IDENTIFICATION OF DISEASE REACTION OF 11 *CAMELINA SATIVA* GENOTYPES TO DOWNY MILDEW (*PERONOSPORA PARASITICA*) AND INVESTIGATION OF STRATEGIES FOR ITS CONTROL

by

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Submitted in partial fulfilment of the requirements

for the degree of Master of Science

at

Dalhousie University

Halifax, Nova Scotia

in co-operation with

Nova Scotia Agricultural College

Truro, Nova Scotia

December 2009

DALHOUSIE UNIVERSITY

NOVA SCOTIA AGRICULTURAL COLLEGE

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DATE: 9 December, 2009

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DEPARTMENT OR SCHOOL: Department of Plant and Animal Science

DEGREE: MSc. CONVOCATION: May YEAR: 2010

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Signature of Author

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Abstract

The SEM observation revealed that *Peronospora parasitica* started to germinate and finish direct penetration between 4 hours and 8 hours after inoculation. Conidiophores were observed growing out through stomata 1 week after inoculation. Eleven camelina (*Camelina sativa*) genotypes were evaluated for their disease reaction to three downy mildew isolates obtained from Truro, NS; Charlottetown, PEI; and Canning, NS. The results showed that CN30478 was the most resistant genotype and CN30475 was the second most resistant. Calena and CN101985 were the most susceptible genotypes. Dithane DG, Bravo 500, Tanos 50 DF and Tattoo C were tested to identify their efficacy for downy mildew control of a single application on four camelina genotypes. In controlled environment experiments, the fungicides were applied either one day before or one week after inoculation. Both the field and growth chamber experiments failed to show any significant effects of these fungicides on downy mildew severity.

List of Abbreviations and Symbols Used

ISR	induced systemic resistance
SAR	systemic acquired resistance
ROS	reactive oxygen species
RNS	reactive nitrogen species
RSS	reactive sulphur species
HR	hypersensitive reaction
RH	relative humidity
PEI	Prince Edward Island
NS	Nova Scotia
NSAC	Nova Scotia Agricultural College
SEM	Scanning Electron Microscopy
AAFC	Agriculture and Agri-Food Canada
PI	post inoculation
LSD	least significant difference
LSMeans	least square means
DI	disease index

Acknowledgements

I would like to thank my supervisor, Dr. Claude Caldwell for giving me this great opportunity to do my Masters study at Nova Scotia Agricultural College. I really appreciate his guidance and help not only on my project, but also on my life. I also thank the other supervisor who is also a friend of mine, Dr. Bruce Gray for his constant support and encouragement throughout my whole project. I thank my committee member, Dr. Kevin Falk, for provision of camelina seed, and for his constructive ideas and comments on my research.

I am grateful to Doug MacDonald for his help and comments during my research, as well as the Crop Development Institute crew and all the people who supported and helped me during my project. I am grateful to Dr. Richard Martin and Chris Fleming of Agriculture and Agri-Food Canada for helping me by setting up the misting system used for field inoculations in Charlottetown.

I would also like to acknowledge the financial support from the Technology Development program of Nova Scotia Agriculture Services Branch.

Last but not the least, I would like to thank my mother, my wife and my friends for their love, support and encouragement in my study.

Chapter 1: Introduction

1.1 Background

Camelina (*Camelina sativa*) has a long history as a crop in agriculture (Föller and Paul, 2002); however, the use of camelina has been limited mostly to limited oil products, such as cooking oil and lamp oil, in the past (Ehrensing and Guy, 2008). In recent years, camelina has attracted greater attention because of its great adaptability and its potential for production of a functional oil; it has good resistance to pests and diseases, and high omega-3 fatty acid content in its seed (Zubr, 1997). Nowadays, the use of camelina is not only for food oil production, but also for biodiesel production, cosmetics, skin care products, soaps, and soft detergents (Ehrensing and Guy, 2008). The advantages possessed by camelina indicate a promising market in the future (Ehrensing and Guy, 2008).

In fields, camelina has been observed to be affected by some diseases (Vollmann *et al.*, 2001). Among these diseases, downy mildew (caused by *Peronospora parasitica*) has been reported most frequently (Vollmann *et al.*, 2001). It is an obligate parasite that can survive only on living hosts (Birch *et al.*, 2006) and overwinters in the form of oospores on host debris (Spencer, 1981). In the spring, the oospores germinate and cause the primary infection by releasing conidia onto host plants (Moss *et al.*, 1994). Leaves, stems and pods of the host can be infected and more conidia will be produced on the infected parts to cause the secondary infection (Spencer, 1981). To control downy mildew in the

field, chemical protection has been applied on crops such as lettuce and cucumber, (Moore *et al.*, 2008; Kim *et al.*, 1999); however, to date, there is no fungicide registered for downy mildew control on camelina (Anonymous, 2007a; McVay and Lamb, 2008). Considering the potential environmental and health side-effects of fungicide application, the development of resistant varieties of camelina is preferred for control of downy mildew.

1.2 Literature Review

1.2.1 *Camelina sativa*

1.2.1.1 Background History

Camelina sativa, which is also named false flax or "gold of pleasure", belongs to the Brassicaceae family or crucifers (Bonjean and Le Goffic, 1999). As an ancient plant species, *C. sativa* was first cultivated in Europe in the Neolithic times and became an important crop in the Iron Age (400 B.C. – 500 A.D.) (Föller and Paul, 2002). From the beginning of the last century, *C. sativa* cultivation spread from Europe to other continents, such as North America (Zubr, 1997). At present, oil products in the market are mainly made from soybean, sunflower and rapeseed (Gehringer *et al.*, 2006). However, in recent years, due to the acknowledgement of the abundant resources of omega-3 fatty acids in *C. sativa* and *C. sativa*'s great capacity for pest and disease resistance, *C. sativa* is becoming an economically important crop (Zubr, 1997). Compared with many other brassicas

investigated previously, *C. sativa* contains a higher level of omega-3 polyunsaturated fatty acids, especially the linolenic acid (30-40% in camelina oil), and lower glucosinolate content (13.2-36.2 μmol/g dry seed) (Budin *et al.* 1995; Crowley, 1999; Gugel and Falk, 2006). The similarly high content of linolenic acid can only be found in linseed and edible fish oils (Crowley and Fröhlich, 1998; Matthäus and Zubr, 2000). In addition, camelina oil is more stable than linseed and edible fish oils (Vollmann *et al.*, 2005). In addition to the valuable oil resources, the lower glucosinolate content in camelina seeds makes the seeds more suitable for feeding animals than some other cruciferous seeds (Schuster and Friedt, 1998). Glucosinolates are not preferred compounds in animal feeds because, although glucosinolates themselves are not toxic, if they are catalyzed by enzymes, some of their metabolic products will show toxic effects (Schuster and Friedt, 1998).

1.2.1.2 Camelina Cultivation

Camelina sativa has been reported to have two forms: spring and winter (Föller and Paul, 2002). For spring forms, the growth of *C. sativa* requires about 120 days in Europe. In Central and Northern Europe, winter forms could be ready for harvesting before the end of July (Zubr, 1997). Compared with camelina growing in Europe, *C. sativa* in Canada takes only about 90 days to mature (Caldwell and MacDonald, 2006). In order to provide a favorable environment for camelina growth, Zubr (1997) suggested that weeds should be removed before sowing *C. sativa* to reduce competition. Before sowing, the use of fertilizers is the other strategy to help *C. sativa*'s growth by improving soil fertility. About 30 kg phosphorous and 50 kg potassium per hectare was reported to improve soil

fertility and the optimum nitrogen supply was about 100 kg per hectare. Compared with other crops, *C. sativa* may be considered as a low-input crop due to its high disease resistance. During its growth, *C. sativa* does not need much chemical protection, such as the use of fungicides. Thus, the cost of camelina cropping is often less than comparable oilseed crops.

