Identification of Blueberry Leaf Rust Pathogen and Quantification of Disease Infection Levels in a Blueberry Plantation in Hastings, NZ

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Abstract

Blueberries (*Vaccinium* spp.) are a favourite fruit and they are produced worldwide. In New Zealand, blueberries are the main export berry fruit and contribute greatly to export income. More than 2,800 tonnes of blueberries were produced in the 2017/2018 harvesting season and earned $34.8 million export income in 2018. Currently, 740 ha of the blueberry plantations can be found in both the South Island and North Island. Otautau is the main growing region in the South Island while Waihopo, the Waikato regions of Ngatea and Ohaupo, the Bay of Plenty and Hastings are the regions in the North Island, producing most of the fresh blueberries in New Zealand.

However, blueberry leaf rust has become a prevalent disease in blueberry production and a concerning issue for blueberry growers. In Hastings production sites, serious infections have been found in recent years. Although fungicides were applied to control blueberry leaf rust, this form of control is incomplete and unsustainable for blueberry production. The deployment of varieties that are naturally resistant would be a better option for managing blueberry leaf rust disease. Currently, few cultivars are available for this purpose, but breeding for rust resistance can address this demand. The main issues preventing the production of resistant varieties are insufficient knowledge about this rust pathogen in New Zealand, and the lack of resistant germplasm sources and efficient resistance screening procedures.

In this study, using the morphological characteristics and genome sequencing results based on the Internal Transcribed Spacer (ITS) regions, *Thekospora minima* was identified as the causal organism of blueberry leaf rust disease in Hastings, Hawke's Bay, New Zealand. Additionally, a field assessment was used for understanding the blueberry rust disease resistance levels in current blueberry cultivars. The disease incidence and disease severity of 23 blueberry cultivars, including five rabbiteye, three northern highbush and fifteen southern highbush, were assessed using *Fiji* software during the 2019 harvesting season. Based on a Tukey Honest Significant Differences

(TukeyHSD) analysis, these observed blueberry cultivars were divided into four infection levels of blueberry leaf rust using the percentage of the infected area on the leaf (PIAL). 'Scintilla' was highly susceptible to blueberry leaf rust disease, while 'Blue Moon' and 'Southern Splendour' were moderately susceptible. Nineteen blueberry cultivars, made up of 'Rahi', 'Centra Blue', 'Centurion', 'Titan', 'Sky Blue', 'Nui', 'Duke', 'Camellia', 'Misty', 'Springhigh', 'Snowchaser', 'Miss Jackie', 'Miss Lily', 'Georgia Dawn', 'Suziblue', 'Kestrel', 'Flicker', 'Sweetcrisp' and 'Palmetto', showed susceptibility to this rust disease, and ''O' Neal' was the one that showed partial resistance to the blueberry rust infection. Furthermore, using 1.5×10^4 concentration inoculum, an inoculation test was completed in a temperature-controlled room at the Plant Growth Unit of Massey University. The inoculum was sprayed on the healthy leaves from detached branches of the 'Sky Blue' blueberry cultivar and they were grown in reverse osmosis water for a 35-day observation on rust symptom development. *Fiji* software was applied in the assessment of disease severity in this inoculation test. A strong correlation (>0.99) was found between the increase in lesion area (ILA) from the inoculation test and the PIAL from field assessment. A preliminary prediction equation was established by a simple linear regression model. This equation can be used to predict the blueberry leaf rust level on different blueberry cultivars and breeding materials under field conditions by using the results from an inoculation test. This model would be an efficient approach for assisting the screening on blueberry leaf rust of blueberries.

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Abbreviations

ALA	average lesion area
ANOVA	analysis of variance
DI	disease incidence
DNA	deoxyribonucleic acid
DS	disease severity
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization of the United Nations
ILA	increase in the lesion area
ITS	internal transcribed spacer
MMIC	Manawatu microscopy and imaging center
NCBI	national center for biotechnology information
NHB	northern highbush
PCR	polymerase chain reaction
PGU	plant growth unit
PIAL	percentage of infection area on the leaf
rDNA	ribosomal DNA
RE	rabbiteye
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SHB	southern highbush
TAE	tris-acetate-ethylenediaminetetraacetic acid
TukeyHSD	tukey honest significant differences
USDA	the U.S. department of agriculture

Chapter 1 Introduction

1.1 Introduction

Blueberries (*Vaccinium* sp.) have health benefits and are appreciated by consumers worldwide. Indeed, with their high concentration of bioactive components, such as anthocyanins, procyanidins and flavonoids, they have the potential to reduce the risk of some chronic diseases, namely, type 2 diabetes mellitus and cardiovascular disease (Carey et al., 2017; Carey, Gomes, & Shukitt-Hale, 2014; Elks et al., 2013; Howard & Hager, 2007; Liu, 2007). With consumers now are more willing to spend on health products, there has been continued growth in the market demand for blueberries in recent years (Food and Agriculture Organization of the United Nations FAO, 2017d). As evidence for this, total blueberry production doubled worldwide in 2017, when compared to the past decade, reaching 596,813 tonnes (FAO, 2017d). According to Statistics NZ, blueberries were the biggest export berry fruit in New Zealand in 2018 (Horticulture New Zealand & Plant & Food Research, 2018). Their export value has doubled since 2010, reaching $35 million in 2018, with over 40% of fresh blueberries exported to Australia during the 2017/18 production season (Australian Government, 2018; Horticulture Innovation Australian Limited, 2019; Horticulture New Zealand & Plant & Food Research, 2010, 2018). To meet this export demand, New Zealand blueberry production areas have increased by 41.7% in the past decade, with 740 ha of land used for blueberry production in 2018 (Horticulture New Zealand & Plant & Food Research, 2010, 2018). Moreover, an increasing number of new southern highbush and rabbiteye cultivars have been propagated and transplanted at new plantation sites to meet this demand (D. Hutchins, personal communication, 13th March 2018).

Unfortunately, leaf rust disease of blueberry has become a constraint for blueberry production, especially in the Hastings region. This disease results in a serious infection on the plant, which can cause yield loss by early or undesirable plant defoliation.

Although fungicides are commonly applied to control the disease, this form of control is often not complete. In New Zealand, Mancozeb is the only fungicide that has been registered for blueberry rust control (Young, 2018/2019). As with any other fungicide, its extensive and/or exclusive use causes fungicide resistance in the pathogen population. Another concern, which is shared by both producers and consumers, is the negative effect that fungicides have on human health and the environment. Indeed, in New Zealand, fungicide residues must not exceed a certain maximum threshold set for fresh blueberry products, as required by the New Zealand Ministry for Primary Industries (Kinsella, 2018). With these points in mind, the use of disease-resistant cultivars is a more sustainable option for blueberry rust control. Notable, though, there are limited amount of blueberry cultivars with naturally resistant to blueberry leaf rust, and no southern highbush or rabbiteye blueberry cultivars have been identified with rust resistance. There are two main reasons for this. One is that plant breeding for new cultivars with resistance to rust disease is impeded by insufficient knowledge about the rust pathogens in New Zealand. The other is the lack of efficient screening procedures for identifying resistant sources.

Therefore, this research contains two parts. The first part is focused on identification of the pathogen in New Zealand, the second part is focused on developing an efficient screening procedure for identifying blueberry accessions with rust resistance. Blueberry leaf rust disease is a common issue in blueberry production, and it is found in blueberry plantations worldwide. In other countries, some research has reported blueberry rust symptoms, pathogen organism identification and present control methods. Therefore, the methods used from previous studies on pathogen identification can be applied to identify the rust pathogens found in New Zealand. Meanwhile, various screening procedures from previous studies used to identify resistant blueberries can be adjusted and used in this study. In order to develop an efficient screening procedure on blueberries, these procedures should take into account blueberry plant characteristics and phenological stages as these factors are important for understanding the pathogen development on the plant and the interaction between pathogen and blueberries.

Hence, taxonomy, botanical characteristics and the phenological stages of the blueberry crop will be introduced in the literature review below as background information. In addition, other relevant information will be reviewed, such as blueberry breeding, present growing cultivars and productions. The previous research on blueberry rust and screening procedures will be described. Finally, the aim and hypotheses of this study will be defined in this chapter.

1.2 Literature review

1.2.1 Taxonomy of blueberry

The blueberry belongs to the subgenus *Cyanococcus* of the genus *Vaccinium* in the Ericaceae family (Polashock, Caruso, Averill, & Schilder, 2017; Rowland, Hancock, & Bassil, 2011). Currently, all commercial blueberry types are from section *Cyanococcus* of *Vaccinium* genus, highbush, rabbiteye and lowbush are the most commonly used in production (Galletta & Ballington, 1996; Polashock et al., 2017; Retamales & Hancock, 2012; Rowland et al., 2011; Sakhanokho, Rinehart, Stringer, Islam-Faridi, & Pounders, 2018). Highbush includes two main species: northern highbush (NHB) (*V. corymbosum* L.) and southern highbush (SHB) (a group of complex hybrids between *V. corymbosum* L.and *V. darrowii*). Rabbiteye (RE) cultivars belong to a species of *V. virgatum* Ait. [syn.*V. ashei* Reade.]. The majority of the lowbush cultivars are from *V. angustifolium* Aiton, and some of them are from *V. myrtilloides* Michx or *V. darrowii* Camp. These cultivars have various genetic backgrounds and polyploid numbers. At least three types of polyploids are found in blueberries, which range from diploid (2N=2X=14) to tetraploid (2N=4X=28) and hexaploid (2N=6X=42) (Table 1.1) (Retamales & Hancock, 2012; Rowland et al., 2011). Therefore, new cultivars developed by interspecific crosses between different ploidy levels may be unsuccessful in producing next generation populations (Pathirana et al., 2015; Rowland et al., 2011).

Table 1.1 Important blueberry species from *Vaccinium* section *Cyanococcus* with ploidy levels and origin.

***Vaccinium* Species**	**Ploidy Levels**	**Origin**
V. angustifolium Ait.	4x	North-east North America
V. boreale Hall & Aald.	2x	North-east North America
V. constablaei Gray	6x	Mountains of South-east North America
V. corymbosum L.	2x	South-east North America
V. corymbosum L.	4x	East North America
V. darrowii Camp	2x	South-east North America
V. elliottii Chapm.	2x	South-east North America
V. fuscatum Ait.	2x	Florida
V. hirsutum Buckley	4x	South-east North America
V. myrsinites Lam.	4x	South-east North America
V. myrtilloides Michx.	2x	Central North America
V. pallidum Ait.	2x,4x	Mid-Atlantic North America
V. simulatum Small	4x	South-east North America
V. tenellum Ait.	2x	South-east North America
V. virgatum Ait. [syn. *V.ashei*]	6x	South-east North America

1.2.2 Botanical characteristics of blueberries

Even though there are differences in their genetic background, some common botanical characteristics are shared among highbush, RE and lowbush cultivars of blueberry (Eck, Gough, Hall, & Spiers, 1990; Polashock et al., 2017; Rowland et al., 2011). For instance, both new vegetative and flower buds are formed on one-year-old branches. Vegetative buds are small, pointed and scalelike, while flower buds are larger and nearly spherical shaped. Flower buds contain six to twelve potential flowers. The flower comprises of sepals (calyx), a spherical or urn-shaped corolla with four- or five-lobed petals, ten stamens and a pistil. After pollination, blueberry fruit is developed. Mature blueberry fruit is in blue colour, and some are light blue with wax on the surface. An ovary with up to 100 seeds is developed in these true berry fruit.

However, there are differences among these three types of cultivars, which are caused by the diversity in genetic backgrounds and plant morphologies (Polashock et al., 2017; Retamales & Hancock, 2012). For example, the fine and fibrous roots of highbush and RE blueberries grow in the upper 0.5 m of the soil, while the rhizomatous roots of lowbush extend between 6.0 cm to 25 cm below the soil surface. Additionally, highbush blueberries range from 1.8 to 4.0 m in height, and RE plants can reach 6.0 m tall, while lowbush may grow under 0.15 m (Figure 1.1). Blueberry leaves also vary from an ovate to a narrowly elliptical shape, with a green mature colour, and changes to yellow, orange or red colouring during defoliation. Likewise, there are different self-fertility levels among blueberries. In general, lowbush cultivars are not self-fertile. Cultivars of RE and SHB maintain a certain level of self-fertility, while NHB cultivars are self-fertile. Hence, in commercial blueberry productions, these similarities and differences of botanical characteristics need to be considered when making crop management decisions.

Figure 1.1 Different types of blueberry cultivars. (A) Plants of southern highbush (SHB) cultivar ‘Biloxi’; (B) fruits of SHB cultivar’ Biloxi’; (C) plant of rabbiteye (RE) cultivar ‘Alapaha’; (D) fruits of RE cultivar ‘Alapaha’; (E) plants of lowbush and (F) fruits of lowbush cultivar ‘Brunswick’ (American Beauties Native Plants, n.d.-a, n.d.-b; Fall Creek Farm & Nursery, n.d.; Georgia's Integrated Cultivar Release System, n.d.).

1.2.3 Phenological stages

Botanical characteristics are important for blueberry crop management. In general, based on botanical characteristics, blueberry can be divided into: (a) deciduous or (b) evergreen plants. Most of the blueberries are deciduous plants, with the yearly cycle beginning from autumn after defoliation (Flora of North America, 1942; Galletta & Ballington, 1996; Hancock, Lyrene, Finn, Vorsa, & Lobos, 2008; Retamales & Hancock, 2012):

After defoliation, most of the vegetative tissues and flower buds can be clearly seen on one-year-old branches. In winter, plants stop growing and become dormant. In spring, flowers take place on the lateral shoots after the plants have received sufficient chilling hours. Flowers may break dormancy before or after vegetative buds break. This variation is due to the difference in the cultivars' genetic background, climate conditions and production practices (Polashock et al., 2017; Rowland et al., 2011). In commercial production, honeybees are used to solve the self-sterile issue on blueberry and increase the fruit set ratio (Eck et al., 1990; Polashock et al., 2017). Although there is different pollination receptivity among cultivars, for most of them the fertilization can be completed in one week after blooming in late spring (Eck et al., 1990; Polashock et al., 2017).

After pollination, most of the blueberry fruits develop during summer (December-March for New Zealand). Fruit development has a double sigmoid growth curve (Figure 1.2) (Collins, Irving, & Barker, 1966; Early, 2010; Godoy, Monterubbianesi, & Tognetti, 2008; Retamales & Hancock, 2012). There is a rapid increase in fresh weight in stage Ⅰ because of continued cell division and endosperm growth. After that, the increase in fruit volume and fresh weight during stage Ⅱ is relatively stable. Later, a rapid increase in fresh weight, sugars and anthocyanins accumulation takes place which is caused by cell enlargement in stage Ⅲ. Meanwhile, fruit colour changes occur during these stages, from immature green to translucent greenish white, greenish pink, blue-red, and finally

a complete blue (Li, Lee, & Wang, 2014). Additionally, berry volume increases almost 50% when the color shifts from greenish pink to blue. Simultaneously, bioactive components like anthocyanins and other compounds accumulate in the epidermis and hypodermis. Due to the accumulation of these pigments, a dark blue colour is shown on the fruit, while the light blue colour blueberries are formed by an extra translucent waxy layer on top of the blue pigments (Ribera, Reyes-Diaz, Alberdi, Zuñiga, & Mora, 2010; Riihinen, Jaakola, Kärenlampi, & Hohtola, 2008).

However, the fruit development period varies between 40 days to five months for different types of cultivars. For instance, the NHB blueberry takes 42-90 days, the SHB blueberry requires 55-60 days, the lowbush blueberry needs 70-90 days and the RE takes 60-135 days (Eck et al., 1990; Polashock et al., 2017).

When the harvest time is over in the autumn, the differentiation of flower buds and leaf buds begins (Eck et al., 1990). Both flower and leaf buds stop development and leaves defoliate when the temperature becomes low in the autumn. Dormancy will be broken and the new yearly cycle will take place in the spring of next season.

Most of the NHB, RE blueberries have similar phenological stages as mentioned above. However, there is an obvious difference between defoliated plants and evergreen plants. Most of the evergreen and low-chilling cultivars are from the species *V. ashei*, *V. angustifolium*, *V. tenellum* Aiton and *V. darrowii* Camp, which can maintain leaves during autumn to winter. When temperature goes up in spring, these evergreen plants can have a faster growth and earlier fruit development than defoliated blueberries.

Summer is the most important season for blueberry production, irrespectively of defoliated or evergreen cultivars. It directly affects the final yield, as the volume and fresh weight of blueberries is determined in this period. A healthy plant in autumn is essential for guaranteeing the flower bud production, which indirectly affects the production of the next season. Therefore, to ensure a high and stable yield, cultivation

management practices during summer and autumn should maintain healthy plants to prevent infection by diseases and damage by insects.

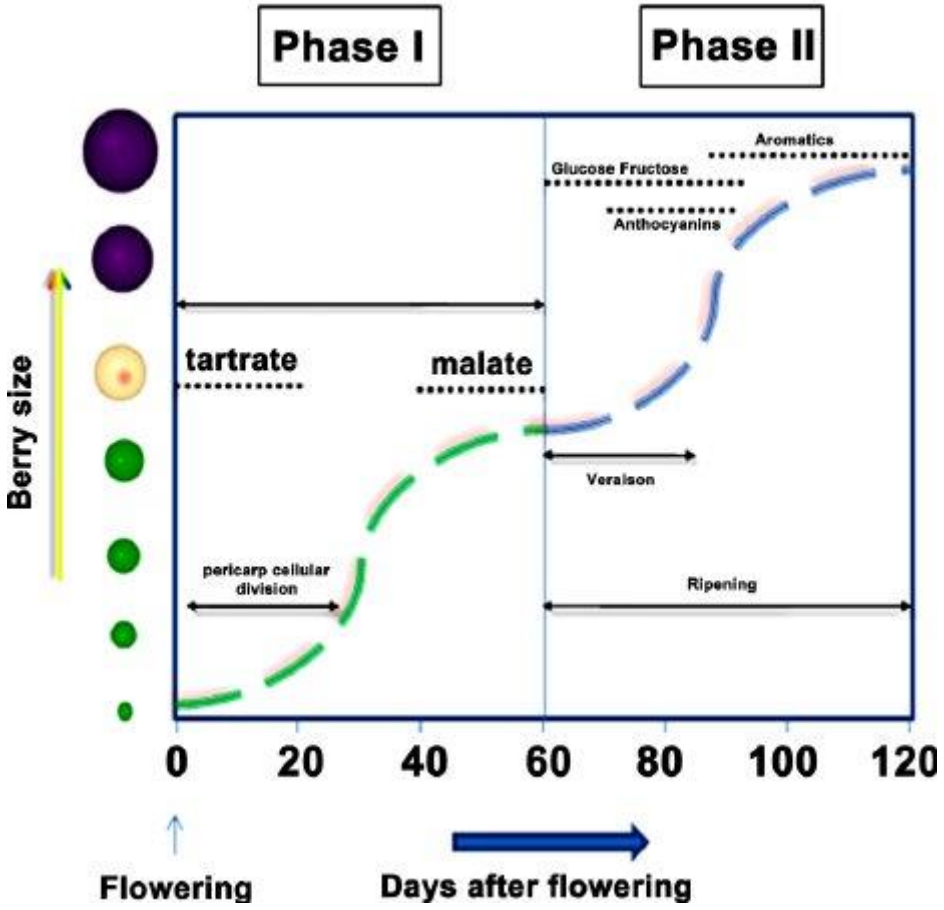


Figure 1.2 Blueberry double sigmoid growth curve with two phases. There is a rapid increase in cell numbers by continued cell division and endosperm growth in stage Ⅰ; a relatively stable fruit volume and weight in stage Ⅱ; and then a rapid increase in fresh weight, sugars and blue pigments accumulation by cell enlargement in stage Ⅲ. Fruit colour changes during these stages, from immature green to translucent greenish white, greenish pink, blue-red, and finally a complete blue (Panagiotis, Aziz, & Roubelakis-Angelakis, 2012, p. 138).

1.2.4 Blueberry breeding history

The plant characteristics of each phenological stage have a direct influence on fruit yield and quality. To increase yield and improve fruit quality, plant breeders have been

working on blueberry cultivar development since the 1900s. Blueberry breeding was first launched by Frederick Vernon Coville at the U.S. Department of Agriculture (USDA) in the 1900s and he domesticated the highbush blueberry at USDA (Galletta & Ballington, 1996). After this, his work was continued by George Darrow, Arlen Draper, Ralph Sharp and Paul Lyrene at the USDA. Through their breeding efforts, new cultivars of NHB, SHB and RE were released in the 19th century (Galletta & Ballington, 1996; Retamales & Hancock, 2012; Rowland et al., 2011).

1.2.4.1 Breeding history of highbush cultivars

Since the 1900s, most of the early highbush cultivars were released from USDA breeding programs (Rowland et al., 2011). In the 1900s, Coville focused on improving plant types for NHB and released new cultivars, which were more adaptable in various climate zones, soil types and production practices. Based on these selections, breeding efforts changed to develop cultivars with flexible chilling requirements, which can allow the production of blueberries in warmer regions. For this purpose, SHB was developed by a combination between NHB *V. corymbosum* and the evergreen species *V. darrowii*. When these new cultivars were grown for production in warmer regions, plant breeders considered the improvement of other characteristics, such as disease resistance, heat tolerance, fruit size and fruit qualities, and early and late harvest. Since the program launched by Coville, successful NHB cultivars, such as ‘Duke’, ‘Elliott’ and ‘Legacy’, were bred and continually used in production (Australian Blueberry Growers' Association, n.d.-b; Blueberries New Zealand, n.d.-b; Fall Creek Farm & Nursery, 2019d). In addition, contemporary NHB cultivars, like ‘Nui’, ‘Puru’ and ‘Reka’, developed from New Zealand HortResearch Inc., are used in New Zealand plantations (Hancock, 2006; Lyrene, 1998, 2002, 2017).

1.2.4.2 Breeding history of RE cultivars

RE breeding started shortly after the highbush breeding program. The RE cultivar was first developed by a cooperative breeding project between Georgia Coastal Plain

Experiment Station and the North Carolina Experiment Station in 1939. After that, RE breeding selection continued in the Georgia, North Carolina and Florida breeding stations. New cultivars, ‘Tifblue’ and ‘Brightwell’, ‘Powderblue’ and ‘Premier’, and ‘Bluegem’ and ‘Bonita’, were released from these breeding stations, respectively. In the same era, two RE cultivars, ‘Maru’ and ‘Rahi’, were bred by New Zealand HortResearch Inc. Fruit quality, fruit colour, size and texture, became important targets for improvement in these breeding programs (Lyrene, 2006; Retamales & Hancock, 2012).

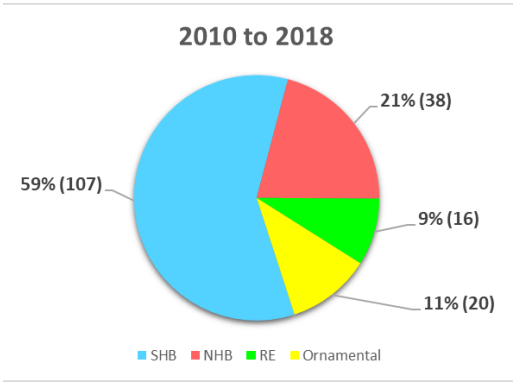
1.2.4.3 Breeding history of lowbush cultivars

Lowbush blueberries’ breeding progress was relatively slower than highbush and RE, as it mainly used as a wild type blueberry for production. Several varieties were bred in past decades by the Canadian Department of Agriculture and the American Agriculture Experiment Stations of Maine, Michigan, Wisconsin, Minnesota and West Virginia. From 1975 to 1988, only six cultivars were developed by the breeding program launched in Nova Scotia (Galletta & Ballington, 1996). As the colourful leaves occur in autumn, such as ‘Burgundy’, ‘North Country’ and ‘Northsky’, etc, these later released cultivars were grown as ornamental plants rather than as commercial fruit production cultivars (Brand, 2015).

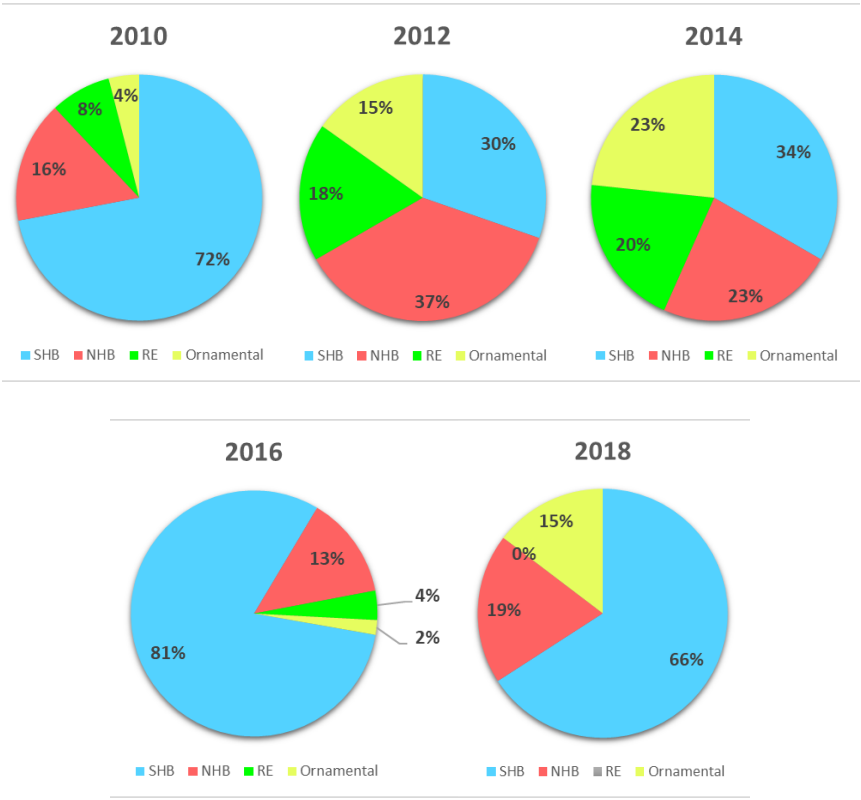
1.2.5 Current breeding progress

Blueberry breeding is a time-consuming and laborious endeavor. The breeding process may take around ten years from a cross combination to a commercial grower trial (Plant & Food Research, 2017a; Retamales & Hancock, 2012). In 100 years of blueberry breeding history, not many cultivars have been released for commercial productions. However, since the number of blueberry plant breeders, institutes and companies working on new cultivar developments has increased, more cultivars have been released in the past decade. From 2010 to 2018, a total of 181 new blueberry varieties were registered, including 107 SHB, 38 NHB, 16 RE and 20 ornamental varieties (Figure

1.3). SHB was the main breeding type in recent years, with 59% of the new cultivars released for this type (Figure 1.3). Most of these new SHB cultivars were released from the USA breeding programs (45 from Florida, eight from Georgia, nine from Fall Creek Farm and Nursery, three from North Carolina) (Table 1.2). Additionally, eleven NHB, four RE and fifteen ornamental varieties were also developed in America (Table 1.2). Hence, America is the biggest blueberry breeding country, releasing more than half of the new cultivars for blueberry production worldwide (Table 1.2). New Zealand is the second new cultivar contributor that has developed eighteen new cultivars in recent years with five SHB, three NHB, seven RE and three ornamental varieties (Table 1.2). Besides, Australia also focused on SHB breeding, while Japan and Europe made efforts on NHB cultivars development (Table 1.2). Additionally, in 2018, China has begun to release new blueberry cultivars that are suitable for the local climate conditions in 2018 (Table 1.2).



