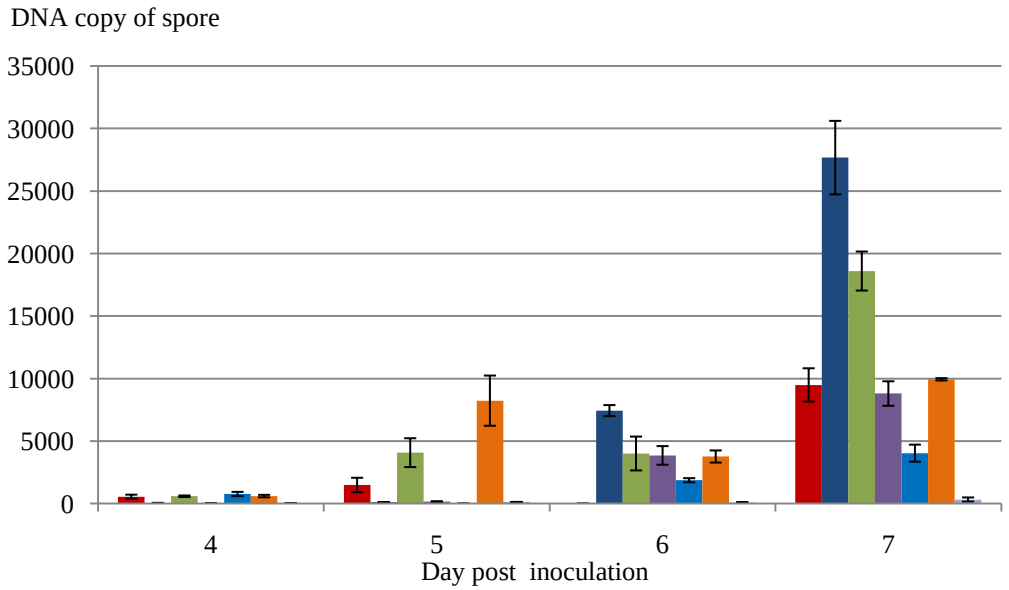



**Figure 6. 13** qPCR results from experiment 1 for BL801 DNA sampled from days 4 to 7 post inoculation. =cv. Cobham Green, =cv. UCDM2, =cv. Valmaine, =cv. UCDM10, =cv. Captain，= cv. Pennlake, =cv. UCDM14.

Figure 6.14 and 6.15 show the results of Experiment 2. The amount of *B. lactucae* DNA extracted from each cultivar was increasing until the 6th dpi before it declined on the 7th dpi. Figure 6.14 shows the DNA amount increased on the 3rd dpi and it increased quickly on the 4th dpi on cv. Valmaine and cv. Pennlake. The data of cv. Cobham Green was missing on the 4th, 6th and 7th dpi. On the 5th day, the *B. lactucae* DNA amount extracted from Cobham Green was lower than the other cultivars. Figure 6.15 shows, the DNA amount on cv. UC DM2 increased quickly on the 6th dpi, and cv. Pennlake had the highest amounts in the middle (4th dpi) and late infection stages (7th dpi). On the 5th day the DNA amount on cv. Captain shows the second highest amount while it was slightly less on cv. Valmaine. On the 6th dpi, the DNA amounts extracted from cv. UC DM2 and cv. Captain were the second highest and it shows slightly lower on cv. Valmaine and cv. UC DM10. The 7th dpi, DNA levels showed similar amounts on cultivars UC DM2, Valmaine and UC DM10, but it declined sharply in cv. Captain. The results show that *B. lactucae* DNA was increasing slightly on cv. UC DM14.

Figure 6.16 shows the result for *B. lactucae* spore count per unit weight of cotyledons BL801 on six selected susceptible cultivars. The results show that fewer spores were counted on cv. Cobham Green. Cultivar Valmaine showed the highest spore count and cv. Pennlake showed the second highest. Cultivars UC DM2, UC DM10 and Captain gave similar counts. Considering the errors, the spore per unit weight of cotyledons from each cultivar might be similar. The outcome of the spore count per unit weight of cotyledons is similar to the results of 5th dpi qPCR in experiment 2.

The results of spore count per unit weight of cotyledons show a possibility that isolate BL801 developed faster than isolate BL806 on these selected cultivars. The 7$^{th}$ dpi spore count per unit weight cotyledons results for BL801 is similar to the results of qPCR on the 5$^{th}$ dpi, while for BL806 it is similar to the qPCR results on the 7$^{th}$ day. The results of the spore count per unit weight of cotyledons and qPCR are different between *B. lactucae* isolates BL801 and BL806.

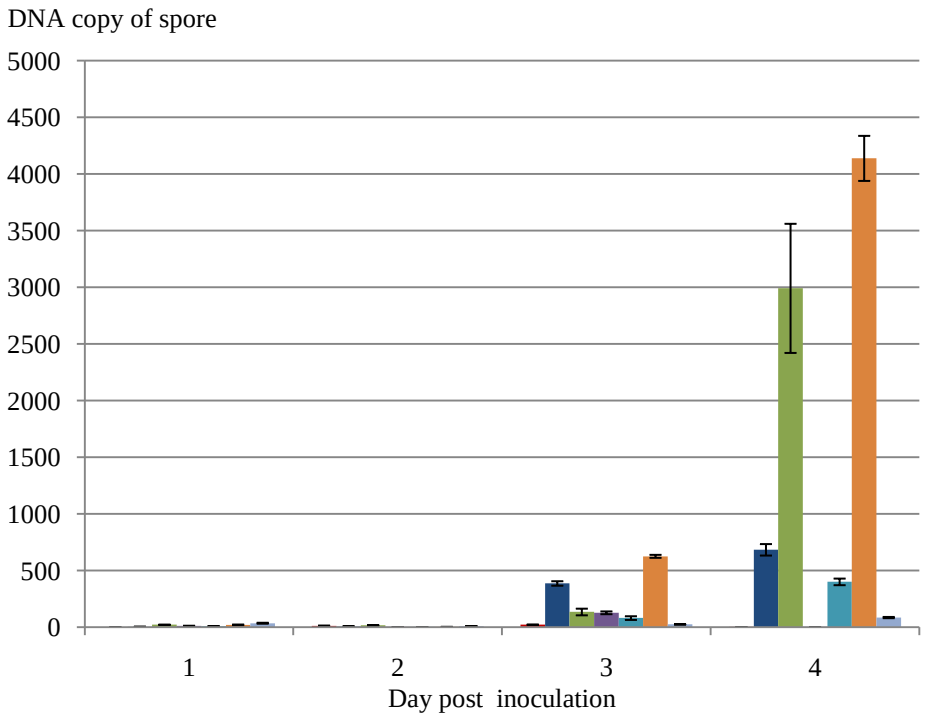



**Figure 6. 14** qPCR results from experiment 2 for BL801 DNA sampled from days 1 to 4 post inoculation. = cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, =cv. Captain， = cv. Pennlake, = cv. UCDM14.

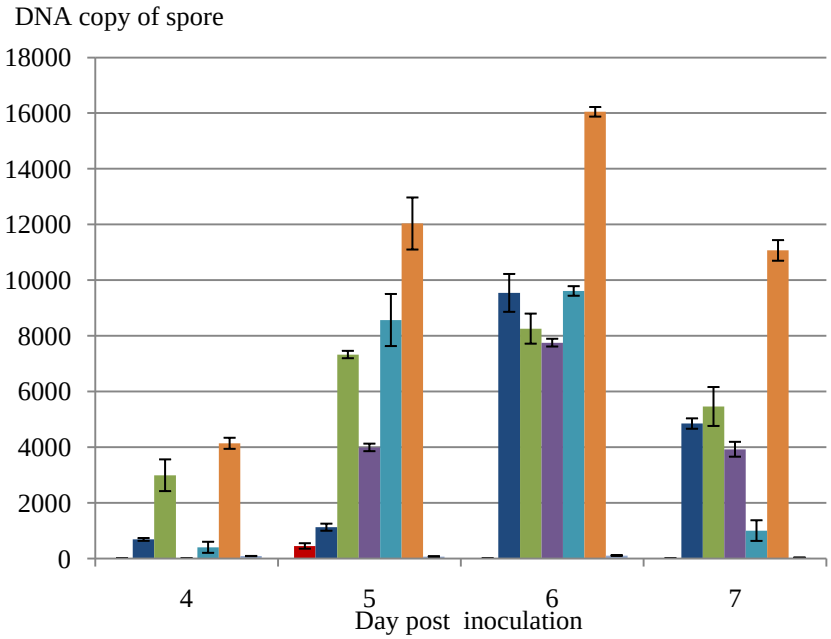



**Figure 6. 15** qPCR results from experiment 2 for BL801 DNA sampled from days 4 to 7 post inoculation. = cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, = cv. Captain， = cv. Pennlake, = cv. UCDM14.

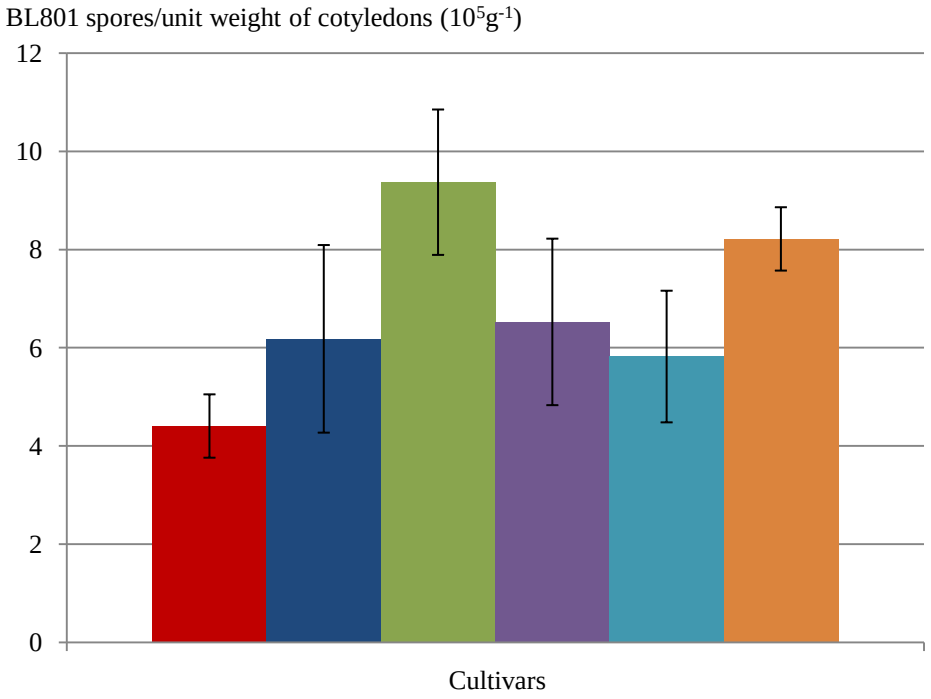



**Figure 6. 16** The results for spore count per unit weight of cotyledons for BL801 on susceptible cultivars.
= cv. Cobham Green, = cv. UCDM2, = cv. Valmaine, = cv. UCDM10, = cv. Captain， = cv. Pennlake.

### 6.3.3 qPCR quantification on resistant cultivars

The results of qPCR quantification on selected resistant cultivars are shown in Table 6.8, where the mean values of DNA of isolates BL801 and BL806 extracted from five resistant cultivars are shown. However the values here are too low to be considered reliable.

**Table 6. 8** qPCR results for BL801 and BL806 from Experiment 1 and 2 on resistant cultivars**.**

| Experiment | Day | Cultivars | PIVT 1309 | LS 102 | Colorado | Ninja | Discovery |
|---|---|---|---|---|---|---|---|
| BL801 qPCR quantification | | | | | | | |
| 1 | 1 | | 0.0[1] | 2.6 | 6.3 | 0.0 | 0.0 |
| | 5 | | 0.0 | 1.3 | 5.0 | 0.7 | 31.4 |
| | 7 | | 0.0 | 1.9 | 4.5 | 0.7 | 13.5 |
| 2 | 1 | | 0.0 | 0.5 | 2.9 | 1.1 | 0.0 |
| | 5 | | 0.0 | 0.4 | 0.9 | 14.3 | 0.0 |
| | 7 | | 0.0 | 2.0 | 1.4 | 13.6 | 0.0 |
| BL806 qPCR quantification | | | | | | | |
| 1 | 1 | | 0.0 | 0.4 | 0.5 | 0.7 | 0.1 |
| | 5 | | 0.0 | 0.5 | 0.0 | 0.1 | 0.0 |
| | 7 | | 0.0 | 0.3 | 0.3 | 0.5 | 0.0 |
| 2 | 1 | | 0.0 | 0.4 | 0.4 | 0.0 | 0.0 |
| | 5 | | 0.0 | 0.6 | 0.5 | 0.0 | 0.0 |
| | 7 | | 0.3 | 0.3 | 0.6 | 0.7 | 0.0 |

[1] Zero value: DNA could not be detected by qPCR. DNA was extracted on the first the fifth and the seventh days post inoculation. 1: 1copy of spore DNA.

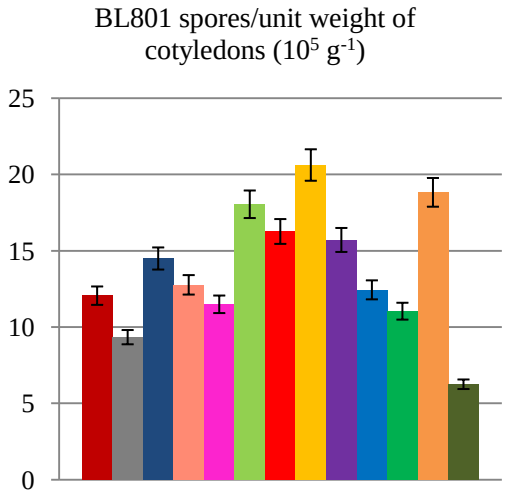
### 6.3.4 Results of Experiment 3 (qPCR quantification of *B. lactucae* in the entire IBEB EU-A set cultivars)

The results of spore per cotyledon unit weight count and qPCR quantification of BL801 and BL806 on susceptible EU-A IBEB cultivars and are shown in Figure 6.17 to Figure 6.16.

Figure 6.17 shows the results for spore count/unit weight of cotyledons and qPCR quantification of *B. lactucae* isolate BL801 on susceptible EU-A IBEB cultivars. Five cultivars show the highest count on them which were Valmaine, Sabine, LSE57/15, UCDM10 and Pennlake. The count for cv. LSE57/15 was the highest in these five cultivars, cultivars Valmaine and Pennlake were lower followed by cv. Sabine and cv. UCDM10. Three futher cultivars also gave greater counts than cv. Cobham Green which were cultivars UCDM2, Dandie and Captain and four cultivars gave smaller counts than cultivar Cobham Green which were cultivars Lednicky, R4T57D, Hilde II and LSE 18. The count on cultivar LSE18 was the smallest.