1.2.1.3 *Camelina sativa* Industry and Economic Benefits

As early as in Bronze Age, camelina seeds were used for oil extraction for food, medicinal use and lamp oil (Ehrensing and Guy, 2008). To date, camelina oil still exists in European markets as salad dressings and cooking oil (Ehrensing and Guy, 2008). Consumers' positive feedback from the market indicates that camelina edible products possess a promising future (Anonymous, 2007a). In addition to edible products, camelina oil is also used in agricultural spraying applications and biodiesel production (Ehrensing and Guy, 2008). Other camelina products in today's market include cosmetics, skin care products, soaps, and soft detergents (Ehrensing and Guy, 2008).

Due to camelina's high nutritional value, there are some investigations focused on camelina meal for feeding animals. In certain European countries, because camelina contains glucosinolates, camelina meal for livestock feeds has been banned. However, the results of McVay and Lamb's (2008) experiments showed a contrary result that no negative impact on animal performance and growth was found after using camelina meal for feed. Moreover, the camelina meal tested in those experiments increased the content of omega-3 fatty acid in beef, dairy and eggs. Due to the exciting results from previous

experiments, McVay and Lamb (2008) are still doing investigations on camelina meals to find out the optimal formulations for omega-3 enriched products.

Although camelina has a long history in European agriculture, its market is still very limited. However, its remarkable features, such as lower input costs and high content of polyunsaturated fatty acids give it a promising potential to be significant in future markets (Anonymous, 2007a).

1.2.1.4 *Camelina sativa* and Diseases

In field experiments, compared with other brassica crops, the occurrence of diseases on *C. sativa* is low (Vollmann *et al.*, 2001). However, there still are some pathogens that can affect *C. sativa* , such as *Pseudomonas syringae* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Rhizoctonia solani* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Sclerotinia sclerotiorum* (Zubr, 1997; Séguin-Swartz et al, 2009), *Peronospora parasitica* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Erysiphe* spp. (Föller *et al.*, 1998), *Albugo candida* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009) and *Botrytis cinerea* (Zubr, 1997). Among these pathogens, *Peronospora parasitica* (downy mildew) occurred the most frequently in *C. sativa* cropping seasons (Vollmann *et al.*, 2001). As a result, many investigations have been carried out so far to understand the interaction between *Peronospora parasitica* (*P. parasitica*) and *C. sativa*.

1.2.2 Downy Mildew

1.2.2.1 Introduction of Downy Mildew

"The term 'mildew', followed by 'downy mildew', was first employed in the United States to denote a group of parasitic fungi with little in common except their appearance as a delicate outgrowth (either white, or highly colored in the case of moulds) caused by the proliferation and fructification of mycelium on the surface of necrotic tissue." (Spencer, 1981, p. 1) Due to their morphology and epidemic characteristics, downy mildew constitutes a fungus-like group which shows great negative impacts on several crops (e.g. vine, tobacco, sunflower and soybean) (Spencer, 1981).

In general, the development of downy mildew depends on the existence of susceptible hosts and proper environmental conditions (Achar, 1998). After successful establishment on hosts, Spencer (1981) proved that the secondary infection caused by downy mildews would produce a huge number of spores; for instance, *Plasmopara halstedii* in a 200 cm^2 area on a sunflower's leaf could produce 200 000 spores per day. Associated with preferred weather conditions, such as heavy rainfall and wind, those spores can be disseminated to other susceptible hosts to enlarge the extension of infected areas and cause large economic loss in a crop field. In addition, human behaviour such as transplanting, grafting and transportation of infected seeds and fruits, has extended the geographical distribution of downy mildew in the world.

1.2.2.2 Introduction of *Peronospora parasitica*

1.2.2.2.1 Symptoms

According to the description from Vollmann *et al.* (2001), the first symptoms of downy mildew (*P. parasitica*) on *C. sativa* are observed at the beginning of flowering. The upper stem is the first part to present symptoms and after that, the infected stem. The white mould is the conidiophore and conidia that could also be found on leaves and developing pods. When the flowering stage ends, the mould turns from white to grey and the infected stem shows a twisted growth.

1.2.2.2.2 Disease Cycle

P. parasitica over-winters in the form of oospores on fallen leaves (Moss *et al.*, 1994). In spring, new infection starts with the germination of oospores in the soil (Slusarenko and Schlaich, 2003). Spencer (1981) reported that the conidia are released to inoculate host plants in the spring. Conidia on the surface of a susceptible host will generate germ tubes from the side. When those germ tubes reach the surface of the host, appressoria will be formed at the tip of germ tubes. The infection hyphae developing from appressoria will then penetrate into host tissues through natural openings, such as stomata, or directly through cuticle by breaking holes (4-5 μm in diameter). *P. parasitica* usually infects a host plant by direct penetration. After entering the host tissues, hyphae will grow

intercellularly. Haustoria developing from hyphae penetrate into host cells through 1-2 μm diameter holes in the cell wall to absorb nutrients from host cells. After 1 to 2 weeks, conidiophores which branch from hyphae will grow out from host tissues through stomata (Slusarenko and Schlaich, 2003). Those conidiophores are tree-like structures that develop several branches and secondary branches. Spherical conidia are generated at the tips of those branches, (Slusarenko and Schlaich, 2003). After maturity, conidia are easily disseminated to other places by rainfall and wind (Spencer, 1981). Then, a new conidia infection cycle will take place in light of the steps described above. This is called secondary infection. The entire disease cycle is shown in Figure 1.1. Because *P. parasitica* possesses the ability to occur as a secondary infection, the disease can be rapidly spread in cropping fields.

To reproduce oospores to over-winter, *P. parasitica* will form spherical oogonia and paragynous antheridia (Spencer, 1981). Both male and female sex organs arise from hyphae, not only from separate hyphae, but also from the same hypha (Spencer, 1981). When the two sex organs make contact with each other, the oogonium will generate a receptive thin walled papillum at the contacting point (Spencer, 1981). Then an antheridium will form a fertilization tube into the oogonium through the receptive papilla (Spencer, 1981). The oosphere will be fertilized inside of the oogonium (Slusarenko and Schlaich, 2003). After that, the fertilized oosphere will develop to an oospore (Slusarenko and Schlaich, 2003). Oospore formation is favored in older leaves. As a result, oospores can be found easier in necrotic or chlorotic leaves rather than in green ones (Spencer,

1981).