(A)



(B)

Figure 1.3 Total number of registered blueberry cultivars and the percentage of each blueberry type in the last eight years, from 2010 to 2018 (A), and the percentage of each registered blueberry type in 2010, 2012, 2014, 2016 and 2018, respectively (B). From Clark and Finn (2010); Finn et al. (2012); Gasic et al. (2014); Gasic, Preece, and Karp (2018); Gasic et al. (2016). (Blue) SHB = Southern Highbush, (Red) NHB = Northern Highbush, (Green) RE = Rabbiteye, (Yellow) Ornamental = Ornamental Blueberries.

Table 1.2 Number of registered blueberry cultivars by country and types from 2010 to 2018. From Clark and Finn (2010); Finn et al. (2012); Gasic et al. (2014); Gasic et al. (2018); Gasic et al. (2016).

Country (Locations)		**Blueberry Types**				**Total Cultivar from Each Breeding Location and Country**	
		SHB	NHB	RE	Ornamental		
USA	Florida	45	0	0	3	48	**95**
	Georgia	8	0	2	2	12	
	Michigan	0	3	0	0	3	
	USDA	0	3	2	5	10	
	Fall Creek	9	5	0	5	19	
	North Carolina	3	0	0	0	3	
New Zealand		5	3	7	3	18	**18**
Australia		14	0	0	0	14	**14**
Japan		0	5	0	0	5	**5**
Europe		0	9	0	0	9	**9**
China		2	4	0	0	6	**6**
Total Blueberry Cultivars from Each Blueberry Type		**86**	**32**	**11**	**18**	**147**	**147**

SHB = Southern Highbush, NHB = Northern Highbush, RE = Rabbiteye.

From 2010 to 2018, the newly developed blueberry cultivars were improved in several aspects, including fruit characteristics, physiological adaptations and disease resistance. These crucial plant characteristics are summarized below (Buck et al., 2012; Hancock, 2006; Lobos & Hancock, 2015; Lyrene, 2006; Retamales & Hancock, 2012; Rowland et al., 2011):

a) Reducing the chilling hours, increasing the cold tolerance and expanding the production in warmer or colder regions. All these factors can be beneficial for extending the harvest time of the year. Such low chilling and evergreen SHB cultivars were released from the Florida breeding program, such as, ‘Kestrel’, ‘Scintilla’, ‘Snowchaser’ and ‘Springhigh’ (Clark & Finn, 2006, 2010; Finn et al.,

2012).

b) Prolonging the harvest season by choosing the early maturing cultivars or late flowering and late maturing cultivars. For instance, recently developed RE varieties from New Zealand, including ‘Rake’, ‘Rahi’, ‘Sky Blue’, ‘Centra Blue’ and ‘Centurion’, were selected for their late harvest characteristics (Buck et al., 2012; Gasic et al., 2014; Patel & George, 1997).

c) Fruit-based selection: (i) market- and customer-driven fruit characteristics such as large fruit size, fruit colour, flavour, firmness, long shelf life; (ii) grower-driven traits such us easy fruit harvest by hand or machine harvesters. For example, ‘Aurora’, ‘Draper’ and ‘Liberty’, new NHB cultivars from Michigan State University, were improved to be later maturing and to give longer shelf life (Okie, 2004). In another example, a $2 coin fruit size jumbo blueberry cultivar, developed by Australian breeder Ridley Bell in 2008, has been used for a field trial in New Zealand since 2016 (Buck et al., 2012; Hutching, 2017). Additionally, high antioxidant content has become one of the important fruit quality parameters in recent years (Plant & Food Research, 2017a, 2018). In order to reach this target, the New Zealand blueberry breeding program is working on extending the harvesting season cultivars, increasing the fruit size, improving fruit color, texture, taste and yield (Plant & Food Research, 2017a).

d) Resistance to diseases caused by fungi and oomycetes, such as mummy berry (*Monilinia vaccinii-corymbosi* (Reade)), *Phytophthora* root rot (*Phytophthora cinnamomi* Rands) and anthracnose fruit rots (*Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc.), are vital to maintaining fruit quality and the yield. ‘Chanticleer’ and ‘Hannah’s Choice’, released from the USDA breeding program, were bred for disease resistance and winter cold tolerance (Okie, 2002). ‘Bluejay’ and ‘Reveille’ were selected for mummy berry resistance, while ‘’O’Neal’ and ‘Springhigh’ showed tolerance to stem blight and stem canker (Retamales &

Hancock, 2012). Some NHB cultivars had rust resistance, such as 'Bluecrop', 'Burlington', 'Collins', 'Dixi', 'Earliblue', 'Gem', 'Ivanhoe', 'Olympia', 'Stanley' and 'Weymouth' (Heidenreich, Fiacchino, & Koeller, n.d.; Nelson, 2008). Only one new SHB cultivar, C00-09, has been mentioned to have blueberry leaf rust resistance during the field trial in Australia (Gasic et al., 2016), which may be caused by *Thekopsora minima* as it had been found in Australia (Biosecurity Tasmania, 2017).

1.2.6 Cultivation requirements for various blueberry types

Since the abovementioned cultivars were bred under dissimilar selection environments and for various breeding purposes, variations in plant habits can be found among different cultivars. Therefore, to match the local environment, each blueberry plantation is using different blueberry cultivars for production. In general, when selecting a suitable cultivar to adapt to local production environment and systems, two vital aspects need to be considered, namely, the chilling requirement and soil types in the growing region. Based on the combination of variations in the chilling hours and soil types, four climate zones are defined for growing blueberries with similar plant characteristics. Additionally, the main blueberry cultivars in each climate type are summarised in Table 1.3:

a) If the climate is mild and wet in summers (<30℃), but is very cold in winters (<0℃), and if the chilling hours are normally over 1000 h, and the soil is organic sand and loam, then lowbush and NHB are the dominant types for commercial production. For example, NHB is the main type growing in Jilin (China) and northern Europe, while lowbush cultivars are suitable to grow in Canada and Eastern Europe.
b) If the climate is mild in both winter and summer, the soil is acid, and the chilling hours are more than 600 h, then fruit can be produced by NHB, SHB and RE in these climate regions.

c) If the summer is hot, but the winter is mild, and if the soil remains acidic, but the chilling hours vary from 0 h to 800 h, NHB, SHB and RE cultivars need to be selected for production becaused of the requirement of chilling hours. For instance, in Australia and Chile, which are both under these climate conditions, these types for blueberry are planted in different production areas. In Mexico and Uruguay, only the low-chilling SHB cultivars are selected for commercial production.

d) If chilling hours are less than 450h, and the summer is hot and dry while the winter is mild, regions with this climate conditions are more suitable for growing SHB cultivars (Retamales & Hancock, 2012).

Table 1.3 Blueberry production regions according to climate conditions and soil types.

Temperature	**Chilling Hours**	**Soil Types**	**Production Regions**	**Blueberry Types**	**Adapted Cultivars**	**References**
Mild, wet summers (<30℃) and very cold winters (<0℃)	>1000 h	Rich organic sands or loams that do not require acidification	Jilin (China), Italy, Germany, Michigan, New Jersey, the Netherlands and Poland, Canada (Maritime, Quebec, Atlantic)	Northern Highbush	'Aurora', 'Bluecrop', 'Duke', 'Draper', 'Elliott', 'Jersey', 'Liberty'	Agriculture and Agri-Food Canada (2011); Brazelton (2013); Fall Creek Farm & Nursery (2019c); New Nouveau Brunswick Canada (2010); Retamales and Hancock (2012); Strik, Moore, and
				Lowbush	'Blomidon', 'Burgundy', 'Brunswick'	

						Finn (2014)
Mild, moist summers and moderate winters (>0℃)	>600 h	Sands and loams with high organic content and low pH to mineral soils that do require acidification	France, Japan, Northern New Zealand, South-central Chile, the Pacific Northwest and Southern Australia	Northern Highbush	‘Bluecrop’, ‘Brigitta’, ‘Duke’, ‘Elliott’, ‘Nui’, ‘Puru’, ’Reka’	Australian Blueberry Growers' Association (n.d.-b); Blueberries New Zealand (n.d.-b); Retamales and Hancock (2012); Retamales et al. (2014)
				Southern Highbush	‘Biloxi’, ‘Legacy’, ‘Misty’, ‘’O’-Neal’, ‘Star’, ‘Sapphire’	
				Rabbiteye	‘Brightwell’, ‘Centurion’, ‘Climax’, ‘Delite’, ‘Maru’, ‘Powderblue’, ‘Rahi’, ‘Tifblue’, ‘Woodard’	
Hot, wet summers (28-30℃) and mild winters (> freezing)	[0 h-(500-800 h)]	Mineral soils with high clay content that do need acidification blueberry cultivars	Argentina, Mexico, northern New South Wales, North-central Chile, South-eastern USA, Uruguay	Southern Highbush	‘Emerald’, ‘Jewel’, ‘’O’-Neal’, ‘Star’	Fall Creek Farm & Nursery (2019a, 2019e, 2019f); Retamales and Hancock (2012); Retamales et al. (2014)
				Rabbiteye	‘Brightwell’, ‘Climax’, ‘Premier’, ‘Powderblue’ ‘Tifblue’	
				Northern Highbush	‘Croatan’	
Hot dry summers (>30℃) and mild winters (<7℃)	250-450 h	Mineral soils with high clay content that do require acidification	North-central Chile, South Africa, Spain	Southern Highbush	‘Emerald’, ‘Jewel’, ‘Star’, ‘Suziblue’	Fall Creek Farm & Nursery (2019b); Retamales and Hancock (2012)

Based on the table above, highbush and RE are the most common blueberry types grown under the four climate conditions, while lowbush cultivars are more suitable for

growth in cold regions of the first climate type. Currently, highbush and RE are mainly for fresh blueberry production, while most lowbush are grown for processed production (Brazelton, 2013; Brazelton & Young, 2017). In the following sections, the cultivation requirements for each type of blueberry grown in the respective regions are described:

1.2.6.1 Production regions of highbush cultivars

Highbush cultivars are grown in all four blueberry production regions, including the Americas, Europe, Asia and the Pacific Rim (Table 1.3). Based on the difference of the chilling requirements, the highbush blueberry is divided into NHB and SHB. In general, NHB needs 800 to 1200 chilling hours for breaking bud dormancy and grows well in a temperate zone with a winter. While SHB blueberries can be planted in warmer regions and their chilling hours can vary from 0 to 800 h. In terms of soil type, SHB cultivars do best in acid soils, while NHB cultivars can adapt to a soil with a relatively higher pH value (Lyrene, 2002, 2006; Retamales & Hancock, 2012; Spectrum Analytic Inc., n.d.). Due to these differences, NHB and SHB have theirown specific production regions.

In commercial production, highbush blueberries are maintained between a 1.8 to 2.5 m plant height and transplanted in the field with aisles between the rows of plants (Polashock et al., 2017; Rowland & Hammerschlag, 2004). ‘Bluecrop’, ‘Duke’, ‘Elliott’, ‘Jersey’, ‘Liberty’, ‘Aurora’ and ‘Draper’ are predominant NHB cultivars grown in cold and long winter areas, such as in Europe, Mid-western and North-eastern USA and Jilin, China. ‘Brigitta’, ‘Duke’, ‘Elliott’, ‘Nui’, ‘Puru’, ’Reka’ can grow under a relatively mild winter with less chilling hours, but in acidic, sandy and loamy soil conditions. The SHB cultvars, such as ‘Biloxi’, ‘Legacy’, ‘Misty’, ‘’O’-Neal’, ‘Star’, ‘Sapphire’, ‘Emerald’ and ‘Jewel’, can be used for production in regions with less chilling hours, but need acidic soil types, such as those found in South America, Europe and the Pacific Rim (Table 1.3).

1.2.6.2 Production regions of RE cultivars

Usually, RE cultivars are extremely vigourous, longevous and productive plants with late fruiting. Most of the RE cultivars have a chilling requirement of 500-700 h and can grow in relatively flexible soil types (Galletta & Ballington, 1996; Retamales & Hancock, 2012). RE can maintain in a similar commercial cultivation manner as highbush for blueberry production (Polashock et al., 2017). Due to its late fruiting character, RE cultivars, such as Brightwell', 'Centurion', 'Climax', 'Delite', 'Maru', 'Powderblue', 'Rahi', 'Tifblue' and 'Woodard', have been grown in the Americas, Australia and New Zealand (Table 1.4).

1.2.6.3 Production regions of lowbush cultivars

Lowbush blueberry types are composed of different species, including 'sweet lowbush blueberry' (*V. angustifolium* Aiton), European blueberry (*V. myrtillus*) and Canadian blueberry (*V. myrtilloides* Michaux) (Galletta & Ballington, 1996; Nestby, Percival, Martinussen, Opstad, & Rohloff, 2011). In general, lowbush blueberry plants are lower than 0.5m, have typical rhizomes, and can grow on dry acidic lands. It is considered to be the most winter-hardy domesticated blueberry type (Galletta & Ballington, 1996; Martinussen, Nestby, & Nes, 2008). Nowadays, lowbush blueberries are produced in Quebec, Nova Scotia, and New Brunswick in Canada, Maine in the USA, Europe and China (Brazelton, 2013; Mclsaac, n.d.; Yarborough, 2015). 'Augusta', 'Blomidon', 'Brunswick', 'Chignecto', 'Cumberland', and 'Fundy' were the first lowbush cultivars released from 1975 through 1988. Later released cultivars of lowbush blueberries included 'Burgundy', 'North Country', 'Northsky', 'Pretty Yellow' 'Spring', 'Tophat' and 'Verde' were used as ornamental plants (Brand, 2015; Galletta & Ballington, 1996).

1.2.6.4 Blueberry production regions in New Zealand

Specifically, New Zealand is in a temperate zone, and has a moderate climate in summer and winter. As a consequence, both highbush and RE are the main blueberry types

planted in New Zealand (Table 1.4). However, the harvest time and growing region for highbush and RE are different. Generally, the first harvest begins for SHB plants from November to mid-December. After that, from December to early January, the mature fruit can be picked on NHB plants (Plant & Food Research, 2017b). In general, fruit harvested in this early season, from November to early December, have good export prices, while fruit picked in the peak harvesting season, from December to January, is more suitable to sell at a lower price for local consumption. In terms of the growing areas of each blueberry type, most of the SHB cultivars are grown in the Waikato-northward areas. 'Marinba', 'Misty', and ''O' Neal', are examples of SHB cultivars that require less chilling hours before flowering and can thus be grown in the Waikato-northward areas. 'Bluecrop', 'Duke', 'Toro', 'Blue Moon', 'Earliblue', 'Nui', 'Puru', 'Reka', 'Sunset Blue', 'Elliott' and 'Jersey' are common NHB varieties grown in the Waikato-southward regions that have adapted to longer chilling hours for their dormancy requirement. Most of the RE cultivars are harvested from mid-January to mid-March when the picking season of highbush cultivars is over (Plant & Food Research, 2017b). As the blueberry harvest goes down from December to February in Australia, it requires an increase of blueberry imports from New Zealand. Therefore, most of the RE blueberries grown in New Zealand are used for export in this period. RE cultivars, such as 'Tifblue', 'Ocean Blue', 'Sky Blue', 'Velluto Blue', 'Centra Blue', 'Centurion', 'Delite', 'Maru', 'Powder Blue' and 'Rahi', are currently used in blueberry productions in the North Island and the Waikato-northward areas for late harvest production (Blueberries New Zealand, n.d.-b; Blueberry Country, 2018; Tharfield Nursery Ltd, 2012). In addition, a new group of blueberry cultivars developed in Australia with a jumbo large fruit size, such as 'Eureka', 'Eureka Sunset', 'Eureka Sunrise', 'Dazzle' and 'Twilight', have grown by BerryCo Limited in the Bay of Plenty for the premium fruit market demand in Asian countries (BerryCo Limited., 2016).

Table 1.4 New Zealand blueberry cultivars and plantation regions (Blueberries New Zealand, n.d.-b; Blueberry Country, 2018; Tharfield Nursery Ltd, 2012).

Blueberry		**Chilling Hours (h)**	**Harvest Period in New Zealand**	**Marketing Targets**	**Suitable Plantation Regions**
Type	**Cultivar**				
NHB	Bluecrop	>800	Mid-Dec - end Jan	Export/local fresh market	Waikato-southwards
NHB	Duke	>800	Mid-Dec - end Jan	Export/local fresh market	Waikato-southwards
NHB	Toro	>800	Mid-Dec - end Jan	Export/local fresh market	Waikato south-wards
NHB	Blue Moon	>1000	Nov - Jan	Export/local fresh market	Waikato south-wards
NHB	Earliblue	>800	Nov - Mid Dec	General good export price	Waikato south-wards
NHB	Nui	>800	Nov - Dec	General good export price	Waikato south-wards
NHB	Puru	>1000	Nov - Mid Dec	General good export price	Waikato south-wards
NHB	Reka	>800	Nov- Dec	General good export price	Waikato south-wards
NHB	Sunset Blue	>1000	Nov - Mid Dec	General good export price	Waikato south-wards
NHB	Elliott	>800	Feb - Mid-Apr	Good export prices	Waikato north-wards
NHB	Jersey	>800	Mid-Dec - end Jan	Process/low price	Waikato north-wards
RE	Ocean Blue	600-750	Feb - Mar	Export/local fresh market	Waikato north-wards
RE	Sky Blue	400-600	Feb - Mar	Export/local fresh market	Waikato north-wards
RE	Velluto Blue	500-700	Mid-Feb - Mar	Export/local fresh market	Waikato north-wards
RE	Centra Blue	600-750	Feb - Mid-Apr	Good export prices	The North Island
RE	Centurion	600-700	Feb - Mid-Mar	Good export prices	Waikato north-wards
RE	Delite	500	Feb - Mid-Apr	Good export prices	Waikato north-wards
RE	Maru	600-700	Feb - Mid-Mar	Good export prices	Waikato north-wards
RE	Powderblue	550-600	Feb - Mid-Apr	Good export prices	All districts
RE	Rahi	600-750	Jan - Feb	Good export prices	The North Island
RE	Tifblue	600-700	Jan - Feb	Good export prices	Waikato north-wards
SHB	Marinba	200	Nov - Mid Dec	General good export price	Waikato north-wards
SHB	Misty	150	Nov - Mid Dec	General good export	Waikato north-wards

				price	
SHB	O'Neal	~400	Nov - Mid Dec	General good export price	Waikato north-wards

SHB = Southern Highbush, NHB = Northern Highbush, RE = Rabbiteye.

1.2.7 Global blueberry production

Blueberry cultivars, as previously discussed, are producing fruit in commercial plantations, globally. In 2017, over 500,000 tonnes of blueberries were supplied for consumption all over the world (FAO, 2017d). Nowadays, fresh blueberries are offered to consumers all year round and production farms can be found on each continent. Due to the different season cycles and climate conditions, the blueberry harvest times vary between the northern hemisphere and the southern hemisphere (Figure 1.4). In the northern hemisphere, the harvest starts in March and ends in later October. In the southern hemisphere, most of the harvest begins in October and ends in March. Australia has developed a year-round production of blueberries, and fruit can be picked throughout the year in different regions.

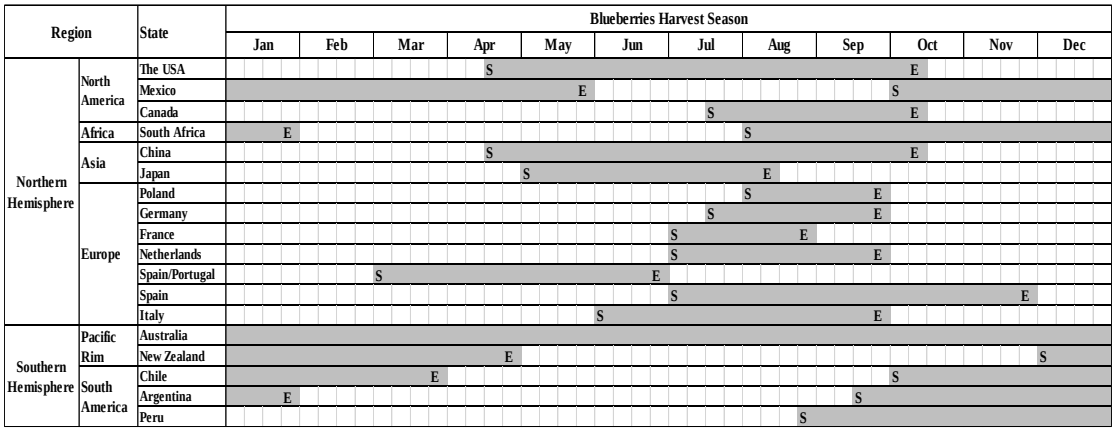


Figure 1.4 The blueberry harvesting seasons in different production countries in the northern and southern hemispheres (Australian Blueberry Growers' Association, n.d.-a; Berry Lovers, 2018; P. Mulderij, 2018; Retamales & Hancock, 2012). S = start of the harvest season, E = end of the harvest season.

1.2.7.1 Blueberry production in the northern hemisphere

In the northern hemisphere, blueberry production is in North America, Europe and Asia. North America is the largest blueberry producer in the world. The main production areas of North America are the states of Georgia, North Carolina, Oregon, Washington and British Colombia (Brazelton, 2013; FAO, 2017b). Canada has had significant growth in blueberry production from both lowbush and highbush cultivars, and is the second largest producer in the world (FAO, 2017b; Fresh Plaza, 2018b). Mexico also has increased blueberry production for American market demands in the past decade (Brazelton, 2013; Fresh Plaza, 2018e; T. Johnson & McClatchy-Tribune, 2015). Meanwhile, in Europe, most of the blueberries are produced in Spain, France, Poland, Germany and the Netherlands. This fresh product from Europe is supplied to the UK market and local consumption, and has an 18.4% share of the total blueberry production in the world (Brazelton, 2013; FAO, 2017b, 2017c). However, Europe still needs to import fresh and frozen blueberries from South America and Canada to meet the demand of the local market (Agriculture and Agri-Food Canada, 2016). In addition, production from Asian countries has been growing over the last decade (Brazelton, 2013; FAO, 2017a; Retamales & Hancock, 2012). China is one of the main producers and importers in Asia (Brazelton, 2013). Due to a huge gap between supply and consumption demands in China, blueberry production and imports have been continuously growing (Asia Fruit Logistica, 2018; Fresh Plaza, 2018d). Additionally, Japan and South Korea also have a shortage of blueberry supply and have to import blueberries from North America and Chile to satisfy their domestic markets (Brazelton, 2013; Fresh Plaza, 2017).

1.2.7.2 Blueberry production in the southern hemisphere

In the southern hemisphere, South America, Africa and the Pacific Rim regions have blueberry productions. In 2018, Chile became the largest exporter of fresh blueberries in the world, with 24% of the share, and it was the biggest blueberry supplier for China (Brazelton, 2013; Fresh Plaza, 2018c). Additionally, in 2018, Peru became the third

largest exporter in the world (12% share), which was only 1% less than Spain, and it was the second biggest supplier of blueberries to China (Fresh Plaza, 2018f, 2018g). In South Africa, blueberry production had been rising sharply over the past few years (Brazelton, 2013). Blueberry production was over 10,000 tonnes in the 2017/2018 production season, and more than 70% of the product was used for export (Jansen, 2017). In Argentina, 21 million kilograms of fresh blueberries were produced by new blueberry cultivars in 2017, and 77% of these products were used for export (Brazelton, 2013; Fresh Plaza, 2018a).

In the Pacific Rim, most of the blueberries are produced by Australia and New Zealand (Brazelton, 2013). Australia produced 9,553 tonnes of blueberries at the end of June 2017. In total, 87% of the blueberries were harvested in the Northern New South Wales region of Coffs Harbour from June to March. January to March was the main harvest season in the Queensland, Tasmania, Victoria and South Australian production regions (Figure 1.5). Even though blueberry producers have used evergreen SHB cultivars for a year-round production, Australia still has a shortage of blueberries to meet local market demand (Australian Blueberry Growers' Association, n.d.-a; R. Mulderij, 2018). Therefore, in 2017, 1,211 tonnes of fresh blueberries were imported from New Zealand to meet the demand in Australia (Australian Government, 2018; Horticulture Innovation Australian Limited, 2018). According to descriptions from the Australian Biosecurity Import Conditions, fresh berries for human consumption can only be imported from New Zealand (Australian Government, 2018). Hence, New Zealand is the only fresh blueberry supplier for Australia.

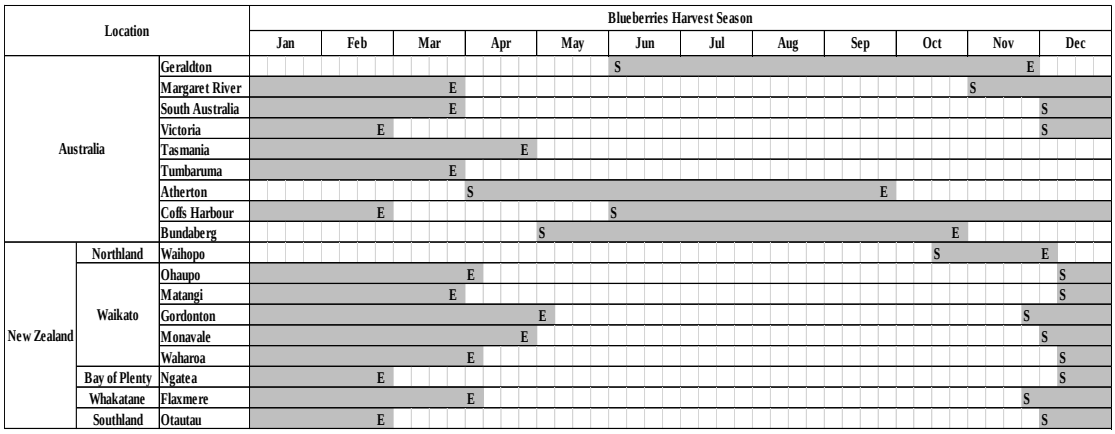


Figure 1.5 Harvest times for on different blueberry production regions in Australia and New Zealand (Blueberries New Zealand, n.d.-a; Horticulture Innovation Australian Limited, 2018; Horticulture New Zealand & Plant & Food Research, 2017). S = start of the harvest season, E = end of the harvest season.