In Figure 6.17 five cultivars gave the highest *B. lactucae* DNA amount on them which were cultivars Valmaine, Sabine, LSE57/15, UCDM10 and Pennlake. Cultivars Valmaine gave the highest amount and cultivar UCDM10 was lower, the other three cultivars gave much smaller DNA extractions. The spore DNA amounts from cultivars Cobham Green, Sabine and Captain were very low. The other cultivars gave higher DNA amounts than cultivar Cobham Green but these were lower than 1000 spores DNA copies.

(a)




(b)

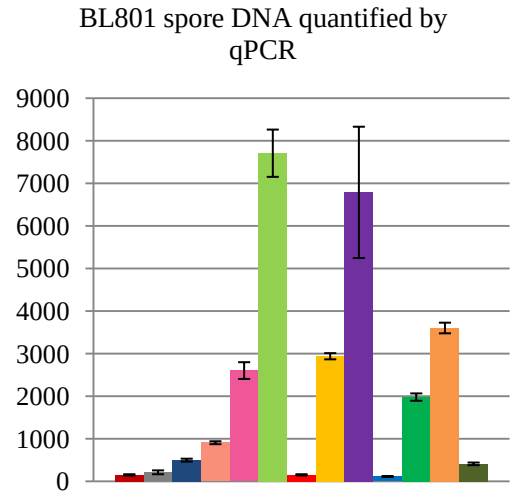



**Figure 6. 17** The results for (a) spore count/unit weight of cotyledons and (b) qPCR quantification of BL801 on susceptible EU-A IBEB cultivars.

Cobham Green, Lednicky, UCDM2, Dandie, RT457D, Valmaine, Sabine, LSE57/15, UCDM10, Captain，Hilde II, Pennlake, LSE 18.

Figure 6.18 shows the results of spore count/unit weight of cotyledons and qPCR quantification of *B. lactucae* isolate BL806 on susceptible EU-A IBEB cultivars. Cultivars UCDM10, Valmaine and Pennlake had the three highest values however cultivars LSE57/15 and Argeles were lower. The count from cv. Cobham Green was the lowest. The highest DNA extraction was from cultivar Valmaine however cultivars R4T57D, Pennlake and UCDM10 were lower. The other cultivars tested gave very low extracted DNA amounts. Cultivars Cobham Green, Hilde II and Argeles were the lowest. However cultivars Valmaine and Pennlake showed consistency in the results of spore count/unit weight of cotyledons and qPCR quantification, but other cultivars differed in this respect.

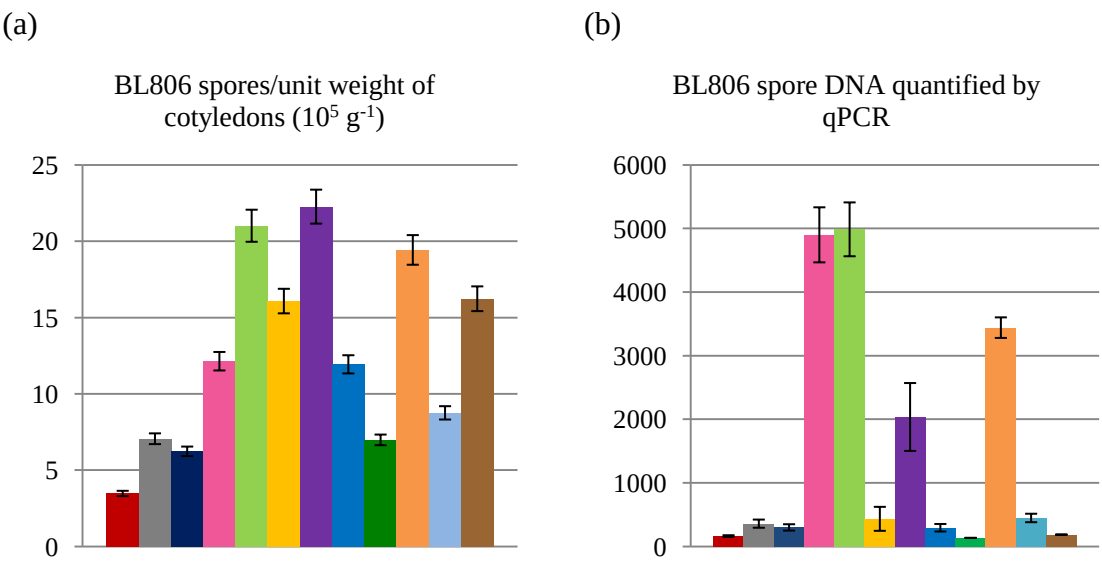



**Figure 6. 18** The results for (a) spore count/unit weight of cotyledons and (b) qPCR quantification of BL806 on susceptible EU-A IBEB cultivars.
Cobham Green, Lednicky, UCDM2, RT457D, Valmaine, LSE57/15, UCDM10, Captain, Hilde II, Pennlake, UCDM 14, Argeles.

Isolate BL801, the 5th day post inoculation gave qPCR quantification results which were similar to the seven day spore count per unit weight of cotyledons. Isolate BL806 results were very similar and generally higher number of BL801 spores were produced than BL806 on susceptible cultivars.

Figure 6.19 shows the *B. lactucae* spore per cotyledon unit weight of six cultivars (same susceptible cultivars as Experiment 1 and 2), for comparison of the results between the 1st and 2nd Experiments (Section 6.3.2) and Experiment 3. Figure 6.19 shows the result of BL801 on the six selected cultivars of Experiment 3 which is similar to the results of 1st and 2nd Experiments (Figure 6.16). Isolate BL806 shows that with the exception of cv. Cobham Green, the results of other cultivars were similar to 1st and 2nd Experiments (Figure 6.11). The variation of spore production for BL801 was not large on these susceptible cultivars while the variation on BL806 was larger between cultivars.

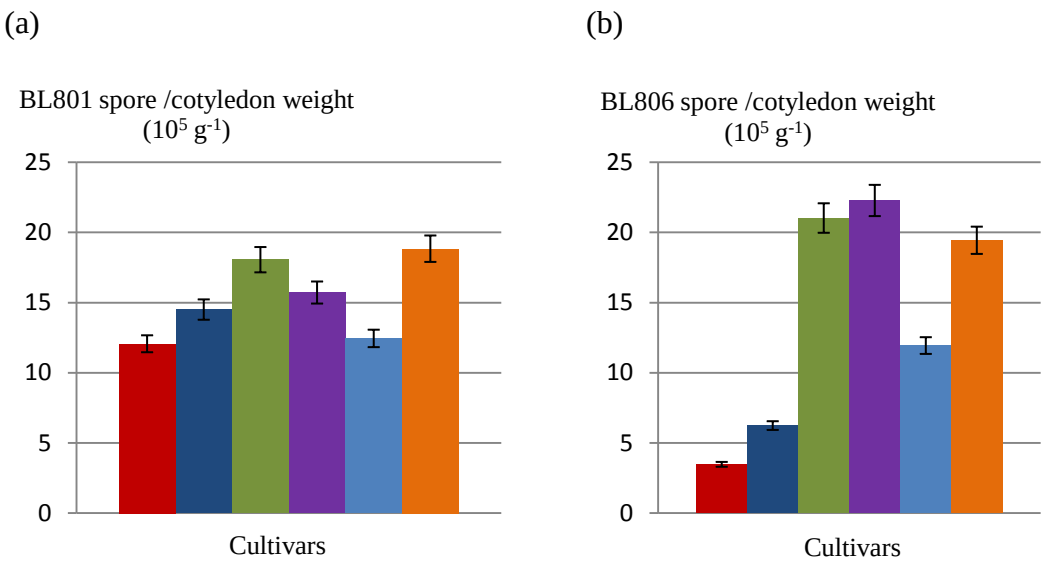



**Figure 6. 19** Results for BL801 (a) and BL806 (b) spore count per unit weight of cotyledons ($10^5$ $g^{-1}$) on selected EU-A IBEB susceptible cultivars**.**
Cobham Green, UCDM2, Valmaine, UCDM10, Captain，Pennlake.

Table 6.9 summarises the results of IBEB identification, spore count per unit weight of cotyledons and qPCR quantification for *B. lactucae* isolate BL801 and BL806. IBEB identification only shows the compatable and incompatable reaction between isolates and cultivars, spore count per unit weight of cotyledons shows the difference of the compatable reaction. However qPCR shows the difference in compatable and incompatable reactions.

**Table 6. 9** Results of IBEB phenotypic identification and qPCR quantification and spore count per unit weight of cotyledons of BL801 and BL806 on the EU-A IBEB cultivars set.

| Isolate | IBEB Lettuce Cultivars | 0. Cobham Green | 1. Lednicky | 2. UC DM2 | 3. Dandie | 4. R4T57D | 5. Valmaine | 6. Sabine | 7. LSE 57/15 | 8. UC DM10 | 9. Capitan | 10. Hilde II | 11. Pennlake | 12. UC DM14 | 13. PIVT 1309 | 14. LSE18 | 15. LS-102 | 16. Cororado | 17. Ninja | 18. Discovery | 19. Argeles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BL801 | IBEB | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | - | - | - |
| | qPCR | 149 | 210 | 496 | 905 | 2600 | 7707 | 150 | 2937 | 6787 | 112 | 1977 | 3600 | 57.4 | 1.6 | 408.3 | 3.8 | 5.9 | 6.1 | 4.4 | 7.8 |
| | S/UWC | 12.1 | 9.3 | 14.5 | 12.8 | 11.5 | 18.0 | 16.3 | 20.6 | 15.7 | 12.4 | 11.0 | 18.8 | 0.0 | 0.0 | 6.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| BL806 | IBEB | + | + | + | - | + | + | - | + | + | + | + | + | + | - | - | - | - | - | - | + |
| | qPCR | 162 | 357 | 298 | 0.9 | 4900 | 4987 | 7.2 | 433 | 2035 | 293 | 133 | 3440 | 446 | 11.3 | 2.6 | 3.1 | 5.6 | 2.2 | 3.3 | 184.0 |
| | S/UWC | 3.5 | 7.1 | 6.2 | 0.0 | 12.1 | 21.0 | 0.0 | 16.1 | 22.3 | 11.9 | 7.0 | 19.4 | 8.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 16.2 |

+: Compatible interaction; -: incompatible interaction; S/UWC: spore count per unit weight of cotyledons ($10^5$ spores $g^{-1}$).

### 6.3.5 Statistical analysis

The data of qPCR quantification were subjected to $\log_{10}$ transformation. For Experiments 1 and 2, analysis of variance looked at effects including: isolate, cultivar, cultivar by isolate, isolate by sample time, cultivar by sample time, cultivar by isolate and cultivar by isolate by sample time. All the effects are significant ($p<0.05$) (see Appendix 2 for further detail), except cultivar by isolate in Experiment 2 is not significant ($p=0.662$). For Experiment 3, samples were only taken on day five post inoculation so sample time was not included in the effects. A difference was considered to be significant if larger than the least significant difference (LSD) of means (at the 95% probability level) (see Appendix 2 for further detail). The cultivars are significantly different from cv. Cobham Green (susceptible control) in Experiments 1, 2 and 3 (see Appendix 2 for further detail).

Tables 6.10 and 6.11 show the general variance of qPCR quantification results. Tables 6.12 and 6.13 show correlation analysis of qPCR quantification and spore count per unit weight of cotyledons. The data of qPCR quantification and spore count per unit weight of cotyledons were analysed by GenStar 12$^{th}$ edition after $\log_{10}$ transformation.

#### 6.3.5.1 Analysis of variance of qPCR quantification results

Table 6.10 shows the mean of spore DNA quantified by qPCR. The difference between isolates BL801 and BL806 is significant in both Experiments 1 and 2 (susceptible and resistant). In Experiment 1, the isolate by cultivar interactions are significantly different between isolates BL801 and BL806, except cultivars Captitan, PIVT 1309 and Ninja. The susceptible interaction on each cultivar is significantly

different compared with susceptible control Cobham Green, except in cultivars Capitan and UCDM10 infected by BL801. In Experiment 2, the isolate by cultivar interactions are significantly different between isolates BL801 and BL806, except on cultivars PIVT1309, LS-102, Colorado and Discovery. The susceptible interaction on each cultivar is significantly different compared with the susceptible control Cobham Green.

**Table 6. 10** Analysis of variance of quantity of spore DNA in lettuce cotyledons at time points 1, 2, 3, 4, 5, 6 and 7 days of Experiments 1 and 2.

| Mean of spore DNA[1] quantified by qPCR | | | | | | | |
|---|---|---|---|---|---|---|---|
| Isolates BL801and BL806 | | Experiment 1 | | | Experiment 2 | | |
| | | BL801 | BL806 | LSD | BL801 | BL806 | LSD |
| | | 1.97 | 1.72 | 0.08 (DF:98) | 2.13 | 1.49 | 0.04 (DF:98) |
| Isolate and susceptible cultivars Interaction | Cobham Green | 1.71 | 0.95 | 0.21 (DF:181) | 0.59 | 0.49 | 0.1 (DF:180) |
| | UC DM2 | 1.97 | 1.42 | | 2.53 | 1.7 | |
| | Valmaine | 2.4 | 2.08 | | 2.81 | 2.07 | |
| | UC DM10 | 1.83 | 1.56 | | 2.08 | 1.04 | |
| | Capitan | 1.7 | 1.76 | | 2.38 | 1.54 | |
| | Pennlake | 2.7 | 2.13 | | 2.96 | 1.81 | |
| | UC DM14 | 1.46 | 2.11 | | 1.6 | 1.75 | |
| Isolatse BL801 and BL806 | | 0.19 | -0.18 | 0.11 (DF:30) | 0.11 | -0.21 | 0.04 (DF:30) |
| Isolate and resistance cultivars interaction | PIVT1309 | -0.3 | -0.23 | 0.22 (DF:54) | -0.3 | -0.3 | 0.11 (DF:44) |
| | LS-102 | 0.15 | -0.12 | | 0.08 | -0.1 | |
| | Colorado | 0.63 | -0.06 | | 0.26 | -0.2 | |
| | Ninja | -0.08 | -0.18 | | 0.83 | -0.17 | |
| | Discovery | 0.56 | -0.3 | | -0.3 | -0.29 | |

[1]Data subjected to $\log_{10}$ transformation offset by 0.5 to avoid losing zero values. Negative values are results of qPCR readings less than 0.5.

Table 6.10 shows the mean of spore DNA quantified by qPCR sampled on the fifth day post inoculation of Experiment 3. The difference between isolates BL801 and BL806 is significant. The isolate by cultivar interactions are significantly different between isolates BL801 and BL806, except on cultivars Cobham Green, Lednicky, UCDM2, R4T57D, Valmaine, Pennlake, LS-102, Colorado and Discovery. The

interaction on each cultivar is significantly different compared with susceptible control Cobham Green, except in cultivars Lednicky, Sabine and Capitan infected by BL801, and in cultivars UCDM2, Capitan, Hilde II and Argeles infected by BL806.