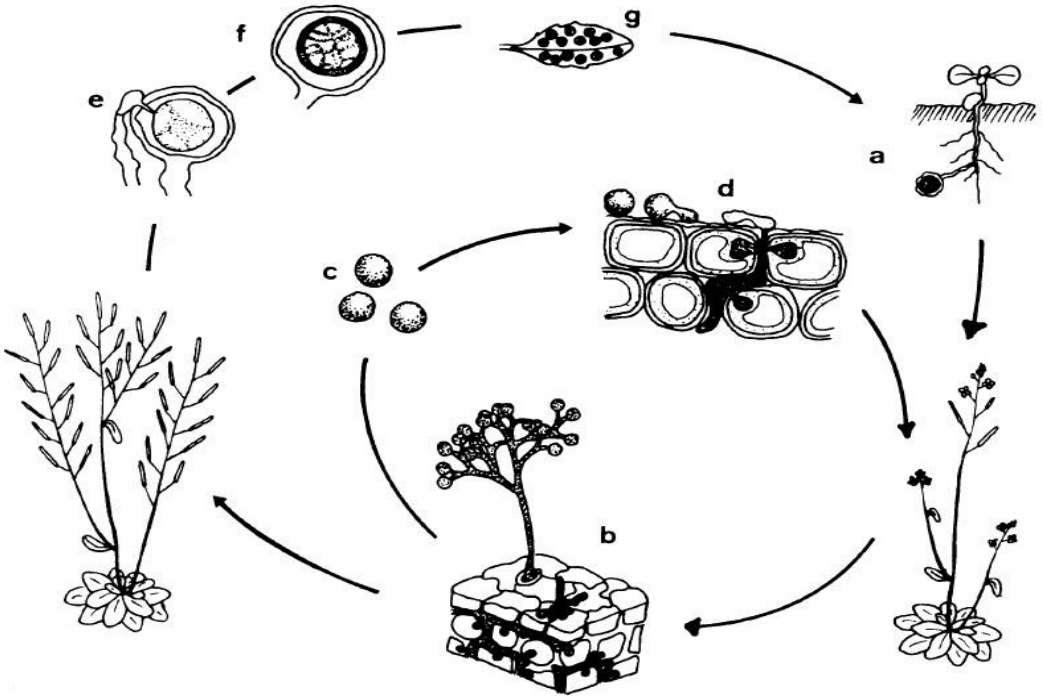

Figure 1.1 Life cycle of *Peronospora parasitica*. (a) infections arise initially from oospores germinating in the soil. (b) Plants are colonized by a coenocytic, intercellularly growing mycelium which swells to fit the intercellular spaces, giving it an irregular appearance. The hyphae put out pear-shaped feeding organs called haustoria into host cells. After a variable period of growth (1–2 weeks) conidiophores, bearing asexual, spherical hyaline conidiospores (c) grow out of stomata. (d) On germination, conidia initiate new rounds of infection. (e–g) Oospores are formed concurrently with asexual spores. (e) The female sexual organs, oogonia, contain an oosphere that is fertilized via a fertilization tube growing through its outer wall from the male antheridium. (f) The fertilized oosphere develops into a mature oospore. (g) Oospores are very profuse in infected leaves (Slusarenko and Schlaich, 2003).

1.2.2.2.3 *P. parasitica* Maintenance

Peronospora parasitica is an obligate pathogen which means that it cannot be cultured without the existence of the living host (Turk, 2002); in other words, it cannot be cultured on an artificial synthetic media (Föller and Paul, 2002). Kiefer *et al.* (2002) used a host-free system to investigate the early stages of downy mildew of grape (*Plasmopara viticola*) development. In the host-free system, the zoospores might generate two germ

tubes, whereas zoospores on the host formed only one germ tube to penetrate through stomata directly. Additionally, it has been found that a few zoospores in the host-free system could form mycelium; however, the mycelium growth was not stable. Most of them were not able to develop into further stages. Because downy mildew cannot be cultured in a host-free system, Spencer (1981) suggested that the most suitable method to maintain *P. parasitica* is to inoculate it on young brassica plants or culture it on detached cotyledons in an incubator under proper environmental condition settings in light of the requirement of different stages in the development of *P. parasitica*.

To find the most suitable way to keep *P. parasitica*, Föller and Paul (2002) also set up an experiment in which the isolates of *P. parasitica* were frozen in glycerine, polyethylene glycol and dimethyl sulfoxide or directly on infected cotyledons. Those isolates were stored at -25 ºC. After one day, seven days, one month and three months, the viability and the growth condition of the isolates were tested. The results showed that the most efficient way was to keep *P. parasitica* directly on infected leaves at -25 ºC. With this method, stored *P. parasitica* was still viable after 10 months.

1.2.2.3 Impact of Environment on *P. parasitica* Development

1.2.2.3.1 Spore Survival

The ability of spores or conidia to remain viable varies among downy mildew species (Spencer, 1981). For the same species, however, spore viability may also vary

under different environment conditions (Spencer, 1981). Conidia of *Peronospora tabacina* and spores of *P. halstedii* can survive for 1 – 2 weeks at around 20 ºC (Spencer, 1981). Conidia of *Bremia lactucae* can remain viable for 1 – 2 weeks at 21 ºC and for about 2 months at 2 – 10 ºC (Spencer, 1981). In general, the spore viability decreases when the temperature rises; however, the optimum temperatures can vary with humidity. In addition to the temperature, spore viability can be decreased with increasing UV irradiation (Spencer, 1981). A similar conclusion was also drawn in Wu *et al*.'s (2000) experiment in which they investigated the effects of temperature, relative humidity and solar radiation on the survival of *B. lactucae*. The results of their experiments revealed that temperature could greatly affect the viability of *B. lactucae*, while relative humidity could not. Sporangia would lose viability much faster at 31 ºC than at 23 ºC. Therefore, lower temperatures are preferred by *B. lactucae*. For the solar radiation investigated in this experiment, they found that the percent germination of *B. lactucae* decreased as the exposure increased under all six treatments: (1) dark; (2) Florescent light (FL); (3) ultraviolet A (UVA1) (6.0 W m–2) peak at 340 to 350 nm; (4) ultraviolet A (UVA2) (12.5 W m^{-2}) peak at 340 to 350 nm; (5) ultraviolet B (UVB1) (1.5 W m^{-2}) peak at 305 to 310 nm; (6) ultraviolet B (UVB2) (7.0 W m^{-2}) peak at 305 to 310 nm. However, only UVB1 and UVB2 could kill *B. lactucae* sporangia.

The relationship between humidity and spore viability is perplexing because different downy mildew species may have completely different requirements; for instance, *Pseudoperonospora cubensis* sporangia remain viable longer at low humidity, whereas

sporangia of *Pseudoperonospora humuli* prefer high humidity to maintain viability. For *B. lactucae* conidia and *P. viticola* sporangia, it has even been reported that the viability of their conidia increased in both high and low humidities (Spencer, 1981).

1.2.2.3.2 Germination

Light has been shown to promote germination in *Peronospora manshurica* and *P. trifoliorum* when it was provided prior to or during spore germination. However, for some other species, such as *P. tabacina*, *P. viciae* and *Sclerospora sacchari*, light had no effect on germination. Moreover, in some cases, such as in *B. lactucae*, continuous light could inhibit the penetration of fungus into the host (Spencer, 1981).

In addition to light, temperature is the other environmental factor that affects germination. *P. viciae* and *B. lactucae* can germinate within the range from 0 - 25 ºC and the optimum temperatures for *P. viciae* are from 4 – 8 ºC. Isolates of Australian *P. tabacina* can germinate in the range from 8 – 27 ºC, whereas *Sclerospora sorghi* isolates from Taiwan can geminate between 10 – 30 ºC and 12 – 32 ºC on sugarcane and maize, respectively. In these examples, the temperature ranges seem to vary with the geographical origin of species and isolates (Spencer, 1981).

1.2.2.3.3 Colonization

After penetration, fungi will grow inter-cellularly within host plants. In this stage,

humidity and temperature still are the two important factors for fungal development. When the temperature increased or decreased from the optimal level, the pathogen's incubation period was extended and the colonization was inhibited; for instance, *P. humuli* is able to grow intercellularly between 7 ºC to 29 ºC. Its growth is fully suppressed when temperature is lower than 7 ºC or higher than 29 ºC. In contrast, a relatively high relative humidity was observed to be associated with the faster growth of a pathogen's hyphae. At the same temperature, *P. viticola* and *B. lactucae* will grow more rapidly at high humidity than at low humidity. As another example, the incubation period of *P. viticola* was 6 days at 13.2 – 13.5 ºC at 100% relative humidity and 11 – 12 days at 80 – 90% relative humidity at the same temperature. However, in some instances, relative humidity had no impact on the incubation period, such as on *P. humuli* (Spencer, 1981).