In New Zealand, blueberries can be grown in both the North and South Islands (Figure 1.5). In the North Island, the main blueberry production is in the Waikato region (Ngatea and Ohaupo), while Waihopo and Hastings also produce blueberries. In the South Island, blueberries are mainly grown in Otautau (Blueberries New Zealand, n.d.-a; Horticulture New Zealand & Plant & Food Research, 2017). The total production area in New Zealand has increased by 23% in the past decade and reached 624 ha in 2017 (FAO, 2017a; Figure NZ, 2018). Simultaneously, the production of blueberries was more than 3,000 tonnes in 2017 (FAO, 2017a; Horticulture New Zealand & Plant & Food Research, 2017). And the export value has doubled since 2010 and reached $32.3 million in 2017 (Horticulture New Zealand & Plant & Food Research, 2010, 2017). As New Zealand is the only fresh blueberry supplier for Australia, over 40% of fresh blueberries were exported during the 2017/18 production season (Australian Government, 2018; Horticulture Innovation Australian Limited, 2018; Horticulture New Zealand & Plant & Food Research, 2018). Meanwhile, New Zealand growers also targeted the premium fruit market in Asia (Japan, Korea and China), with the export value was estimated to be more than $8 million in the 2017/18 harvest season (Brown,

2017). In 2018, a new joint venture between Maori collective and government scientists chose blueberries as a foundation crop and established a $1 billion goal for blueberry exports. Due to the increased financial and technical support from this new joint venture, the Zealand blueberry industry is expected to develop rapidly in the future (Fox, 2018; Timewell, 2017).

1.2.8 Challenges of New Zealand blueberry industry

Since a $1 billion export target was defined, there is no doubt that there will be an expansion of blueberry production in New Zealand. Based on the cultivar characteristics mentioned previously, SHB and RE cultivars are suitable for this expansion. Due to the low chilling requirement of SHB cultivars, they can be planted in the North of Auckland for early harvest market. RE can be used for the late harvest export market in Australia. Fresh fruit harvested during these periods can gain a higher profit than the fruit picked in the peak season (D. Hutchins, personal communication, 13th March 2018). Additionally, jumbo large fruit cultivars can be used for the premium markets in Asian countries (BerryCo Limited., 2016; Brown, 2017; Hutching, 2017).

However, currently used cultivars and cultivating practices are insufficient to complete this task. One of the main barriers to this expansion is blueberry leaf rust, as it causes several impediments on blueberry production. First, a heavy infection of blueberry leaf rust can result in yield loss (European and Mediterranean Plant Protection Organization, EPPO, 2017; Polashock et al., 2017; Simpson, Wilk, Collins, Robertson, & Daniel, 2017). This is caused by premature leaf drop and early plant defoliation, which reduces fruit bud production and nutrient supply for fruit development (Figure 1.6). Additionally, infected fruit causes a direct decrease in yield and fruit quality. (Plant Biosecurity & Product Integrity, 2016). Finally, these infected products may cause quarantine issues or biosecurity restrictions when exported to other countries. As mentioned earlier, fresh blueberry products are mainly exported to Australia and Asian countries, and blueberry rust is one of the quarantine diseases that needs to be checked

in these countries. For instance, the Australian import conditions of fresh blueberries states that “Imports of *Vaccinium* spp. into South Australia, Western Australia, Victoria and Tasmania must be free from Blueberry Rust” (Australian Government, 2018, para. 9).

Figure 1.6 Blueberry rust disease symptoms in commercial plantations in the Hastings. Rust disease causes early defoliation of cultivar ‘Centra Blue (A) plant; a severe infection on cultivar ‘Centra Blue’(B) plants.

1.2.9 Solutions for blueberry rust control

One solution to blueberry leaf rust in the short term is to apply production management for disease control, which includes several aspects. The first one is to remove all the potential hosts of blueberry rust, such as plants from *Pernettya*, *Pieris, Lyonia, Menziesia, Hugeria*, *Leucothoe* and *Tsuga* spp. within a half mile radius (Brocklands Pty Ltd., 2018; Plant Biosecurity & Product Integrity, 2016). The second is to use cultivating and hygiene practices for rust control. This includes avoiding the use of any infected plants, materials and tools in the field, to control humidity by applying a drip irrigation system, to regulate plant growth by pruning and fertilizing appropriately, and

to maintain a clean field by following orchard hygiene and sanitation management (Biosecurity Tasmania, 2017; Plant Biosecurity & Product Integrity, 2016). The third is to spray fungicides, which is the most common option chosen by producers to control rust disease. Mancozeb is the only fungicide that has been registered in New Zealand and shown in the catalog of Novachem for the control of blueberry rust disease in New Zealand. The recommended application for this fungicide is that it is used as a protectant, which can form a coating on leaf surfaces to prevent the disease establishment. Spraying every 10-14 days can reduce the spores' ability to germinate (Young, 2018/2019).

Even though growers followed these measures for preventing the infection of blueberry by rust disease, it remains a major issue on blueberry production in the North Island (Barlow, 2014; NZ Herald, 2004). In addition, blueberry rust disease has spread to all of the blueberry production regions in New Zealand, which include the Waikato, Auckland, the Bay of Plenty and Mid-Canterbury (New Zealand Fungi, 2019a, 2019b). Likewise, blueberry rust disease has become prevalent in plantations in Hastings, Hawke's Bay (D. Hutchins, personal communication, 13th March 2018). It seems that these management techniques are ineffective at controlling blueberry rust disease in New Zealand blueberry productions. In the long term, the deployment of varieties with natural resistance towards blueberry rust would be a sustainable option for managing this disease during fruit production.

1.2.10 The lack of blueberry rust-resistant cultivars and the issues impede on the breeding of rust-resistance varieties

In New Zealand, presently cultivated varieties lack resistance to blueberry leaf rust (Table 1.4 and Table 1.5). In most of the plantations, no SHB and RE cultivars with natural rust resistance are used for blueberry commercial production in New Zealand. Even though there two NHB cultivars ('Bluecrop' and 'Earliblue') are rust-resistant, they are only planted in a small production region in New Zealand (Blueberries New

Zealand, n.d.-b; Blueberry Country, 2018; Clark & Finn, 2006, 2010; Finn et al., 2012; Gasic et al., 2014; Gasic et al., 2018; Gasic et al., 2016; Heidenreich et al., n.d.; Nelson, 2008; Okie, 2002, 2004; Tharfield Nursery Ltd, 2012). Moreover, no resistant cultivars are grown in Hastings for blueberry production (D. Hutchins, personal communication, 13th March 2018). Even though breeding materials for blueberry rust resistance were selected by breeders at Plant & Food Research, no cultivar with rust disease resistance has been released for commercial production in recent years (Buck et al., 2012; Finn et al., 2012; Gasic et al., 2014; Gasic et al., 2018; Gasic et al., 2016). The main issues preventing the breeding of resistant varieties are insufficient knowledge about the rust pathogen in New Zealand and the lack of efficient resistance screening procedures for blueberry rust disease. In the following sections, detailed information about these two issues is given.

Table 1.5 Blueberry rust-resistant cultivars and screening approaches.

Reaction	**Blueberry Cultivar(s)**	**Blueberry Type**	**Disease Screening Approach**	**Reference(s)**
Resistance	C00-09	SHB	Cultivar description	Gasic et al. (2016)
	"O' Neil' (possibly referring to "O'Neal')	SHB	Two-year-old seedling inoculation	Zheng et al. (2017)
	MS1718, PI638745	SHB	Plant inoculation	Babiker, Stringer, Smith, and Sakhanokho (2018)
	'Bluecrop', 'Burlington', 'Collins', 'Dixi', 'Earliblue', 'Gem', 'Ivanhoe', 'Olympia', 'Stanley', 'Weymouth'	NHB	Field observation	Heidenreich et al. (n.d.); Ivey (2016)
Moderate	'Powderblue'	RE	A two-year	Scherm and

resistance			survey on the field	Krewer (2008)

SHB = Southern Highbush, NHB = Northern Highbush, RE = Rabbiteye.

1.2.11 Blueberry rust disease

1.2.11.1 What is blueberry rust disease?

To understand blueberry rust disease in New Zealand, the first step is to identify clearly the relevant disease's symptoms in the field. In general, early symptoms of blueberry rust are recognized by roughly circular red-purple spots on the leaf surface. Then, reddish to brown centred necrotic spots (Figure 1.7 A) develop on the adaxial leaf surface, with yellow to orange colored rust pustules (uredinia) (Figure 1.7 B) found on the underside of these spots (Plant Biosecurity & Product Integrity, 2016; Polashock et al., 2017). This yellow to an orange colored pustule is a specific feature for diagnosing blueberry rust disease in the field.

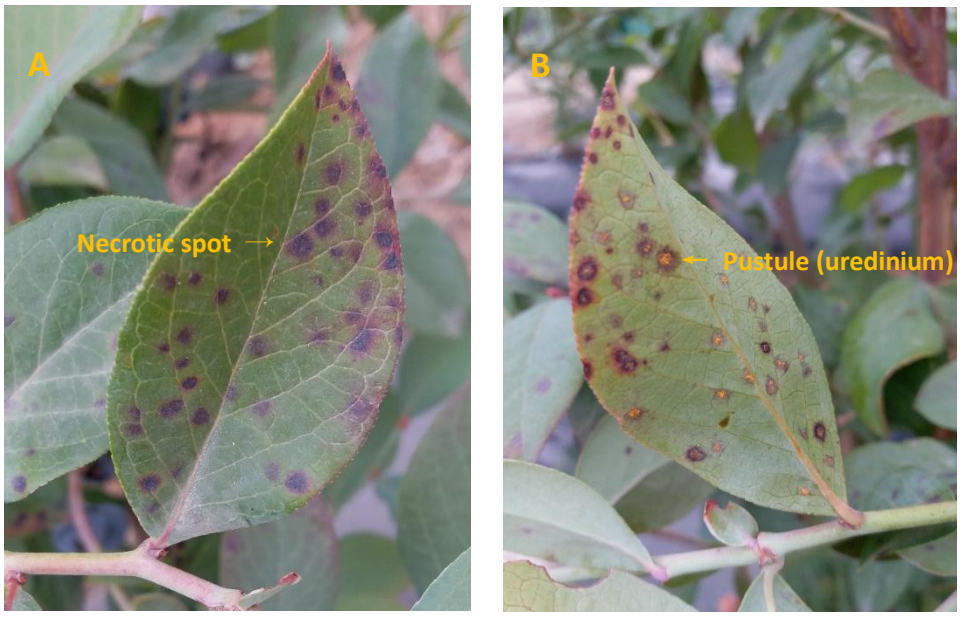


Figure 1.7 Blueberry leaf rust symptoms on cultivar 'Rahi'. (A) Blueberry rust symptom of upper leaf surface with reddish to brown centred necrotic spots; (B)

blueberry rust symptom of lower leaf surface with yellow to orange colored rust pustules (uredinia).

1.2.11.2 Rust disease causal organism

To have a further understanding of blueberry leaf rust, an accurate identification of the causal organism(s) in New Zealand is needed. Then its pathogenicity can be understood, and an efficient screening procedure can be set up for breeding resistant cultivars. Two kinds of pathogens are reported to be the causal organisms of rust disease on blueberries. One is *Naohidemyces vaccinii* (*N. vaccinii*), and the other is *Thekopsora minima* (*T. minima*) (Polashock et al., 2017). Some confusion was caused by various synonymic names for blueberry leaf rust. Currently, there are three commonly used names: *Pucciniastrum vaccinii* (*P. vaccinii*)*, T. minima and N. vaccinii* (Table 1.6). Hence, to avoid this issue in this study, the species identifications and morphology descriptions from Sato, Katsuya, and Hiratsuka (1993) are used:

- *T. minima* was a separated species from *P. vaccinii*, which peridermioid aecia was found in eastern North America and Japan.

- *N. vaccinii* is synonymic as *P. vaccinii*, *Thekopsora hakkodensis* and *Thekopsora vaccinii* (Table 1.6).

Table 1.6 Synonymic binomial names of blueberry rust causal fungi and the bold letters of species names used in this thesis (New Zealand Fungi, 2019a, 2019b; Sato et al., 1993).

Name Used in This Thesis	Synonymic Names
***Naohidemyces vaccinii* (Jørst.) S. Sato, Katsuya & Y. Hirats. (1993)**	*Melampsora vaccinii* (Alb. & Schwein.) G. Winter (1880) (stat. anam.)
	Melampsora vaccinii G. Winter (1881) [1884] (nom. illegit.)
	Melampsora vacciniorum J. Schröt. (1887) [1889]
	Naohidemyces vaccinii (Jørst.) S. Sato, Katsuya & Y. Hirats. (1993) (nom. inv.)
	Naohidemyces vaccinii (Jørst.) S. Sato, Katsuya & Y. Hirats. ex Wanderweyen & Fraiture (2007)
	Naohidemyces vacciniorum (J. Schröt.) Spooner (1999)
	Pucciniastrum myrtilli (Schumach.) Arthur (1906)
	Pucciniastrum vaccinii Jørst. (1952) [1951]
	Pucciniastrum vacciniorum (DC.) Dietel (1897)
	Thekopsora hakkodensis S. Ito et Hirat. f., (1927)
	Thekopsora hakkodensis S. Ito et Hirat. f. ex Hirat. f., (1936)
	Thekopsora vaccinii (Jørst.) Hirats. f. (1955)
	Thekopsora vacciniorum (DC.) P. Karst. (1879)
	Uredo pustulata var. *vaccinii* Alb. & Schwein. (1805)
	Uredo vacciniorum DC. (1815)
***Thekopsora minima* (Arthur) P. Syd. & Syd. (1915)**	*Pucciniastrum minimum* Arthur (1906)

As blueberry rust may be caused by *N. vaccinii* or *T. minima* or both in New Zealand, a clear distinction between these two species is necessary for pathogen species identification. Based on the descriptions from Sato et al. (1993), images and a summary of distinctive features in these two species are shown below:

- The structure of aecia on the *Tsuga deversifolia* host is the most distinctive morphological feature between *N. vaccinii* and *T. minima*. The aecia of *N. vaccinii*

are uredo-type (Figure 1.8 A), with a dome-like peridium and well-developed ostiolar cells (Figure 1.9 A), while the characters of *T. minima* are cylindrical with fragile peridial cells (Figure 1.8 B). *N. vaccinii*. has echinulate, borne singly on inconspicuous pedicels aeciospores (Figure 1.10 A), while *T. minima* has verrucose subglobose aeciospores (Figure 1.10 B).

- There is a difference between *T. minima* and *N. vaccinii* with regards to the uredinium structure. Both uredinium has dome-like peridium, but *N. vaccinii* has a clearer ostiolar cells structure than *T. minima* (Figure 1.11). However, the urediniospores are not easily discriminated by the surface sculpture (Figure 1.12).

- The most differential character of teliospores between these two species is the germ pore location in the cell. The germ pore of *N. vaccinii* is in the centre of each teliospore cell, while *T. minima* has germ pores at the corner of each cell at the centre of the spore balls. However, the images from this paper do not clearly show this difference.

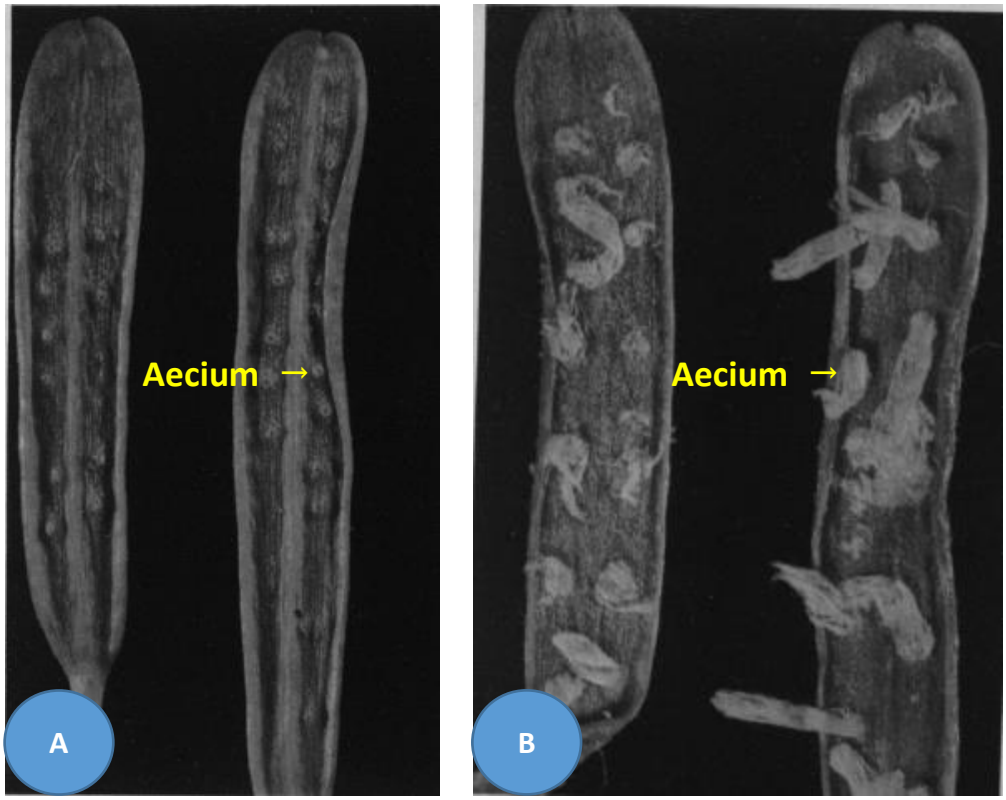


Figure 1.8 Aecia structures of *N. vaccinii* and *T. minima* on *Tsuga deversifolia* (*T. deversifolia*). (A) Aecia of *N. vaccinii* on *T. deversifolia* from Japan; (B) aecia of *T. minima* on *T. deversifolia* from Japan Sato et al. (1993).

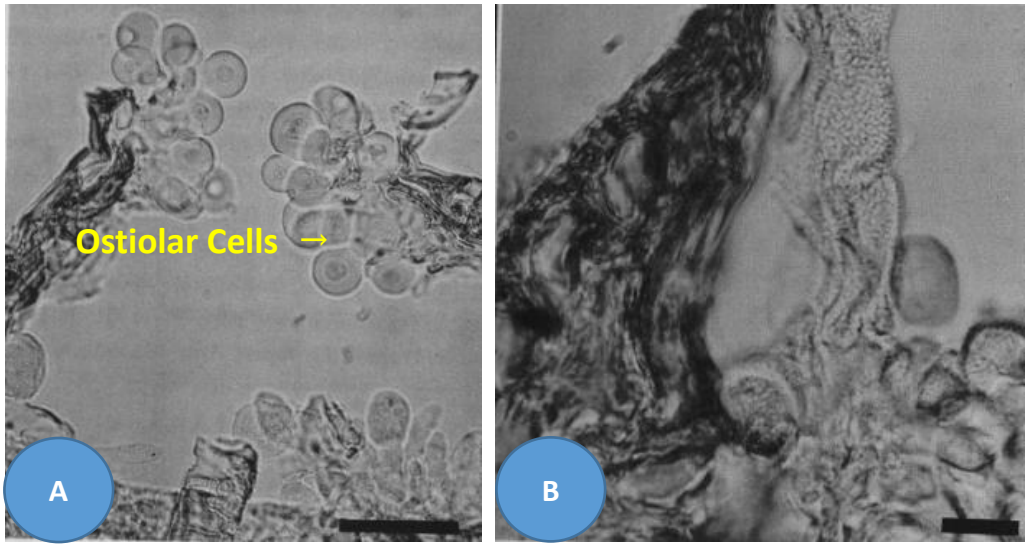


Figure 1.9 Aecium cross-section of *N. vaccinii* and *T. minima* on different *Tsuga* spp. hosts. (A) Aecium cross section of *N. vaccinii* with well-developed ostiolar cells on

Tsuga deversifolia from Japan; (B) aecium cross section of *T. minima* on *Tsuga canadensis* from Eastern USA (Sato et al., 1993).

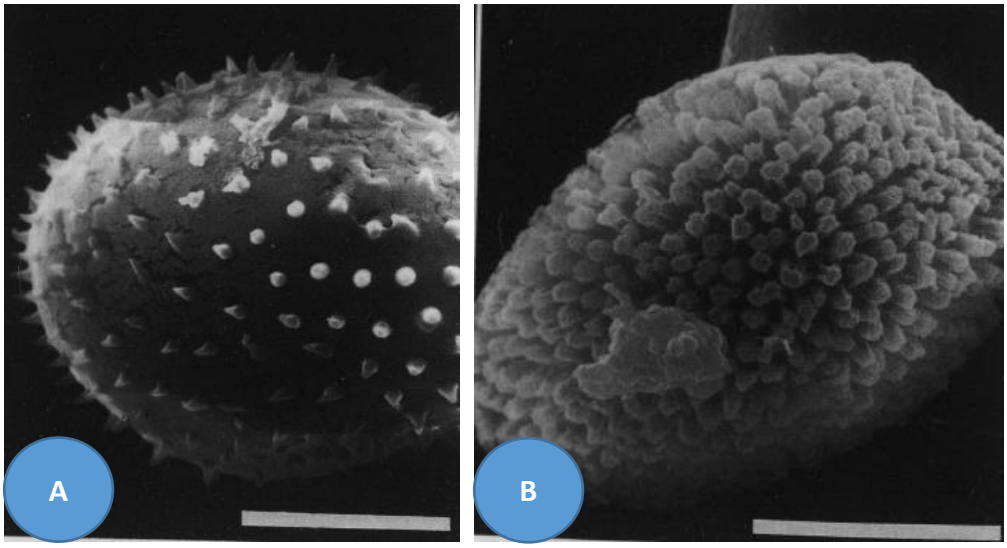


Figure 1.10 Aeciospore surface from *N. vaccinii* and *T. minima* on different *Tsuga* spp. hosts. (A) Aeciospore of *N. vaccinii* on *Tsuga heterophylla* from Western Canada; (B) aeciospore of *T. minima* on *Tsuga canadensis* from Eastern USA (Sato et al., 1993).

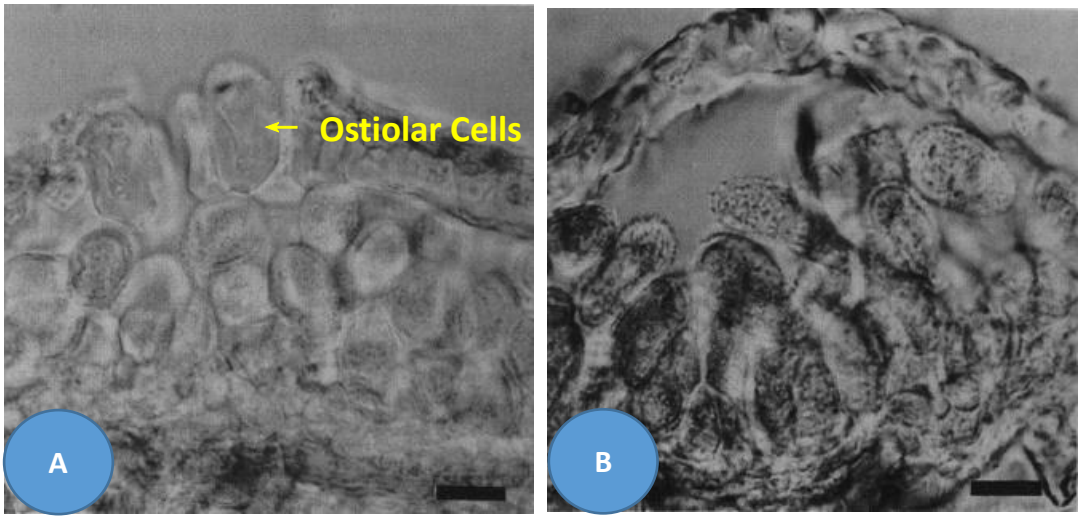


Figure 1.11 Uredinium cross-section of *N. vaccinii* and *T. minima* from different hosts. (A) Cross-section of uredinium of *N. vaccinii* on *Vaccinium membranaceum* from Western Canada shows well-developed ostiolar cells; (B) cross section of uredinium of

T. minima on *Lyonia neziki* from Japan without ostiolar cells (Sato et al., 1993).

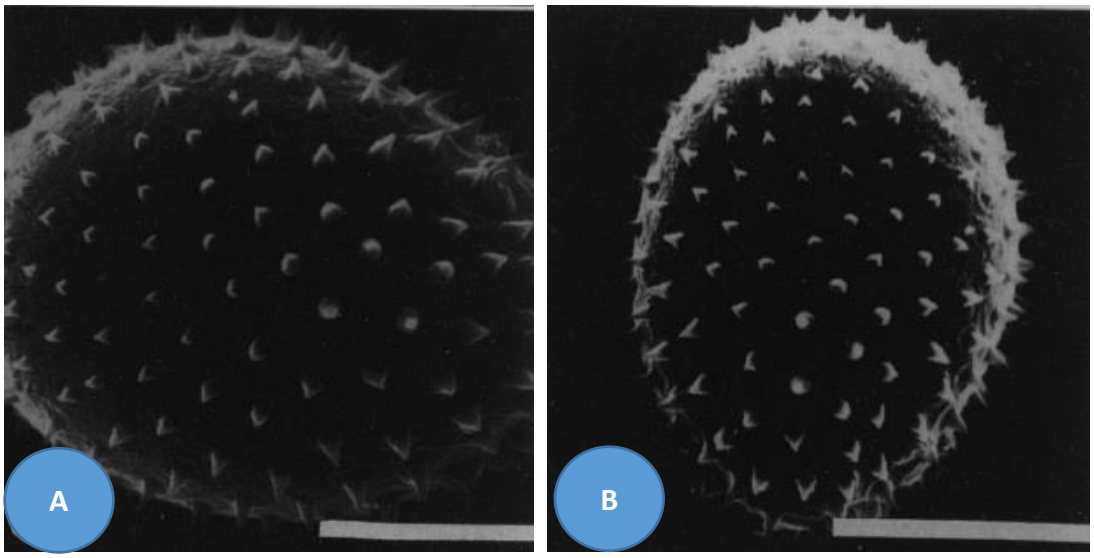


Figure 1.12 Urediniospore surface image from *N. vaccinii* and *T. minima* on different hosts. (A) Urediniospore of *N. vaccinii* on *Leucothoe grayana var. intermedia* from Japan; (B) urediniospore of *T. minima* on *Lyonia neziki* from Japan (Sato et al., 1993).