**Table 6. 11** Analysis of variance of quantity of spore DNA in lettuce cotyledons sampled on the fifth day post inoculation of Experiment 3.

| Mean of spores DNA[1] quantified by qPCR | | | | |
|---|---|---|---|---|
| Isolate BL801and BL806 | | BL801 | BL806 | LSD |
| | | 2.21 | 1.9 | 0.06 |
| Isolates and EU-A IBEB cultivars interactions | Cobham Green | 2.17 | 2.21 | 0.29 |
| | Lednicky | 2.3 | 2.54 | |
| | UC DM2 | 2.69 | 2.46 | |
| | Dandie | 2.96 | 0.12 | |
| | R4T57D | 3.41 | 3.69 | |
| | Valmaine | 3.89 | 3.7 | |
| | Sabine | 2.17 | 0.69 | |
| | LSE57/15 | 3.47 | 2.5 | |
| | UC DM10 | 3.8 | 3.28 | |
| | Capitan | 2.05 | 2.45 | |
| | Hilde II | 3.3 | 2.13 | |
| | Pennlake | 3.56 | 3.54 | |
| | UC DM14 | 1.76 | 2.64 | |
| | PIVT 1309 | 0.32 | 1.07 | |
| | LSE18 | 2.61 | 0.47 | |
| | LS102 | 0.58 | 0.56 | |
| | Colorado | 0.8 | 0.79 | |
| | Ninja | 0.78 | 0.43 | |
| | Discovery | 0.69 | 0.49 | |
| | Argeles | 0.88 | 2.27 | |

[1]Data subjected to $\log_{10}$ transformation.

### 6.3.5.2 Correlation analysis between qPCR quantification and spore count per unit weight of cotyledons

Figure 6.20 and Table 6.12 show the correlation of qPCR results (sampled 4, 5, 6 and 7 dpi) and spore count per unit weight of cotyledons (sampled 7 dpi). In experiment 1, qPCR of isolate BL806 DNA sampled on 4 and 7 dpi were significantly correlated to spore count/unit weight of cotyledons (R>0.811). Although not significant, qPCR of isolate BL801 DNA and spore weight count had the highest R value at 5dpi qPCR. No results of isolates BL801 and BL806 were significant in Experiment 2.

**Table 6. 12** Analysis of correlation coefficient (R) from Experiments 1 and 2, comparing qPCR quantification of *B. lactucae* DNA (sampled on days 4, 5, 6 and 7 after inoculation) and spore count per unit weight of cotyledons (sampled on 7 dpi).

| Day after inoculation | Correlation coefficient (R) of Exp.1 | | Correlation coefficient (R) of Exp.2 | | 95% Critical value for Pearson correlation ($N^1$=6) |
|---|---|---|---|---|---|
| | BL806 | BL801 | BL806 | BL801 | 0.811 |
| 4 | 0.867 | 0.136 | 0.474 | 0.084 | |
| 5 | 0.607 | 0.657 | -0.098 | 0.316 | |
| 6 | 0.705 | 0.450 | -0.172 | 0.204 | |
| 7 | 0.904 | 0.255 | 0.278 | 0.284 | |

[1]Degree of freedom (DF) = N-2.
Negative value represents the regression line goes down in Figure 6.20

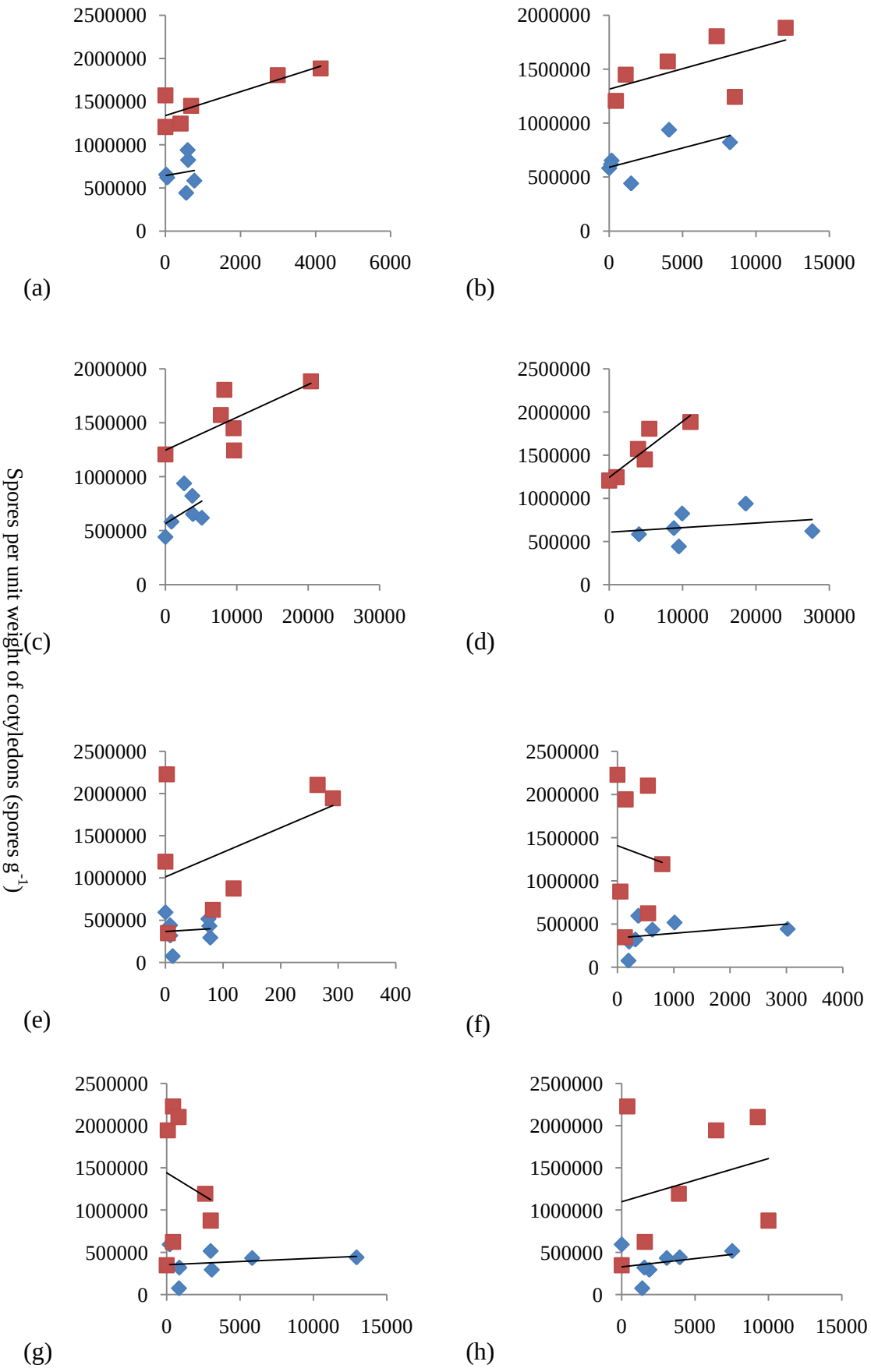

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Spores per unit weight of cotyledons (spores g-1)
qPCR quantification (copy number of spore DNA)

**Figure 6. 20** Regression lines from Experiments 1 and 2 for correlation analysis of qPCR of DNA from two *Bremia lactucae* isolates (BL801 and BL806; sampled days 4, 5, 6 and 7 post inoculation) and the spore count per unit weight of cotyledons (sampled on 7 dpi). Experiment 1: (a) on 4 dpi, (b) on 5 dpi, (c) on 6 dpi, (d) on 7 dpi; experiment 2: (e) on 4 dpi, (f) on 5 dpi, (g) on 6 dpi, (h) on 7 dpi. BL801: ◆ ; BL806: ■ , N = 20.

Figure 6.21 and Table 6.13 show the correlation analysis results of qPCR results from Experiment 3 and spore count per unit weight of cotyledons at 7 dpi. The 95% critical value for Pearson correlation (N=20) is 0.444. There was a significant correlation between the qPCR and spore count per unit weight of cotyledons for both isolates BL801 and BL806 (R> 0.444).

**Table 6. 13** Analysis of correlation coefficient (R) from Experiment 3, comparing qPCR quantification of *B. lactucae* DNA (sampled on 5 days after inoculation) and spore count per unit weight of cotyledons (sampled on 7 dpi).

| Day post inoculation | Correlation coefficient (R) of Exp.3 | | 95% Critical value for Pearson correlation (N=20) |
|---|---|---|---|
| | BL806 | BL801 | 0.444 |
| 5 | 0.675 | 0.612 | |

[1]Degree of freedom (DF=N-2) is 18.

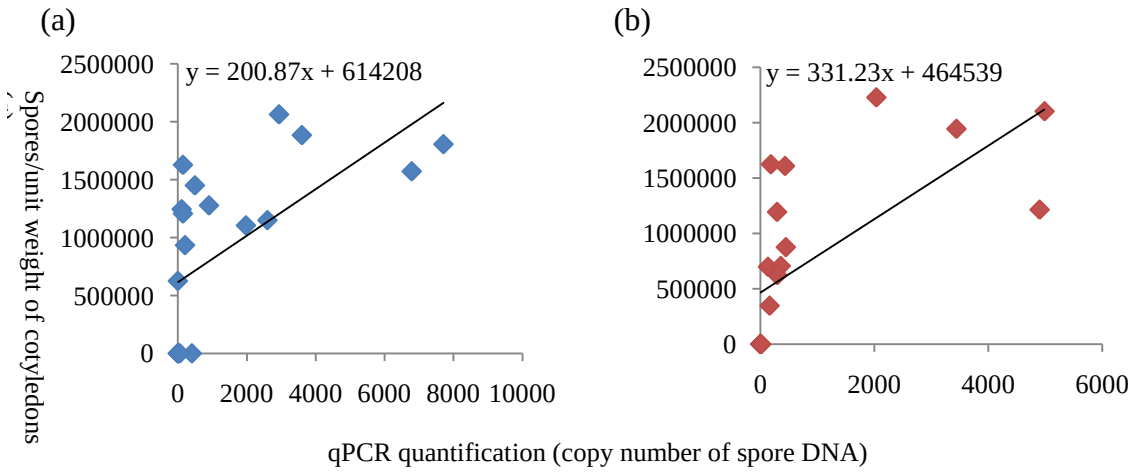



**Figure 6. 21** Regression lines from Experiment 3 for correlation analysis of qPCR of DNA from two *Bremia lactucae* isolates (BL801 and BL806; sampled day 5 post inoculation) and the spore count per unit weight of cotyledons (sampled on 7 dpi) . (a) BL801: ◆ , (b)BL806: ◆ , N = 20.

## 6.4 DISCUSSION

In ChapterS 3 and 5 BL801 and BL806 were differentiated by the IBEB EU-A set of lettuce cultivars and the RxLR1 marker. In this chapter, the differences between these two isolates have been observed by classical microscopic methods and had also been detected by qPCR molecular methods. It is possible that qPCR technique can be used to study the lettuce-*Bremia* interaction. The host-pathogen interaction has been classified in three categories: incompatibility (no visible symptom to an extensive necrotization), incomplete resistance (limited sporulation) and full compatibility (visible profuse sporulation) (Lebeda *et al.*, 2002), which could be determined and be quantified by qPCR in further studies.

### 6.4.1 The development of isolate BL801 and BL806 on resistant cultivars

The microscopy observation results show that BL801 and BL806 behaved differently when they were trying to colonize the same resistant cultivars. Isolate BL801 was more aggressive than BL806, which means more infection structures of BL801 had been observed under the microscope than BL806. Figures 6.5 and 6.6 show the development of the infection structures by these two isolates on six resistant cultivars. These figures show clearly that a higher percentage of secondary vesicle hyphae (short, medium and long) occur on these cultivars when infected by BL801 than by isolate BL806. Hyphal development is an indication of the pathogens growth, at the late infection stage. More long hyphae will appear with further development into mycelium. Isolates BL801 and BL806 could not produce mycelium in these resistant cultivars, however more than 80% of the BL801 infection in cultivar Discovery could develop long hyphae with approximately 20% occurrence on cultivar Ninja and 5% in cultivar LS-102 at the $7^{th}$ day post inoculation. For isolate BL806, less

than 5% of the infection could produce long hyphae in cultivar Discovery, and approximately 10% of the infection could produce medium hyphae in cultivars Ninja and Discovery.

These results show the incompatible reaction varies, which cannot be observed by the IBEB identification set. The differences are shown on some cultivars (like PIVT 1309 and Colorado) where the pathogen stopped developing at the stage of producing short hypha, while in cultivar Discovery development stopped before mycelia were produced. It would be worth investigating the reason why pathogen development stopped and why the pathogen could develop further on certain IBEB cultivars regarded as resistant. Cultivar PIVT1309 and Colorado contained resistance genes *Dm*15 *Dm*18 while cultivars LS-102, Ninja and Discovery contained *Dm*17 *Dm*36 and *Dm*37. In this case, genetically *Dm*15 *Dm*18 are more resistant to these two isolates, *Dm*17 *Dm*36 and *Dm*37 are less resistant to these isolates.

The statistical analysis of microscopy results showed that the difference between BL801 and BL806 is significant ($p<0.05$). Cultivars Discovery and Ninja are regarded as resistant cultivars to isolate BL801 and BL806 according to the IBEB system. However, isolate BL801 can produce long hypae and haustoria in this cultivar. As Tables 6.2, 6.3, 6.5 and 6.6 show, these two cultivars showed less resistance than the other cultivars. Cultivar Discovery may have weaker resistance to BL801 because it produced more long hyphae compare to other resistant cultivars. In the future studies, it would be interesting to observe this pathogen's further development over a longer period in cultivar Discovery. Another finding was isolates could be differentiated by hyphal development at the later infection stage. Tables 6.4,

6.5 and 6.6 show BL801 produced more hyphae than BL806, and the differences of short hyphae are significant ($p<0.05$) between isolate by cultivar interactions in each cultivar, indicating that these two isolates could be differentiated by measureing the short hyphae produced without taking other measures (medium and long hyphae) at the later infection stage.

In Tables 6.5 and 6.6, the zero results have no variances so they do not meet the assumption required for analysis of variance. As a result of this the data could be reanalyzed omitting treatment with means of zero. This could be without data for cultivars Cobham Green, Colorado, PIVT1309 with isolate BL806 in further analysis.