1.2.2.3.4 Sporulation

Sporulation occurs in a wet and dark environment after a period of light (Spencer, 1981). Light is a very important factor regulating downy mildew sporulation (Spencer, 1981); only few or no downy mildew fungi will sporulate under continuous light or darkness (Spencer, 1981). Suppression of sporulation of *P. tabacina* caused by continuous light has been proven in Heist *et al.*'s (2001) experiment. For *P. tabacina*, Spencer (1981) suggested that the optimum photoperiod was 13 hours. After that period, sporulation will reach the peak in 5 hours under moist dark conditions.

Relative humidity and temperature are the other two important factors affecting

sporulation according to Spencer (1981). Some species can start to sporulate when leaves are covered by a film of water, whereas some other species need more water, such as dew on the plant surface, to sporulate. The lowest requirement of relative humidity for most downy mildew sporulation varies from 91 to 100% according to different species, whereas some species can start to sporulate when relative humidity is as low as 80%. For temperature, it varies among different species of downy mildew. For example, the minimum, optimum and maximum temperatures are respectively 7 ºC, 10 – 16 ºC and 22 ºC for *Peronospora destructor* and 4 ºC, 8 – 16 ºC, 20 ºC for *P. viciae* (Spencer, 1981).

For the sporulation of *B. lactucae*, Su *et al.* (2003) investigated the impact of temperature, relative humidity and wind speed. They found that temperature had a significant effect on sporulation and the optimum temperature range for sporulation was from 10 to 20 ºC. Sporulation of downy mildew pathogens might be affected by temperature via the impact of temperature on vegetative growth of sporangiophores; for instance, at suboptimal and supraoptimal temperatures, *P. destructor* sporangiophore will grow shorter than at the optimum temperature. In addition to temperature, sporulation also decreased significantly when relative humidity dropped below a certain level which depends on the downy mildew species. Wind speeds were found to have a negative impact on sporulation in this experiment. The result showed that even a low wind speed could reduce relative humidity near the leaf surface to a level that is not suitable for sporulation; for instance, sporulation was found to start to decrease when wind speed exceeded 0.1 m s^{-1}. Furthermore, when wind speeds exceeded 0.5 m s^{-1}, sporulation was

completely inhibited.

1.2.2.3.5 Spore Release and Spread

Su *et al.* (1999) also studied *B. lactucae* spore release; they investigated the impact of light initiation and relative humidity decrease on spore release. They found that both of the two factors showed a positive impact on spore release, although for relative humidity, it decreased only 5% from 100% to 95%. Therefore, the results also suggest that *B. lactucae* spores are very sensitive to changes in relative humidity. The authors postulated that the spore release may be related to electrical charges which resulted from the two factors; however, this needs to be tested in further experiments.

1.2.3 Disease management

1.2.3.1 Fungicides

With different crops, the damage caused by downy mildew varies with different growth stages (Spencer, 1981); for example, downy mildew has the greatest impact on some crops when infection occurs during their seedling stage (Spencer, 1981). Hence, the timing of a fungicide application is important (Spencer, 1981). On the other hand, the efficacy of different fungicides also varies depending on different plant-pathogen interactions; for instance, Khalil *et al.* (1992) reported that copper oxychloride and mancozeb were effective for downy mildew control on muskmelon. Kagadi *et al.* (2002) also found that chlorothalonil could control downy mildew effectively on ridge gourd,

while Gupta and Shyam (1996) reported that chlorothalonil was the least effective fungicide controlling downy mildew on cucurbits. To date, although chemical control is still the most effective way for most downy mildew species control in the field (Gisi and Sierotzki, 2008), no fungicide has been registered for camelina (Anonymous, 2007a; McVay and Lamb, 2008).

From the mid-1940s to the mid-1960s, sprays and dusts of fungicides, such as chloranil (spergon), copper-based materials and zineb, were the most common forms of fungicides used in fields. However, these early fungicides were replaced by captafol, daconil, dichlofluanid and propineb. Among the new fungicides, dichlofluanid had a strong ability of downy mildew control on the cotyledons of cabbage and cauliflower. Furthermore, it also showed an impact on increasing the size and dry weight of the plants. In the late 1970s, the use of prothiocarb prior to sowing was found to dramatically decrease the disease in over-wintered cauliflower plants (Spencer, 1981). Crowley and Fröhlich (1998) suggested that a mixture of carbendazim and metalaxyl fungicides could be sprayed in the field to control downy mildew.

In today's fungicide market, the old multi-site fungicides, including dithiocarbamates, phthalimides, chloronitriles and copper formulations still account for 50% of the fungicide market (Gisi and Sierotzki, 2008). Application of multi-site fungicides is able to create a barrier at the surface to protect plants from pathogen invasion by affecting multiple metabolic steps of pathogens (Gisi and Sierotzki, 2008). Quinone outside inhibitors (inhibits mitochondrial respiration), the phenylamides (inhibit ribosomal RNA

synthesis) (Davidse, 1995), the carboxylic acid amides (target on phospholiphid biosynthesis and cell wall deposition) and cyanoacetamid-oximes are the four fungicides dominating the single-site fungicide market (Gisi and Sierotzki, 2008). Single-site fungicides can only affect one specific metabolic process of the pathogen with only a few side effects (Gisi and Sierotzki, 2008). However, because they can only affect one step of pathogen metabolism, they may be easily overcome by the pathogen (Gisi and Sierotzki, 2008).

In addition to the application of traditional fungicides, due to environmental issues, biocontrol is a newer and preferred method (Perazzolli *et al.*, 2008). The fungi of the *Trichoderma* genus have been investigated mostly for biocontrol and TRICHODEX© which contains *Trichoderma harzianum* T39 is being used commercially for grape downy mildew control (Vinale *et al.*, 2008; Elad, 1994). Deng *et al.* (2007) and Djonovic *et al.* (2007) found that *Trichoderma* spp involves an enzymatic determinant of antagonistic action and a proteinaceous elicitor that contributes to the induction of resistance. However, the effect of *T. harzianum* on defensive mechanisms differs in different host-pathogen systems, such as competition for nutrients and space (Elad, 1996), the negative impact on other pathogens' pathogenicity enzyme activities (Kapat *et al.*, 1998), direct effect on other pathogens growth and induction of plant disease resistance (Elad and Freeman, 2002; Howell, 2003).

Application of non-pathogenic microorganisms is another way to induce a plants defense mechanism which is known as induced systemic resistance (ISR; Van Loon,

2007). The inducer of ISR can be used on some parts of a plant to activate a systemic resistance in the whole plant (Pieterse *et al.*, 2001). This is similar with the defense genes regulated systemic acquired resistance (SAR; Pieterse *et al.*, 2001). However, their signaling pathways are different (Verhagen *et al.*, 2004). In addition to non-pathogenic microorganisms, ISR can also be induced by natural and synthetic compounds, such as b-aminobutyric acid (BABA; Hamiduzzaman *et al.*, 2005), plant volatile organic compounds (Conrath *et al.*, 2006) and low doses of SAR inducer (Thulke and Conrath, 1998).

1.2.3.2 Cultural Methods

Fungicides have been effective in controlling downy mildew. However, the use of fungicides also can be a threat to our environment. For this reason, standards have been developed to regulate fungicide production and many fungicides have been banned. In this situation, efficient cultural methods are considered to be a better way to control downy mildew.

Spencer (1981) suggested that, because of the importance of water in *P. parasitica'*s development, the reduction of the relative humidity around plants was an important aspect of downy mildew control. In order to do this, growers should avoid dense sowing and weeds should be removed. In addition, infected debris should be removed and continuous cropping should be avoided. Crop rotation is recommended according to Spencer (1981). However, in camelina fields, according to the observation in our trials, a two-year rotation with non-brassica plants seemed not to be enough for downy mildew control. In addition,

Spencer (1981) mentioned that a six-year-rotation was not effective for pea crops to prevent the invasion from *P. pisi* whose oospores might remain viable for 10-15 years in the soil. Therefore, the duration of crop rotation cannot be determined without further experiments.