In conclusion the difference in aecium and aeciospore structures has the most distinctive characteristic between *N. vaccinii* and *T. minima* (Figure 1.8, Figure 1.9 and Figure 1.10). Besides this feature, the ostiolar cells from the uredinium also show the difference between these two species (Figure 1.11). However, the structure of the spores may be various on different hosts. The urediniospore of *N. vaccinii*, *T. minima* and the aeciospore of *N. vaccinii* are similar and difficult to be distinguished (Figure 1.12). Fortunately, both *N. vaccinii* and *T. minima* are heteroecious species and have a similar macrocyclic life cycle. In this kind of life cycle, a certain type of spore only occurs on particular hosts and under specific environmental conditions. Therefore, the life cycle of blueberry rust will be clarified in the following section in order to assist the further identification of blueberry leaf rust field samples.

1.2.11.3 Life cycle of blueberry rust disease pathogens

As *N. vaccinii* and *T. minima* have similar macrocyclic life cycle, in this section, *T. minima* is used as an example to understand the life cycle of blueberry rust pathogens. In the macrocyclic life cycle of *T. minima*, four different kinds of spores (urediniospores, teliospores, basidiospores, and aeciospores) are necessary to complete the asexual and sexual stages in this cycle. As a heteroecious species, it needs two hosts (Ericaceae and *Tsuga* spp.) for these two stages. Crops from the Ericaceae family are primary hosts for *T. minima* (Landcare Research, 2019a; Plant Biosecurity & Product Integrity, 2016). These hosts can be infected by two kinds of spores. The first are the aeciospores, produced from hemlock (*Tsuga* spp.), during the first infection of these primary hosts. The second are the urediniospores, which multiply from the primary hosts to maintain the asexual cycle. *Tsuga* spp. (West – *T. heterophylla* and *mertensiana*, East – *T. canadensis* and *caroliniana*, Asia - *T. diversifolia*, *T. sieboldii*, *T. chinensis* and *T. dumosa*) are considered to be the secondary hosts for the sexual stage (EPPO, 2017; Sato et al., 1993). In these species, host status is found on *T. heterophylla*, *T. mertensiana*, *T. canadensis*, *T. diversifolia*, and *T. sieboldii*. To complete the sexual stage on these secondary hosts, three kinds of spores are produced. The first ones are teliospores, which are produced on primary hosts in a cold winter. The second ones are basidiospores which germinate from teliospores to infect the secondary hosts. After that, aeciospores are produced from secondary hosts and the sexual process is completed.

To complete the macrocyclic life cycle of the *T. minima* blueberry rust pathogen, blueberry and *Tsuga* spp. are needed (Figure 1.13). Blueberry, as a primary host for the asexual stage, is infected by airborne dikaryotic aeciospores in early summer. After that, dikaryotic uredinia develop and germinate on the lower surface of blueberry leaves. Numerous urediniospores spread from uredinia rapidly reinfect blueberry during summer. When the temperature goes down in the late autumn, telia develop on blueberry leaves and overwinter. When the temperature goes up in spring, basidia germinate from dikaryotic teliospores to produce haploid basidiospores. These

basidiospores are released to infect the secondary host, hemlock pine (*Tsuga* spp.), for the sexual stage. After infection, haploid spermogonia are developed on hemlock pine needles. After dicaryotisation, dikaryotic aeciospores are produced for the infection on blueberries for the next asexual cycle in spring (Cummins & Hiratsuka, 2003; Polashock et al., 2017).

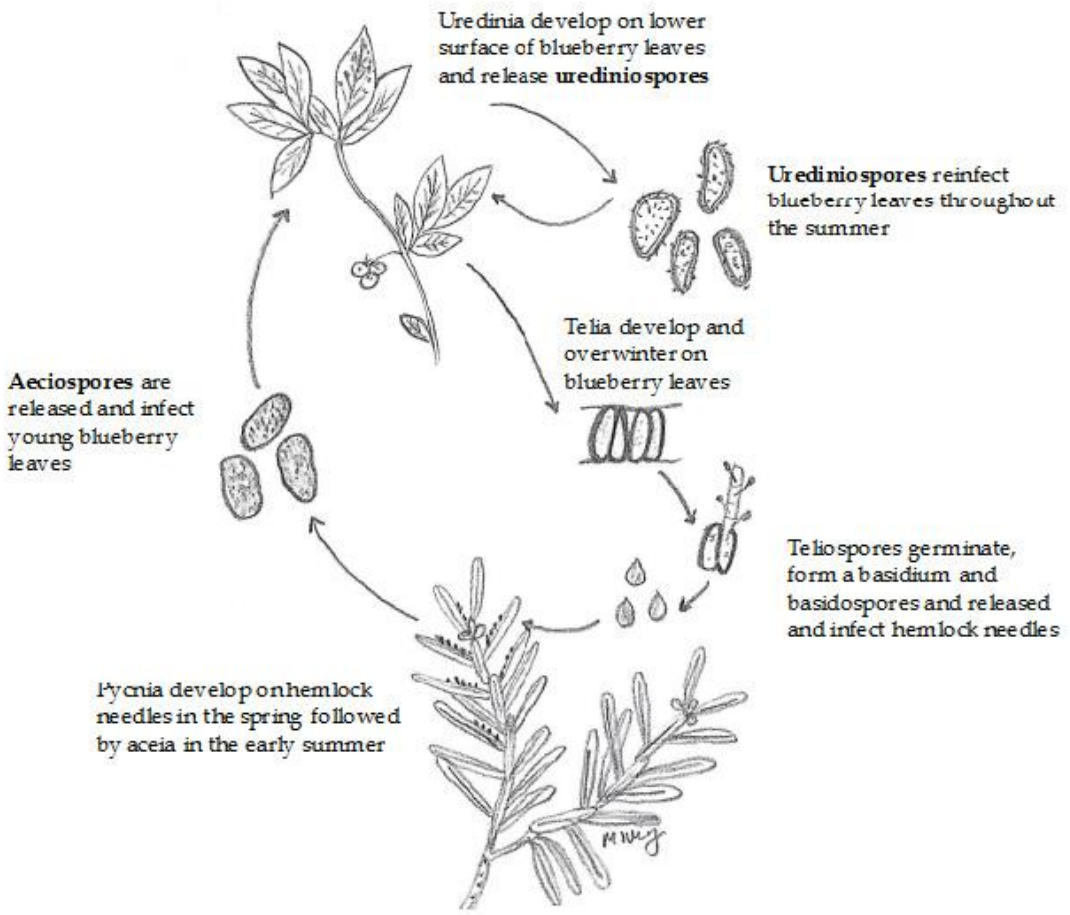


Figure 1.13 Blueberry leaf rust disease life cycle. Both aeciospores and urediniospores can infect blueberry to cause lesion symptoms (Ivey, 2016).

As mentioned above, both aeciospores and urediniospores can infect blueberries resulting in rust disease. In New Zealand, blueberries are mainly infected by urediniospores, which are multiplied by asexual life cycles. The reasons are: (1) blueberry rust does not require a complete macrocyclic life cycle in New Zealand.

Under a mild winter of 12-16℃, urediniospores can survive over winter by persisting on the leaves of low chilling or evergreen blueberry cultivars and can infect blueberry in the next season (100% Pure New Zealand, n.d.; Nelson, 2008; Pfister, Halik, & Bergdahl, 2004; Plant Biosecurity & Product Integrity, 2016); (2) the winter temperature is not low enough for the development of telia (EPPO, 2017); (3) the secondary hosts of *Tsuga* spp. are not widely grown in New Zealand (Polashock et al., 2017; Sato et al., 1993). Only *T. heterophylla* was found in Canterbury (near Castle Hill village), which is about 600 km to the closest blueberry orchard in Otautau (Heenan, de Lange, Cameron, Ogle, & Champion, 2004; Landcare Research, 2019b). Therefore, under microscopy, urediniospores and uredinia should be observed from lesions on leaf samples suspected of having blueberry rust disease in New Zealand.

1.2.12 Blueberry rust species in New Zealand

In New Zealand, blueberry rust was first described on highbush and RE blueberry cultivars in the Waikato region in 2004 (Barlow, 2014; NZ Herald, 2004). Between 2004 to 2005, eight rust specimens of *N. vaccinii* were recorded in the Landcare Research database, which were collected from the Waikato, Auckland and the Bay of Plenty (New Zealand Fungi, 2019a). However, in 2014 and 2015, three rust samples were collected from Mid-Canterbury, Auckland and the Bay of Plenty, and all of them were identified to be *T. minima* by Landcare Research (New Zealand Fungi, 2019b). Hence, both species of blueberry rust were identified in New Zealand.

Based on the identification results from Landcare Research, *N. vaccinii* was once found to be prevalent in Auckland and the Bay of Plenty, while only *T. minima* was detected in these regions in the recent years. Currently, rust disease has become prevalent on blueberry plants in the main blueberry plantation, Gourmet Blueberries. Ltd, in Hastings (D. Hutchins, personal communication, 13th March 2018). However, causal agent of rust disease in this production area has not yet been identified, which means blueberry rust can be caused by either *N. vaccinii*, *T. minima*, or both. This ambiguous

situation may affect blueberry export. As already noted, the Australian import conditions description of fresh blueberry products says, “Imports of *Vaccinium* spp. into South Australia, Western Australia, Victoria and Tasmania must be free from Blueberry Rust” (Australian Government, 2018). Additionally, the condition description also states that this blueberry rust is *N. vaccinii* (Australian Government, 2018). Therefore, the blueberry rust species identification is beneficial for blueberry exports. If the blueberry rust is *N. vaccinii* in Hastings, certain treatment procedures need to be applied to meet the Australia import criterion in advance to avoid the economic loss caused by these quarantine issues.

1.2.13 Identification of blueberry rust species in Hastings

1.2.13.1 Morphological identification

To solve this problem in Hastings, a clear identification of blueberry rust species is imperative. As previously mentioned, urediniospores and uredinia are common in New Zealand, and the yellow to orange colored rust pustules (uredinia) (Figure 1.7 B) have been found in the main plantation, Gourmet Blueberries. Ltd, in Hastings. Notably, morphological characteristics, such as size and shape of urediniospores, as well as structures of uredinia, can be examined by a bright-field light microscopy and scanning electron microscopy (SEM) from blueberry lesions on leaves. Through these observations, the identification can follow the morphological characters mentioned above. However, there is no obvious difference for urediniospores between *N. vaccinii* and *T. minima* (Figure 1.12). Only depending on the structure of the ostiolar cell of the uredium using cross-section images, it may be insufficient to distinguish the differences between *N. vaccinii* and *T. minima* (Figure 1.11). Therefore, a genome sequence homology of the Internal Transcribed Spacer (ITS) region can be used to provide another evidence to support species identification.

1.2.13.2 The ITS region identification for rust species

It is a common approach to use the genome sequence homology of the ITS region for rust species identification. The advantages and procedures of this approach are explained in this section. Several advantages of using this approach are:

- The ITS region is part of the ribosomal deoxyribonucleic acid (rDNA). rDNA is present as multiple copies and can be easily amplified by Polymerase Chain Reaction (PCR), which means a small amount of relatively crude DNA can be used as a sample for the PCR reaction (0.1-10 ng per amplification) (Gardes, White, Fortin, Bruns, & Taylor, 1991; White, Bruns, Lee, & Taylor, 1990).

- The structure of rDNA is already known for many organisms. Figure 1.14 shows that, in general, it contains nuclear small rDNA, ITS1, 5.8S rDNA, ITS2 and nuclear large rDNA, in general (Gardes & Bruns, 1993).

- The ITS region is one of the fastest evolving units in the nucleus, which has a higher variation ratio than nuclear small rDNA sequences and mitochondrial rRNA genes, and this variation can be found among species from the same genus (Gardes et al., 1991). Because of these reasons, it has become a suitable approach for analyzing the phylogenetic relationships among species within a genus or among populations (Gardes & Bruns, 1993; Gardes et al., 1991; White et al., 1990).

As the ITS region contains a high variation among species, many primers (Figure 1.14) have been developed in this region for amplifying specific fragments by PCR. Through comparing the nucleotide sequences from these amplified fragments, the indeterminate fungal species within or without the same genus of rust can be differentiated. For instance, four rust specimens from cereals and grasses were distinguished by the PCR primer pair ITS1rustF10d/ITS1rustR3c, and a parsimonious tree among 13 rust families was built up by analyzing the results from two primer pairs (Rust2inv/LR06 and

Rust18S-R/NS1) (Table 1.7).

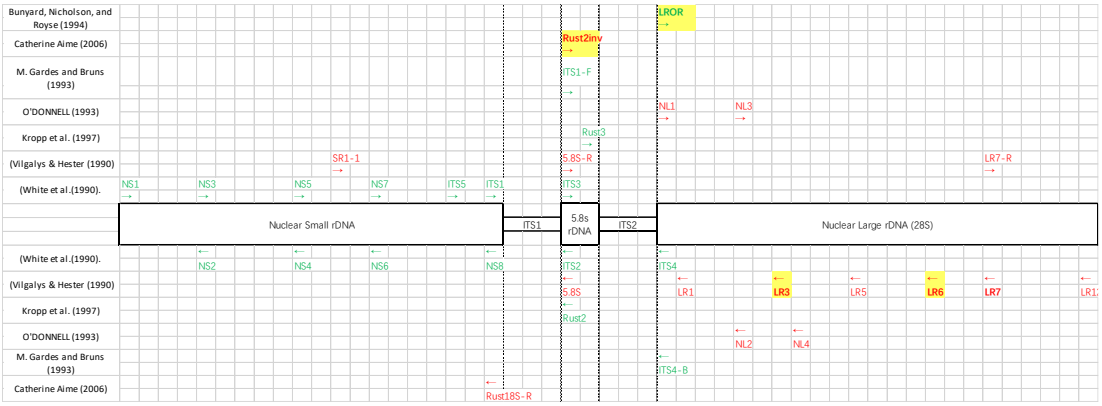


Figure 1.14 Structure of the ITS regions and the primer locations (Aime, 2006; Bunyard, Nicholson, & Royse, 1994; Kropp, Hansen, Wolf, Flint, & Thomson, 1997; O'donnell, 1993; Vilgalys & Hester, 1990; White et al., 1990). PCR primers used on rust families analysis are highlighted in yellow.

Table 1.7 PCR primers in the ITS regions that have been developed for fungal species distinction in blueberries and other crops.

Materials	**Primers**	**Sample**	**Purpose**	**Reference**
Morels (*Morchella* spp.)	LROR/LR12	DNA	To distinguish black morels (three species) and yellow morels (three species) into separated taxonomic groups.	Bunyard et al. (1994)
Puccinia spp.	ITS1rustF10d/ ITS1rustR3c	DNA	To distinguish four common rust pathogens of cereals and grasses.	Barnes and Szabo (2007)
Puccinia spp.	Rust 1/ITS4 Rust 2/Rust 3	DNA	To identify rust on dyer's woad and its relationship with other Puccinia species which infect the	Kropp et al. (1997)

			Brassicaceae family.	
13 rust families	Rust2inv/LR06 Rust18S-R/NS1	DNA	To establish the parsimonious tree among 13 rust families.	Aime (2006)
13 ascomycetes and 14 basidiomycetes	ITS-F/ITS4-B	DNA	To use a specific primer for identification of mycorrhizae and rusts.	Gardes and Bruns (1993)

As previously mentioned, some of these PCR primers were also applied to blueberry rust species identification. The commonly used primer pair for blueberry rust identification is Rust2inv/LR6, which covers part of 5.8S, ITS2 and part of the nuclear large rDNA (Figure 1.14). This primer pair is used to identify both *T. minima* and *N. vaccinii* on blueberries or other plant hosts (Table 1.8). Additionally, nested PCR primer pairs, such as LROR/LR6, LROR/LR3 and NL1/NL4, which are inside the amplified region from PCR primer Rust2inv/LR6, were also used for blueberry rust species identification. Combining the results from these two kinds of primer pairs can increase the sequencing accuracy. Hence, Rust2inv/LR6 and LROR/LR3 would be the suitable PCR primer pairs for identifying the blueberry rust species in Hastings.

Table 1.8 PCR primers specific to the ITS region that have been developed for rust species identification.

Crop	Species	Primer(s)	Location(s)	Reference
Blueberries	*T. minima*	Rust2inv/LR6; Rust18S-R/NS1	California	Shands, Crandall, Ho, and Miles (2018)
Blueberries	*T. minima*	Rust2inv/LR6	Oregon	Wiseman, Gordon, and Putnam (2016)
Blueberries	*T. minima*	ITS3/ITS4	Michigan	Schilder and Miles (2011)
Blueberries	*T. minima*	No details for primers	South Africa	Mostert et al. (2010)
Blueberries	*T. minima*	Rust2inv/LR7; LROR/LR6	Queensland and New South Wales	McTaggart, Geering, and Shivas (2013)
Blueberries	*T. minima*	Rust2inv/LR6	Mexico	Rebollar-Alviter et al. (2011)
Blueberries	*T. minima*	Rust2inv/LR6	China	Zheng et al. (2017)
Blueberries	*T. minima*	ITS/ITS1-F KYO2 / ITS4	Brazil	Pazdiora et al. (2018)
Group of crops	*N. vaccinii*	Rust2inv/LR6; Rust18SR/NS1	Not mentioned	Aime, Bell, and Wilson (2018)
Group of crops	*N. vaccinii*	LROR/LR6; NL1/NL4	Not mentioned	Maier, Begerow, Weiß, and Oberwinkler (2003)
Group of crops	*N. vaccinii*	Rust2inv/LR6; LROR/LR3; Rust18S-R/NS1	Not mentioned	Aime (2006)

In conclusion, following the morphology and genetic homology analysis, it is feasible to distinguish *N. vaccinii* and *T. minima*. Consequently, the blueberry rust species in Hastings should be identified from these experiments. One of the issues for preventing rust disease-resistant cultivar development can be solved after a clear understanding of the rust pathogens in Hastings.

1.2.14 Issues from current screening procedures on blueberry rust disease

As mentioned previously, the other issue restricting the breeding process on rust

resistance is the lack of efficient resistance screening procedures for identifying resistant germplasm sources. Both field assessments and inoculation tests were used on resistance screening on blueberry leaf rust. Several studies attempted to identify the resistance level by assessing different cultivar reactions to blueberry rust disease in the field. For instance, from the field observations, several NHB cultivars, namely 'Bluecrop', 'Burlington', 'Collins', 'Dixi', 'Earliblue', 'Gem', 'Ivanhoe', 'Olympia', 'Stanley', and 'Weymouth', showed resistance to blueberry rust disease (Heidenreich et al., n.d.; Ivey, 2016). From a field survey by Zheng et al. (2017), three SHB cultivars, namely 'Sharpblue', 'Misty', and 'Bluegold', were identified to be susceptible to blueberry rust. In fact, most of the rust disease resistance was identified in NHB cultivars. While in other types, only one SHB cultivar, 'C00-09', was identified with resistance to rust disease, and one RE cultivar, 'Powderblue', was found with moderate resistance (Table 1.5). Hence, with this information on resistance sources, it is insufficient to support the breeding of new SHB and RE cultivars.

In addition, inoculation test results could ideally be used as an indicator of plant disease severity in the field. This is the other option for identifying the resistance levels in different cultivars, even to find out any resistance sources. However, only two SHB accessions were identified with rust disease resistance, and eleven commercial cultivars ('Windsor', 'Pearl', 'Bobolink', 'Ventura', 'Suziblue', ''O'Neal', 'Springhigh', 'Snowchaser', 'Biloxi', 'Sharpblue' and 'Star') showed susceptibility in this inoculation test (Babiker et al., 2018). Moreover, from all these inoculation tests, only 'Biloxi', 'Mistry' and 'Sharpblue' showed a consistent result between the inoculation tests and field assessments, while ''O'Neal' demonstrated a controversial result from the inoculation test by Babiker et al. (2018) and Zheng et al. (2017). However, there are no further field assessment results to confirm the rest of the studied materials ('Windsor', 'Pearl', 'Bobolink', 'Ventura', 'Suziblue', 'Springhigh', 'Snowchaser', 'Star') (Table 1.5). By combining these inoculation results with field assessments results is possible, it could mean a better understanding of the cultivars' reactions to rust disease.

In conclusion, two aspects are important for establishing blueberry rust disease screening procedures for identifying resistant germplasm sources. In one aspect, field assessments on current blueberry cultivars, especially from SHB and RE, are essential. The reason for this is not only because few resistance sources are identified in these types, but also because these blueberries are important for New Zealand blueberry industry expansion, as previously mentioned. Understanding the rust resistance levels under field conditions can assist in selecting suitable cultivars for further commercial production in Hastings or in other areas of New Zealand. In the other aspect, the correlation between inoculation tests and field assessments needs to be figured out. If both results are highly correlated in these commercial cultivars, plant breeding materials could use inoculation results to predict disease severity under field conditions. The advantages of inoculation tests are that they are easier to manage in that they are conducted in an environmental control room with lower environmental variations, have fewer space restrictions for the test, and are less time-consuming. Therefore, it will be an efficient and economical way for disease severity identification. The feasibility of setting up a standard resistance screening procedure for blueberry rust will be discussed in the following sections.

1.2.15 Development of efficient resistance screening procedures for blueberry rust disease

1.2.15.1 Field assessment protocols

As mentioned above, more information is required on rust disease reactions, especially on SHB and RE cultivars. Fortunately, most of the popular cultivars from these two types are growing in selection field trials in Hastings. It is important to decide which approach should be applied to this field assessment. In general, disease incidence (DI) and disease severity (DS) are used for plant disease assessments (McRoberts, Hughes, & Madden, 2003; Seem, 1984). DI is a qualitative measurement, recording whether the plant is either with infection or without infection. It is a discrete (binary) variable and normally takes values of either 0 or 1. DS, on the other hand, is a

quantitative measurement, whereby data of proportion or percentage of the infected plant tissue is assessed. The severity scale is a continuous variable takes values between 0 and 1, inclusive for proportions or 0 to 100 for percentages. On blueberries, these approaches were used on the study of blueberry epidemiology of *Septoria* leaf spot disease (Ojiambo & Scherm, 2004). Moreover, DI and DS were previously applied in blueberry rust research. DI data were used to distinguish the difference between rust disease reactions from four SHB cultivars (Zheng et al., 2017). The percentage of the infected area on the leaf (PIAL) was used for rust DS on blueberries to explain the efficacy test of different fungicides on a blueberry cultivar, 'C99-42' (Ingram et al., 2016). Since both DI and DS were used in the previous studies of blueberry rust, these will be also applied in this research for describing the reaction from different blueberry cultivars to blueberry rust disease. In addition, this study will focus on assessing the rust disease resistance level for different cultivars of SHB and RE. Therefore, a total of 23 cultivars (15 SHB, five RE and three NHB) will be used for recording the data in the field during the harvest season of 2019. DI will be indicated by the percentage of infected leaves, and DS will be shown by the average lesion area (ALA) and the PIAL on the leaf.

1.2.15.2 Plant inoculation tests

Additionally, plant inoculation test, as another efficient and economical option, can be used to indicate the resistance level of different cultivars in a short time. According to previous studies, the inoculation test includes two kinds of approaches. One is to brush the fresh urediniospores on the leaf surface for a direct inoculation, such as the pathogenicity tests launched by Barrau, de los Santos, and Romero (2002), Zheng et al. (2017) and Rebollar-Alviter et al. (2011). While the other uses inoculum with a certain concentration (1×10^3 to 3.8×10^5) of urediniospores for a spray inoculation, such as the tests by Pazdiora et al. (2018), Keith et al. (2008), Dal Bello and Perelló (1998), Mostert et al. (2010) and Babiker et al. (2018). In fact, in these tests, controversial results were shown on the test of ''O'-Neal'. These contradictory results were from two different

inoculation approaches. When the urediniospores on the undersides of the leaves were brushed for the inoculation, ‘‘O’ Neal’ showed resistance to blueberry rust (Zheng et al., 2017). In contrast, using 3×10^5 spores/mL as a spray-based inoculum. ‘‘O’ Neal’ showed susceptibility to the disease (Babiker et al., 2018). Comparing these approaches, inoculation by inoculum has a controlled concentration and an even infection on the leaf, which gives a better effect for the infection process and improve the repeatability of the experiment.

1.2.15.3 Development of rating scale by using *Fiji* image analysis software

Field assessments and inoculation tests will be used to develop the resistance screening procedures for blueberry rust disease in this study. The percentage of infected leaves, PIAL and lesion numbers on the leaf represent DI and DS for the field assessments, respectively. Inoculum with urediniospores is used for the inoculation test. Nevertheless, the rating scale for these tests affect the accuracy of the results, directly. In previous studies on blueberry rust, a rating scale for field assessment and inoculation test was based on the observation by researchers, such as the tests from Zheng et al. (2017) and Babiker et al. (2018). The only one rating scale on blueberry leaf rust was developed by Babiker et al. (2018) for describing the rust symptoms on the leaf. These scale levels were set from 0 to 4: (0): When there was no sporulation on the leaf surface, it was considered as immune. (1): When necrotic flecks occurred without sporulation, it was considered as a resistant material. (2): when the symptom area was less than 50% of the leaf, these kinds of the materials were classified as susceptible. (3): when the infected area was between 50%-75% of leaf surface, these materials had higher susceptibility than the previous one. (4) in this final scale, materials were highly susceptible for rust infection with more than 75% leaf surface was infected and defoliation also were observed. As the DS was scored by observation, a personal error may occur, with inconsistent results gained by different observers for defining the PIAL on the leaf.