Although microscopy can give reliable visual results, this assessment is very time consuming. In this chapter, it was difficult to quantify the cell death only by visualizing. Hydrogen peroxide accumulation in infected cells has been demonstrated due to the enhanced activity of $H_2O_2$-scavenging enzymes during hypersensitivity in resistance reactions by *Lactuca* spp. (Sedlářová *et al*., 2007). Nitric oxide also plays an important role in the resistance response to infection (Petřivalský *et al.*, 2007). These findings provide an alternative way to detect resistance reactions to *B. lactucae.*

### 6.4.2 qPCR assay quantification of the development of BL801 and BL806

qPCR is a very sensitive method of detecting DNA in the sample and can be sensitive and reliable with high throughput. In this project, spore count per unit weight of cotyledons was used to evaluate whether qPCR results correlated to the actual spore

number on the cultivars. The results of qPCR quantification show the differences between BL801 and BL806 as well. For both of the isolates one thing in common is that the DNA amount started to increase at the 4$^{th}$ day after inoculation.

The DNA accumulation by BL806 in the selected inoculated cultivars developed slower in comparison to BL801. The results show the increase in BL806 DNA started at the 6$^{th}$ day while BL801 started at the 5$^{th}$ day, and this may explain the difference. The DNA quantity of BL806 extracted on the 7$^{th}$ day showed similarity to the results of the spore count per unit weight of cotyledons on the 7$^{th}$ day, while the DNA quantity of BL801 extracted on the 5$^{th}$ day showed similarity to the spore count per unit weight of cotyledons. The results of these observations are not very consistent, and it is possibly caused by losing spores during sampling. Therefore the result might be more reliable before heavy sporulation. However this might be difficult to standardise.

The qPCR assessments of attempted infection of resistant cultivars by *B. lactucae* were not reliable as the values were very low. The qPCR quantification method is potentially very sensitive and the DNA in the plant tissue could be detected even in very low amounts. The low value is possibly caused by DNA losses during the extraction. The lysis procedure could not fully disturpt the cell. This might be the reason why some DNA data of cultivar Cobham Green was missing. A better tissue disturption method is needed in future work.

The qPCR results for isolate BL801 on cultivar Discovery (more BL801 DNA was produced from cv. Discovery than other cultivars) showed the similarity to the

microscopy results (more long hyphae were produced in cv. Discovery than other cultivars). Another finding is cultivar UC DM 14 is a resistant cultivar in IBEB system to BL801 but showed DNA increasing in these experiments. The DNA amount from cv. UCDM14 was more than what was detected in cultivar Discovery; therefore, cultivar UCDM14 might have weaker resistance to BL801 than cv. Discovery. In the experiment of entire IBEB EU-A cultivars, a consistent result was observed, when these two cultivars (UCDM14 and Discovery) were inoculated and cultured in the same system and measured using the same DNA extraction method. Therefore it will be worth carrying out microcopic observations on both of these cultivars, to explain the differences observed by qPCR quantification. In Table 6.9, cultivar UC DM14 infected by BL801, showed incompatible reaction with 57.4 copies of spore DNA was detected. Cultivar Captain was susceptible to BL801 and 112 copies of spore DNA were detected. It would be worth investigating if qPCR quantification could be used to examine the cultivars were susceptible or resistant by the DNA amount of *B. lactucae* in the tissue.

It is possible that the qPCR method can be applied to field commercial lettuce cultivars. The laboratory qPCR results would show the interaction between the isolate or isolates of the pathogen and the field cultivars. It would show whether the field cultivars are susceptible to the isolate present or if the cultivars are resistant. It is possible that this information could be used to quantify the development of pathogen. The information could determine whether pathogens are very aggressive or nonaggressive to the cultivars grown. These laboratory results can provide suggestions and advice to the growers such as when they should use fungicides by predicting the early stage of epidemic under conducive environmental conditions.

Furthermore it is also possible to contribute these results to model the pathogen development and the host genetic interactions.

The regression lines (for both isolates BL801 and BL806) in Figures 6.20 and 6.21 show that they are not two parallel lines which means isolates BL801 and BL806 behaved differently, therefore parallelism analysis is worth investigating in the future work. It will be interesting to compare other isolates by analyzing the intercepts to see whether the isolates behaved differently, which could be an alternative method to differentiate isolates.

# CHAPTER 7 GENERAL DISCUSSION

*Bremia lactucae* is a highly variable plant pathogen (Lebeda *et al*., 2002; Petrželová and Lebeda, 2004), which causes difficulty for disease control in lettuce production, and also for *B. lactucae* population studies. This oomycete has been studied by plant biologists for a century using conventional physiological pathology techniques and more recently using the molecular techniques for genetic investigations. In this current era of biology, molecular technologies provide powerful tools for pathology research in topics such as the phylogeny of pathogens based on molecular evidence, the genetic investigation of the molecular basis of pathogen virulence and host resistance, and potential application of research discoveries in the control of the disease. In this PhD project, molecular techniques have been evaluated for potential use in *B. lactucae* population studies to further the understanding of this oomycete pathogen.

## 7.1 THE CLASSICAL PHENOTYPIC STUDY OF *B. LACTUCAE* POPULATIONS

### 7.1.1 IBEB differential lettuce cultivars

The genus *Bremia* was established by Regel in 1843. In studies in the early 20$^{th}$ century the existence within *B. lactucae* of a number of physiologic races were demonstrated by showing that the various forms differed in their pathogenicity towards varieties of the cultivated lettuce (Jagger and Chandler, 1933; Schultze and Roder, 1938; Ling and Tai, 1945). Early studies focused on the morphology of *B. lactucae* (such as the size and shape of the sporangia) for the taxonomy of the race investigations (Schweizer, 1919; Ling and Tai, 1945). After Flor's hypothesis (1942, 1956 and 1971) on the gene-for-gene relationship, which developed from genetic

analysis of natural variation in flax-*Melampsora* rust interactions, the host-parasite interaction controlled by matching gene pairs of pathogen avirulence (*Avr*) and host resistance (*R*) genes was applied to other pathosystems including lettuce-*Bremia* downy mildew (Crute and Johnson, 1976; Ilott *et al.*, 1989). The International *Bremia* Evaluation Board (IBEB) differential lettuce set is a *Bremia* denomination and identification system based on the gene-for-gene hypothesis. A set of lettuce varieties, each of them carrying different *Dm* (Downy mildew) resistance gene/factors demonstrated the interaction with matching *Avr* (Avirulence) gene/factors in the *Bremia* isolate by showing the visualized + sporulation or - no symptom. The + and - scoring will give a sextet code for the isolate for race identification. The IBEB differential set (or other similar systems) is a phenotypic identification system for *B. lactucae* population and until now it is the most acceptable method for race differentiation as the (interaction) symptom can be observed macroscopically. This kind of coded system has an advantage for investigating pathogen population variation as the code only changes one digit if the pathotype changes from avirluence (incompatible) to virulence (compatible) or vice versa, and this change can be visualized by the incompatible and compatible patterns shown on the cultivars (Limpert *et al*., 1994).

In this project, the 37 isolates of *B. lactucae* that were collected have been differentiated and identified by EU-A IBEB lettuce cultivars set and each of the isolates has been given an IBEB sextet code (Table 3.7 Table 3.8 and Table 3.9). These results indicated that these samples belong to 37 races. However, are these isolates single line race, as they are field samples and have not been isolated from single spores? Can the IBEB differentiation be completely reliable for *B. lactucae*

race identification?

There are several questions that have to be considered. Firstly, what is a *B. lactucae* isolate collected from the field? Normally a sample isolated from a single line of lettuce cultivars in the field, is called an unknown isolate. In most cases this field collected sample is regarded as a race, but it may be comprised of several races. However, it is difficult to investigate the actual races within a field sample. Single spore isolation can only isolate part of the population, and IBEB can only show the virulence-phenotypes that match the limited *Dm* genes in the system. My results show that two different single-spore isolates (S1 and S2) that were isolated from a Lincolnshire 2009 collected field sample Caugin4 (BL815). Sample BL805 was assigned with a sextet code according to the IBEB differential set as a race, but actually it was comprised of two races (S1 and S2 were isolated from BL815 and had been differentiated by the IBEB set, see chapter 3). It is likely that in practice, field samples are heterozygous and/or mixtures of different races. Therefore the IBEB sextet code could not be completely accurate in showing the real identity of the samples without use of single spore techniques to define the population structure in field samples. Two single spore isolates sharing one IBEB sextet code may actually be different races if they contain different *Avr* genes which are not included in the IBEB set. When more than one isolate shows the same - and + patterns, but actually the degree of sporulation varies between them, this may indicate that they are in fact different, but would be considered as one isolate within the IBEB system. Some studies (Dickson and Crute, 1974; Crute and Norwood, 1978; Eenink and Dejong, 1982) have developed different scoring systems based on the level of sporulation. These scoring systems are helpful as a supplement to show the degree of virulence of

*Bremia* instead of a + symbol to differentiate isolates. The description of isolates using IBEB therefore depends on the number of tests conducted and the amount of sub sampling from the original isolation. Both those variables are not described or designated within the IBEB system. Therefore if an 'isolate' is used to represent the epidemiology of *B. lactucae* in the field without being single spore isolated there may be an underestimate of the realistic population variation and virulence of the pathogen and the competition between races may be ignored. The level of sporulation is likely to be important, but is not used within the IBEB system. The description of isolates using IBEB therefore depends on laborious preparation and tests including sub-sampling from the original field isolate to provide a reliable evaluation of race(s) present in a particular field.

Secondly, how do the races compete with each other to dominate the population, and how does the population vary? *B. lactucae* is highly variable, and the mechanism of the variation is still not well understood. Excluding the impact of environmental conditions, the avirulence of *B. lactucae* might change because of the sexual production of oospores or, if some aggressive races dominate the population, the less virulent races might disappear due to competition. The field investigation carried out in Lincolnshire demonstrated the changes of virulence factors which occurred. Studies of *Bremia* populations based on v-phenotypes investigation could be useful (Crute, 1992a; Lebeda and Schwinn, 1994; Lebeda and Zinkernagel, 2003). In these studies the virulence diversity of the pathogen population structure was examined by determining the v-phenotypes using the IBEB method. However, a simulation system would need to be set up to investigate race competition. In such a system, certain races would be combined in equal ratio and used to inoculate a mixture of lettuces

cultivars under the same environmental conditions. The final ratio of the races would show whether one had dominated the population. Subsequent investigation of the genes associated with the dominant race would be essential to enhance the understanding of the genetic virulence of *Bremia*.

Further investigation would require a lot of labour and time particularly if races had to be identified using the IBEB system. Therefore the investigation of *Avr* gene associated markers is essential for the study of *B. lactucae* populations.

### 7.1.2 Microscopic observation

The initiation of infection of *B. lactucae* is the penetration of the plant surface by forming the germ tubes and appressoria and penetration pegs (reviewed in Lebeda *et al*., 2008b and Latijnhouwers *et al*., 2003). Once the pathogen has overcome the barrier of the host cell wall, a primary vesicle is formed within the epidermal cell. This vesicle then swells to form the secondary vesicle from which hyphae develop and exit the cell. The hyphae branch and elongate and penetrate more host cells, and obtaining nutrients from the host to enable growth and colonization by haustoria.

In the lettuce - *Bremia* interaction, the recognition of disease development is based upon the specific *Dm/Avr* gene combination (Mansfield *et al*., 1997). The hypersensitive response is considered as a programmed cell death, triggered by the resistance reaction (Kamoun *et al*., 1999), and it is important for race- specific resistance of lettuce to *B. lactucae* (Lebeda *et al*., 2001). Woods *et al.* (1988a,b) found that variation also existed between isolates in the timing of cell death, as indicated by irreversible membrane damage, according to the *Dm/Avr* gene combination. An estimate of this timing was made by observing the degree of

expansion of the primary vesicle or the initiation of the secondary vesicle. Thus isolates can be distinguished by using microscopy to identify differences between disease developments.

The microscopy results give in Chapter 6 show that isolates BL801 and BL806 behaved differently when they were trying to colonize the same IBEB resistant cultivars. Isolate BL801 was more virulent than BL806 on these cultivars, as BL801 developed more infection structures than BL806. Interestingly the degree of incompatibility of the cultivars varied in this experiment. Such variation could not be shown by testing with the IBEB differential set. Therefore, potential high through put methods, such as *B. lactucae* avirulence related molecular markers might be necessary to be developed in further works.

## 7.2 THE DEVELOPMENT OF MOLECULAR MARKERS FOR *B. LACTUCAE* POPULATION STUDIES

This PhD project aimed to find the molecular markers which can differentiate *B. lactucae* isolates which vary in IBEB designations. ITS markers and microsatellite (SSR and ISSR) markers development were used.

### 7.2.1 SSR markers

Microsatellites are considered as highly polymorphic DNA markers with discrete loci and co-dominant alleles (Schlotterer, 1998), and the hybridization method has been successfully used to construct libraries and determine flanking sequences of microsatellite DNA loci in different organisms including animals fungi and plants such as amphibians, birds, fish, mammals, reptiles, insects, fungi, plants (Glen and

Schable, 2005). However, it was found that SSR markers could not be developed for *Bremia* using this protocol, (Chapter 4) possibly because the primer design was not suitable for the *Bremia* microsatellite regions. The results of the microsatellite repeats found in the Pv SSR primers amplified sequence are repeats of TA, TCG and CGA, which were not included in the 5' anchored biotinylated primers in this protocol. Also, in the study of Delmotte *et al*. (2006) 5'-biotinylated $(CT)_{10}$ and $(GT)_{10}$ oligonucleotides were used as probes, supporting the conclusion that the unsuccessful results might have been caused by the 5′-biotinylated primers design not being suitable for oomycete microsatellite regions. An optimized protocol is necessary for microsatellite marker development.

The isolation of microsatellite loci from *Plasmopara viticola* is an example for use as a *Bremia* microsatellite marker development as *B. lactucae* had been successfully amplified by Pv14, Pv16 and Pv39 (Delmotte *et al*., 2006). However microsatellites isolation from *P. viticola* was difficult with a density of non-redundant positive clones of 1.6%, and the microsatellite sequences were often short and yielded low variability in markers (Delmotte *et al*., 2006). Nevertheless, during the present study, these pimers did successfully amplify some microsatellite repeats of *Bremia* and showed some polymorphism between isolates (Chapter 4). Therefore it may be worth carrying out further work to obtain more genetic information for microsatellite marker development.