1.2.3.3 Host Resistance

In the process of interacting with microbes, plants evolved their own strategies to resist pathogens' infection; for example, Kortekamp and Zyprian (1999) found that leaf hairs on grape leaves can serve as the first barrier to protect plants from infection. This is due to leaf hairs confining water droplets in which zoospores (*Plasmopara viticola*) are carried from leaf surfaces so that zoospores cannot infect leaves.

Besides physical defense barriers, plants also employ more important defense strategies which are vertical resistance and horizontal resistance (Crute, 1992). They are the two most common categories of host resistance (Crute, 1992). Vertical resistance, which is determined by a dominant gene (R gene), shows strong resistance to some pathogenic races (Crute, 1992); however, hosts with vertical resistance may be very susceptible to other races (George, 2005). In addition, when compared with other resistance patterns, vertical resistance is easier to overcome by the pathogen (George, 2005). In contrast, horizontal resistance is determined by several genes (Lebeda *et al.*, 2002) and therefore more difficult to overcome (Mysore and Ryu, 2004). Moreover, a host with horizontal resistance is able to be resistant to a spectrum of pathogen races (Mysore

and Ryu, 2004). However, the level of their disease resistance is not as great as those with vertical resistance (Lebeda *et al.*, 2008).

When plants are attacked by pathogens, they can release reactive oxygen (ROS), nitrogen (RNS) and sulphur (RSS) species intermediates which are considered to be the early chemical signals in plant defense systems (Lebeda *et al.*, 2008). ROS are able to trigger plant resistance mechanisms, such as programmed cell death (Kamoun *et al.*, 1999). Programmed cell death, which is termed a hypersensitive reaction (HR), is one of the most important defensive strategies in vertical resistance, although it may also be observed in horizontal resistance (Lebeda *et al.*, 2002). As biotrophic pathogens, oomycetes need to protect the integrity of the plasma membrane to ensure the nutrient supply from living host cells (Lebeda *et al.*, 2008). Hence, cell death caused by HR may cut the nutrient supply for the pathogen, which leads to the death of pathogen and the inhibition of disease spread (Lebeda *et al.*, 2008). In resistant hosts, the concentration of hydrogen peroxide (H_2O_2) will dramatically increase during the onset of pathogen attack (Sedlářová *et al.*, 2007). In contrast, there are no significant changes on H_2O_2 concentration in susceptible host species (Sedlářová *et al.*, 2007). However, the accumulation of H_2O_2 at infected sites may not be sufficient for host cell death (Hückelhoven and Kogel, 2003). It may require nitric oxide (NO) functioning together to regulate cell death (Dellendone *et al.*, 2003). In addition to H_2O_2, $O_2^{\cdot-}$ is another ROS found in plant defense system (Beligni and Lamattina, 1999). However, in contrast to H_2O_2, it suppresses cell death by reacting with $NO^{\cdot}$ to generate $ONOO^-$ so that cell death

cannot be triggered because of the lack of NO (Beligni and Lamattina, 1999). It is interesting to note that the opposite effects of H_2O_2 and $O_2^{\cdot-}$ found in plant defense system are also found in mammalians (Irani *et al.* 1997; Clément *et al.* 1998).

Except ROS generation, rapid rearrangement of cytoskeletal components in epidermal cells, links to the relocation of cytoplasm, nuclei and other organelles, will also occur when host cells are invaded by oomycetes (Koh *et al.*, 2005; Takemoto *et al.*, 2003); for instance, in lettuce, actin filaments cannot be detected in epidermal cells after pathogen invasion (Sedlářová *et al.*, 2001); and ER and Golgi bodies aggregated at infected sites contacting with the pathogen (Lebeda *et al.*, 2008). During a pathogen's colonization stage, haustoria penetration can also induce similar architectural changes in mesophyll cells (Spencer-Phillips, 1997).

In addition to the plant defense mechanisms mentioned above, there are other mechanisms employed by plants to protect themselves from pathogen infection, such as salicylic acid (SA) synthesis and transportation (Vasyukova and Ozeretskovskaya, 2007). Compared with the two crop protection methods discussed in section 1.2.3.1 and section 1.2.3.2, finding or "creating" a resistant variety is a long-term solution for downy mildew control with only little, or no, environmental impact (Farnham *et al.*, 2002).

1.3 Objectives

The overall goal of this research was to evaluate the potential impact of downy mildew on production of *Camelina sativa*, to identify breeding lines with superior

resistance to *P. parasitica* and to determine the most efficacious fungicide on downy mildew control. In order to achieve this overall goal, the following objectives were met:

1. The reaction of 11 genotypes (one cultivar and ten breeding lines) to three isolates of *P. parasitica* (two from Nova Scotia and one from Prince Edward Island (PEI)) were evaluated under controlled environment conditions.

2. The reaction of the 11 genotypes to natural infection in the field was evaluated using a visual severity scale.

3. The efficacy of four fungicides (mancozeb, chlorothalonil, famoxadone/cymoxanil, propamocarb/chlorothalonil) for downy mildew control under both field and controlled environment conditions were tested.

4. The infection process on camelina was examined by the use of Scanning Electron Microscopy (SEM).

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Chapter 2: Maintenance of *Peronospora parasitica* isolates and SEM observations on *P. parasitica* structures

2.1 Abstract

Peronospora parasitica, the causal pathogen of camelina downy mildew, is an oomycete that cannot be cultured on artificial synthetic media. Some researchers found that, although *P. parasitica* was able to show some initial growth on synthetic media, no further development took place. Therefore, *P. parasitica* must be maintained on a compatible host. To obtain a successful infection, three conditions of the plant disease triangle (a susceptible host; a virulent pathogen and favourable environmental conditions) must be satisfied. In our project, *P. parasitica* collected from three camelina fields in varying geographic regions and environments was maintained in a controlled environment on Calena, a susceptible *Camelina sativa* cultivar. Scanning electron microscopy observation showed that *P. parasitica* penetrated directly into plant tissues through the cuticle. It germinated and accomplished penetration between 4 hours and 8 hours after inoculation. The conidiophores grew out through stomata 1 week after inoculation.

2.2 Introduction

Downy mildew caused by *Peronospora parasitica* is the most frequently observed disease in camelina (Vollmann *et al*., 2001). It is an obligate parasite that can only survive on living hosts (Turk, 2002); it cannot be cultured in an artificial synthetic medium (Föller and Paul, 2002). A successful infection requires three factors: 1) a susceptible host; 2) a virulent pathogen and 3) favourable environmental conditions according to the disease triangle theory (Schumann and D'Arcy, 2006). As a confirmed susceptible camelina genotype in preliminary experiments, Calena was chosen as the susceptible host. Downy mildew (*P. parasitica*) conidia on infected plants in the field were collected as the inoculum sources. During *P. parasitica* developmental stages, it can tolerate the temperature range from 5ºC - 30ºC (Kofoet and Fink, 2007); however, a cool environment (defined by Slusarenko and Schlaich, 2003, as from 8ºC to 24ºC) is preferred. The other two most important environmental factors that have great influence on downy mildew growth are light and relative humidity (RH) (Sutton and Hildebrand, 1984 and 1985). Light is a very important factor that can affect almost all steps of downy mildew's development (details were described in section 1.2.2.3). Rumbolz *et al*. (2002) found that continuous white light could inhibit grape downy mildew (*Plasmopara viticola*) sporangium formation. In addition, darkness was hypothesized to be an essential requirement to promote full sporangial differentiation. The importance of high relative humidity (RH) during downy mildew development was recognized a few decades ago

(Spencer, 1981); however, continuously high RH can also suppress downy mildew sporulation and spore release (Kitz, 2008; Su *et al*., 1999 and Byrne *et al*., 2005). Kitz (2008) evaluated the impact of three different RH settings on downy mildew (*P. farinose*) infection on quinoa (*Chenopodium quinoa*). There were three groups of quinoa plants. After inoculation, the first group of plants was kept under an environment with RH >90% for 10 days. The second group was maintained at RH ≥ 95% on the first day and sixth day. For the other days, the RH was maintained at 60-70%. The total experimental period of this group was 10 days. The third group was tested with the same RH conditions of the second group, but with an additional humidity cycle until day 16. The results showed that no symptoms and sporulation showed up in the first group. However, in both the second and third groups, sporulation took place. This observation suggests that a relatively low RH period is also important for downy mildew's development, probably for host colonization. In this chapter, the method used for *P. parasitica* maintenance on living camelina plants will be described in detail.