In order to reduce this impact, a standardized screening procedure is vital to be

developed, which can give a consistent result, even testing by different researchers. Fortunately, many tools and software have been developed for rust disease analysis. For example, fluorescence and spectroradiometers have been used for wheat rust early detection (Ashourloo, Mobasheri, & Huete, 2014; Huang et al., 2007). Additionally, a spectroradiometer was also used on early blueberry rust detection on 'Sharpblue' (Ahlawat, Jhorar, Kumar, & Backhouse, 2011). *ImageJ* is one of the most popular open-source software used on rust disease research. This software has been used to count the uredinia number on the photographed leaves for the wheat leaf rust inoculation test (Panwar, McCallum, & Bakkeren, 2013). In addition, *ImageJ* has been used to analyze the rust disease impact on the highbush cranberry. By calculating the size, density and coverage of telia on the photographed leaves, it figured out the correlation between quality and quantity for cranberry reduction and rust disease infection (Daust, 2013). Recently, this software was used for fungicide efficacy tests of blueberry rust disease. For instance, Simpson et al. (2017) used *Fiji*, a distribution of *ImageJ*, for analyzing the percentage of infected leaf areas on blueberry cultivar 'C99-42'. In a 12-week experiment period, 20 leaf samples were collected every 14 days and processed by this software. Based on the DS results among the fungicide treatments, fenbuconazole and tebuconazole had a better rust control effect than mancozeb under an evergreen production system at Wollongbar, New South Wales. In addition, from 2014 to 2016, there were other blueberry fungicide trials in Australia (Daniel, 2018). Using *ImageJ*, the total leaf area and the infected area on the blueberry cultivar 'C99-42' were calculated for DS analysis. Results showed that under severe disease pressure in 2015, azoxystrobin + cyproconazole, fenbuconazole and tebuconazole gave the greatest reduction in DS Through these fungicide tests, image-analysis software can be used for calculating the size and the number of the lesions, the total leaf area and the infected leaf area on the photographed leaves. It offers a convenient approach for the analysis of photos representing symptomatic leaves with lesions taken from the field and a consistent scoring procedure on the infected area measurements (Schindelin, Rueden, Hiner, & Eliceiri, 2015). Based on these advantages, *Fiji* is a consistently feasible approach for disease severity analysis in both field assessments and inoculation tests

for blueberry rust disease.

1.3 The aim of this study and research objectives

The growth of blueberry production in New Zealand has led to the increasing demand for new blueberry plantations. Due to the low-chilling requirement of SHB cultivars, and a long fruit development in RE varieties, these two types of blueberries are suitable to focus on a profitable market. SHB blueberries can have an early harvest, while RE blueberries can have a late harvest, so the market price in these periods is higher than the harvest peak season. However, blueberry rust is the impeding issue for using these cultivars in production. Yield losses are caused due to early or undesirable plant defoliation in summer and autumn on the infected plants, which reduces fruit productivity in the present season and flower bud formation for the coming season. Moreover, fruit can be infected by rust pathogen (Biosecurity Tasmania, 2017). These infected blueberries will cause quarantine issues during export. Therefore, blueberry rust control is necessary for New Zealand production. Instead of spraying fungicides for blueberry plants for rust control, resistant blueberry cultivars would be a better option for rust disease management. Unfortunately, there are no commercial cultivars with rust disease resistance at present, especially in these two types. One reason is insufficient knowledge about the rust pathogens in New Zealand. The other reason is the lack of standard and efficient screening procedures for identifying resistant sources.

Therefore, two aims are set out in this study. One is to identify rust species in the commercial plantations in Hastings, Hawke's Bay. Another is to establish a efficient screening procedure for identifying rust-resistance sources in the blueberry germplasm. To achieve these objectives, several feasible approaches are mentioned in the literature review. According to these approaches, the research questions are listed below:

- What is the causal organism of blueberry rust in Hastings? Is blueberry leaf rust in Hastings caused by *N. vaccinii*, *T. minima* or both?

- Are there any commercial cultivars that have resistance to blueberry leaf rust under field disease pressure?

- Is it possible to set up a disease rating scale for different blueberry cultivars based on photographed leaves using *Fiji* software?

- Is it possible to develop a standard inoculation protocol? Do these inoculation results have any correlation with field assessment results?

Chapter 2 Identification of rust species from Hastings blueberry production regions

2.1 Introduction and aims

Blueberry leaf rust was first found on highbush blueberries and RE in the Waikato region, in the Northern Island of New Zealand, in 2004 (Barlow, 2014; NZ Herald, 2004). In 2004 and 2005, eight rust specimens of *N. vaccinii* were recorded in the Landcare Research database, which were collected from the Waikato, Auckland and the Bay of Plenty (New Zealand Fungi, 2019a). In addition, between 2014 and 2015, three rust samples were collected from Mid- Canterbury, Auckland and the Bay of Plenty, and all of them were identified to be *T. minima* by Landcare Research (New Zealand Fungi, 2019b). Therefore, both species of blueberry rust have been identified in New Zealand.

In recent years, rust disease has become prevalent in the main blueberry production area, Gourmet Blueberries. Ltd, in Hastings (D. Hutchins, personal communication, 13th March 2018). However, the causal organism of rust in this production area has not yet been identified, which means that the blueberry rust could be caused either *N. vaccinii, T. minima* or both. As fresh blueberry product with *N. vaccinii* not being allowed to be exported to South Australia, Western Australia, Victoria and Tasmania, this ambiguous situation may affect blueberry export (Australian Government, 2018). Blueberry rust species identification is necessary for the blueberry industry in Hastings. If the blueberry rust is found to be *N. vaccinii*, certain treatment procedures need to be applied to meet the Australian import criterion in order to avoid the economic loss caused by quarantine issues.

In order to solve this issue, this study will make a clear identification of blueberry leaf rust in Hastings using morphology and genome sequence homology analysis. Therefore,

this chapter includes two experiments. One is the identification from morphological examination of urediniospore and uredinium structures. The other is from genome homology analysis in the ITS region.

2.2 Morphological identification

2.2.1 Materials

Diagnosed rust leaves, with the clear orange-color blueberry rust uredinia, such as the symptoms shown in Chapter 1 Figure 1.7, were randomly collected from each cultivar in two separate plantation net houses of Gourmet Blueberries. Ltd, Hastings, in June 2018 (Figure 2.1). In total, fresh symptomatic leaf samples were randomly picked from 8-10 different plants of seven different cultivars in three growing locations (Figure 2.1). During this sample collection, fresh yellow-orange colored pustules and urediniospores of ‘Centra Blue’ and ‘Rahi’ were found on the downside leaf surface, while ‘Ono’ and ‘Nui’ were found with light yellow to gray color urediniospores and only brown color pustules were seen on the downside leaf surface of ‘Snowchaser’, ‘Georgia Dawn’ and ‘Centurion’. These infected leaves were immediately sent to the Manawatu Microscopy and Imaging Centre (MMIC) for morphological identification by scanning electron microscopy (SEM) and light microscopy.

Figure 2.1 Collection sites of leaf samples with rust lesions at blueberry plantation net houses of Gourmet Blueberries. Ltd, Hastings, Hawkes Bay, New Zealand (source from Google Satellite Map). 1 = Growing location of SHB blueberry 'Snowchaser' and 'Georgia Dawn'; 2 = Growing location of RE blueberry 'Centra Blue', 'Centurion', 'Rahi', and 'Ono'; 3 = Growing location of NHB blueberry 'Nui'.

Table 2.1 The growing location of seven selected cultivars for diseased leaves for sample collection in Gourmet Blueberries. Ltd's production net houses.

Field No.	Blueberry Type	Blueberry Cultivar	Growing Location on Google Satellite Map
R4-24	Southern Highbush	Snowchaser	No.1
R4-41	Southern Highbush	Georgia Dawn	No.1
C2-52	Rabbiteye	Centra Blue	No.2
C3-47	Rabbiteye	Centurion	No.2
D2-3	Rabbiteye	Rahi	No.2
E2-23	Rabbiteye	Ono	No.2
F3-18	Northern Highbush	Nui	No.3

2.2.2 Methods

Leaf samples were processed by the MMIC for SEM and light microscopy. SEM was used to collect images of both the upper and lower leaf surfaces from all seven cultivar samples. Urediniospore size was calculated with minimum 8 individual spores from clear SEM images of each cultivar. Additionally, the structure of a uredinium on the leaf surface was examined by SEM. Light microscopy was used to get cross-section images of the uredinia structure. Uredinium size was calculated minimum of 3 individual uredinia cross-section images. The images and the size of the urediniospores and uredinia are shown in the results below.

2.2.3 Results

The rust urediniospores were detected on all the infected leaf samples picked from the seven cultivars. However, the color of these pustules was dissimilar. Fresh yellow-orange color pustules and urediniospores were found on 'Centra Blue' and 'Rahi', while 'Ono' and 'Nui' were found with light yellow to gray color urediniospores and only brown color pustules were seen on the downside leaf surface of 'Snowchaser', 'Georgia Dawn' and 'Centurion'. (Figure 2.2 A1-A7).

From the SEM observation, the average size of urediniospore from different cultivars varies from 10.29 μm to 15.52 μm in width and 13.91 μm to 17.07 μm in length. In total, the average size of these 78 urediniospores is 11.97 μm x 15.05 μm (Table 2.2). Urediniospores are an obovoid to elliptical in shape and the surface were finely verrucose in these different cultivars (Figure 2.2 B1-B7).

The rust uredinium was detected in all samples from light microscopy cross-section slices, while it was only found on 'Snowchaser', 'Georgia Dawn', 'Centra Blue', 'Rahi' and 'Nui' by SEM (Figure 2.2 C1-C7 and D1-D7). In these cases, more samples may need to be processed from lesion areas to find the uredinium under SEM.

Based on the images from light microscopy and SEM, uredinia are hypophyllous and mostly clustered with a dome shape and protrude from stomata (Figure 2.2 C1-C7). The peridia are clearly shown on the cross-sections from light microscopy images. They grow within the epidermal cells of the host and have a lack of conspicuous ostiolar cells (Figure 2.2 D1-D7). In a total of 36 uredinium examinations, the average diameter is 146.88 μm (Table 2.2), and the average diameter of uredinium in these cultivars varies from 82.95 μm to 218.4 μm in width and 47.95 μm to 84.28 μm in height. In all these samples, no telia or teliospores were detected.

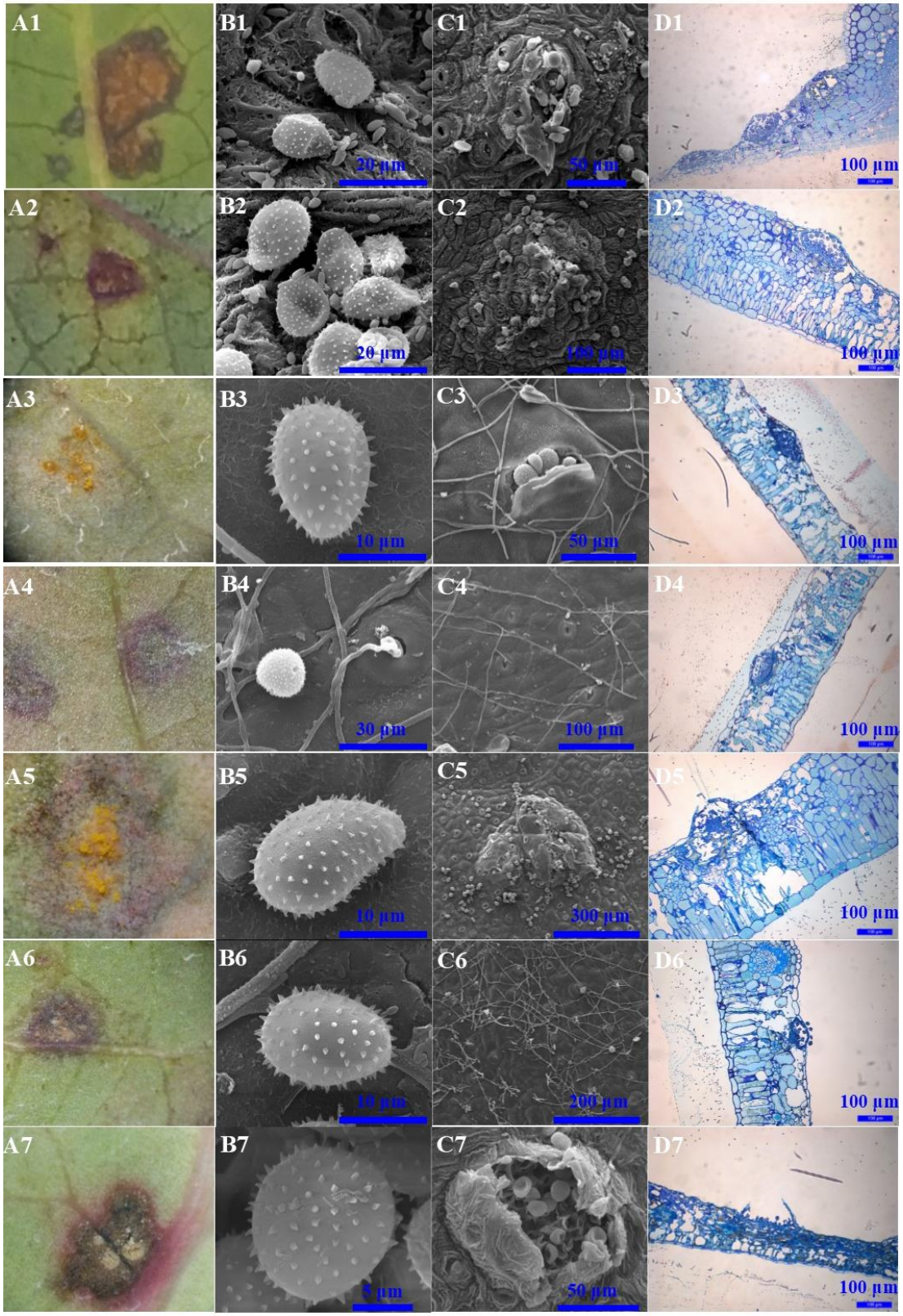


Figure 2.2 Images of rust pustules, urediniospores and uredinia on seven different cultivars, observed by scanning electron microscopy (SEM) and light microscopy. (A1-A7) Rust pustule on the abaxial leaf surface of seven blueberry cultivars; (B1-B7) SEM

images of urediniospore(s) from seven blueberry cultivars; (C1-C7) SEM images of uredinium from seven blueberry cultivars; (D1-D7) light microscopy images of uredinium cross-section from seven blueberry cultivars; (A1-D1) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of southern highbush (SHB) 'Snowchaser'; (A2-D2) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of SHB 'Georgia Dawn'; (A3-D3) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of rabbiteye (RE) 'Centra Blue'; (A4-D4) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of RE 'Centurion'; (A5-D5) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of RE 'Rahi'; (A6-D6) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of RE 'Ono'; (A7-D7) images of a symptom of the leaf lesion, SEM of urediniospore, SEM of uredinium and cross-section of light microscopy of northern highbush (NHB) 'Nui'.

Table 2.2 Average urediniospore and uredinium size on of seven blueberry cultivars.

Field No.	**Blueberry**		**Urediniospore Average Size (µm)**		**Uredinium Average Size (µm)**	
	Type	**Cultivar**	**Width**	**Length**	**Width**	**Height**
R4-24	SHB	Snowchaser	11.97	14.19	113	76.5
R4-41	SHB	Georgia Dawn	12.67	14.09	165	84.28
C2-52	RE	Centra Blue	10.29	16.17	178.44	76.02
C3-47	RE	Centurion	15.52	17.07	113.7	64.5
D2-3	RE	Rahi	11.19	16.37	218.4	83.475
E2-23	RE	Ono	10.62	14.43	82.95	47.95
F3-18	NHB	Nui	11.71	13.91	156.66	71.26
Average Size of All the Observations			11.97	15.05	146.88	72

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye.

2.3 Genome sequence homology analysis

2.3.1 Materials

Fresh urediniospores from symptomatic leaf samples of blueberry cultivar ‘Rahi’ were used for genome sequence analysis by PCR. These leaf samples were picked at location No.2 of the plantation net house at Gourmet Blueberries. Ltd in June 2018 (Figure 2.1).

2.3.2 Methods

2.3.2.1 DNA extraction

DNA was extracted from 100-200 fresh urediniospores from five different leaves which randomly picked in the field. These urediniospores were brushed from a single pustule (uredinium) from each leaf by pipette tip and put on a sterile glass slide. These spores were crushed between two glass slides. After that, these slides were checked by microscopy to confirm that the cell wall of urediniospores had been completely destroyed (Figure 2.3 B) before being suspended in a 20 µl extraction buffer. This buffer is comprised of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), and 0.01% Proteinase K. This suspension was incubated at 37°C for 60 minutes and then transferred to a 95°C hot bath for 10 minutes. At the end, these five DNA suspensions were stored at -20°C until required for use in PCR experiments (see below) (Liang, Tian, & Kakishima, 2006; Virtudazo, Nakamura, & Kakishima, 2001; Yang, Tian, Liang, & Kakishima, 2014; Yang, Tian, Lu, Liang, & Kakishima, 2015).

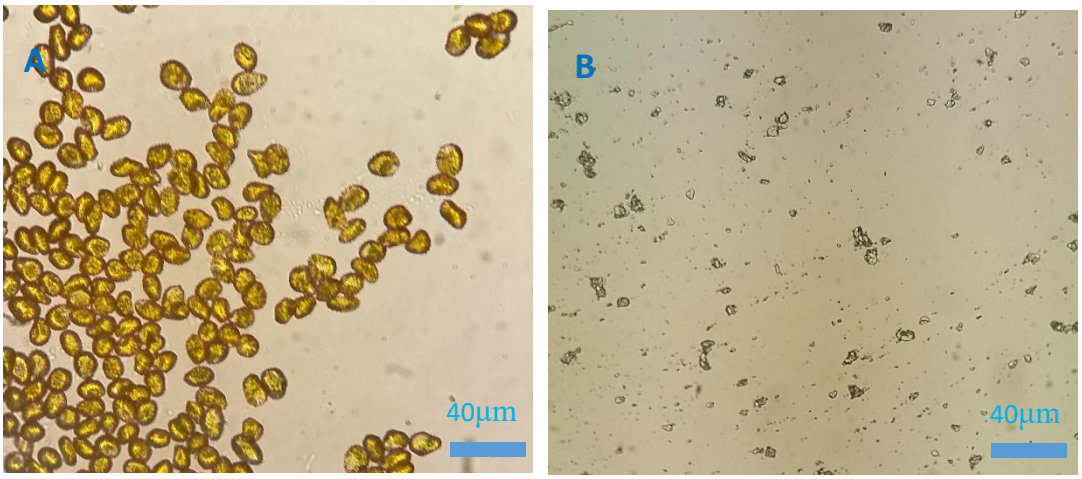


Figure 2.3 Urediniospores crushed for DNA extraction, as confirmed by light microscopy. (A) Before being crushed, urediniospores were visualized by light microscopy to possess a complete cell structure; (B) after being crushed, the cell wall of the urediniospores was observed to be completed destroyed.

2.3.2.2 PCR amplification and sequencing

PCR was performed in 20-μl reaction volumes with 10 μl PCR Master Mix (Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix), 1μl of each 10 μM primers (forward and reverse), and 3 μl (10- to 100-fold) DNA template from the DNA suspension. The ITS region was amplified by two pairs of primers (Table 2.3). One is the primer pair Rust2inv/LR6, which results in an amplicon of approximately 1400 bp and covers the 5.8S subunit, the ITS-2 region, and part of the large 28S subunit. The other primer pair is LROR/LR3, which results in an amplicon of around 600 bp inside the large 28S subunit. The amplification by both primer pairs was achieved with an initial denaturation step of 10s at 98℃; 30 cycles of 1s at 98℃; 5s at 55℃; 23s at 72℃, and a final extension of 1 minute at 72℃.

Table 2.3 Nucleotide sequence of four primers used for PCR amplification.

Primer Name	**Nucleotide Sequence**	**Reference**
LROR	5'-ACCCGCTGAACTTAAGC-3'	(Bunyard et al., 1994)
LR3	5'-GGTCCGTGTTTCAAGAC-3'	(Vilgalys & Hester, 1990)
Rust2inv	5'-GATGAAGAACACAGTGAAA-3'	(Aime, 2006)
LR6	5'-CGCCAGTTCTGCTTACC-3'	(Vilgalys & Hester, 1990)

Once the amplification was completed, 20 µl of each PCR sample was added to 4 µl of 6X loading dye and resolved by 1% Tris-acetate-ethylenediaminetetraacetic acid (TAE) agarose gel electrophoresis at 100 V for 35-40 minutes. After confirmation of the correct 1400 bp and 600 bp amplicons by agarose gel electrophoresis, the PCR products were excised from the gel and purified using an OMEGA E.Z.N.A. Gel Extraction Kit D2500-01. The clean PCR products were then sent for sequencing at the Massey Genome Service Sequencing was performed in both the forward and reverse directions using the Rust2inv and LR6 PCR primers (1400-bp amplicon), and the LROR and LR3 PCR primers (600-bp amplicon). Five resulting nucleotide sequences were aligned by Geneious v 9.0.5 (Kearse et al., 2012) to generate a single contiguous sequence and deposited in GenBank (GenBank Accession: MK604179).

2.3.2.3 Phylogenetic analyses

Based on ITS sequences, a phylogenetic tree was constructed using a neighbor joining tree method, with a 5% maximum sequence difference in Blast Tree View. The purpose of this tree was to determine whether the ITS nucleotide sequence from above is more similar to ITS nucleotide sequences from the rust species *T. minima* or *N. vaccinii*. Therefore, the phylogenetic tree was constructed with the rust ITS nucleotide sequence from above and all *T. minima* and *N. vaccinii* ITS nucleotide sequences present in the National Center for Biotechnology Information (NCBI) database.

2.3.3 Results

The alignment result shows a higher shared nucleotide identity and coverage to ITS

sequences from *T. minima* than from *N. vaccinii*. The detected rust sample in this study has a more than 99% of identity with several *T. minima* isolates, which were identified from blueberries in other countries. For example, the ITS sequence from 'PREM 60245', which was from a rust infecting *V. corymbosum* in South Africa (GenBank Accession: GU355675), has 100% of query coverage and 99.79% sequence identity to the ITS sequence obtained in this study (Figure 2.4). Notably, it contains three mismatched nucleotides in the alignment, which may be the result of a mutation, or could be from the matching allele in the dikaryotic urediniospore, which may differ slightly in sequence. In support of the latter, two possible nucleotides were identified in the sequenced ITS nucleotide sequence at positions 144 and 1244. In addition, 'LD 1081', which caused rust symptoms on *V. corymbosum* in Mexico (GenBank Accession: HM439777), has 97% of query coverage and 99.86% shared sequence identity; '001', which caused rust lesions on *V. corymbosum L.* x *V. darrowii* Camp in California (GenBank Accession: KY991374), has 96% of query coverage and 99.57% shared sequence identity; and *T. minima* environmental samples, which were collected from *V. corymbosum* leaves in Oregon (GenBank Accessions: KU136371, KU136372, and KU136373), has 96%, 96% and 95% of query coverage and 99.57%, 99.78% and 99.78% shared sequence identity, respectively. However, the identity of *N. vaccinii* was 99.18% in 67% of query coverage. Moreover, the phylogenetic tree shows that the rust sample collected in Hastings is more closely related to *T. minima* than *N. vaccinii* (Figure 2.5). Therefore, based on the alignment results and the phylogenetic tree structure, *T. minima* is the rust species that was found in the blueberry plantation of Gourmet Blueberries. Ltd, Hastings, Hawke's Bay.

```
Rahi   1    AGATTCAGTGATCATCGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCATGAAACCCTCTCA   90
60245  1    AGATTCAGTGATCATCGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCATGAAACCCTCTCA   90

Rahi   91   TCATAATTCTTTATTTAGTTAAGGAGTTGGTGATGGATGTTGAGTGTTGCYGTCATTGGCTCACTTTAAATACATAAGTACTTTTATTGA   180
60245  91   TCATAATTCTTTATTTAGTTAAGGAGTTGGTGATGGATGTTGAGTGTTGCCGTCATTGGCTCACTTTAAATACATAAGTACTTTTATTGA   180

Rahi   181  GGAAACAATATAAGAGTGGAGAAATACTTGGTGTGATATTTATTATTCATCAAGGAGTGTGGTGGGGTTTACCCTACTGCAGCCATTGTT   270
60245  181  GGAAACAATATAAGAGTGGAGAAATACTTGGTGTGATATTTATTATTCATCAAGGAGTGTGGTGGGGTTTACCCTACTGCAGCCATTGTT   270

Rahi   271  TATTGATTTTTTGATAAATAGCTTCCTAACCTTTAGAAAGTTGACCCTTTAGACCTCAAATCAGGTGGGACTACCCGCTGAACTTAAGCA   360
60245  271  TATTGATTTTTTGATAAATAGCTTCCTAACCTTTAGAAAGTTGACCCTTTAGACCTCAAATCAGGTGGGACTACCCGCTGAACTTAAGCA   360

Rahi   361  TATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACGGCGAGTGAAGAGGGAAAAGCCCAAATTTGTAATCTGGCTCTT   450
60245  361  TATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACGGCGAGTGAAGAGGGAAAAGCCCAAATTTGTAATCTGGCTCTT   450

Rahi   451  TTTATAAGGGTCCGAGTTGTAATTTTGAGAACTGTTTTCTGTGCTGGACCATGTACAAGTCTGTTGAAAAACAGCATCATTGAGGGTGAA   540
60245  451  TTTATAAGGGTCCGAGTTGTAATTTTGAGAACTGTTTTCTGTGCTGGACCATGTACAAGTCTGTTGAAAAACAGCATCATTGAGGGTGAA   540

Rahi   541  AATCCCGTACATGATATGGACTACCAGGGCAATATGATACAGTCTCTAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAA   630
60245  541  AATCCCGTACATGATATGGACTACCAGGGCAATATGATACAGTCTCTAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAA   630

Rahi   631  ATTCCATCTAAGGCTAAATATAGGTGAGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGATGAAAAGAACTTTGGAAAGAGAGTTAAC   720
60245  631  ATTCCATCTAAGGCTAAATATAGGTGAGAGACCGATAGCAAACAAGTACCGTGAGGGAAAGATGAAAAGAACTTTGGAAAGAGAGTTAAC   720

Rahi   721  AGTACGTGAAATTGCTAAAAGGGAAACAGTTAAAGTTAGACTTGTTATTGTTGGATCAGCTTCTTTATTGAGGTGTACTCTGATGATTAA   810
60245  721  AGTACGTGAAATTGCTAAAAGGGAAACAGTTAAAGTTAGACTTGTTATTGTTGGATCAGCTTCTTTATTGAGGTGTACTCTGATGATTAA   810

Rahi   811  CAGACCAGCATCAATTTTTGAGTGTTGGATAAGGGTTTTGGGAATGTGGCAGCTTTGGTTGTGTTATAGACCATTACTTTGATACAATGC   900
60245  811  CAGACCAGCATCAATTTTTGAGTGTTGGATAAGGGTTTTGGGAATGTGGCAGCTTTGGTTGTGTTATAGACCATTACTTTGATACAATGC   900

Rahi   901  TTAGGATTGAGGAACGCAGTAAGCTTTGTTGCAGATTTTTTCGGAAATCTCCTTGCTACGGATGTTGGTGTAATAGCTTTAAATGACCCG   990
60245  901  TTAGGATTGAGGAACGCAGTAAGCTTTGTTGCAGATTTTTTCGGAAATCTCCTTGCTACGGATGTTGGTGTAATAGCTTTAAATGACCCG   990

Rahi   991  TCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTATTTGGGTGTTGAAACCCTTATGCGTAATGAAAGTGAATGTAAATGGGAT   1080
60245  991  TCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTATTTGGGTGTTGAAACCCTTATGCGTAATGAAAGTGAATGTAAATGGGAT   1080

Rahi   1081 CTGTAAAAGGTGCACCATTGACCAGTCCAGATTATTTATATGATGGTACTGAGTAAGAGCAAGTATGTTGGGACCCGAAAGATGGTGAAC   1170
60245  1081 CTGTAAAAGGTGCACCATTGACCAGTCCAGATTATTTATATGATGGTACTGAGTAAGAGCAAGTATGTTGGGACCCGAAAGATGGTGAAC   1170

Rahi   1171 TATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAAGCTCGTAGCGGTTCTGACGTGCAAATCGATCGYTGAATTTGGGTATAGG   1260
60245  1171 TATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAAGCTCGTAGCGGTTCTGACGTGCAAATCGATCGTTGAATTTGGGTATAGG   1260

Rahi   1261 GGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAGACTCATGTCAGTTTTATGAGGTAAA   1350
60245  1261 GGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAGACTCATGTCAGTTTTATGAGGTAAA   1350

Rahi   1351 GCGAATGATTAGAGGCCTTGGGAATGTAACATTCTTAACCTATTCTCAAACTTTAAATATGTAAGACGTTCTTGTTTCTTAATTGAACT-   1439
60245  1351 GCGAATGATTAGAGGCCTTGGGAATGTAACATTCTTAACCTATTCTCAAACTTTAAATATGTAAGACGTTCTTGTTTCTTAATTGAACTT   1440

Rahi   1440 GGACATGCG  1448
60245  1441 GGACATGCG  1449
```

Figure 2.4 Alignment between the partial sequence of the 5.8S ribosomal RNA gene, the complete sequence of internal transcribed spacer 2, and the partial sequence of the 28S ribosomal RNA gene from *Thekopsora minima* isolate "PREM 60245" isolated from blueberry in South Africa (GenBank Accession: GU355675), and the same region sequenced from the rust pathogen isolated form blueberry cultivar 'Rahi' in the Hawke's Bay of New Zealand.