### 7.2.2 Inter simple sequence repeat (ISSR) marker development

ISSR has been considered as a new type of DNA marker that involves the use of microsatellite sequences directly in PCR for DNA amplification (Zietkiewicz *et al.*, 1994). These enable amplification of genomic DNA and provide information about many loci simultaneously. SSR primers were developed to determine the genetic variability of *B. lactucae* isolates by Wagner and Idczak (2004). Thirty-one ISSR primers were tested to distinguish 17 races of *B. lactucae*. In this PhD project, the 5´-anchored tri-nucleotide and un-anchored tri-nucleotide ISSR primers were chosen from the Wagner and Idczak (2004) primer list. The results show that ISSR primers can differentiate three *B. lactucae* isolates. The cluster analysis of banding patterns scoring indicated that the three isolates were genetically distinct (Figure 4.13). However, it was found that the ISSR results were not repeatable and there were also difficulties in the isolation of the specific loci in the amplified DNA product. A recent study of *Sclerospora graminicola* (pearl millet downy mildew) characterization using ISSR markers showed that 22 isolates of *S. graminicola* were assessed by using 20 ISSR primers and the results of ISSR fingerprints revealed significant genetic diversity among the *S. graminicola* isolates (Sudisha *et al*., 2010) . Therefore, ISSR markers could be a powerful tool for fingerprinting and diversity analysis for fungal pathogens (Sudisha *et al*., 2010). *S. graminicola* is closely related to *B. lactucae* and, like *Bremia*, the populations are highly variable leading to breakdown of resistance (Hess *et al*., 2002). Furthermore suitable and efficient ISSR markers have been presented for discriminating other oomycete pathogens like *Phytophora* and *Pythium* (Cooke and Lees, 2004). Therefore, despite the technical difficulties, ISSR markers should be regarded as a promising tool for *B. lactucae* population study in future work.

### 7.2.3 The ribosomal internal transcribed spacer (ITS) primer

Choi *et al*. (2007) indicated that the long length and repetitions of the ITS region may be a very common event in *B. lactucae.* ITS2 evolution and repetitions are a major factor in the huge size and sequence variability of ITS among and within species which are subject to high rates of evolution, therefore these repetitions could show considerable sequence variation between *Bremia* isolates. For this reason specific *B. lactucae* primers designed by Choi *et al*. (2007) from the ITS2 region were used in this project. Br 1234F and Br 1586 had been chosen from which specific primers were redesigned which could amplify smaller products for qPCR quantification studies. Although, these Br primers did not show polymorphisms between *B. lactucae* isolates, they are nonetheless highly specific for *B. lactucae*. It is likely that some SNPs exist in the sequences of the repeats amplified by these primers which could be developed into markers. In summary, it has proven to be difficult to develop a set of molecular markers that can identify differences between *B. lactucae* isolates using these methods but they show promise for the SNP polymorphisms investigation.

## 7.3 THE COMPARISON OF RXLR EFFECTORS AND QPCR QUANTIFICATION AND THE IBEB SET

As RxLR effector proteins have been described as a class of genes which include examples of avirulence elicitors (based on R-gene mediated resistance in the host) (Fabro *et al*., 2011), it is likely that natural variation should be easy to detect in RxLR effectors from *B. lactucae* pathogen. My results showed that RxLR1 primers could be used to differentiate isolates BL801 and BL806. The field samples show more polymorphisms when amplified with other RxLR primers. Surprisingly, it was

not possible to detect variation in other examples of RxLR effectors from *B. lactucae*, which may indicate that the level of RxLR variation of *B. lactucae* might not be as high as other downy mildews.

The results of Fabro *et al.* (2011) show that high polymorphism was found in SNPs in effector candidates between seven *Hyaloperonospora arabidopsidis* (*Hpa*) isolates. The sequencing of the Hpa isolate Emoy2 genome revealed its potential to encode at least 140 RxLRs (or HaRxLs) and 56% of these showed polymorphism between 1to 10 SNPs. In my results, 8 SNPs were found between 3 isolates amplified by RxLR5, which indicates that it would be worth sequencing all the candidates to look for SNP polymorphisms. From the 17 RxLRs sequenced in this study, only field samples showed polymorphism. This is less than the 56%, but this may be because only 17 RxLRs were investigated, compared to the 140 candidates investigated. It may therefore be worth putting further effort into RxLR marker development.

The qPCR quantification could not quantify the DNA extracted from resistant cultivars. This it might have been because the scale of the standards for the calibration curve was too high. This calibration curve was more suitable for the high spore production of the susceptible cultivars, not the resistant cultivars. However considering the findings from using the RxLR1 primers, it may be possible to use these primers in qPCR to quantify the DNA extracted from the resistant cultivars and show the degree of the resistance to *B. lactucae*.

Statistical analysis of the results showed that both RxLR1 markers and qPCR quantification could differentiate the isolates BL801 and BL806 indicating that both

these methods could be useful for future diagnostic work

## 7.4 FUTURE WORK FOR POPULATION STUDIES

The results of this project indicated that it is very difficult to develop the race specific markers for *B. lactucae*. However, the methods I used in this project show the potential for developing such markers with future work focusing on the use of RxLR primers and ISSR primers. The qPCR application shows an alternative way for population studies. It will take time for the marker development, but what could we do to investigate race competition without certain race specific markers? The results of this project not only provide useful information for the future studies but also inspired another way of conducting population competition study by using qPCR methods.

Xu (2011) developed a simulation system to investigate epidemic development of a race-specific pathogen in cultivar mixtures. This study shows a potential to develop a simulation system for *B. lactucae* population competition studies for future work if race-specific markers have not been developed. The cultivars mixture will force the pathogen to survive in a spatially heterogeneous host environment. Some studies (Frey *et al*., 1977; Mundt and Browning, 1985; Mundt *et al.*, 1996; Ntahimpera *et al.*, 1996; Burdon and Thrall, 1999; Mundt, 2002; Cox *et al*., 2004; Didelot *et al.*, 2007) have shown that the rate of disease increase can be reduced significantly in mixtures compared to monocultures. The systematic change in spatial position of individual mixture components is a feasible option for disease management (Xu, 2011).

Thus a simulation system with mixtures lettuce cultivars for *Bremia* population study is feasible for future work. For example, two *B. lactucae* races A and B could be inoculated separately with the same lettuce cultivars arrangement under the same conditional simulation system (separate experiment). They could be sampled on the $4^{th}$ or $5^{th}$ day post inoculation and DNA could be extracted separately from each cultivar for qPCR quantification. Ideally, the polymorphisms detected from DNA extracted from each isolate would be different from races (pattern A or pattern B). If the patterns would be significantly different, then Race A and B could be mixed equally and inoculated on the same cultivars as the separate experiment. If the final qPCR quantification patterns might show the similarity to A or B that would indicate which race dominated the system. This system might show how combinations of races interact.

The results in Chapter 5 showed a possibility of using RxLR markers to quantify the development of *B. lactucae* in host tissue during the early infection stages of susceptible cultivars and later stage of the resistant cultivars. As the RxLR effectors are likely to be secreted by haustoria and in most instances the resistance response is triggered after the formation of haustoria to enable the pathogen to develop and establish a limited biotropy before recognition (Tör *et al*., 2002). This suggested that the quantity of RxLR effectors represent the development of the pathogen infection structure. Therefore shorter (around 100-150 bp) primers can be designed from *Bremia* RxLR markers for qPCR quantification to investigate the variation of *B. lactucae* during the colonization. This potential technique can be a complemantary method to the microscopic observation.

## CONCLUSION

In this PhD project, I mainly focus on developing reliable and efficient molecular methods for studying *B. lactucae*. The potential use of different types of molecular markers (microsatellite, ITS, ISSR and RxLR) were investigated and have provided useful information for future studies. The IBEB system does not appear to be completely reliable for race identification, because IBEB resistance reactions are variable, and in particular, the class (+ or -) designation cannot adequately describe the true interaction between pathogen and host. RxLR primers are very promising for detecting potential variation in the avirulence of the *B. lactucae*. Although the race specific markers have not yet been fully developed, alternative methods like qPCR quantification and microscopic observation can show major differences between isolates. However, the variation in *B. lactucae* based on the set of RxLR effectors used in this investigation was surprisingly very low, especially in comparison with downy mildew pathogens of *A. thaliana* and *B. oleracea.* Further exploration of RxLR effector genes and other genes in *B. lactucae* that encode secreted virulence proteins will be necessary to better explain the tremendous variation in pathogenicity of this lettuce pathogen.

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# APPENDICES

## APPENDIX 1. ADDITIONAL INFORMATION

**Table A1.** The IBEB phenotypic classification of BL801 and BL806.

| IBEB EU-A lettuce varieties | Reaction to isolates | |
|---|---|---|
| | BL801 | BL806 |
| Cobham Green * | + | + |
| Lednicky (*Dm* 1) | + | + |
| UC DM2 (*Dm* 2) | + | + |
| Dandie (*Dm* 3) | + | - |
| R4T57D (*Dm* 4) | + | + |
| Valmaine (*Dm* 5/8) | + | + |
| Sabine (*Dm* 6) | + | - |
| LSE57/15 (*Dm* 7) | + | + |
| UC DM10 (*Dm* 10) | + | + |
| Capitan (*Dm* 11) | + | + |
| Hilde II (*Dm* 12) | + | + |
| Pennlake (*Dm* 13) | + | + |
| UC DM14 (*Dm* 14) | - | + |
| PIVT 1309 (*Dm* 15) | - | - |
| LSE18 (*Dm* 16) | + | - |
| LS102 (*Dm* 17) | - | - |
| Colorado (*Dm* 18) | - | - |
| Ninja (*Dm* 36) | - | - |
| Discovery (*Dm* 37) | - | - |
| Argeles (*Dm* 38) | - | + |

*: Cobham Green is a universal susceptible cultivar (contains no known *Dm* gene).
+: visible sporulation, -: no visible sporulation.

**Table A 2.** The calculations of percentages of infection structures and host cell death.

| | Calculation |
|---|---|
| Secondary vesicle | (secondary vesicle without hypha developed/total secondary vesicle) x100% |
| Short hypha | (short hypha/ total secondary vesicle) x100% |
| Medium hypha | (medium hypha/total secondary vesicle) x 100% |
| Long hypha | (long hypha/total secondary vesicle) x 100% |
| Cell death | (cell death/total secondary vesicle) x 100% |

Figures 6.5 and 6.6 were generated followed the formular in this table.

## APPENDIX 2. OUTPUT OF GENSTAT (12th EDTITION) ANALYSIS

### A 2.1 The output of GenStat 12th edition of microscopic observation

**Secondary vesicle:**

Variate: SQRT(secondary_vesicle)

| Source of variation | d.f.(m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| day.block stratum | | | | | |
| sample | 6 | 183.2030 | 30.5338 | 66.15 | <.001 |
| Residual | 14 | 6.4625 | 0.4616 | 0.85 | |
| day.block.leaf stratum | | | | | |
| isolate | 1 | 1103.7471 | 1103.7471 | 2028.54 | <.001 |
| cultivar | 5 | 1964.0324 | 392.8065 | 721.93 | <.001 |
| isolate.cultivar | 5 | 118.8071 | 23.7614 | 43.67 | <.001 |
| isolate.sample | 6 | 16.2399 | 2.7067 | 4.97 | <.001 |
| cultivar.sample | 30 | 342.0284 | 11.4009 | 20.95 | <.001 |
| isolate.cultivar.sample | 30 | 150.3740 | 5.0125 | 9.21 | <.001 |
| Residual | 140(14) | 76.1751 | 0.5441 | | |
| Total | 237(14) | 3862.0221 | | | |

* SQRT: square root transformation.

Mean of isolate by cultivar of secondary vesicle

| Isolate | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | 8.168 | 15.8 | 9.973 | 6.209 | 10.09 | 8.624 |
| BL806 | 3.708 | 11.744 | 3.995 | 4.725 | 5.067 | 4.512 |

Mean of isolate by sample of secondary vesicle

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 8.219 | 9.205 | 9.921 | 10.257 | 11.066 | 10.235 | 9.772 |
| BL806 | 3.886 | 4.461 | 6.184 | 6.099 | 6.237 | 5.997 | 6.51 |

Mean of cultivar by sample of secondary vesicle

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 5.74 | 5.908 | 6.111 | 5.633 | 6.063 | 5.811 | 6.3 |
| Control | 8.051 | 9.694 | 15.731 | 15.731 | 15.731 | 15.731 | 15.731 |
| Discovery | 6.298 | 6.507 | 6.186 | 7.45 | 7.914 | 7.358 | 7.176 |
| LS-102 | 3.066 | 5.449 | 5.496 | 5.055 | 6.585 | 6.353 | 6.265 |
| Ninja | 6.535 | 6.567 | 7.481 | 8.577 | 9.318 | 7.363 | 7.207 |
| PIVT1309 | 6.624 | 6.876 | 7.311 | 6.62 | 6.296 | 6.082 | 6.167 |

Mean of isolate by cultivar by sample of secondary vesicle

| Isolate | Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 8.56 | 8.26 | 8.08 | 7.72 | 8.53 | 7.75 | 8.28 |
| | Control | 10.86 | 13.13 | 17.32 | 17.32 | 17.32 | 17.32 | 17.32 |
| | Discovery | 9.56 | 9.20 | 8.22 | 11.00 | 11.42 | 10.64 | 9.78 |
| | LS-102 | 2.58 | 6.91 | 6.52 | 5.29 | 7.34 | 7.35 | 7.48 |
| | Ninja | 7.66 | 8.25 | 9.25 | 12.26 | 14.35 | 10.73 | 8.13 |
| | PIVT1309 | 10.10 | 9.48 | 10.14 | 7.96 | 7.43 | 7.62 | 7.65 |
| BL806 | Colorado | 2.92 | 3.56 | 4.14 | 3.54 | 3.60 | 3.87 | 4.32 |
| | Control | 5.24 | 6.26 | 14.14 | 14.14 | 14.14 | 14.14 | 14.14 |
| | Discovery | 3.04 | 3.82 | 4.15 | 3.90 | 4.41 | 4.07 | 4.57 |
| | LS-102 | 3.56 | 3.99 | 4.47 | 4.82 | 5.83 | 5.36 | 5.05 |
| | Ninja | 5.41 | 4.89 | 5.71 | 4.90 | 4.29 | 4.00 | 6.28 |
| | PIVT1309 | 3.15 | 4.27 | 4.49 | 5.28 | 5.16 | 4.55 | 4.69 |

Least significant differences of means (5% level) of secondary vesicle

| | Isolate | cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126 | 42 | 36 | 21 |
| d.f. | 140 | 140 | 14 | 140 |
| l.s.d. | 0.1837 | 0.3182 | 0.3435 | 0.4501 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar by sample |
|---|---|---|---|
| rep. | 18 | 6 | 3 |
| l.s.d. | 0.4731 | 0.8308 | 1.1823 |
| d.f. | 58.35 | 148.72 | 153.31 |
| Except when comparing means with the same level(s) of | | | |
| sample | 0.4861 | 0.8420 | 1.1907 |
| d.f. | 140 | 140 | 140 |
| isolate.sample | | | 1.1907 |
| d.f. | | | 140 |
| cultivar.sample | | | 1.1907 |
| d.f. | | | 140 |

**Hyphae:**

Variate: SQRT(hyphae)