2.3 Materials and Methods

2.3.1 Disease Triangle Theory

Peronospora parasitica obtained from infected plants in the field was used to inoculate and maintain on a susceptible camelina genotype, Calena. Growth chambers were set at 17ºC and 14 hours photoperiod per day. The air RH was regulated using the

method of the alternation of high RH/relatively low RH (details in section 2.3.4). Thus the three factors of the disease triangle are satisfied.

2.3.2 Downy Mildew Inocula

Infected plants in the fields were collected in July, 2008 from Agriculture and Agri-Food Canada (AAFC) Research Station in Charlottetown, PEI (PEI isolate), Lyndhurst Farms Ltd. in Canning, NS (Canning isolate) and the Plumdale Farm of Nova Scotia Agricultural College (NSAC) in Truro, NS (Truro isolate). They were brought to the lab and conidia of *P. parasitica* on the infected parts were collected using a vacuum spore collector (Figure 2.1). Collected conidia were either used for new inoculations or stored at -20ºC.

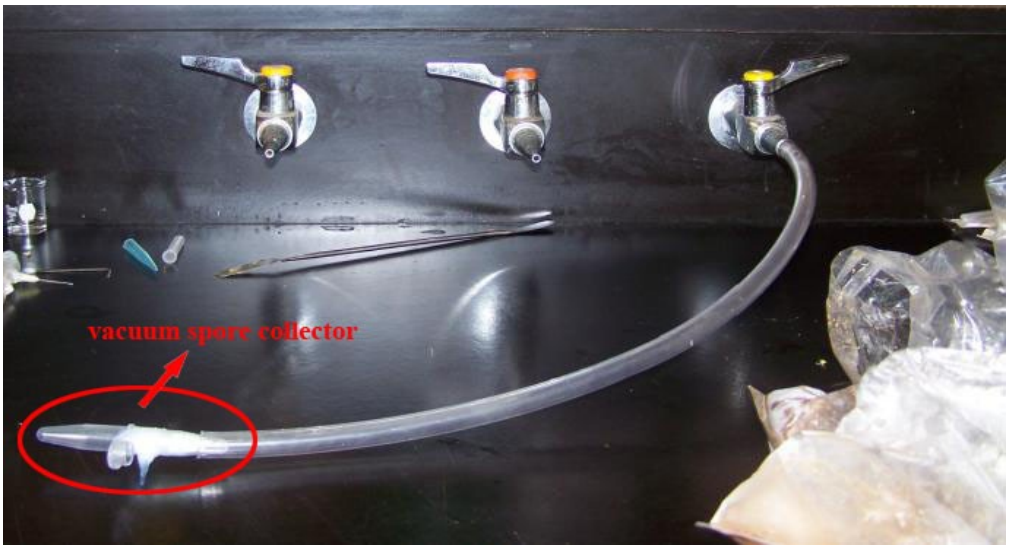

Figure 2.1: The vacuum spore collector used for conidia collection

2.3.3 Plant Preparation

Fifteen to twenty seeds of *Camelina sativa* cv Calena were sown in Pro-mix, a

commercial peat, vermiculite and perlite mix adjusted to pH 6.5 with lime in plastic pots (15 cm diameter) and placed in a greenhouse at 18 - 25ºC (day-night). Standard greenhouse sodium vapour lamps were set at 7 am/7 pm day/night regime everyday. The light intensity fluctuated in the range from 140 - 880 µmol s^{-1} m^{-2} depending on the weather and time of day. Pots were watered thoroughly daily. Plants in each pot were thinned to four to seven plants per pot after 2 or 3 weeks. Plants aged between 4 and 6 weeks were inoculated with a *P. parasitica* spore suspension as described below.

2.3.4 Inoculation

Conidia collected from infected plants were used to make 50 000-70 000 spores/mL spore suspensions. Depending on the size of the leaf, 1-3 drops of spore suspension were dropped onto each leaf of prepared plants using a 3 mL syringe. After inoculation, plants were put into plastic bags (180 mm × 75 mm × 510 mm) misted with water by a hand sprayer until water drops adhered to the inside surface of the bags. They were then placed into one of three growth chambers depending on the source of inoculum. All the growth chambers were set at 17ºC, 14 hours photoperiod under incandescent light and cool white fluorescent light combination (Light intensities of the three chambers (one each for the Truro, PEI and Canning isolates) were 90 µmol s^{-1} m^{-2}, 82 µmol s^{-1} m^{-2} and 70 µmol s^{-1} m^{-2}, respectively). The second day after inoculation, the bags were removed and plants in each growth chamber were placed into three sealed transparent plastic cages (500 mm × 500 mm × 750 mm; Figure 2.2) and returned to their respective growth chambers. In each

cage, there was a 1-2 cm layer of water on the bottom in order to maintain high RH. With this method, RH could be maintained at a level ranging from 70% to 80% measured by Nexxtech^{TM} Indoor Thermo/Hygrometer. Every 5 days, the plants were put into misted plastic bags overnight to promote sporulation until conidia on infected plants were used in experiments or harvested. Some of the harvested conidia were used to inoculate the next group of camelina plants and the remainder frozen at -20ºC.

Figure 2.2: The cage used for maintaining inoculated host plants (RH ranges from 70% - 80% inside). 1. A thin layer of water; 2. Insect sticker.

2.3.5 Scanning Electron Microscopy (SEM)

The objectives of SEM observation were as follows:

(1) To study the appearance and size of *P. parasitica* conidia, infection structures and reproduction structures and how each related to the host.

(2) To find out if *P. parasitica* infects host by direct penetration or through stomata.

(3) To obtain a general idea about the timing of conidium germination and the production of infection structures.

In this experiment, five pots with five 4-week-old healthy camelina plants (Calena) in each and one pot with five 1-week-old healthy camelina seedlings (Calena) were inoculated with the Truro downy mildew isolate. The concentration of spore suspension was 70 000 spores/mL. On each plant, large and healthy leaves which were usually the fourth to the seventh leaves from the bottom were chosen. Each chosen leaf was covered by a cheese cloth which had been soaked in spore suspension. In light of the size of the leaf, the size of cheese cloth varied from approximate 10 mm × 10 mm to approximate 20 mm × 20 mm. This cheese cloth method was not applied for 1-week-old seedling inoculation. The seedlings were inoculated by directly placing a drop of spore suspension on each cotyledon with a hypodermic needle. The inoculated plants were then incubated according to the method described in section 2.3.4.