Three mismatched nucleotides in this alignment. Position 141: The nucleotide sequence from the rust on 'Rahi' shows a Y, while the nucleotide sequence from *T. minima* isolate "PREM 60245" shows a C, which means that the PCR amplicon had a

mixed product with both C and T at the same locus. This may be caused by a single locus mutation from the tested sample, or that the dikaryotic urediniospore maintains multiple alleles of this ITS sequence at this locus; position 1244: The nucleotide sequence from the rust on 'Rahi' shows a Y, while the nucleotide sequence from *T. minima* isolate "PREM 60245" shows a T, which means that the PCR amplicon had a mixed product with both C and T at the same locus. This may be caused by a single locus mutation from the tested sample, or that the dikaryotic urediniospore maintains multiple alleles of this ITS sequence at this locus; position 1439: There is a deletion of a T in the nucleotide sequence from the rust on 'Rahi'. This may be caused by a mutation from the tested sample.

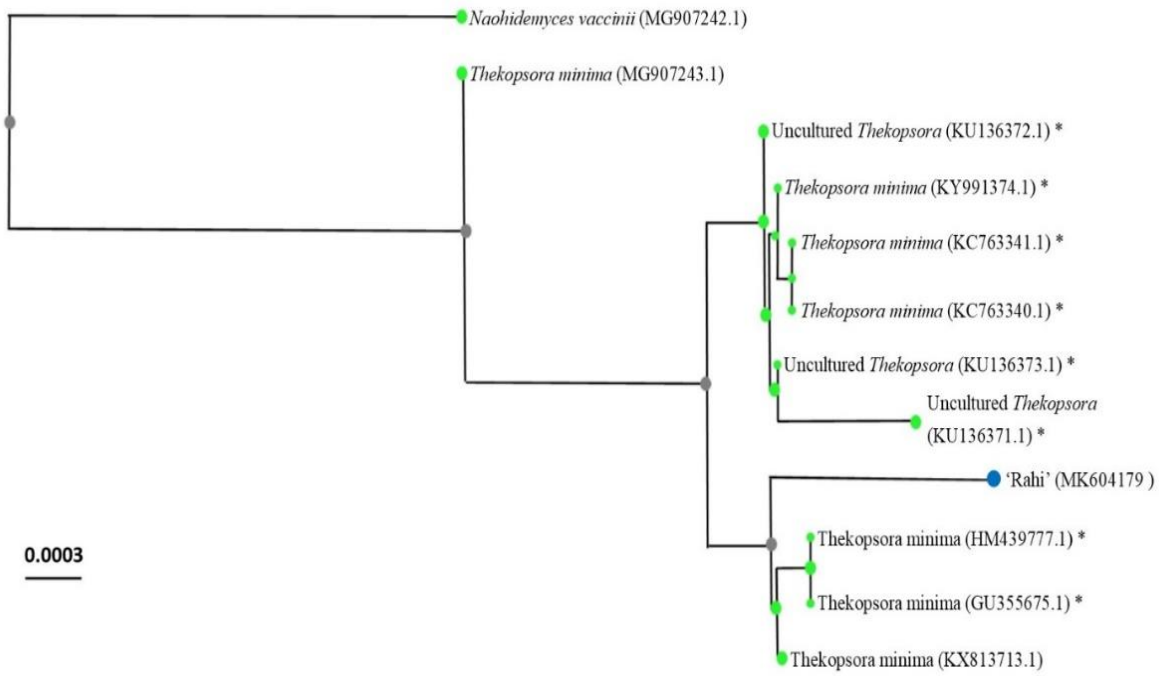


Figure 2.5 A phylogenetic tree was constructed using all the *T. minima* and *N. vaccinii* internal transcribed spacer (ITS) sequences from the NCBI database and the rust sample sequencing result was constructed using a neighbor joining tree method with a 5% maximum sequence difference. Green spot = rust fungi; blue spot = rust sample collected from symptomatic leaves of rabbiteye blueberry cultivar, 'Rahi', at Gourmet Blueberries. Ltd, Hastings, Hawke's Bay, in June 2018. * The plant hosts of *Thekopsora*

minima are blueberry (*Vaccinium* spp.) cultivars.

2.4 Discussion

In previous studies, both *N. vaccinii* (*P. vaccinii*) and *T. minima* have been identified as the blueberry rust causal organism in many countries. Based on the morphological characteristics of urediniospores and a pathogenicity test, *N. vaccinii* (*P. vaccinii*) was identified in Hawaii, Argentina and Spain (Barrau et al., 2002; Dal Bello & Perelló, 1998; Keith et al., 2008). Nevertheless, combining the results from the morphology and genome sequence homology of the ITS region, recent reports showed that blueberry rust was caused by *T. minima* in America (California, Oregon, Michigan), Brazil, Mexico, South Africa, Australia (Bundaberg, Queensland), China (Sichuan), and Europe (Belgium, Germany, Portugal and the Netherlands) (EPPO Global Database, 2019; Mostert et al., 2010; Pazdiora et al., 2018; Rebollar-Alviter et al., 2011; Schilder & Miles, 2011; Shands et al., 2018; Wiseman et al., 2016; Zheng et al., 2017). In New Zealand, the database of Landcare Research showed that in Auckland and the Bay of Plenty, *N. vaccinii* was detected from the samples collected in 2004 and 2005. Ten years later, only *T. minima* was detected in these regions (New Zealand Fungi, 2019a, 2019b). Correspondingly, based on the morphology and genome sequence homology analysis from two experiments in this study, the rust samples collected at Gourmet Blueberries. Ltd were confirmed to be *T. minima* rather than *N. vaccinii*.

Even though the color of the pustules was dissimilar, from fresh yellow-orange color to light yellow, gray and brown color, morphological results showed that *T. minima* was found on NHB, SHB and RE blueberries in Hastings blueberry plantations. Meanwhile, the genome sequence homology analysis also confirmed the rust pathogen from 'Rahi' was *T. minima*. Due to the specimen for genome sequence homology analysis was only collected from 'Rahi' cultivar, more specimens from other cultivars should be collected and identified to have a confirmed result of the prevalent rust pathogen species in this region. Overall, this identification is useful for producers to diagnose the disease in the

field and to control the disease in production.

Chapter 3 Rust disease field assessment and pathogenicity test

3.1 Introduction and aims

In New Zealand, blueberry leaf rust was first found on highbush blueberries and RE in the Waikato region in 2004 (Barlow, 2014; NZ Herald, 2004). In the same year, blueberry rust was also found in Auckland and the Bay of Plenty (New Zealand Fungi, 2019a). Ten years later, blueberry leaf rust was also identified in Mid-Canterbury in the South Island (New Zealand Fungi, 2019b). In recent years, blueberry leaf rust has become prevalent in Hastings, which has impeded blueberry production. However, only one SHB cultivar, 'C00-09', and two accessions were mentioned with rust disease resistance (Babiker et al., 2018; Gasic et al., 2016). In addition, only one RE blueberry cultivar, 'Powderblue', was found to have moderate resistance to blueberry rust by field assessment (Scherm & Krewer, 2008). For most of the SHB and RE commercial cultivars, the resistance level of blueberry leaf rust is unclear (Clark & Finn, 2006, 2010; Finn et al., 2012; Gasic et al., 2014; Gasic et al., 2018; Gasic et al., 2016). The insufficient information about rust disease on these cultivars results in difficulty in cultivars selection for production. Additionally, the lack of efficient screening procedures for rust disease resistance causes confusion when comparing the result from different researches. For example, ''O'Neal' showed susceptibility to blueberry rust, *T. minima*, from the inoculation test by Babiker et al. (2018), while Zheng et al. (2017) had a contradictory result from the inoculation test of *T. minima* on 2-year-old plants. Therefore, more information is needed on blueberry leaf rust on present cultivars, together with a standard procedure for blueberry leaf rust screening.

This study has two aims to solve the problems mentioned above. The first aim is to conduct a field assessment of commercial cultivars to establish a leaf rust disease scale for blueberries. Based on the correlation between the disease incidence (DI) and disease severity (DS) level detected among cultivars under field disease pressure, a rating scale for blueberry leaf rust can be developed. The second aim of this study is to investigate

an artificial inoculation test in a temperature-controlled room. An inoculation test can be an efficient and economical option for disease susceptibility/resistance identification. It is easier to manage with fewer environmental variations that need to be controlled, and it requires less space and is less time-consuming. Still, the correlation between the results from the inoculation test and level of disease under field disease pressure needs to be identified. In order to understand this, inoculation test data about disease incidence and disease severity for the same cultivar is used for a correlation analysis for a further model establishment. Based on this model, the rust disease level of different blueberry cultivars or breeding materials in the field can be predicted by the inoculation test result in the controlled room. Hence, this chapter includes two experiments. One is the field assessment of 23 different cultivars growing at Gourmet Blueberries. Ltd in Hastings, Hawke's Bay. The other experiment is the inoculation test on 'Sky Blue' in a temperature-controlled room.

Additionally, to reduce the error effect caused by the observers, an image-analyzed software, *Fiji*, will be used in this study. Photographed leaves will be used as data analysis objectives for field assessments and the inoculation tests. Using *Fiji* Software, the date of the lesions and their number and size, infected leaf area, the increase in lesion size and the percentage of infection leaf area will be calculated by processing these photographed leaves. After that, R Studio will be used for data statistical analyses, including an analysis of variance (ANOVA) test, Tukey Honest Significant Differences (TukeyHSD) analysis, a correlation analysis of disease incidence and disease severity on these 23 different cultivars from field assessment, and a correlation analysis between the inoculation test and field assessments.

3.2 Materials

3.2.1 Field assessment

A total of 23 cultivars were selected from one commercial plantation net house (approximately 333,122 m^2) of Gourmet Blueberries. Ltd (Figure 3.1). It includes five

RE cultivars (‘Rahi’, ‘Centra Blue’, ‘Centurion’, ‘Titan’ and ‘Sky Blue’) cultivated at location C, three NHB cultivars (‘Nui’, ‘Blue Moon’ and ‘ Duke’) grown at location B, and 15 SHB cultivars (‘Camellia’, ‘Palmetto’, ‘Misty’, “O’ Neal’, ‘Springhigh’, ‘Scintilla’, ‘Snowchaser’, ‘Miss Jackie’, ‘Miss Lily’, ‘Georgia Dawn’, ‘Southern Splendour’, ‘Suziblue’, ‘Kestrel’, ‘Flicker’ and ‘Sweetcrisp’) planted at location A (Table 3.1.). Ten one-year-old branches were chosen from ten different plants in the field, randomly in each block A, B and C. Two healthy leaves of each branch were labelled in the field and were used for rust symptom assessment records from January to March in the 2019 harvesting season.

Figure 3.1 Three growing locations (A, B and C) of selected cultivars for field assessment at Gourmet Blueberries. Ltd, Hasting, Hawke’s Bay. Image from Google Satellite. Blueberry cultivars were selected at three different growing blocks (A, B and C) in the blueberry production net house with the total area around 333,122 m^2; A =

Southern highbush growing location; B = Northern highbush growing location; C = Rabbiteye growing location.

Table 3.1 The growing location of 23 selected blueberry cultivars in the Gourmet Blueberries. Ltd's net house for the use in the field assessment from January to March 2019.

Field No.	Blueberry Cultivar	Blueberry Type	Sampling Block
M3-49	Rahi	RE	C
P3-02	Centra Blue	RE	C
P3-03	Centurion	RE	C
P3-52	Titan	RE	C
P3-53	Sky Blue	RE	C
Q3-54	Nui	NHB	B
Q4-42	Blue Moon	NHB	B
Q4-43	Duke	NHB	B
R2-03	Camellia	SHB	A
R2-04	Palmetto	SHB	A
R3-03	Misty	SHB	A
R3-04	O'Neal	SHB	A
R4-09	Springhigh	SHB	A
R4-23	Scintilla	SHB	A
R4-24	Snowchaser	SHB	A
R4-26	Miss Jackie	SHB	A
R4-40	Miss Lily	SHB	A
R4-41	Georgia Dawn	SHB	A
R4-44	Southern Splendour	SHB	A
R4-47	Suziblue	SHB	A
R4-50	Kestrel	SHB	A
R4-51	Flicker	SHB	A
R4-57	Sweetcrisp	SHB	A

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye.

3.2.2 Inoculation test

One-year-old detached branches from 'Sky Blue' were selected for the inoculation test. Five detached branches with 6-8 healthy leaves were picked for the inoculation test.

Three detached branches with 6-8 healthy leaves were selected as a control group. After the inoculation, these branches were observed for six week and images of each leaf was taken weekly.

3.3 Methods

3.3.1 Field assessment

A photo of the upper leaf surface for each labelled leaf was taken once per week for recording the rust symptom development. A ruler with the scale was horizontally put next to the leaf in each photo (Figure 3.2 A). This assessment was continually done for eight weeks during the harvest season from January to March of 2019. As Chapter 2 morphological pictures showed that the color of pustules is dissimilar. This can be caused by the different stage of the symptom development and the effect from different weather condition. Therefore, the lesion area calculation starts with the reddish pustule color as this is the main lesion color found during early autumn in Hastings.

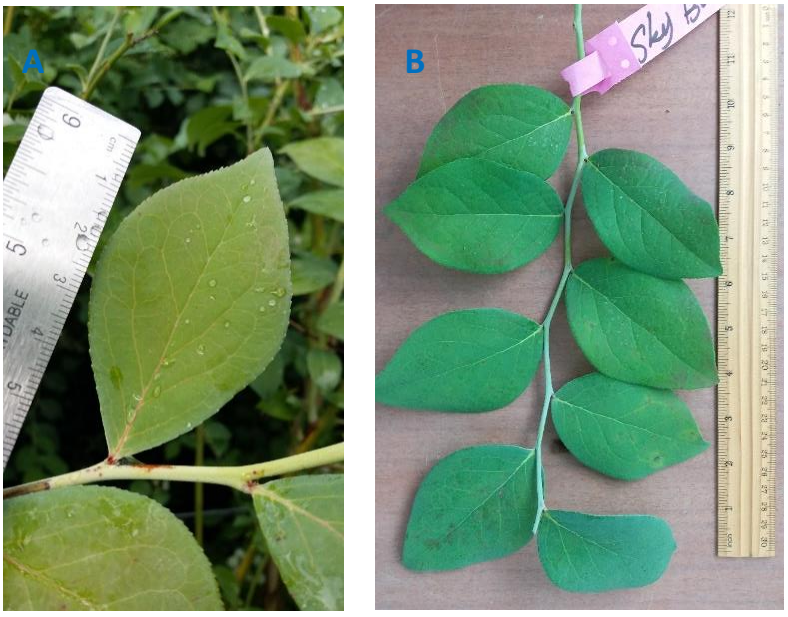


Figure 3.2 Photographed leaves of ‘Sky Blue’ blueberry cultivar for disease severity analysis in the field and in the temperature-controlled room. (A) The photo was taken of the upper side of the selected leaf at Gourmet Blueberries. Ltd as part of the field

assessment analysis during the 2019 harvesting season; (B) the photo was taken of the upper side of the selected leaves as part of the inoculation test in a temperature-controlled room at the Plant Growth Unit at Massey University from February to March in 2019.

3.3.2 Inoculation test

In a temperature-controlled room, an inoculation test was launched at the Plant Growth Unit (PGU) of Massey University. Using a total of eight detached branches (five branches used for the inoculation test and three branches used as a control), this test was completed in 35 days as follows:

1) Rust leaves with the typical orange pustules (rust symptoms) were picked from Gourmet Blueberries. Ltd, Hastings, one day before inoculation. These fresh leaves were kept in paper bags and stored at 4℃ overnight.

2) Fresh urediniospores were brushed off from these infected leaves with a camel's hairbrush and suspended in a 0.05% Tween 20 solution. The inoculum was then adjusted to 1.5×10^4 concentration by hemacytometer and prepared for the inoculation.

3) The inoculum was sprayed by using a hand-held sprayer on both sides of the fully expanded healthy leaves and the control branches were sprayed with 0.05% Tween 20 solution.

4) After spraying, these branches were grown in reverse osmosis water and maintained at 20-23°C in a plastic chamber in the temperature-controlled room. Relative humidity was kept at approximately 90% during the whole symptom observation period.

5) Each leaf was considered as one individual sample and a photo record was taken for each leaf once a week for the symptom development on the upper leaf surface. All the photographed leaves were measured with a ruler at the same level as an indicator scale (Figure 3.2 B).

3.3.3 Data analysis

All the images were collected from both the field assessment and inoculation tests. Appling the *ROI Manager* function in *Fiji* software, these images were then used for the calculation of lesion numbers, lesion area, and leaf area (Figure 3.3). The processed data were used for a DS analysis presented by the average lesion area (ALA) (cm^2) of each cultivar and the percentage of infected area on the leaf (PIAL) [PIAL = (ALA/average leaf area)$\times$100%]. In addition, DI (DI = number of infected leaves/total observed leaves) was also recorded from photographed leaves. Finally, R Studio was used to analyze these data in several aspects:

- ANOVA of the indicator of rust DI, ALA and PIAL variables was performed to test the effect of the different blueberry cultivars and cultivars in the same type.

- The significant effects of DI, ALA and PIAL from the field assessment were analyzed by multiple comparisons of means using TukeyHSD analyses.

- The relationship between DS and DI from the field assessment results by correlation analyses.

- The relationship between the DS and DI of the inoculation tests, and the field assessment of ‘Sky Blue’ by correlation analyses.

- A rust disease prediction model developed by using simple linear regression model analysis.

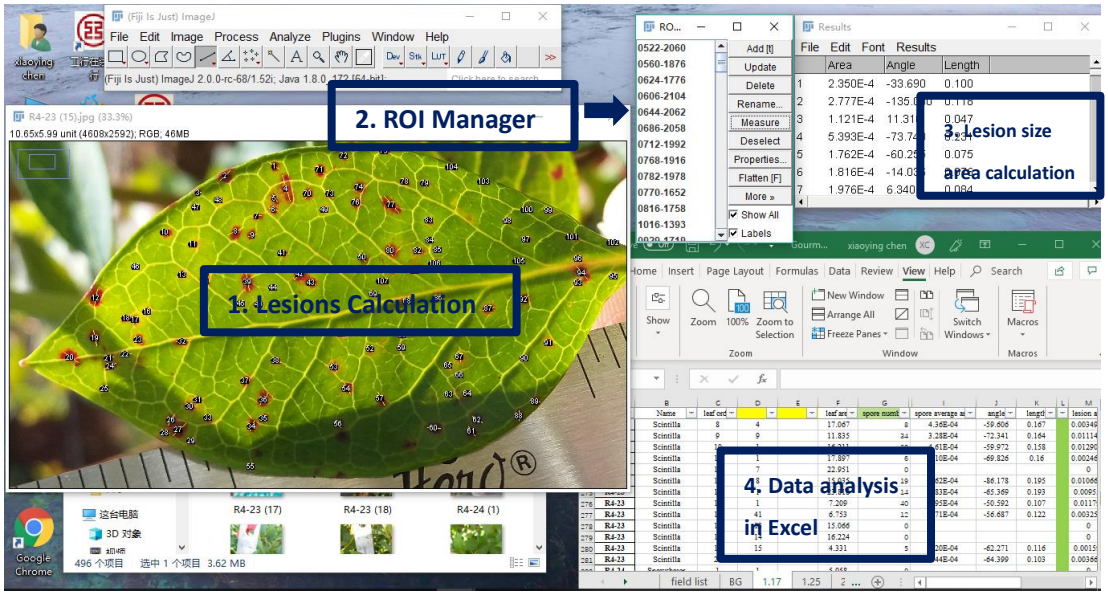


Figure 3.3 The *ROI Manager* function of *Fiji* software was used for lesion size area calculation and the data was inserted into MS Excel software for analysis.

3.4 Results

3.4.1 ANOVA results of DI and DS from the field assessments

In the eight weeks of field assessment from January to March in 2019, processed data of DI, ALA and the PIAL, as three variable factors, were for ANOVA tests. Significant differences were observed, based on the ANOVA test results (Table 3.2). More specifically, in DI, significantly different results ($p<0.05$) were observed among different observation date, different blueberry cultivars and different cultivars within the same type. In contrast, no difference was found among these three blueberry types ($p=0.879$). In the ALA, except for the observation date, significantly different results ($p<0.05$) were observed among these three blueberry types, different blueberry cultivars and different cultivars within the same blueberry type. In the PIAL, significant differences were found among all the blueberry cultivars and different cultivars in the same blueberry type ($p<0.05$), while no significantly different results were obtained among these three blueberry types ($p=0.0686$) and different observation date ($p=0.17$). From this ANOVA test, the significant differences in DI, ALA and the PIAL were

always observed among different blueberry cultivars and different cultivars within each type.

Table 3.2 P-value of ANOVA test results from DI, ALA and the PIAL variable factors from the field assessment data.

ANOVA Test Factor	DI	ALA	PIAL
Blueberry Types	0.879	0.0313	0.0686
All Blueberry Cultivars	<2E-16	<2E-16	<2E-16
Observation Date	2.19E-09	0.156	0.17
RE Cultivars	6.57E-08	2.11E-06	2.00E-07
NHB Cultivars	5.28E-09	1.23E-06	4.13E-07
SHB Cultivars	5.87E-12	<2E-16	<2E-16

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye; DI = Disease Incidence; ALA = Average Lesion Area; PIAL = The Percentage of the Infected Area on the Leaf. Green color: is p-value less than 0.05, which means there is a significant difference in these testing factors.

3.4.2 TukeyHSD analysis of the DI from the field assessments

According to the ANOVA test results on the DI, the TukeyHSD was used to analyze the difference among these three blueberry types, different cultivars from the same type and all blueberry cultivars, respectively (Table 3.3).

In different blueberry types, the TukeyHSD results show that the DI has no difference among RE, NHB and SHB (Table 3.3, TukeyHSD (1)), which consistent with ANOVA test results above (Table 3.2).

In RE, the DI on ‘Centra Blue’ is higher than other cultivars, while ‘Titan’ and ‘Sky Blue’ have a relatively lower DI on blueberry rust infection (Table 3.3, TukeyHSD (2)). In NHB, ‘Nui’ and ‘Duke’ have a lower blueberry rust DI than ‘Blue Moon’ (Table 3.3, TukeyHSD (2)). In these 15 SHB, the difference in DI is not easy to be separated by the TukeyHSD result. When the TukeyHSD results share with the same letter, there are

no significant difference between two cultivars. The TukeyHSD result of cultivars ‘Suziblue’ is ‘abcdef’, it means that there has no difference between ‘Suziblue’ and other SHB cultivars on DI. The results of DI value show that ‘Scintilla’ is the cultivar with a higher DI value in an earlier stage than other SHB cultivars, while ‘‘O’Neal’’ is the cultivar remains the lowest DI value in SHB type.

In all observed blueberry cultivars, rust disease was observed on ‘Centra Blue’ ‘Blue Moon’ and ‘Scintilla’ in the first observation, meanwhile, these cultivars maintain a faster infection than other blueberry cultivars (Table 3.3). In contrast, ‘‘O’Neal’’ shows a partial resistance to blueberry rust disease infection than others, which the increase of DI is slow, and the value of DI is also lower than others on the final observation (Table 3.3). Due to there is no significant difference between two cultivars, when their TukeyHSD results share with the same letter. Therefore, it is not easy to distinguish these cultivars into a separated reaction level on rust disease infection (Table 3.3, TukeyHSD (3)).