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| day.block stratum | | | | | | |
| sample | 6.00 | | 501.70 | 83.62 | 144.64 | <.001 |
| Residual | 14.00 | | 8.09 | 0.58 | 0.90 | |
| day.block.leaf stratum | | | | | | |
| isolate | 1.00 | | 949.04 | 949.04 | 1484.91 | <.001 |
| cultivar | 5.00 | | 3442.01 | 688.40 | 1077.11 | <.001 |
| isolate.cultivar | 5.00 | | 346.45 | 69.29 | 108.41 | <.001 |
| isolate.sample | 6.00 | | 5.44 | 0.91 | 1.42 | 0.21 |
| cultivar.sample | 30.00 | | 478.89 | 15.96 | 24.98 | <.001 |
| isolate.cultivar.sample | 30.00 | | 78.65 | 2.62 | 4.10 | <.001 |
| Residual | 140.00 | -14.00 | 89.48 | 0.64 | | |
| Total | 237.00 | -14.00 | 5766.75 | | | |

Mean of isolate by cultivar of hyphae

| Isolate | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | 3.365 | 14.911 | 8.847 | 3.781 | 8.629 | 3.833 |
| BL806 | 0.964 | 11.341 | 2.136 | 2.633 | 1.274 | 1.732 |

Mean of isolate by sample of hyphae

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 4.134 | 5.959 | 7.327 | 8.459 | 8.759 | 8.216 | 7.74 |
| BL806 | 0.549 | 2.403 | 3.658 | 4.225 | 4.492 | 4.078 | 4.02 |

Mean of cultivar by sample of hyphae

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 0.902 | 2.101 | 1.412 | 2.756 | 2.658 | 2.562 | 2.761 |
| Control | 5.85 | 7.375 | 15.731 | 15.731 | 15.731 | 15.731 | 15.731 |
| Discovery | 3.785 | 5.433 | 4.794 | 5.795 | 6.411 | 6.322 | 5.901 |
| LS-102 | 0 | 3.213 | 2.734 | 2.975 | 4.955 | 4.338 | 4.233 |
| Ninja | 3.512 | 4.144 | 4.906 | 6.864 | 6.072 | 4.963 | 4.2 |
| PIVT1309 | 0 | 2.821 | 3.379 | 3.933 | 3.923 | 2.968 | 2.454 |

Mean of cultivar by isolate by sample of hyphae

| Isolate | Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 1.81 | 4.20 | 2.82 | 3.51 | 3.32 | 3.52 | 4.38 |
| | Control | 8.41 | 9.37 | 17.32 | 17.32 | 17.32 | 17.32 | 17.32 |
| | Discovery | 7.57 | 8.64 | 7.16 | 9.29 | 10.29 | 9.61 | 9.37 |
| | LS-102 | 0.00 | 3.26 | 3.09 | 3.57 | 5.75 | 5.42 | 5.37 |
| | Ninja | 7.02 | 6.87 | 8.76 | 11.77 | 10.86 | 8.98 | 6.13 |
| | PIVT1309 | 0.00 | 3.41 | 4.80 | 5.29 | 5.02 | 4.45 | 3.86 |
| BL806 | Colorado | 0.00 | 0.00 | 0.00 | 2.00 | 2.00 | 1.61 | 1.14 |
| | Control | 3.30 | 5.38 | 14.14 | 14.14 | 14.14 | 14.14 | 14.14 |
| | Discovery | 0.00 | 2.23 | 2.43 | 2.30 | 2.54 | 3.03 | 2.43 |
| | LS-102 | 0.00 | 3.16 | 2.38 | 2.38 | 4.16 | 3.26 | 3.09 |
| | Ninja | 0.00 | 1.41 | 1.05 | 1.96 | 1.29 | 0.94 | 2.27 |
| | PIVT1309 | 0.00 | 2.23 | 1.96 | 2.58 | 2.83 | 1.48 | 1.05 |

Least significant differences of means (5% level) of hyphae

| | Isolate | Cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126 | 42 | 36 | 21 |
| d.f. | 140 | 140 | 14 | 140 |
| l.s.d. | 0.1991 | 0.3449 | 0.3844 | 0.4878 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar sample |
|---|---|---|---|
| rep. | 18 | 6 | 3 |
| l.s.d. | 0.5211 | 0.9049 | 1.2844 |
| d.f. | 55.31 | 147.1 | 153.59 |
| Except when comparing means with the same level(s) of sample | 0.5269 | 0.9125 | 1.2905 |
| d.f. | 140 | 140 | 140 |
| isolate.sample | | | 1.2905 |
| d.f. | | | 140 |
| cultivar.sample | | | 1.2905 |
| d.f. | | | 140 |

**Cell death:**

Variate: SQRT(cell_death)

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| day.block stratum | | | | | | |
| sample | 6 | | 114.528 | 19.088 | 14.38 | <.001 |
| Residual | 14 | | 18.581 | 1.327 | 0.95 | |
| day.block.leaf stratum | | | | | | |
| isolate | 1 | | 865.563 | 865.563 | 620.53 | <.001 |
| cultivar | 5 | | 416.040 | 83.208 | 59.65 | <.001 |
| isolate.cultivar | 5 | | 264.553 | 52.911 | 37.93 | <.001 |
| isolate.sample | 6 | | 11.443 | 1.907 | 1.37 | 0.233 |
| cultivar.sample | 25 | (5) | 221.782 | 8.871 | 6.36 | <.001 |
| isolate.cultivar.sample | 25 | (5) | 209.088 | 8.364 | 6.00 | <.001 |
| Residual | 120 | (34) | 167.385 | 1.395 | | |
| Total | 207 | (44) | 1925.592 | | | |

Mean of isolate by cultivar of cell death

| Isolate | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | 7.807 | 4.348 | 10.652 | 6.814 | 10.857 | 8.55 |
| BL806 | 3.838 | 3.609 | 4.272 | 5.311 | 5.081 | 4.676 |

Mean of isolate by sample of cell death

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 6.865 | 8.072 | 7.734 | 8.58 | 9.455 | 7.977 | 8.516 |
| BL806 | 2.938 | 4.47 | 4.555 | 4.611 | 4.945 | 4.55 | 5.185 |

Mean of cultivar by sampleof cell death

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 5.912 | 5.758 | 4.918 | 5.893 | 6.072 | 5.953 | 6.251 |
| Control | 0.691 | 5.708 | 3.824 | 4.275 | 4.88 | 3.943 | 4.53 |
| Discovery | 6.47 | 6.709 | 6.523 | 7.889 | 8.353 | 7.712 | 8.577 |
| LS-102 | 3.066 | 5.513 | 5.73 | 5.125 | 7.873 | 7.52 | 7.611 |
| Ninja | 6.31 | 6.638 | 7.951 | 9.128 | 9.55 | 8.24 | 7.967 |
| PIVT1309 | 6.959 | 7.298 | 7.922 | 7.264 | 6.471 | 4.214 | 6.167 |

Mean of isolate by cultivar by sample of cell death

| Isolate | Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 8.91 | 7.91 | 5.69 | 7.87 | 8.50 | 7.71 | 8.05 |
| | Control | 1.38 | 5.82 | 3.93 | 4.76 | 5.66 | 4.18 | 4.71 |
| | Discovery | 9.90 | 9.34 | 8.66 | 11.59 | 11.83 | 11.19 | 12.07 |
| | LS-102 | 2.58 | 7.00 | 6.71 | 5.16 | 8.45 | 8.60 | 9.20 |
| | Ninja | 7.66 | 8.33 | 10.19 | 13.10 | 14.81 | 12.48 | 9.43 |
| | PIVT1309 | 10.77 | 10.03 | 11.23 | 9.00 | 7.48 | 3.71 | 7.65 |
| BL806 | Colorado | 2.92 | 3.60 | 4.14 | 3.91 | 3.64 | 4.20 | 4.45 |
| | Control | 0.00 | 5.60 | 3.72 | 3.79 | 4.11 | 3.71 | 4.35 |
| | Discovery | 3.04 | 4.08 | 4.39 | 4.19 | 4.88 | 4.24 | 5.09 |
| | LS-102 | 3.56 | 4.02 | 4.75 | 5.09 | 7.29 | 6.44 | 6.02 |
| | Ninja | 4.96 | 4.95 | 5.71 | 5.16 | 4.29 | 4.00 | 6.51 |
| | PIVT1309 | 3.15 | 4.57 | 4.62 | 5.53 | 5.46 | 4.72 | 4.69 |

Least significant differences of means (5% level) of cell death

| | Isolate | Cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126 | 42 | 36 | 21 |
| d.f. | 120 | 120 | 14 | 120 |
| l.s.d. | 0.2946 | 0.5103 | 0.5824 | 0.7216 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar by sample |
|---|---|---|---|
| rep. | 18 | 6 | 3 |
| l.s.d. | 0.7803 | 1.3436 | 1.9035 |
| d.f. | 52.17 | 129.74 | 133.12 |
| Except when comparing means with the same level(s) of | | | |
| sample | 0.7795 | 1.3501 | 1.9093 |
| d.f. | 120 | 120 | 120 |
| isolate.sample | | | 1.9093 |
| d.f. | | | 120 |
| cultivar.sample | | | 1.9093 |
| d.f. | | | 120 |

**Short hyphae:**

Variate: SQRT(short_hyphe)

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| day.block stratum | | | | | | |
| sample | 6.00 | | 22.17 | 3.69 | 5.94 | 0.00 |
| Residual | 14.00 | | 8.71 | 0.62 | 1.06 | |
| day.block.leaf stratum | | | | | | |
| isolate | 1.00 | | 340.47 | 340.47 | 579.69 | <.001 |
| cultivar | 5.00 | | 227.51 | 45.50 | 77.47 | <.001 |
| isolate.cultivar | 5.00 | | 144.18 | 28.84 | 49.10 | <.001 |
| isolate.sample | 6.00 | | 26.98 | 4.50 | 7.66 | <.001 |
| cultivar.sample | 30.00 | | 374.88 | 12.50 | 21.28 | <.001 |
| isolate.cultivar.sample | 30.00 | | 157.37 | 5.25 | 8.93 | <.001 |
| Residual | 140.00 | -14.00 | 82.23 | 0.59 | | |
| Total | 237.00 | -14.00 | 1306.99 | | | |

Mean of isolate by cultivar of short hyphae

| Isolate | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | 3.35 | 1.201 | 4.702 | 3.113 | 6.455 | 3.834 |
| BL806 | 0.964 | 0.471 | 1.837 | 2.428 | 1.274 | 1.732 |

Mean of isolate by sample of short hyphae

| Isolate | sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 4.134 | 4.069 | 3.713 | 4.172 | 3.946 | 3.605 | 2.79 |
| BL806 | 0.549 | 1.432 | 1.301 | 1.868 | 2.007 | 1.477 | 1.522 |

Mean of cultivar by sample of short hyphae

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 0.902 | 2.061 | 1.412 | 2.756 | 2.698 | 2.528 | 2.741 |
| Control | 5.85 | 0 | 0 | 0 | 0 | 0 | 0 |
| Discovery | 3.785 | 4.981 | 3.972 | 3.096 | 3.506 | 2.477 | 1.069 |
| LS-102 | 0 | 2.885 | 2.696 | 2.876 | 4.135 | 3.513 | 3.289 |
| Ninja | 3.512 | 3.758 | 3.585 | 5.459 | 3.596 | 3.758 | 3.382 |
| PIVT1309 | 0 | 2.821 | 3.379 | 3.933 | 3.923 | 2.969 | 2.454 |

Mean of isolate cultivar by sample of short hyphae

| Isolate | Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 1.81 | 4.12 | 2.82 | 3.51 | 3.40 | 3.45 | 4.34 |
| | Control | 8.41 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Discovery | 7.57 | 8.20 | 5.52 | 3.89 | 4.93 | 2.80 | 0.00 |
| | LS-102 | 0.00 | 2.58 | 3.01 | 3.37 | 4.43 | 4.36 | 4.04 |
| | Ninja | 7.02 | 6.10 | 6.12 | 8.96 | 5.90 | 6.57 | 4.49 |
| | PIVT1309 | 0.00 | 3.41 | 4.80 | 5.29 | 5.02 | 4.45 | 3.86 |
| BL806 | Colorado | 0.00 | 0.00 | 0.00 | 2.00 | 2.00 | 1.61 | 1.14 |
| | Control | 3.30 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Discovery | 0.00 | 1.76 | 2.43 | 2.30 | 2.08 | 2.16 | 2.14 |
| | LS-102 | 0.00 | 3.19 | 2.38 | 2.38 | 3.84 | 2.67 | 2.54 |
| | Ninja | 0.00 | 1.41 | 1.05 | 1.96 | 1.29 | 0.94 | 2.27 |
| | PIVT1309 | 0.00 | 2.23 | 1.96 | 2.58 | 2.83 | 1.48 | 1.05 |

Least significant differences of means (5% level) of short hyphae

| | Isolate | Cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126.00 | 42.00 | 36.00 | 21.00 |
| d.f. | 140.00 | 140.00 | 14.00 | 140.00 |
| l.s.d. | 0.19 | 0.33 | 0.40 | 0.47 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar sample |
|---|---|---|---|
| rep. | 18.00 | 6.00 | 3.00 |
| l.s.d. | 0.52 | 0.88 | 1.24 |
| d.f. | 48.60 | 141.93 | 153.98 |
| Except when comparing means with the same level(s) of | | | |
| sample | 0.51 | 0.87 | 1.24 |
| d.f. | 140.00 | 140.00 | 140.00 |
| isolate.sample | | | 1.24 |
| d.f. | | | 140.00 |
| cultivar.sample | | | 1.24 |
| d.f. | | | 140.00 |

**Medium hyphae:**

Variate: SQRT(medium_hyphe)

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| day.block stratum | | | | | | |
| sample | 6.00 | | 66.85 | 11.14 | 22.92 | <.001 |
| Residual | 14.00 | | 6.81 | 0.49 | 1.42 | |
| day.block.leaf stratum | | | | | | |
| isolate | 1.00 | | 184.68 | 184.68 | 539.66 | <.001 |
| cultivar | 5.00 | | 233.71 | 46.74 | 136.58 | <.001 |
| isolate.cultivar | 5.00 | | 198.20 | 39.64 | 115.83 | <.001 |
| isolate.sample | 6.00 | | 56.14 | 9.36 | 27.34 | <.001 |
| cultivar.sample | 30.00 | | 217.82 | 7.26 | 21.22 | <.001 |
| isolate.cultivar.sample | 30.00 | | 187.79 | 6.26 | 18.29 | <.001 |
| Residual | 140.00 | -14.00 | 47.91 | 0.34 | | |
| Total | 237.00 | -14.00 | 1017.31 | | | |

Mean of isolate by cultivar of medium hyphae

| Isolate | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | 0.062 | 1.136 | 3.847 | 1.624 | 4.839 | 0.005 |
| BL806 | 0 | 0 | 0.529 | 0.7 | 0.011 | 0 |

Mean of isolate by sample of medium hyphae

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 0 | 2.518 | 1.948 | 2.632 | 2.969 | 2.282 | 1.083 |
| BL806 | 0 | 0.064 | 0 | 0 | 0.417 | 0.524 | 0.442 |