Samples were obtained 4 hours post inoculation (PI), 8 hours PI, 24 hours PI, 48 hours PI and 1 week PI. One sample was obtained from each inoculated leaf. Each sample was cut from the area covered by the cheese cloth. Samples of 1 week PI obtained from cotyledons were an exception because they were obtained from infected cotyledons without cheese cloth. The whole infected cotyledons were cut from seedlings for

preparation for SEM.

Samples were fixed in 3% gluteraldehyde in phosphate buffer followed by 2% osmium tetroxide. Gluteraldehyde in phosphate buffer stops cellular processes, whereas osmium tetroxide fixes lipid membranes. After each fixative, samples were washed twice in phosphate buffer. All fixatives and buffer washes were at pH 6.8. After the final phosphate buffer wash, samples were dehydrated gradually in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% ethanol. The process used for SEM sample preparation is described in Table 2.1.

The samples were critical point dried with liquid CO_2, on a Polaron E3100 Jumbo Series II critical point dryer, then mounted on stubs with carbon tape and sputter coated with gold palladium in a SC7620 Polaron Mini Sputter Coater. The samples were observed under the Hitachi FE-SEM 4700.

Table 2.1: Fixation/Dehydration of biological samples for SEM.

Treatment	Time (hours)
3% gluteraldehyde in phosphate buffer*	3
phosphate buffer wash	0.5
phosphate buffer wash	0.5
2% osmium tetroxide	2
phosphate buffer wash	0.5
phosphate buffer wash	0.5
10% ethanol	0.5
20% ethanol	0.5
30% ethanol	0.5
40% ethanol	0.5
50% ethanol	0.5
60% ethanol	0.5
70% ethanol*	0.5
80% ethanol	0.5
90% ethanol	0.5
100% ethanol	2

**Sample can be stored for long periods at these stages*

2.4 Results and Discussion

The method introduced above maintained downy mildew conidia on living hosts very well for the two year duration of the experimentation. Since this is the method for maintaining the continuous inocula sources, one may not need to promote the sporulation every 5 days by putting infected plants into misted bags. Thus, the duration of downy

mildew conidia on living hosts can be extended; for instance, if one promotes the sporulation every 5 days, downy mildew conidia can be maintained on living hosts for around 1 month, whereas by reducing the frequency of the sporulation promotion, the downy mildew conidia can be maintained for more than two months. Therefore, according to the experimental plan arrangement, the frequency of sporulation promotion can be adjusted for a longer or a shorter duration of downy mildew conidia maintenance. However, the first sporulation promotion 5 days post inoculation should not be avoided because it will provide the initial sporulation.

Prior to adopting this method, several different ways were attempted for the maintenance of downy mildew conidia. However, none were effective. One of these methods was designed to put inoculated plants into a misted transparent plastic garbage bag (Figure 2.3). The bag was sealed to provide a high RH near 100%. With this method, the sporulation did occur on inoculated plants, however, it was not able to produce as many conidia as were expected and the duration of downy mildew conidia maintenance was short. This may be because the RH was kept too high, the temperature went up because of plants' respiration and the sealed space and there was no air circulation in the sealed bag.

Figure 2.3: inoculated host plants in the sealed misted bag

In general, to have a successful infection, after inoculation, plants should be maintained in a cool and wet environment with the alternation of light and dark. A high RH environment is created to promote the sporulation. One can reduce the frequency of sporulation promotion after the first one to extend the duration of downy mildew conidia maintenance.

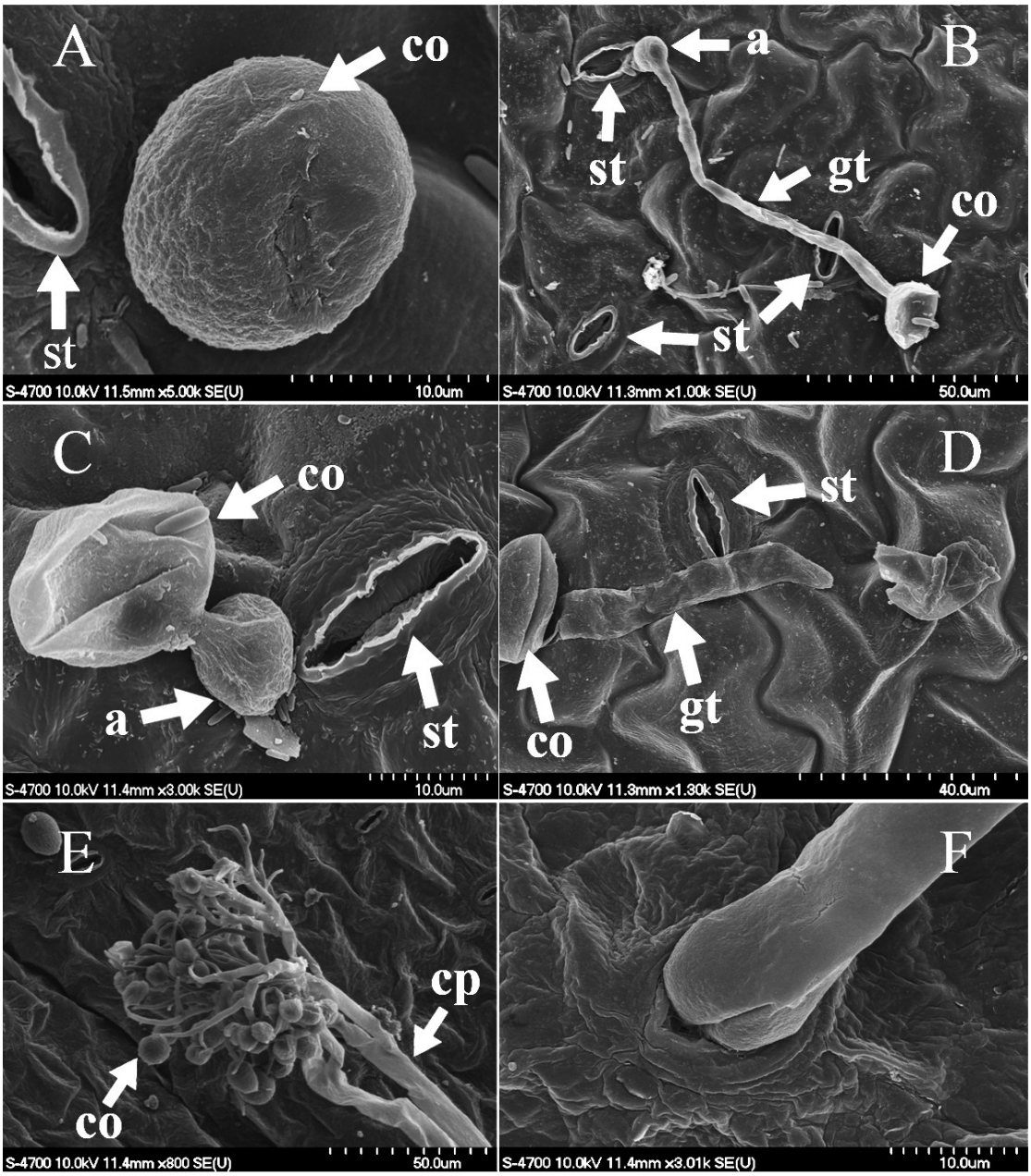

Figure 2.4: SEM images (**A**) An ungerminated conidium (4 hours PI). (**B**) A germinated conidium with a germ tube and an appressorium (8 hours PI). (**C**) A flattened conidium with an appressorium (8 hours PI). (**D**) A flattened conidium with a flattened germ tube (24 hours PI). (**E**) Conidiophore bearing mature conidia (1 week PI). (**F**) A conidiophore growing out of a stoma (1 week PI). (co=conidium; st=stoma; gt=germ tube; a=appressorium; cp=conidiophore)