Table 3.3 Disease incidence (DI) (%) results of 23 blueberry cultivars under field assessment at Gourmet Blueberries. Ltd, Hastings, Hawke's Bay from January to March in 2019.

Blueberry		2019 Harvesting Season								TukeyHSD		
Cultivar	Type	17-Jan	25-Jan	1-Feb	8-Feb	15-Feb	22-Feb	1-Mar	10-Mar	(1)	(2)	(3)
Rahi	RE	0.600	0.600	0.667	0.688	0.778	0.688	0.688	0.722		b	abcde
Centra Blue	RE	0.900	0.900	1.000	0.950	1.000	0.947	0.947	1.000		a	a
Centurion	RE	0.300	0.100	0.350	0.400	0.556	0.778	0.833	0.900	a	bc	cdef
Titan	RE	0.050	0.150	0.389	0.444	0.316	0.474	0.632	0.650		c	def
Sky Blue	RE	0.300	0.350	0.300	0.300	0.350	0.400	0.400	0.500		c	ef
Nui	NHB	0.150	0.400	0.350	0.400	0.400	0.550	0.550	0.550		b	def
Blue Moon	NHB	0.850	0.950	0.850	1.000	1.000	1.000	1.000	1.000	a	a	a
Duke	NHB	0.050	0.050	0.400	0.300	0.350	0.400	0.450	0.500		b	ef
Camellia	SHB	0.200	0.200	0.350	0.350	0.450	0.500	0.350	0.500		cdef	ef
Palmetto	SHB	0.100	0.250	0.100	0.150	0.300	0.350	0.200	0.500		ef	f
Misty	SHB	0.400	0.200	0.300	0.150	0.300	0.500	0.550	0.500		cdef	ef
O'Neal	SHB	0.000	0.150	0.150	0.150	0.200	0.300	0.300	0.350		f	f
Springhigh	SHB	0.188	0.188	0.188	0.500	0.625	0.750	0.688	0.875		bcdef	cdef
Scintilla	SHB	0.700	0.750	0.833	1.000	1.000	1.000	1.000	1.000		a	ab
Snowchaser	SHB	0.050	0.150	0.250	0.150	0.350	0.450	0.550	0.786		def	ef
Miss Jackie	SHB	0.250	0.650	0.650	0.700	0.750	0.750	0.800	0.857	a	abcd	abcde
Miss Lily	SHB	0.050	0.250	0.650	0.700	0.800	0.850	0.800	0.900		abcde	abcde
Georgia Dawn	SHB	0.150	0.600	0.550	0.700	0.700	0.850	0.900	0.950		abcd	abcde
Southern Splendour	SHB	0.100	0.800	0.950	0.900	1.000	1.000	1.000	1.000		ab	abc
Suziblue	SHB	0.150	0.250	0.550	0.600	0.750	0.750	0.700	0.750		abcdef	bcdef
Kestrel	SHB	0.300	0.500	0.800	0.850	0.800	0.900	0.889	0.900		abc	abcd
Flicker	SHB	0.000	0.150	0.500	0.421	0.600	0.600	0.600	0.650		cdef	def
Sweetcrisp	SHB	0.300	0.850	0.900	0.850	0.950	0.950	0.950	0.950		ab	abc

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye. Green-Yellow-Red: Indicating the DI value increases from 0 to 100% which means no leaf was infected to all the leaves were infected. Green: 0-34%, which means no leaf was infected to 34% of the leaves were infected. Yellow: 35-68%, which means 35 to 68 percentage of the leaves were infected. Red: 69-100%, which means 69 to 100 percent of the leaves were infected. When the Tukey Honest Significant Differences (TukeyHSD) results share with the same letter, there are no significant difference between two cultivars. TukeyHSD (1) shows no differences among different blueberry types. TukeyHSD (2) shows differences among cultivars within the same type. TukeyHSD (3) shows differences among all 23 studied cultivars.

3.4.3 TukeyHSD analysis of the ALA from the field assessment

According to the ANOVA test results of the ALA, TukeyHSD was used to analyze the difference among different blueberry types, cultivars from the same type and all blueberry cultivars, respectively (Table 3.4).

The TukeyHSD analysis results show that the ALA is different among RE, NHB and SHB. NHB obtains a higher ALA value than RE, while SHB has similar ALA value between NHB and RE (Table 3.4, TukeyHSD (1)).
In RE, the ALA of 'Centra Blue' is higher than other cultivars (Table 3.4, TukeyHSD (2)). In NHB, 'Nui' and 'Duke' have a lower ALA value than 'Blue Moon' (Table 3.4, TukeyHSD (2)). In these 15 SHB, 'Scintilla' is the cultivar with the highest value of ALA than other SHB cultivars, following by 'Southern Splendour' (Table 3.4, TukeyHSD (2)).

When using the TukeyHSD analysis in all observed blueberry cultivars, 'Scintilla' has the highest ALA value, while ''O'Neal'' has the lowest ALA value (Table 3.4). Due to there is a significant difference between two cultivars when TukeyHSD results with no letter in common, these cultivars can be distinguished into separated reaction levels on rust disease infection (Table 3.4, TukeyHSD (3)).

A similar increasing pattern was found on 'Blue Moon', 'Scintilla', and 'Southern Splendour' between DI and the ALA (Table 3.3 and Table 3.4). The value of DI and the ALA are both increasing. However, this similar pattern doesn't show on 'Centra Blue'. Even though there more leaves are infected by blueberry leaf rust, the value changing of ALA is slow.

Table 3.4 Average lesion area (ALA) (cm^2), multiplied by 100,000 times, of 23 blueberry cultivars under field assessment at Gourmet Blueberries. Ltd, Hastings, Hawke's Bay from January to March in 2019.

Blueberry		2019 Harvesting Season								TukeyHSD		
Cultivar	Type	17-Jan	25-Jan	1-Feb	8-Feb	15-Feb	22-Feb	1-Mar	10-Mar	(1)	(2)	(3)
Rahi	RE	42.90	81.38	106.97	46.70	80.14	85.36	101.08	112.33		b	cd
Centra Blue	RE	145.00	280.97	112.59	101.12	131.76	127.54	79.56	212.61		a	cd
Centurion	RE	11.66	5.38	13.64	9.33	24.00	41.03	96.55	137.76	b	b	cd
Titan	RE	2.41	15.98	21.82	24.39	20.53	27.15	39.75	58.15		b	cd
Sky Blue	RE	53.98	60.11	38.99	40.34	27.53	44.62	42.83	43.85		b	cd
Nui	NHB	16.33	56.50	114.28	151.20	199.07	242.47	236.50	272.34		b	cd
Blue Moon	NHB	470.76	551.12	319.04	451.79	395.17	754.87	806.99	1100.78	a	a	b
Duke	NHB	6.69	6.69	17.09	22.03	18.94	36.73	52.30	100.03		b	cd
Camellia	SHB	17.87	33.63	25.14	25.83	27.87	36.30	28.60	60.76		c	cd
Palmetto	SHB	4.16	8.82	1.70	3.38	4.40	9.02	5.60	31.10		c	d
Misty	SHB	23.45	19.97	18.03	18.21	23.60	36.69	30.48	40.79		c	cd
O'Neal	SHB	0.00	6.74	5.51	4.73	8.13	10.52	10.26	15.85		c	d
Springhigh	SHB	8.88	6.71	7.14	20.83	23.99	49.50	70.60	128.49		c	cd
Scintilla	SHB	410.92	724.91	1109.36	1264.83	1270.62	1355.86	1506.07	1644.18		a	a
Snowchaser	SHB	2.90	5.29	4.02	2.76	12.76	20.53	21.02	140.60		c	cd
Miss Jackie	SHB	180.82	138.36	76.26	80.54	87.76	77.27	107.35	139.01	ab	c	cd
Miss Lily	SHB	1.25	14.19	32.60	33.08	38.98	134.50	162.84	314.41		c	cd
Georgia Dawn	SHB	11.31	45.18	54.86	55.29	76.91	172.33	180.51	145.14		c	cd
Southern Splendour	SHB	3.99	355.97	955.38	954.94	821.48	881.21	912.01	1562.83		b	b
Suziblue	SHB	3.90	17.58	31.34	32.06	50.20	62.13	71.00	86.08		c	cd
Kestrel	SHB	13.15	30.99	61.09	59.81	99.59	193.48	347.84	370.20		c	cd
Flicker	SHB	0.00	13.04	35.43	19.08	38.19	49.19	73.74	101.89		c	cd
Sweetcrisp	SHB	12.00	94.01	172.43	260.29	277.58	483.67	391.19	812.24		c	c

SHB = Southern Highbush; NHB = Highbush; RE = Rabbiteye. Green-Yellow-Red: Indicating the value of ALA increases from 0 to the highest value (1644.18 cm^2). Green: is about 0 - 45 cm^2 of infected area; Yellow: is about 46 - 400 cm^2 of infected area; Red: is about 400 – 1644.18 cm^2 infected area. When the Tukey Honest Significant Differences (TukeyHSD) results share with the same letter, there are no significant difference between two cultivars. TukeyHSD (1) shows differences among different blueberry types. TukeyHSD (2) shows differences among cultivars within the same type. TukeyHSD (3) shows differences among all 23 studied cultivars.

3.4.4 TukeyHSD analysis of the PIAL from the field assessment

According to the ANOVA test results of the PIAL, TukeyHSD was used to analyze the difference among different blueberry types, cultivars from the same type and all blueberry cultivars, respectively (Table 3.5).

From the TukeyHSD analysis, the values of PIAL show no difference among RE, NHB and SHB (Table 3.5, TukeyHSD (1)). This is consistent with the ANOVA test above (Table 3.2).

In RE, the PIAL of ‘Centra Blue’ is higher than others (Table 3.5, TukeyHSD (2)). In NHB, ‘Nui’ and ‘Duke’ have a lower value of the PIAL than ‘Blue Moon’ (Table 3.5, TukeyHSD (2)). In these 15 SHB, ‘Scintilla’ is the cultivar with the highest value of the PIAL than other SHB cultivars (Table 3.5, TukeyHSD (2)).

When using TukeyHSD analysis in all observed blueberry cultivars, the PIAL of rust disease on ‘Scintilla’ has a higher value than other cultivars, following by ‘Blue Moon’ and ‘Southern Splendour’ (Table 3.5). In contrast, ‘‘O’Neal’’ has the lowest value of the PIAL than other cultivars (Table 3.5).

Due to there is no significant difference between two cultivars, when their TukeyHSD results share with the same letter. Therefore, these cultivars can be distinguished into separated reaction levels on rust disease infection (Table 3.5, TukeyHSD (3)). A similar increasing pattern was found on ‘Blue Moon’, ‘Scintilla’, and ‘Southern Splendour’ in DI, the ALA and the PIAL (Table 3.3, Table 3.4 and Table 3.5). The value of DI, ALA and PIAL are all increasing during the observation period. However, this similar pattern doesn’t show on ‘Centra Blue’. Even though there more leaves were infected by blueberry leaf rust, the value changing on ALA and PIAL is slow.

Table 3.5 The percentage of the infected area on the leaf (PIAL), multiplied by 100,000 times, of 23 blueberry cultivars under field assessment at Gourmet Blueberries. Ltd, Hastings, Hawke's Bay from January to March in 2019.

Blueberry		2019 Harvesting Season								TukeyHSD		
Cultivar	Type	17-Jan	25-Jan	1-Feb	8-Feb	15-Feb	22-Feb	1-Mar	10-Mar	(1)	(2)	(3)
Rahi	RE	3.16	5.99	7.87	3.44	5.90	6.28	7.44	8.27		b	cd
Centra Blue	RE	12.76	24.72	9.91	8.90	11.59	11.22	7.00	18.71		a	cd
Centurion	RE	0.81	0.37	0.94	0.64	1.66	2.83	6.67	9.51	a	b	cd
Titan	RE	0.17	1.15	1.57	1.76	1.48	1.96	2.87	4.19		b	cd
Sky Blue	RE	3.87	4.31	2.80	2.89	1.97	3.20	3.07	3.14		b	cd
Nui	NHB	0.68	2.37	4.79	6.34	8.35	10.17	9.92	11.42		b	cd
Blue Moon	NHB	32.08	37.56	21.74	30.79	26.93	51.44	54.99	75.01	a	a	b
Duke	NHB	0.52	0.52	1.32	1.70	1.46	2.84	4.04	7.73		b	cd
Camellia	SHB	1.55	2.92	2.18	2.24	2.42	3.15	2.49	5.28		c	cd
Palmetto	SHB	0.38	0.80	0.15	0.31	0.40	0.82	0.51	2.82		c	cd
Misty	SHB	2.76	2.35	2.12	2.14	2.78	4.32	3.58	4.80		c	cd
O'Neal	SHB	0.00	0.48	0.40	0.34	0.58	0.75	0.74	1.14		c	d
Springhigh	SHB	0.74	0.56	0.60	1.74	2.01	4.15	5.91	10.76		c	cd
Scintilla	SHB	29.36	51.80	79.26	90.37	90.79	96.88	107.61	117.48		a	a
Snowchaser	SHB	0.28	0.51	0.39	0.27	1.23	1.97	2.02	13.51		c	cd
Miss Jackie	SHB	11.94	9.14	5.04	5.32	5.80	5.10	7.09	9.18	a	c	cd
Miss Lily	SHB	0.06	0.65	1.49	1.51	1.78	6.13	7.42	14.33		c	cd
Georgia Dawn	SHB	0.68	2.70	3.27	3.30	4.59	10.29	10.77	8.66		c	cd
Southern Splendour	SHB	0.25	22.37	60.04	60.01	51.62	55.38	57.31	98.21		b	b
Suziblue	SHB	0.29	1.31	2.33	2.39	3.74	4.63	5.29	6.41		c	cd
Kestrel	SHB	1.14	2.69	5.29	5.18	8.63	16.77	30.14	32.08		c	cd
Flicker	SHB	0.00	0.93	2.53	1.36	2.72	3.51	5.26	7.27		c	cd
Sweetcrisp	SHB	0.77	6.05	11.10	16.76	17.87	31.14	25.18	52.29		c	c

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye. Green-Yellow-Red: Indicating the value of the PIAL increases from 0 to the highest value (117.48). Green: is the PIAL value between 0 - 2.92; Yellow: is the PIAL value between 3.0 - 30; Red: is the PIAL value between 31 - 117.48. When the Tukey Honest Significant Differences (TukeyHSD) results share with the same letter, there are no significant difference between two cultivars. TukeyHSD (1) shows no differences among different blueberry types. TukeyHSD (2) shows differences among cultivars within the same type. TukeyHSD (3) shows differences among all 23 studied cultivars.

3.4.5 Correlation analyses of the field assessment results

Analyzing the data set from all the cultivars and each blueberry type representing RE, NHB and SHB, the correlation between the variable factors of DI, the ALA and the

PIAL are showed into four blocks (Table 3.6). The correlation is relatively low between DI and other factors (ALA and the PIAL), when analyzes the data set from all the cultivars (Table 3.6, Block A). In RE, NHB and SHB, the correlations between DI and other factors (ALA and the PIAL) vary from 0.6 to 0.86, which show relatively higher correlations.

However, a strong correlation is found between the ALA and the PIAL, which the correlation value is over 0.99 (Table 3.6, Block A). Additionally, this strong correlation between the ALA and the PIAL is also found in RE, NHB and SHB, which the correlation values are all more than 0.99 (Table 3.6, Block B, C and D). As PIAL = (ALA / average leaf area)×100%., the average leaf area should have effects on the PIAL's results. The ANOVA test also shows that the average leaf area has significant difference among different blueberry types ($p<4.01e-06$) and different blueberry cultivars ($p<2e-16$). However, in this study, the ALA and the PIAL always maintain a strong correlation without the effect caused by the different leaf size from different blueberry types and blueberry cultivars.

Table 3.6 Analysis of the correlation between every two factors of disease incidence (DI), average lesion area (ALA) and the percentage of infected area on the leaf (PIAL), using the data from all cultivars and from each type.

A			
Factor	**DI**	**ALA**	**PIAL**
DI	1.0000	-	-
ALA	0.5928	1.0000	-
PIAL	0.5956	0.9952	1.0000

B			
Factor	**DI**	**ALA**	**PIAL**
DI	1.0000	-	-
ALA	0.7712	1.0000	-
PIAL	0.7607	0.9932	1.0000

C			
Factor	**DI**	**ALA**	**PIAL**
DI	1.0000	-	-
ALA	0.8593	1.0000	-
PIAL	0.8602	0.9925	1.0000

D			
Factor	**DI**	**ALA**	**PIAL**
DI	1.0000	-	-
ALA	0.6144	1.0000	-
PIAL	0.6054	0.9962	1.0000

DI = Disease Incidence; ALA = Average Lesion Area; PIAL = The Percentage of the

Infected Area on the Leaf. When the correlation is 0, this means there is no correlation between two variable factors, and 1 means that there is a strong positive correlation between two factors. Block A: correlation analysis from the all cultivar data sets; block B: correlation analysis from the rabbiteye data sets; block C: correlation analysis from the northern highbush data sets; Block D: correlation analysis from the southern highbush data sets.

3.4.6 Rating scale of rust disease infection from these 23 observed blueberry cultivars

In this study, DI is indicated by the percentage of the infected leaves and DS is shown by the ALA and the PIAL. DI, the PIAL and the ALA indicate different aspects of rust disease development on blueberry cultivars. Most of the cultivars have correlation between DI and DS, while 'Centra Blue' shows irrelative pattern between DI and DS (Table 3.7). The increase in infected leaves number of 'Centra Blue' is faster than the growth in lesion numbers and sizes (Table 3.4, Table 3.5 and Table 3.6).

In order to have a distinguish level on rust disease infection among these 23 blueberry cultivars, the TukeyHSD results are combined among DI, the PIAL and the ALA. As there is no significant difference when the TukeyHSD results share with the same letter, the TukeyHSD result of DI has a difficulty for separating cultivars into different groups, as most of them share the same letters. In the TukeyHSD results of ALA and PIAL, when the cultivars share with same letters, they are grouped into a same infection level of blueberry rust disease. The grouping result is similar between ALA and the PIAL, as there is a strong correlation between the ALA and the PIAL (Table 3.6 Block A). At present, the PIAL is one of common indexes for disease assessment on blueberry leaf rust, therefore, PIAL is also used for rating scale development in this study.

Based on the TukeyHSD result of the PIAL, four levels of disease infection were differentiated in this study. When the TukeyHSD result is 'a', it equals to a highly

susceptible (HS) group. When the TukeyHSD result is ‘b’, it equals to a moderately susceptible (MS) group. When the TukeyHSD result is ‘c’ and ‘cd’, it equals to a susceptible (S) group. When the TukeyHSD result is ‘d’, it equals to a partially resistant (PR) group.

Therefore, ‘Scintilla’ belongs to the HS group; second, ‘Blue Moon’ and ‘Southern Splendour’ belong to the MS group; third, nineteen blueberry cultivars, made up of ‘Rahi’, ‘Centra Blue’, ‘Centurion’, ‘Titan’, ‘Sky Blue’, ‘Nui’, ‘Duke’, ‘Camellia’, ‘Misty’, ‘Springhigh’, ‘Snowchaser’, ‘Miss Jackie’, ‘Miss Lily’, ‘Georgia Dawn’, ‘Suziblue’, ‘Kestrel’, ‘Flicker’ ‘Sweetcrisp’ and ‘Palmetto’ are in the S group, and finally, ‘‘O’ Neal’ belongs to PR group (Table 3.7).

Table 3.7 Rust disease infection level on 23 blueberry cultivars deduced from combining the TukeyHSD results of disease incidence (DI), average lesion area (ALA) and the percentage of infected area on the leaf (PIAL) on 10th March 2019.

Blueberry		DI	Tukey HSD	PIAL	Tukey HSD	Rust Disease Infection Level*	ALA (cm^2)	Tukey HSD	Rust Disease Infection Level*
Cutlivar	Type								
Rahi	RE	0.722	abcde	0.000083	cd	S	0.001123	cd	S
Centra Blue	RE	1.000	a	0.000187	cd	S	0.002126	cd	S
Centurion	RE	0.900	cdef	0.000095	cd	S	0.001378	cd	S
Titan	RE	0.650	def	0.000042	cd	S	0.000582	cd	S
Sky Blue	RE	0.500	ef	0.000031	cd	S	0.000438	cd	S
Nui	NHB	0.550	def	0.000114	cd	S	0.002723	cd	S
Blue Moon	NHB	1.000	a	0.000750	b	MS	0.011008	b	MS
Duke	NHB	0.500	ef	0.000077	cd	S	0.001000	cd	S
Camellia	SHB	0.500	ef	0.000053	cd	S	0.000608	cd	S
Palmetto	SHB	0.500	f	0.000028	cd	S	0.000311	d	PR
Misty	SHB	0.500	ef	0.000048	cd	S	0.000408	cd	S
O'Neal	SHB	0.350	f	0.000011	d	T	0.000159	d	PR
Springhigh	SHB	0.875	cdef	0.000108	cd	S	0.001285	cd	S
Scintilla	SHB	1.000	ab	0.001175	a	HS	0.016442	a	HS
Snowchaser	SHB	0.786	ef	0.000135	cd	S	0.001406	cd	S
Miss Jackie	SHB	0.857	abcde	0.000092	cd	S	0.001390	cd	S
Miss Lily	SHB	0.900	abcde	0.000143	cd	S	0.003144	cd	S
Georgia Dawn	SHB	0.950	abcde	0.000087	cd	S	0.001451	cd	S
Southern Splendour	SHB	1.000	abc	0.000982	b	MS	0.015628	b	MS
Suziblue	SHB	0.750	bcdef	0.000064	cd	S	0.000861	cd	S
Kestrel	SHB	0.900	abcd	0.000321	cd	S	0.003702	cd	S
Flicker	SHB	0.650	def	0.000073	cd	S	0.001019	cd	S
Sweetcrisp	SHB	0.950	abc	0.000523	c	S	0.008122	c	S

SHB = Southern Highbush; NHB = Northern Highbush; RE = Rabbiteye; DI = Disease

Incidence; ALA = Average Lesion Area; PIAL = The Percentage of the Infected Area on the Leaf. Green –Yellow- Red: The value descriptions of DI, the ALA and the PIAL are as same as the descriptions on Table 3.3, Table 3.4 and Table 3.5 for DI, ALA and the PIAL, respectively. When the Tukey Honest Significant Differences (TukeyHSD) results share with the same letter, there are no significant difference between two cultivars. The rust disease infection level* describes each blueberry cultivar infection level of rust disease according to the TukeyHSD results. HS: high susceptiblity, MS: moderate susceptiblity, S: susceptiblity, PR: partial resistance.

3.4.7 Correlation analyses between the field assessment results and the inoculation tests on 'Sky Blue' blueberry cultivar

In this study, the results analysis excluded the data from control branches, as no disease lesions were observed on their leaves. The inoculation on the 'Sky Blue' was completed in the temperature-controlled room and the observation for the symptom development was last for six weeks, while the field assessment continued for eight weeks (Table 3.8 and Table 3.9). DI, ALA and the PIAL from the temperature-controlled room inoculation tests have a faster development, compared to the field assessment (Table 3.8 and Table 3.9).

Table 3.8 Disease incidence (DI), average lesion area (ALA) and the percentage of infected area on the leaf (PIAL) of the inoculation test on the 'Sky Blue' detached branches in a temperature-controlled room at Plant Growth Unit of Massey University in 2019.

Blueberry Cultivar	Inoculation Data Types	2019					
		5-Feb	12-Feb	17-Feb	26-Feb	5-Mar	12-Mar
Sky Blue	DI	0	0.200	0.733	0.833	0.867	1
	ALA	0	0.000263	0.002518	0.004252	0.004136	0.004302
	PIAL	0	0.00000663	0.00006345	0.0001072	0.0001042	0.0001084

DI = Disease Incidence; ALA = Average Lesion Area; PIAL = The Percentage of the Infected Area on the Leaf.

Table 3.9 Disease incidence (DI), average lesion area (ALA) and the percentage of infected area on the leaf (PIAL) ‘Sky Blue’ field assessment at Gourmet Blueberries. Ltd, Hastings, Hawke’s Bay in 2019.

Blueberry Cultivar	Field Data Types	2019							
		17-Jan	25-Jan	1-Feb	8-Feb	15-Feb	22-Feb	1-Mar	10-Mar
Sky Blue	DI	0.3	0.350	0.300	0.300	0.350	0.400	0.400	0.500
	ALA	0.0005398	0.000601	0.000390	0.000403	0.000275	0.000446	0.000428	0.000438
	PIAL	3.87E-05	4.30984E-05	2.7956E-05	2.892E-05	1.974E-05	3.199E-05	3.07109E-05	3.14361E-05

DI = Disease Incidence; ALA = Average Lesion Area; PIAL = The Percentage of the Infected Area on the Leaf.

In order to determine the correlation between the field assessment and the inoculation test on ‘Sky Blue’, these analyses were completed by the R Studio correlation coefficient analysis. The DI indicates the percentage of infected leaves. In the inoculation test, 100% of DI was received at the end of the experiment due to all the leaves were inoculated by the blueberry leaf rust inoculum. Hence, data from DI is excluded from the correlation coefficient analysis, while data from ALA, the PIAL and the increase in lesion area ratio (ILA = [(present ALA – the first ALA) / the first ALA)]) ×100%) was used for correlation coefficient analysis.

In the ALA, no strong correlation is found between the inoculation test and the field assessment. Likewise, there is no strong correlation between the ALA from the inoculation test and the PIAL from the field assessment. Additionally, in the PIAL, no strong correlation is found between the inoculation test and the field assessment. Interestingly, there is always a strong correlation between the ILA from the inoculation test and other two factors (ALA and the PIAL) from the field assessment. Both of them have a strong positive correlation value over 0.99 (Table 3.10). Based on this strong correlation, two prediction equations were developed by a simple linear regression model.

Table 3.10 Correlation analyses among average lesion area (ALA), the percentage of infected area on the leaf (PIAL) and the increase in lesion area ratio (ILA) of the field assessment results and the inoculation test on the 'Sky Blue'.

Factors	ALA Inoculation	ALA Field	PIAL Inoculation	PIAL Field	ILA Inoculation
ALA Inoculation	1	-	-	-	-
ALA Field	0.3341	1	-	-	-
PIAL Inoculation	1.0000	0.3341	1	-	-
PIAL Field	0.3355	1.0000	0.3355	1	-
ILA Inoculation	1.0000	0.9980	1.0000	0.9981	1

ALA = Average Lesion Area; PIAL = The Percentage of the Infected Area on the Leaf; ILA = The Increase in Lesion Area Ratio. Green: Indication of the correlation value is higher than 0.9, which means there is a strong correlation between the two factors.