Mean of cultivar by sample of medium hyphae

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 0 | 0.236 | 0 | 0 | -0.024 | 0.019 | -0.012 |
| Control | 0 | 3.976 | 0 | 0 | 0 | 0 | 0 |
| Discovery | 0 | 1.579 | 2.081 | 3.909 | 4.573 | 2.764 | 0.408 |
| LS-102 | 0 | 0.427 | 0.763 | 0.524 | 1.715 | 2.537 | 2.168 |
| Ninja | 0 | 1.525 | 2.999 | 3.464 | 3.896 | 3.079 | 2.014 |
| PIVT1309 | 0 | 0 | 0 | 0 | 0 | 0.019 | 0 |

Mean of cultivar by isolate by sample of medium hyphae

| Isolate | Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 0.00 | 0.47 | 0.00 | 0.00 | -0.05 | 0.04 | -0.02 |
| | Control | 0.00 | 7.95 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Discovery | 0.00 | 2.69 | 4.16 | 7.82 | 8.01 | 4.25 | 0.00 |
| | LS-102 | 0.00 | 0.94 | 1.53 | 1.05 | 2.06 | 3.21 | 2.58 |
| | Ninja | 0.00 | 3.05 | 6.00 | 6.93 | 7.79 | 6.16 | 3.95 |
| | PIVT1309 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 |
| BL806 | Colorado | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Control | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Discovery | 0.00 | 0.47 | 0.00 | 0.00 | 1.14 | 1.28 | 0.82 |
| | LS-102 | 0.00 | -0.09 | 0.00 | 0.00 | 1.37 | 1.87 | 1.76 |
| | Ninja | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 |
| | PIVT1309 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

Least significant differences of means (5% level) of medium hyphae

| | Isolate | Cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126 | 42 | 36 | 21 |
| d.f. | 140 | 140 | 14 | 140 |
| l.s.d. | 0.1457 | 0.2524 | 0.3525 | 0.3569 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar by sample |
|---|---|---|---|
| rep. | 18.00 | 6.00 | 3.00 |
| l.s.d. | 0.43 | 0.69 | 0.96 |
| d.f. | 38.73 | 127.74 | 152.98 |
| Except when comparing means with the same level(s) of sample | 0.39 | 0.67 | 0.94 |
| d.f. | 140.00 | 140.00 | 140.00 |
| isolate.sample | | | 0.94 |
| d.f. | | | 140.00 |
| cultivar.sample | | | 0.94 |
| d.f. | | | 140.00 |

**Long hyphae:**

Variate: SQRT(long_hyphae)

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| day.block stratum | | | | | | |
| sample | 6 | | 428.074 | 71.3457 | 243.26 | <.001 |
| Residual | 14 | | 4.1061 | 0.2933 | 1.25 | |
| day.block.leaf stratum | | | | | | |
| isolate | 1 | | 98.4061 | 98.4061 | 419.27 | <.001 |
| cultivar | 5 | | 4662.85 | 932.571 | 3973.35 | <.001 |
| isolate.cultivar | 5 | | 88.98 | 17.796 | 75.82 | <.001 |
| isolate.sample | 6 | | 55.2777 | 9.213 | 39.25 | <.001 |
| cultivar.sample | 30 | | 1300.54 | 43.3512 | 184.7 | <.001 |
| isolate.cultivar.sample | 30 | | 136.239 | 4.5413 | 19.35 | <.001 |
| Residual | 140 | -14 | 32.8589 | 0.2347 | | |
| Total | 237 | -14 | 6713.25 | | | |

Mean of isolate by cultivar of long hyphae

| | Cultivar | | | | | |
|---|---|---|---|---|---|---|
| Isolate | Colorado | Control | Discovery | LS-102 | Ninja | PIVT1309 |
| BL801 | -0.021 | 13.079 | 3.325 | 0.654 | 1.374 | -0.002 |
| BL806 | 0 | 10.87 | 0.067 | -0.026 | -0.001 | 0 |

Mean of isolate by sample of long hyphae

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | 0 | 0.982 | 3.209 | 3.525 | 4.673 | 4.18 | 4.908 |
| BL806 | 0 | 0.896 | 2.357 | 2.357 | 2.406 | 2.357 | 2.355 |

Mean of cultivar by sample of long hyphae

| | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| Cultivar | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Colorado | 0 | 0 | 0 | 0 | -0.089 | -0.008 | 0.022 |
| Control | 0 | 5.165 | 15.731 | 15.731 | 15.731 | 15.731 | 15.731 |
| Discovery | 0 | 0 | 0.354 | 0.736 | 2.212 | 3.887 | 4.686 |
| LS-102 | 0 | 0.233 | 0 | 0 | 1.105 | 0 | 0.86 |
| Ninja | 0 | 0.236 | 0.612 | 1.179 | 2.279 | 0.007 | 0.49 |
| PIVT1309 | 0 | 0 | 0 | 0 | 0 | -0.008 | 0 |

Mean of cultivar by isolate by sample of long hyphae

| | | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Isolate | Cultivar | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| BL801 | Colorado | 0 | 0 | 0 | 0 | -0.178 | -0.015 | 0.045 |
| | Control | 0 | 4.949 | 17.321 | 17.321 | 17.321 | 17.321 | 17.321 |
| | Discovery | 0 | 0 | 0.707 | 1.471 | 3.952 | 7.773 | 9.372 |
| | LS-102 | 0 | 0.471 | 0 | 0 | 2.387 | 0 | 1.721 |
| | Ninja | 0 | 0.471 | 1.225 | 2.357 | 4.558 | 0.014 | 0.99 |
| | PIVT1309 | 0 | 0 | 0 | 0 | 0 | -0.015 | 0 |
| BL806 | Colorado | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Control | 0 | 5.38 | 14.142 | 14.142 | 14.142 | 14.142 | 14.142 |
| | Discovery | 0 | 0 | 0 | 0 | 0.471 | 0 | 0 |
| | LS-102 | 0 | -0.006 | 0 | 0 | -0.178 | 0 | 0 |
| | Ninja | 0 | 0 | 0 | 0 | 0 | 0 | -0.01 |
| | PIVT1309 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Least significant differences of means (5% level) of long hyphae

| | Isolate | Cultivar | Sample | Isolate by cultivar |
|---|---|---|---|---|
| rep. | 126.00 | 42.00 | 36.00 | 21.00 |
| d.f. | 140.00 | 140.00 | 14.00 | 140.00 |
| l.s.d. | 0.12 | 0.21 | 0.27 | 0.30 |

| | Isolate by sample | Cultivar by sample | Isolate by cultivar sample |
|---|---|---|---|
| rep. | 18.00 | 6.00 | 3.00 |
| l.s.d. | 0.35 | 0.56 | 0.79 |
| d.f. | 42.64 | 134.63 | 153.77 |
| Except when comparing means with the same level(s) of | | | |
| sample | 0.32 | 0.55 | 0.78 |
| d.f. | 140.00 | 140.00 | 140.00 |
| isolate.sample | | | 0.78 |
| d.f. | | | 140.00 |
| cultivar.sample | | | 0.78 |
| d.f. | | | 140.00 |

## A 2.2. Output of GenStat 12th edition of qPCR quantification

**Experiment 1 (susceptible cultivars)**

Variate: LOG10((qPCR_experiment_1_DNA_concentrat+0.5))

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Day.Block stratum | | | | | |
| Sample | 6 | 310.6805 | 51.7801 | 360.74 | <.001 |
| Residual | 14 | 2.0095 | 0.1435 | 1.40 | |
| | | | | | |
| Day.Block.Leaf stratum | | | | | |
| Cultivar | 6 | 33.7955 | 5.6326 | 54.87 | <.001 |
| Cultivar.Sample | 36 | 57.9628 | 1.6101 | 15.68 | <.001 |
| Residual | 84 | 8.6231 | 0.1027 | 0.79 | |
| | | | | | |
| Day.Block.Leaf.*Units* stratum | | | | | |
| Isolate | 1 | 4.6396 | 4.6396 | 35.88 | <.001 |
| Cultivar.Isolate | 6 | 14.3924 | 2.3987 | 18.55 | <.001 |
| Isolate.Sample | 6 | 23.5618 | 3.9270 | 30.37 | <.001 |
| Cultivar.Isolate.Sample | 36 | 63.3249 | 1.7590 | 13.60 | <.001 |
| Residual | 98 | 12.6737 | 0.1293 | | |
| Total | 293 | 531.6638 | | | |

Mean of Cultivar by isolate

| Cultivar | Isolate | |
|---|---|---|
| | Bl801 | Bl806 |
| Capitan | 1.701 | 1.764 |
| Cobham Green | 1.711 | 0.949 |
| Pennlake | 2.701 | 2.125 |
| UC DM10 | 1.83 | 1.557 |
| UC DM14 | 1.457 | 2.108 |
| UC DM2 | 1.969 | 1.424 |
| Valmaine | 2.399 | 2.081 |

Mean of Cultivar by sample

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Capitan | 0.335 | 0.724 | 1.092 | 2.38 | 1.121 | 3.045 | 3.427 |
| Cobham Green | 0.338 | 0.642 | 1.458 | 1.203 | 2.787 | 1.049 | 1.834 |
| Pennlake | 1 | 1.001 | 1.819 | 2.323 | 3.337 | 3.667 | 3.742 |
| UC DM10 | 0.32 | 0.257 | 1.243 | 0.882 | 2.349 | 3.25 | 3.554 |
| UC DM14 | 0.474 | 0.863 | 0.987 | 1.767 | 2.558 | 2.713 | 3.117 |
| UC DM2 | 0.47 | -0.017 | 0.87 | 1.35 | 2.186 | 3.226 | 3.791 |
| Valmaine | 0.309 | 1.332 | 1.8 | 1.698 | 2.953 | 3.655 | 3.93 |

Mean of isolate by sample

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Bl801 | 0.511 | 0.659 | 1.613 | 2.17 | 2.413 | 2.606 | 3.794 |
| Bl806 | 0.416 | 0.713 | 1.035 | 1.145 | 2.527 | 3.282 | 2.89 |

Mean of Cultivar by isolate by sample

| Cultivar | Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Capitan | Bl801 | 0.74 | 0.54 | 1.63 | 2.87 | -0.07 | 2.60 | 3.60 |
| | Bl806 | -0.07 | 0.91 | 0.56 | 1.89 | 2.31 | 3.49 | 3.26 |
| Cobham Green | Bl801 | 0.08 | 0.41 | 1.89 | 2.71 | 3.10 | -0.18 | 3.97 |
| | Bl806 | 0.59 | 0.87 | 1.03 | -0.30 | 2.48 | 2.28 | -0.30 |
| Pennlake | Bl801 | 1.16 | 0.96 | 2.57 | 2.77 | 3.88 | 3.57 | 4.00 |
| | Bl806 | 0.84 | 1.05 | 1.07 | 1.88 | 2.79 | 3.77 | 3.49 |
| UC DM10 | Bl801 | 0.94 | 0.17 | 1.16 | 0.82 | 2.21 | 3.57 | 3.94 |
| | Bl806 | -0.30 | 0.35 | 1.33 | 0.94 | 2.49 | 2.93 | 3.17 |
| UC DM14 | Bl801 | 0.48 | 1.00 | 0.64 | 1.66 | 2.11 | 1.95 | 2.36 |
| | Bl806 | 0.47 | 0.72 | 1.34 | 1.87 | 3.01 | 3.48 | 3.88 |
| UC DM2 | Bl801 | 0.47 | 0.27 | 1.40 | 1.59 | 2.08 | 3.53 | 4.44 |
| | Bl806 | 0.47 | -0.30 | 0.34 | 1.11 | 2.29 | 2.92 | 3.14 |
| Valmaine | Bl801 | -0.30 | 1.27 | 2.02 | 2.77 | 3.57 | 3.20 | 4.27 |
| | Bl806 | 0.92 | 1.39 | 1.58 | 0.63 | 2.34 | 4.11 | 3.59 |

Least significant differences of means (5% level)

| | Cultivar | Isolate | Sample | Cultivar by isolate |
|---|---|---|---|---|
| rep. | 42 | 147 | 42 | 21 |
| l.s.d. | 0.139 | 0.0832 | 0.1773 | 0.2074 |
| d.f. | 84 | 98 | 14 | 181.74 |
| Except when comparing means with the same level(s) of | | | | |
| cultivar | | | | 0.2202 |
| d.f. | | | | 98 |

| | Cultivar by sample | Isolate by sample | Cultivar by isolate by sample |
|---|---|---|---|
| rep. | 6 | 21 | 3 |
| l.s.d. | 0.3775 | 0.2295 | 0.5553 |
| d.f. | 96.33 | 45.33 | 193.12 |
| Except when comparing means with the same level(s) of | | | |
| Sample | 0.3679 | 0.2202 | 0.5487 |
| d.f. | 84 | 98 | 181.74 |
| cultivar.Sample | | | 0.5827 |
| d.f. | | | 98 |
| Isolate.Sample | | | 0.5487 |
| d.f. | | | 181.74 |

**Experiment 2 (susceptible Cultivars)**

Variate: LOG10((qPCR_experiment_2_DNA_concentrat+0.5))

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Day.Block stratum | | | | | |
| Sample | 6 | 248.11064 | 41.352 | 2013.9 | <.001 |
| Residual | 14 | 0.28747 | 0.0205 | 0.68 | |
| Day.Block.Leaf stratum | | | | | |
| cultivar | 6 | 106.48789 | 17.748 | 588.9 | <.001 |
| cultivar.Sample | 36 | 109.02786 | 3.0286 | 100.49 | <.001 |
| Residual | 84 | 2.53155 | 0.0301 | 1.03 | |
| Day.Block.Leaf.*Units* stratum | | | | | |
| Isolate | 1 | 30.83053 | 30.831 | 1053 | <.001 |
| cultivar.Isolate | 6 | 14.85185 | 2.4753 | 84.54 | <.001 |
| Isolate.Sample | 6 | 13.09611 | 2.1827 | 74.55 | <.001 |
| cultivar.Isolate.Sample | 36 | 48.65415 | 1.3515 | 46.16 | <.001 |
| Residual | 98 | 2.86927 | 0.0293 | | |
| Total | 293 | 576.74732 | | | |

Mean of cultivar by isolate

| Cultivar | Isolate | |
|---|---|---|
| | Bl801 | Bl806 |
| Capitan | 2.3797 | 1.542 |
| Cobham Green | 0.586 | 0.4939 |
| Pennlake | 2.9599 | 1.812 |
| UC DM10 | 2.076 | 1.042 |
| UC DM14 | 1.5995 | 1.7455 |
| UC DM2 | 2.5254 | 1.7018 |
| Valmaine | 2.8097 | 2.0654 |