3.4.8 Rust disease prediction models

As mentioned above, there were two strong correlations between the inoculation test and the field assessment results. One is between the ILA from the inoculation test and the ALA from the field assessment, the other is between the ILA from the inoculation test and the PIAL from the field assessment. Therefore, based on these strong correlations, two rust disease prediction models were developed. Using ILA from the inoculation test as a predictor, the value of ALA and the PIAL under field conditions can be predicted. Two equations were developed by a simple linear regression model:

- ALA Field = (6.159e-05) + (2.491e-05) × ILA of Inoculation (Adjusted R-squared: 0.9939, p-value: 0.002045)

- PIAL Field = (4.437e-06) + 1.786e-06 × ILA of Inoculation (Adjusted R-squared: 0.9942, p-value: 0.001941)

These two models show correlations between the predictor and response factor ($p<0.05$). Based on these equations, the value of ALA and the PIAL in the field can be predicted

by the ILA results from the inoculation test. In addition, both equations cover over 99% of the possibilities in the prediction. Since the PIAL is one of common indexes for disease assessment on blueberry leaf rust, and it is used for rating scale development in this study, the equation of the PIAL is the recommended one to be used on predicting the disease infection level on blueberry leaf rust. In future studies, through the ILA result from an inoculation test on the blueberry materials, which are unknown about the rust disease infection level, the PIAL value in the field may be predicted by this equation. Hence, based on this PIAL result, a TukeyHSD analysis may be used for grouping the unknown materials, such as blueberry breeding selections and new blueberry cultivars, into different disease infection levels on blueberry leaf rust. These results could assist in the blueberry breeding program and the cultivar selection for commercial production in New Zealand.

3.5 Discussion

No cultivar was found to be immune to blueberry rust disease under field pressure during the harvest season in 2019. In addition, more than 70% of the blueberry cultivars were shown to be seriously infected by rust disease during this observation period, with a DI value over 0.5. This result demonstrated the prevalence of blueberry leaf rust in the Hastings production region, and that the cultivars used for commercial production do not have resistance to this disease.

Additionally, it did have differences on blueberry leaf rust infection among different cultivars, as there are significant differences in DI, ALA and the PIAL when analyzing the data sets from the all blueberry cultivars, and the cultivars from each type. These differences may be caused by the genetic variation from different blueberry cultivars. Therefore, these data were used for further analysis on grouping the cultivars into different disease infection levels by the TukeyHSD results on DI, ALA and the PIAL.

This is the first study to use statistical analysis results in the grouping of different

blueberry cultivars into different rust disease infection levels. In the TukeyHSD result for DI, ALA and the PIAL, both ALA and the PIAL can give a relatively clearer distinguish about rust disease infection levels, when comparing the results from DI. However, the PIAL is one of the common indexes on blueberry rust disease assessment, which reduce the effect from the leaf size variation among different cultivars. Therefore, based on the results of the PIAL, these 23 cultivars were divided into four rust disease infection levels in this study. As there was a lack of resistant material in this study, the criteria to describe these infection levels are highly susceptible (HS)， moderately susceptible (MS), susceptible (S), and partially resistant (PR). In these 23 cultivars, ‘‘O’ Neal’ is partially resistant to rust disease infection, while ‘Scintilla’ shows highly susceptible to blueberry rust disease infection with a most rapid symptom development in the observation period.

In this study, the distinctive infection level on blueberry leaf rust developed by the TukeyHSD result of the PIAL is inconsistent with the field assessment results from previous study. In the study of Zheng et al. (2017), ‘‘O’ Neal’ was defined as a resistant SHB cultivar with 0% of the DI from a random field survey and no rust symptom was found in the inoculation test. However, the DI of ‘‘O’ Neal’ was 35% in this study and it was grouped as a partially resistant cultivar by the TukeyHSD results of the PIAL. In addition, the DI of ‘Mistry’ from his study was 5.1% while it was 50% in this study. The difference between these results may be caused by the different rust disease pressure in the field or the blueberry leaf rust may be caused by a different physiologic race from China and New Zealand.

Additionally, inconsistent result also found in the inoculation test from Babiker et al. (2018), which ‘‘O’ Neal’ was grouped as a susceptible cultivar with a leaf rust rating score of 3.4 ± 0.52, which means the brown spots and sporulating lesions were detected in more than 50% and less than 75% of the leaf surface. In our field assessment, the PIAL result of ‘‘O’ Neal’ was less than 1%. Moreover, ‘Suziblue’, ‘Springhigh’, ‘Snowchaser’ were defined to be susceptible SHB, and all of them with a leaf rust rating

score of 4 in the inoculation test. When the score was 4, the lesions were detected more than 75% of the leaf surface and defoliation were observed. Even though these three cultivars were also grouped as susceptible blueberry cultivars in this study, the PIAL is far more less than 1%. Although using the same index for describing the infection level of blueberry leaf rust, the value is too different to be compared between the study of Babiker et al. (2018) and this study. These inconsistent results may be caused by the lack of the resistant materials which the rating scale is incomplete in this study. In addition, it also can be caused by the difference on the rating scales between the field assessment and the inoculation test. This study found that DI developed faster in the inoculation test, and ALA and PIAL were also higher in the inoculation test. Therefore, even recording the same index for rust disease assessment, the rating scale should be different between the inoculation test and the field assessment. If the data of ILA is available from the study of Babiker et al. (2018), it can be used for the PIAL calculation by the prediction equation from this study. These inoculation results from this previous study may become available for comparison with the field assessment results in this study.

There nineteen cultivars were first time to be identified with a rust disease infection level under field assessment. These are made up of three NHB blueberry cultivars [‘Nui’ (S), ‘Blue Moon’ (MS) and ‘Duke’ (S)], five RE cultivars [‘Rahi’ (S), ‘Centra Blue’ (S), ‘Centurion’ (S), ‘Titan’ (S) and ‘Sky Blue’ (S)] and ten SHB cultivars [‘Scintilla’ (HS), ‘Southern Splendour’ (MS) ‘Camellia’ (S), ‘Miss Jackie’ (S), ‘Miss Lily’ (S), ‘Georgia Dawn’ (S), ‘Kestrel’ (S), ‘Flicker’ (S) and ‘Sweetcrisp’ (S) and ‘Palmetto’ (PR)]. Due to no cultivar tested in this study appears to be immune to blueberry rust, future work needs to test more cultivars, germplasm resources and breeding materials to establish a full scale from resistant to susceptible to blueberry rust disease.

In addition, this study establishes a correlation between the inoculation test result and the field assessment. A strong correlation (>0.99) was found between the ILA of the inoculation test and the PIAL in the field. Based on this high correlation value, a

prediction model was generated by a simple linear regression model which can use inoculation data to predict the cultivar infection level by blueberry leaf rust in the field. This model may offer an efficient way for preliminary evaluation of blueberry rust disease resistance based on the inoculation test results.

However, there may have limitations when using this model for predicting the rust disease infection level on other blueberries. In this study, 'Sky Blue' was the only one cultivar that was used for the inoculation test. The model was developed based on this inoculation test result with a limited data, which was from five observations. Therefore, this model may not suitable to have a prediction on other blueberry cultivars. Hence, in order to establish a better prediction model, it is necessary to have more cultivars, germplasm resources and breeding materials for the inoculation test and the field assessments.

In this study, *Fiji*, an open-source platform for biological-image software was used to establish a repeatable screening scale for blueberry rust disease. Using *Fiji,* photographed leaves can be processed under the same procedures, and the ALA was calculated which became a quantitative index for DS. This is different from the rating scale set by Babiker et al. (2018), which is based on the observation of the percentage of the infected area on the leaf. Five scales (0-4) were set for describing the DS on the infected leaves, from no symptom (0) to the leaves that were highly susceptible to the rust infection with more than 75% of infected leaf area (4). Even though the results of the rust disease infection level from this study are inconsistent with the previous ones, the photographed leaves processing procedure can be repeated by *Fiji* for DS analysis on different blueberries from different studies. Then, these DS data can be used for a comparison. In addition, using *Fiji* for a DS analysis can reduce variance during the rating score by different observers. Hence, *Fiji* is a feasible tool to use on DS analysis for blueberry rust.

There are some issues need to be concerned when using *Fiji* for the future researches.

First, the image quality affects the *Fiji*'s processing results. As blueberry leaf has a wax layer on the surface, light reflection causes overexposure on the leaf, especially on a sunny day. This effect was obvious between the first observation on 17th January and the second observation on 25th January. The first observation was taken during a cloudy day, while the second was taken during a sunny day. It can be seen that both the ALA and the PIAL have decreased between these two observations (Table 3.4 & Table 3.5). Hence, it is better to have a shade when taking the photo in the field under sunshine. Second, there was a similar effect caused by the dew when taking the photo in the morning. Finally, the photographing angle also influences the calculation. In this study, the DS is recording on the same leaf. Even though the camera is relatively parallel when taking the pictures, the difference in the photographing angle still exists between two observations by a hand-held camera. This difference can affect the calculation of the leaf area and the lesion area, especially for those cultivars with a slow disease development. For example, the decrease on the ALA of 'Sky Blue' on 15th February is caused by these two factors. It is better to avoid these drawbacks when using *Fiji* for the DS analysis on blueberry leaf rust disease.

Although no resistant cultivar was identified in this study, this information remains useful for growers when they select cultivars for blueberry production. Additionally, this study demonstrated the feasibility of using image software, *Fiji*, for a quantitative measurement of DS and using statistical analysis results on grouping different blueberry cultivars into different rust disease infection levels. In order to have a better understanding of the cultivars infection level by blueberry rust, to establish a full screening scale from susceptible to resistant, and to build up a better equation for predicting the field assessment by the inoculation test result, it is necessary to have more cultivars, germplasm resources and breeding materials for both the inoculation test and the field assessments in future work.

Chapter 4 Discussion

Global climate change causes effects on both plant hosts and the rust fungal pathogen, especially in natural plant-rust systems. In the geographical aspect, when blueberries are grown in a new region, the plant-rust system will change to adapt to the new environmental conditions. In blueberries, current cultivars can grow in originally colder regions (Lobos & Hancock, 2015). Meanwhile, due to the breeding efforts of developing low-chilling SHB cultivars in past decades, there are available cultivars with 0 to1000 chilling hours that have been used for expanding the plantations into warmer regions (Clark & Finn, 2006, 2010; Finn et al., 2012; Gasic et al., 2014; Gasic et al., 2018; Gasic et al., 2016; Okie, 2002, 2004). On the blueberry rust pathogens, the expansion of blueberry plantations may mean a higher frequency of overlap with the secondary host, hemlock pine's (*Tsuga* spp.), growing regions. This will result in the possibility of the completion of the blueberry rust heteroecious macrocyclic life cycle (Lobos & Hancock, 2015). When this life cycle is completed, a new rust genotype may be produced in the sexual stage, which may develop a new pathotype. A new plant- rust system may need to rebuild under this circumstance. In addition, the influence of temperature and the composition of atmosphere from global climate change also affects the plant-rust system. Blueberries may grow under the stress of heat and drought or in cold and wet environments, and may have damage caused by the increase of UV light radiation when the ozone layer has being destroyed by the chlorofluorocarbons (Lobos & Hancock, 2015). However, the environmental tolerance for rusts as a group in the face of global climate change is large. They can thrive in high-humidity environments and they can also survive in desert habitats (Helfer, 2014). Considering the effects caused by global climate change, blueberry breeding for rust disease resistance is vital for the blueberry industry, globally.

New Zealand, one of several blueberry production countries, is also influenced by this climate change. Extreme weather has already become more frequent, as seen for the

2017/18 summer, which resulted in the warmest year on record in New Zealand (Gattey, 2018). As a result of a changing climate, the blueberry production area has expanded in New Zealand in the past decade (Horticulture New Zealand & Plant & Food Research, 2010, 2018). New orchard farms were set up in Northland, which is more north of the old production area in Auckland (D. Hutchins, personal communication, 13th March 2018). In this region, SHB cultivars are the main blueberry type selected for the early harvest production. Due to the mild winter in this region, SHB cultivars can maintain their leaves throughout the year without defoliation. Using these SHB cultivars for blueberry production, blueberry leaf rust can be easier maintained a lot on the plant and cause a rapid infection under suitable temperature, which can reduce the production. Fungicidal control may be an option for blueberry rust disease control. However, only one fungicide was registered in New Zealand for blueberry rust control. Additionally, no SHB cultivar has been identified with resistance to blueberry leaf rust in this study. Hence, blueberry, especially SHB, breeding with natural resistance to rust disease is necessary for blueberry production in New Zealand.

To develop a SHB cultivar with rust disease resistance, several factors are important for this process. It includes the understanding of rust pathogen, the identification of resistant germplasm sources, and the developing of efficient and repeatable rust disease screening procedures. In this study, through morphological characteristics and genetic sequence analysis on the ITS region, *T. minima* was identified to be the causal organism of blueberry rust disease on blueberry in Hastings region. This is the preliminary result for knowing this rust pathogen on blueberry. However, to have a better understanding of blueberry rust disease, two aspects need to be considered for the further research.

The first aspect is that more work needs to be done on this pathogen. Even though this study has identified the blueberry rust from ‘Rahi’ is caused by *T. minima* in the Hastings region, this result is limited to this cultivar in this region. More rust samples from other cultivars should be identified to confirm the blueberry rust species in this region, which can support the blueberry breeding of rust disease resistance. In addition,

more specimens need to be collected and detected from other blueberry plantations, including the old productions regions of the Waikato (Ngatea and Ohaupo), the Bay of Plenty, Waihopo and Hastings in the North Island, and Otautau in the South Island (Blueberries New Zealand, n.d.-a; Horticulture New Zealand & Plant & Food Research, 2017). Additionally, new plantations in Northland and south of Christchurch also need to be sampled for the rust specimens (D. Hutchins, personal communication, 13th March 2018). It is essential to clarify the rust species in New Zealand before launching into further research on this pathogen. As *N. vaccinii* was identified as the causal organism of blueberry rust in previous rust specimens collected from the Waikato, Auckland and the Bay of Plenty, and new rust spores may have been introduced from Australia by wind currents, like the rust species *Phragmidium violaceum* on blackberry (McKenzie, 1998; New Zealand Fungi, 2019a). Regular field survey is necessary as it may has more than one rust species exist in New Zealand.

After the rust species is confirmed, further research can study the physiological race structure of *T. minima* on blueberry. In this study, a pathogenicity test was only used to confirm the species identity, as well as the symptom development on 'Sky Blue' (i.e. as a DS analysis under environmentally controlled conditions). As using the mixed urediniospores collected from the plantation were inoculated on one blueberry cultivar, it is impossible to detect any physiologic race of *T. minima* in this test. Even though the study from Babiker et al. (2018) showed that there was no host specificity as there no difference was found in pathogenicity or virulence of *T. minima* isolates from either of *V. pallidum* or *V. corymbosum.* However, comparing the field assessment result between Zheng et al. (2017) and this study, ''O' Neal' and 'Mistry' show differences on DI which may be caused by a different physiologic race from blueberry leaf rust, different environmental conditions and plant conditions. Hence, further research needs to detect more specimens of blueberry rust in blueberry production regions in New Zealand and in other countries. Meanwhile, a host panel for screening different physiologic races of *T. minima* needs to be established as well.

Figuring out the physiological race structure of the blueberry rust pathogen is important for understanding the blueberry-rust interaction to assist disease resistance breeding. Plant resistance can be in the form of either vertical or horizontal resistance. A vertical resistance is based on gene-for-gene interactions between resistance genes of the host and the avirulence genes of the pathogen. This system was identified using flax and the flax rust (*melampsora lini*) by Flor (1956). In this type of qualitative resistance, it may be overcome by the evolution of new virulent races of the pathogen (i.e. through deletion or modification of the matching avirulence genes), such as when stem rust disease-resistant cultivars became ineffective against the new race Ug99 (Singh et al., 2011). A horizontal resistance is a quantitative, non-race-specific slow resistance. A group of resistance genes provide a certain level of protection over the growing period, which shows durable resistance to the rust infection (R. Johnson, 1984). Even though the infection is not completely stopped, the spread of the disease is delayed until the adult plant stage (McIntosh, Wellings, & Park, 1995). Based on these two kinds of resistant reactions, the selection approach may vary. As the resistance response of gene-for-gene reactions would be faster, and because these reactions have a clearly distinguishable scale for resistance (as opposed to non-race-specific reactions), an inoculation test may be suitable to identify vertical resistance, while a field assessment may be suitable to identify horizontal resistance. Interestingly, different interactions between blueberry and rust were found in this study. The HS blueberry cultivars, like 'Scintilla', 'Blue Moon' and 'Southern Splendour', showed relatively consistent development among DI, ALA and the PIAL. When DI increases, both ALA and the PIAL increase. While in 'Centra Blue', a MS blueberry cultivar, the increase in DI does not cause a corresponding increase in ALA and the PIAL. The infected leaves number increases in 'Centra Blue' while the lesion area grows relatively slow. This may be caused by different interaction system between blueberry and rust. To understand these differences, it needs to find out the resistant source, test more cultivars, germplasm resources and breeding materials under field disease pressure or by inoculation tests. Then the resistance mechanism against blueberry rust disease on blueberry can be studied.

Inoculation tests have several advantages for disease resistance research, such as ease of testing in an environmentally controlled room with fewer environmental variations, space restrictions occupations, and time-constraints. Thus, the inoculation tests has become an efficient and economical way for disease severity identification. Nowadays, inoculation tests haves been used as common approaches on wheat rust disease research. These inoculation tests were used for virulence races identification, and genetic analysis of rust resistance at both seedling and adult plant stages (Hovmøller, Rodriguez-Algaba, Thach, & Sørensen, 2017; Lan, Randhawa, Huerta-Espino, & Singh, 2017). However, to complete an inoculation test, it is important to have a stable supply of urediniospores and reliable test procedures for the screening test on blueberries.

Blueberry rust is regarded as an obligate parasite. There are no studies about artificial media cultivation for *T. minima.* Blueberry rust inoculation tests are commonly carried out with fresh urediniospores collected from host plants (Babiker et al., 2018; Zheng et al., 2017). However, some studies showed that rust fungi can be produced by an axenic culture, like apple rust, *Gymnosporangium juniperi-virginianae* Schw., and wheat rust, *Puccinia graminis* f. sp. *tritici* (Hahn, 2000; Staples, 2000). Hence, there may be possibilities to develop suitable artificial media for the culturing of *T. minima*. In addition, a stable method for urediniospore production in a greenhouse would also be another option, which requires suitable cultivars and growing conditions for both host plants and pathogens.

In addition, it is essential to develop a reliable inoculation approach for screening blueberry leaf rust on different materials. In this study, only one concentration of inoculum was used on blueberry detached branches for the inoculation test in a temperature-controlled room. In previous studies, the concentration of inoculum varied from 1×10^3 to 3.8×10^5 spores/ml (Keith et al., 2008; Mostert et al., 2010; Pazdiora et al., 2018). In some studies, fresh urediniospores were brushed from the symptomatic leaves for direct inoculation (Rebollar-Alviter et al., 2011; Zheng et al., 2017). In this study, using 1.5×10^4 spores/ml inoculum, rust symptoms appeared on ‘Sky Blue’ in two

weeks and using liquid inoculum can have produced a more even infection on the leaf. In previous research, young plants were commonly used for pathogenicity tests on blueberry rust disease (Babiker et al., 2018; Zheng et al., 2017). Even though detached leaves were used for other rust disease inoculation tests, such as the inoculation test of wheat yellow rust by Hubbard, Pritchard, and Holdgate (2016) and crown rust by Paczos-Grzęda, Sowa, Boczkowska, and Langdon (2019), in our preliminary test, blueberry detached branches were easier to maintain in order to have the leaves in a healthy condition for at least 45 days by reverse osmosis water (data not shown). Considering less place is occupied in the limited size of a temperature-controlled room, and detached branches are easier to collect from one plant, detached branches were selected as the plant material for inoculation. However, the difference between inoculation on the plant and detached branches was not examined, the variance in different concentration inoculums was also not been studied, and the disease infected reactions on other blueberry cultivars were also undetected in this study. All these factors are important for a stable inoculation approach development and a better prediction equation establishment, which are worth clarifying when time permits.

Moreover, the disease screening scale influences the results. In blueberry rust, observation by the researchers is the main approach for disease screening in the inoculation test and the field assessment. This may cause personal error and various standards. Therefore, it is difficult to compare the result between different experiments, even if the test is on a same cultivar. In order to reduce these effects, *Fiji* was used for processing the photographed leaves, and the ALA and the PIAL were calculated. Analyzing these data by R Studio, ANOVA test results showed that there was a significant difference among 23 observed blueberry cultivars for DS from field assessment. Moreover, based on the TukeyHSD result of the PIAL in the field, these observed blueberry cultivars were divided into four groups. This is the first study to describe different blueberry cultivars with different infection levels of rust disease using statistical analysis results, which can give some preliminary test results and an analysis approach for blueberry cultivars' rust disease infection level. Based on these statistical

analysis approaches, new data from other cultivars, germplasm resources and breeding materials, or from the same cultivars but different observation years, can be grouped together to analyze the difference among infection levels. When testing more blueberries, and more repeated data from different years, there will be a clearer definition for each blueberry's rust disease resistance level. In addition, this study also found a correlation between the inoculation test and the field assessment, which gives an idea about how to connect an inoculation test result with the field assessment results. After an improvement in the prediction model by analyzing more data, it may offer an efficient way for the preliminary evaluation of blueberry rust disease resistance based on the inoculation test results.

However, as mentioned before, it has some drawbacks when using *Fiji* image processing software on DS analysis. To overcome these drawbacks, automated image analysis of scanned symptomatic leaves picked from the field can be an option for a quantitative measures on blueberry rust DS (Karisto et al., 2018). Furthermore, new phenotyping tools with field-based, high-resolution and high-throughput sensor-based tools, would be valuable to use on blueberry rust disease phenotyping (Shakoor, Lee, & Mockler, 2017; Simko, Jimenez-Berni, & Sirault, 2016). These new phenotyping tools will offer more precise, objective and reproducible results.

Furthermore, some other aspects need to be considered in a further study. In this study, 23 blueberry cultivars were observed during the 2019 harvest season. To have a better understanding about the effect caused by blueberry leaf rust on blueberry production, a longer observation covering the whole production period and repeats of the observations in different production years are essential for obtaining better field assessment results. This can draw a better picture of the blueberry rust reactions on the same cultivars from different cultivation years, which are under different weather conditions, and the pressure of rust disease varies in the field. In this study, the weather variation was observed. From the late summer to early autumn, dew easily to forms on

both sides of the leaf in the morning, with the temperature varying from 10 to 25℃ (Figure 4.1). As rust infection is through stomas, and a higher number of stomas are found on the downside of the leaf, so rust infection becomes more active under this high humid micro-environment. In addition, the temperature in this period is also suitable for the infection process as the favorable temperature for urediniospore germination is between 15 to 25℃ (Daniel, 2018). It is also mentioned that the rust symptoms are more serious from autumn to spring rather than in the summer (D. Hutchins, personal communication, 13th March 2018). It may because by the dew increases the DS on the blueberry or it may because in summer the vegetative growth is faster than the rust infection on the plant. To understand these interactions in the environment, plant host and pathogen, it is worth having a further observation on the rust disease during the whole production period and repeats under different weather conditions in different years. These results will be more representative on blueberry leaf rust infection levels of different cultivars.

Figure 4.1 Morning dew observed on blueberry in Hastings at 8:20am on the 27th of February in 2019. The dew was clearly seen on both side of the blueberry leaves.

Chapter 5 Conclusion and limitations

In conclusion, through the morphology and genetic sequencing of the ITS region, *T. minima* was identified to be the causal agent of blueberry rust in Hastings, Hawke's Bay. Additionally, a screening procedure for identifying resistant sources of blueberries was developed using TukeyHSD results. *Fiji* was used for ALA calculations and R Studio for statistical analysis. This is the first study to use these tools to describe blueberry rust infection levels among different blueberry cultivars under rust disease pressure in the field. In this study, based on the results produced by the *Fiji* calculations and statistical analyses, 23 blueberry cultivars were divided into four groups, including highly susceptible, moderately susceptible, susceptible and tolerant, by the TukeyHSD results on the PIAL. Following this procedure, new data from other cultivars, or from the same cultivars in different survey years, can be compared in future studies. In addition, based on the statistical analyses, a strong correlation was found between the PIAL in the field and the ILA from the inoculation test. As the PIAL in the field can be used for developing the rating scale of blueberry leaf rust disease, the inoculation test result from the ILA can be used for predicting the cultivar infection level in the field.

However, there are several limitations in this study that can affect the results. Regarding the pathogen identification test, the rust symptomatic leaves from other blueberry production regions were not collected for species identification. The difference between inoculation on the plants and on the detached branches was not examined. The variance in different concentration inoculums was also not studied and only one blueberry cultivar, 'Sky Blue', was used for the inoculation test which was not enough to establish a reliable prediction equation. The disease infected reaction on other blueberry cultivars was also undetected in this study. In the field assessment, there are three main limitations. First, as the available cultivars were limited at Gourmet Blueberries. Ltd, only 23 blueberry cultivars were observed for blueberry rust infection levels under rust disease pressure in the field. Even though it includes cultivars from three NHB, fifteen

SHB and five RE, this number is still a small part of the current blueberry cultivars. This information is insufficient for understanding the blueberry germplasm resources on blueberry leaf rust disease. Second, the observation for the field assessment was only in the 2019 harvest season. Even though differences were found on DI, ALA and the PIAL among different cultivars, it might be not enough for describing the infection level of blueberry leaf rust disease. Due to blueberry leaf rust becoming serious from autumn to winter, the results we found during the harvest season might change in later seasons. Finally, the phenotyping approach by using *Fiji* software can be improved to reduce the errors that are caused by the image quality and inconsistent photographing angles.

Therefore, future work can include the identification of rust specimens from different production regions in New Zealand or maybe in other countries. In addition, field assessments and inoculation tests on more blueberry materials to understand germplasm resources on blueberry leaf rust disease. Furthermore, it needs a longer observation period and more repeats during different years to establish a better correlation coefficient and a prediction equation. Finally, new phenotyping tools with a field-based, high-resolution and high-throughput sensor-based tools would be valuable to use on blueberry rust disease phenotyping to receive more precise, objective and reproducible results. All these improvements will support blueberry plant breeding in relation to rust disease resistance and blueberry industry development in New Zealand.

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