Mean of cultivar by sample

| Cultivar | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Capitan | 0.6871 | 0.6252 | 0.8538 | 1.1499 | 3.4151 | 3.7006 | 3.2944 |
| Cobham Green | -0.1264 | 0.5218 | 1.0601 | 0.3616 | 2.331 | -0.2158 | -0.1527 |
| Pennlake | 0.7908 | 0.6858 | 2.0649 | 3.0395 | 3.118 | 3.0767 | 3.926 |
| UC DM10 | 0.655 | 0.1651 | 1.6318 | 0.2604 | 1.8658 | 3.259 | 3.0759 |
| UC DM14 | 0.9798 | 0.3467 | 1.176 | 2.0026 | 1.7349 | 2.7513 | 2.7161 |
| UC DM2 | 0.371 | 0.5787 | 1.8439 | 2.3755 | 2.8892 | 3.2994 | 3.4374 |
| Valmaine | 0.7916 | 1.0223 | 1.7626 | 2.9385 | 3.2973 | 3.4037 | 3.8467 |

Mean of isolate by sample

| Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Bl801 | 0.9458 | 0.7445 | 1.9597 | 2.0805 | 3.2845 | 3.1387 | 2.7824 |
| Bl806 | 0.2396 | 0.3828 | 1.0098 | 1.3846 | 2.0445 | 2.3684 | 2.9729 |

Mean of Cultivar by isolate by sample

| Cultivar | Isolate | Sample | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Capitan | Bl801 | 0.94 | 0.31 | 1.89 | 2.60 | 3.93 | 3.98 | 3.00 |
| | Bl806 | 0.43 | 0.94 | -0.18 | -0.30 | 2.90 | 3.42 | 3.59 |
| Cobham Green | Bl801 | -0.30 | 1.05 | 0.81 | 0.05 | 2.64 | -0.13 | 0.00 |
| | Bl806 | 0.05 | 0.00 | 1.31 | 0.68 | 2.03 | -0.30 | -0.30 |
| Pennlake | Bl801 | 1.32 | 0.56 | 2.80 | 3.62 | 4.08 | 4.31 | 4.04 |
| | Bl806 | 0.27 | 0.81 | 1.33 | 2.46 | 2.16 | 1.84 | 3.81 |
| UC DM10 | Bl801 | 1.06 | 0.21 | 2.10 | 0.08 | 3.60 | 3.89 | 3.59 |
| | Bl806 | 0.25 | 0.12 | 1.16 | 0.45 | 0.13 | 2.63 | 2.56 |
| UC DM14 | Bl801 | 1.55 | 0.99 | 1.42 | 1.93 | 1.84 | 2.03 | 1.43 |
| | Bl806 | 0.41 | -0.30 | 0.94 | 2.07 | 1.63 | 3.48 | 4.00 |
| UC DM2 | Bl801 | 0.70 | 0.85 | 2.59 | 2.83 | 3.04 | 3.98 | 3.69 |
| | Bl806 | 0.04 | 0.30 | 1.10 | 1.92 | 2.73 | 2.62 | 3.19 |
| Valmaine | Bl801 | 1.35 | 1.24 | 2.11 | 3.46 | 3.87 | 3.92 | 3.73 |
| | Bl806 | 0.24 | 0.81 | 1.41 | 2.42 | 2.73 | 2.89 | 3.96 |

Least significant differences of means (5% level)

| | Cultivar | Isolate | Sample | Cultivar by Isolate |
|---|---|---|---|---|
| rep. | 42 | 147 | 42 | 21 |
| s.e.d. | 0.03788 | 0.01996 | 0.03127 | 0.05319 |
| d.f. | 84 | 98 | 14 | 180.48 |
| Except when comparing means with the same level(s) of | | | | |
| cultivar | | | | 0.05281 |
| d.f. | | | | 98 |

| | Cultivar by Sample | Isolate by Sample | Cultivar by isolate Sample |
|---|---|---|---|
| rep. | 6 | 21 | 3 |
| s.e.d. | 0.09792 | 0.0487 | 0.1391 |
| d.f. | 96.68 | 63.85 | 194.68 |
| Except when comparing means with the same level(s) of | | | |
| Sample | 0.10023 | 0.05281 | 0.14073 |
| d.f. | 84 | 98 | 180.48 |
| cultivar.Sample | | | 0.13971 |
| d.f. | | | 98 |
| Isolate.Sample | | | 0.14073 |
| d.f. | | | 180.48 |

**Experiment 1 (resistant cultivars)**

Variate: LOG10((qPCR_experiment_1DNA_concentrati+0.5))

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Day.Block stratum | | | | | |
| Sample | 2 | 0.593 | 0.296 | 1.66 | 0.267 |
| Residual | 6 | 1.072 | 0.179 | 3.9 | |
| Day.Block.Leaf stratum | | | | | |
| Cultivar | 4 | 3.317 | 0.829 | 18.12 | <.001 |
| Cultivar.Sample | 8 | 2.102 | 0.263 | 5.74 | <.001 |
| Residual | 24 | 1.098 | 0.046 | 0.76 | |
| Day.Block.Leaf.*Units* stratum | | | | | |
| Isolate | 1 | 3.016 | 3.016 | 49.97 | <.001 |
| Cultivar.Isolate | 4 | 2.831 | 0.708 | 11.73 | <.001 |
| Isolate.Sample | 2 | 0.051 | 0.025 | 0.42 | 0.662 |
| Cultivar.Isolate.Sample | 8 | 2.18 | 0.273 | 4.52 | 0.001 |
| Residual | 30 | 1.811 | 0.06 | | |
| Total | 89 | 18.07 | | | |

Mean of cultivar by isolate

| Cultivar | Isolate | |
|---|---|---|
| | BL801 | BL806 |
| Colorado | 0.627 | -0.058 |
| Discovery | 0.563 | -0.301 |
| LS-102 | 0.147 | -0.116 |
| Ninja | -0.083 | -0.178 |
| PIVT1309 | -0.301 | -0.225 |

Mean of cultivar by sample

| Cultivar | Sample | | |
|---|---|---|---|
| | Day 1 | Day 5 | Day 7 |
| Colorado | 0.343 | 0.135 | 0.376 |
| Discovery | -0.301 | 0.271 | 0.423 |
| LS-102 | 0.166 | -0.008 | -0.112 |
| Ninja | -0.301 | -0.172 | 0.081 |
| PIVT1309 | -0.301 | -0.301 | -0.187 |

Mean of isolate by sample

| Isolate | Sample | | |
|---|---|---|---|
| | Day 1 | Day 5 | Day 7 |
| BL801 | 0.071 | 0.188 | 0.313 |
| BL806 | -0.229 | -0.217 | -0.081 |

Mean of cultivar by isolate by sample

| Cultivar | Isolate | | | | | |
|---|---|---|---|---|---|---|
| | BL801 | | | BL806 | | |
| Sample | Day 1 | Day 5 | Day 7 | Day 1 | Day 5 | Day 7 |
| Colorado | 0.805 | 0.374 | 0.702 | -0.12 | -0.105 | 0.051 |
| Discovery | -0.301 | 0.843 | 1.146 | -0.301 | -0.301 | -0.301 |
| LS-102 | 0.452 | 0.063 | -0.075 | -0.12 | -0.079 | -0.149 |
| Ninja | -0.301 | -0.042 | 0.093 | -0.301 | -0.301 | 0.068 |
| PIVT1309 | -0.301 | -0.301 | -0.301 | -0.301 | -0.301 | -0.073 |

Least significant differences of means (5% level)

| | Cultivar | Isolate | Sample | Cultivar by isolate |
|---|---|---|---|---|
| rep. | 18 | 45 | 30 | 9 |
| l.s.d. | 0.1472 | 0.1058 | 0.267 | 0.2177 |
| d.f. | 24 | 30 | 6 | 53.96 |
| Except when comparing means with the same level(s) of | | | | |
| Cultivar | | | | 0.2365 |
| d.f. | | | | 30 |

| | Cultivar by Sample | Isolate by sample | Cultivar by isolate by sample |
|---|---|---|---|
| rep. | 6 | 15 | 3 |
| l.s.d. | 0.3245 | 0.2795 | 0.4235 |
| d.f. | 19.48 | 10.5 | 45.14 |
| Except when comparing means with the same level(s) of | | | |
| Sample | 0.2549 | 0.1832 | 0.3771 |
| d.f. | 24 | 30 | 53.96 |
| Cultivar.Sample | | | 0.4096 |
| d.f. | | | 30 |
| Isolate.Sample | | | 0.3771 |
| d.f. | | | 53.96 |

**Experiment 2 (resistant cultivar)**

Variate: LOG10((qPCR_experiment_2DNA_concentrati+0.5))

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Day.Block stratum | | | | | |
| Sample | 2 | 0.130449 | 0.065224 | 4.11 | 0.075 |
| Residual | 6 | 0.095293 | 0.015882 | 0.84 | |
| Day.Block.Leaf stratum | | | | | |
| Cultivar | 4 | 4.953502 | 1.238375 | 65.26 | <.001 |
| Cultivar.Sample | 8 | 0.999224 | 0.124903 | 6.58 | <.001 |
| Residual | 24 | 0.455395 | 0.018975 | 2.14 | |
| Day.Block.Leaf.*Units* stratum | | | | | |
| Isolate | 1 | 2.402069 | 2.402069 | 271.31 | <.001 |
| Cultivar.Isolate | 4 | 3.224655 | 0.806164 | 91.05 | <.001 |
| Isolate.Sample | 2 | 0.347054 | 0.173527 | 19.60 | <.001 |
| Cultivar.Isolate.Sample | 8 | 1.393707 | 0.174213 | 19.68 | <.001 |
| Residual | 30 | 0.265613 | 0.008854 | | |
| Total | 89 | 14.266960 | | | |

Mean of cultivar by isolate

| Cultivar | Isolate | |
|---|---|---|
| | BL801 | BL806 |
| Colorado | 0.2638 | -0.1978 |
| Discovery | -0.301 | -0.2914 |
| LS-102 | 0.0754 | -0.1037 |
| Ninja | 0.8299 | -0.1726 |
| PIVT1309 | -0.301 | -0.301 |

Mean of cultivar by sample

| Cultivar | Sample | | |
|---|---|---|---|
| | Day 1 | Day 5 | Day 7 |
| Colorado | 0.2078 | -0.1546 | 0.0459 |
| Discovery | -0.2866 | -0.301 | -0.301 |
| LS-102 | -0.1007 | -0.0521 | 0.1104 |
| Ninja | 0.0734 | 0.4486 | 0.4639 |
| PIVT1309 | -0.301 | -0.301 | -0.301 |

Mean of isolate by sample

| Isolate | Sample | | |
|---|---|---|---|
| | Day 1 | Day 5 | Day 7 |
| BL801 | 0.0023 | 0.0991 | 0.2389 |
| BL806 | -0.1652 | -0.2431 | -0.2316 |

Mean of cultivar by isolate by sample

| Cultivar | Isolate | | | | | |
|---|---|---|---|---|---|---|
| | Bl801 | | | Bl806 | | |
| Sample | Day 1 | Day 5 | Day 7 | Day 1 | Day 5 | Day 7 |
| Colorado | 0.526 | -0.008 | 0.274 | -0.110 | -0.301 | -0.182 |
| Discovery | -0.301 | -0.301 | -0.301 | -0.272 | -0.301 | -0.301 |
| LS-102 | -0.102 | -0.059 | 0.388 | -0.099 | -0.045 | -0.167 |
| Ninja | 0.191 | 1.165 | 1.134 | -0.044 | -0.268 | -0.207 |
| PIVT1309 | -0.301 | -0.301 | -0.301 | -0.301 | -0.301 | -0.301 |

Least significant differences of means (5% level)

| | Cultivar | Isolate | Sample | Cultivar by Isolate |
|---|---|---|---|---|
| rep. | 18.000 | 45.000 | 30.000 | 9.000 |
| l.s.d. | 0.095 | 0.041 | 0.080 | 0.112 |
| d.f. | 24.000 | 30.000 | 6.000 | 43.960 |
| Except when comparing means with the same level(s) of | | | | |
| Cultivar | | | | 0.091 |
| d.f. | | | | 30.000 |

| | Cultivar by Sample | Isolate by sample | Cultivar by isolate by sample |
|---|---|---|---|
| rep. | 6.000 | 15.000 | 3.000 |
| l.s.d. | 0.160 | 0.087 | 0.191 |
| d.f. | 29.860 | 13.700 | 53.280 |
| Except when comparing means with the same level(s) of | | | |
| Sample | 0.164 | 0.070 | 0.194 |
| d.f. | 24.000 | 30.000 | 43.960 |
| Cultivar.Sample | | | 0.157 |
| d.f. | | | 30.000 |
| Isolate.Sample | | | 0.194 |
| d.f. | | | 43.960 |

**Experiment 3**

Variate: LOG10((qPCR_experiment_3DNA_concentrati+0.5))

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| Block stratum | 2 | 0.00393 | 0.00197 | 0.06 | |
| | | | | | |
| Block.Leaf stratum | | | | | |
| Cultivar | 19 | 135.08543 | 7.10976 | 212.10 | <.001 |
| Residual | 38 | 1.27377 | 0.03352 | 1.16 | |
| | | | | | |
| Block.Leaf.*Units* stratum | | | | | |
| Isolate | 1 | 2.89046 | 2.89046 | 99.68 | <.001 |
| Cultivar.Isolate | 19 | 28.95845 | 1.52413 | 52.56 | <.001 |
| Residual | 40 | 1.15990 | 0.02900 | | |
| | | | | | |
| Total | 119 | 169.37193 | | | |

Mean of cultivar by isolate

| Cultivar | Isolate | |
|---|---|---|
| | BL801 | BL806 |
| Argeles | 0.883 | 2.266 |
| Capitan | 2.047 | 2.446 |
| Cobham Green | 2.171 | 2.207 |
| Cororado | 0.8 | 0.787 |
| Dandie | 2.956 | 0.116 |
| Discovery | 0.686 | 0.487 |
| Hilde II | 3.295 | 2.125 |
| Lednicky | 2.297 | 2.537 |
| LS102 | 0.578 | 0.557 |
| LSE18 | 2.609 | 0.468 |
| LSE57/15 | 3.468 | 2.497 |
| Ninja | 0.778 | 0.425 |
| Pennlake | 3.556 | 3.536 |
| PIVT 1309 | 0.324 | 1.07 |
| R4T57D | 3.413 | 3.687 |
| Sabine | 2.173 | 0.688 |
| UC DM10 | 3.804 | 3.275 |
| UC DM14 | 1.762 | 2.64 |
| UC DM2 | 2.694 | 2.463 |
| Valmaine | 3.885 | 3.695 |

Least significant differences of means (5% level)

| | Cultivar | Isolate | Cultivar by isolate |
|---|---|---|---|
| rep. | 6 | 60 | 3 |
| l.s.d. | 0.214 | 0.0628 | 0.2874 |
| d.f. | 38 | 40 | 77.26 |
| Except when comparing means with the same level(s) of | | | |
| cultivar | | | 0.281 |
| d.f. | | | 40 |