SEM images showed that conidia have an elliptical shape and each conidium

produced one germ tube and one appressorium for penetration. The size of a conidium is approximately 15 μm long and 13 μm wide. Most conidia on samples of 4 hours PI have not germinated (Figure 2.4 A). However, the penetration seemed to be accomplished within 8 hours after inoculation (Figure 2.4 B and C). This can be seen from the formation of germ tube and appressorium in Figure 2.4 B and C. We do not know if the fungus had actually penetrated the host cell but the conidia were flattened, perhaps implying that primary vesicles had been formed inside the host cell and the cytoplasm in the conidia had been transferred to the primary vesicles (Spencer, 1981). In addition, Figure 2.4B and C showed that *P. parasitica* penetrated directly into plants through the cuticle and epidermal cells rather than via stomata. These observations of *P. parasitica* are similar to those Achar (1998) and Slusarenko and Schlaich (2003) found on other plant species. The SEM observation on 4 hours PI samples and 8 hours PI sample suggested that most conidia germinate and finish the penetration from 4 to 8 hours after the inoculation. Then, external penetration structures collapsed (Figure 2.4 D) and became difficult to distinguish. 1 week after the inoculation, conidiophores grew out through host stomata (Figure 2.4 F) and mature conidia were produced on the tips of conidiophores (Figure 2.4 E).

This SEM experiment revealed that under our environmental settings (section 2.3.4), *P. paracitica* started germinating after 4 hours PI and finished penetration before 8 hours PI. According to this, if the process of penetration structure formation is of interest, in future experiments samples could be collected every hour or even every half hour within 4 and 8 hours post inoculation. The observations for the whole process may help us to

understand when the differentiation takes place and why germ tubes displayed various lengths in our SEM images (Figure 2.4 B and C). For further experiments, the cross section of infected leaves and stems would be needed to observe intercellular hyphae and haustoria within infected plants.

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Chapter 3: Evaluation of downy mildew resistance of 11 Camelina genotypes

3.1 Abstract

Camelina (*Camelina sativa*) has been reported to possess good plant disease resistance. However, there still are some diseases observed in camelina fields. Among these diseases, downy mildew caused by *Peronospora parasitica* has been reported the most frequently. To find resistant genotypes, 11 *C. sativa* genotypes were tested in environment controlled growth chambers with three downy mildew isolates. Disease severity was evaluated on the 12^{th} day after inoculation. Camelina genotype CN30478 displayed the best resistance to downy mildew and CN30475 was the second best. In contrast, Calena and CN101985 were the most susceptible genotypes. Among these 11 genotypes, CN30476, CN101982 and SRS933 were special genotypes because their resistance varies among the three isolates. This indicates that the three isolates were acting differently from one another. This conclusion is also supported by the greater aggressiveness showed by the PEI isolate.

3.2 Introduction

False flax (*Camelina sativa*), also named "gold-of-pleasure", once considered by North Americans as a weed, belongs to the family Brassicaceae (Gugel and Falk, 2006; Putnam et al., 1993). In European agriculture, it has a long history as an oilseed crop (Putnam et al., 1993). Recently, more and more attention is being paid to this "old" oilseed crop because of its high omega-3 polyunsaturated fatty acid content (Crowley and Fröhlich, 1998) and for its properties as a feedstock for biofuel (McVay and Lamb, 2008). The potential for *C. sativa* products in the oilseed market is therefore widely recognized by agriculturists (Crowley and Fröhlich, 1998). In addition to high oil content, *C. sativa* is also characterized by its great disease resistance (Paul et al., 1998). However, there are still some diseases reported in camelina (Vollmann et al., 2001). Among these diseases, downy mildew (*Peronospora parasitica*) has been reported the most frequently (Vollmann et al., 2001).

P. parasitica is an oomycete (Turk, 2002) and as it is an obligate parasite, it can only be maintained on living host tissues (Turk, 2002). It overwinters in the form of oospores on the fallen leaves (Moss et al., 1994). In spring, oospores start to germinate and produce conidia which are further spread onto host plants by rain splash (Spencer, 1981). Thus, a new infection cycle starts. Conidia landing on a host surface penetrate directly into host tissues and grow intercellularly (Spencer, 1981). Haustoria, the feeding structure, will then be formed by *P. parasitica* for nutrient uptake from the host (Spencer, 1981). After a

week, conidiophores bearing new conidia will be observed on the underside of the leaves or on the infected stems (Slusarenko and Schlaich, 2003). The conidia will be spread by wind or rain, causing the secondary infection (Slusarenko and Schlaich, 2003).

Although downy mildew is the most frequently reported disease in camelina, to date, there is no registered fungicide for downy mildew control on camelina (Anonymous, 2007a; McVay and Lamb, 2008). Therefore, selecting downy mildew resistant genotypes of *C. sativa* may be the best way for downy mildew control. Furthermore, the resistant genotypes will also be useful in breeding programs. The overall objective of this study was to evaluate 11 camelina genotypes for downy mildew resistance under controlled environmental conditions. However, because of a different aggressiveness observed among the three isolates used, a different spore suspension concentration of the PEI isolate was used. A sub-experiment was set up in order to understand if different spore suspension concentrations would significantly affect camelina reaction to downy mildew.

3.3 Experiment 1: Growth Chamber Evaluation of 11 Camelina Genotypes for Reaction to Three Isolates of Downy Mildew

3.3.1 Materials and Methods

3.3.1.1 Plant Preparation

Seed of 11 camelina genotypes, CN30475, CN30476, CN30478, CN30479, CN101981, CN101982, CN101985, CN101988, CN101989, SRS933 and Calena, was provided by Dr. Kevin Falk, Agriculture and Agri-Food Canada, Saskatoon,

Saskatchewan. All genotypes were seeded in multi-cell trays with 4 replications for each. The growing medium used in this experiment was Pro-mix, a commercial peat, vermiculite and perlite mix adjusted to pH 6.5 with lime. Each tray contained 14 80-mm diameter cells in three rows (5 cells × 4 cells × 5 cells). However, only 12 cells, four from each of the three rows in each tray were used for seeding. The seeding rate was 12 seeds per cell. All multi-cell trays were placed into a greenhouse at 18 - 25ºC (day-night). Standard greenhouse sodium vapour lamps were set at 7 am/7 pm day/night regime everyday. The light intensity fluctuated in the range from 140 – 880 μmol s^{-1} m^{-2} depending on the weather and time of day. The seeded trays were watered thoroughly daily until the sixth day after seeding, when most plants had reached the cotyledon stage and were ready for inoculation. There are a couple of reasons for choosing seedlings at cotyledon stages to inoculate. Föller and Paul (2002) stated that inoculation at the cotyledon stage was the most efficient method for the identification of camelina susceptibility against downy mildew. Additionally, using seedlings at the cotyledon stage would save much time and labour (Coelho and Monteiro, 2003). Before the inoculation, the number of seedlings was thinned to seven seedlings per cell.

3.3.1.2 Inoculation

Fresh conidia were collected from infected plants and mixed with sterile water to make spore suspensions. For both the Truro and Canning isolates, the concentration of spore suspensions was 70 000 spores/mL. However, because the PEI isolate showed a greater aggressiveness than the Truro and Canning isolates in preliminary experiments,

the concentration of the spore suspension of the PEI isolate was reduced to 10 000 spores/mL. After the preparation of the spore suspension, a 10 µL standard pipette (Eppendorf Digital Pipette^{TM} 4710) was used to drop a 10 µL spore suspension onto each cotyledon. Therefore, each seedling was inoculated with 20 µL spore suspension. After the inoculation, the inoculated seedlings were put into plastic bags (180 mm × 75 mm × 510 mm) misted by hand sprayer until dew formed on the interior bag surface to create a high RH nearly 100% around the inoculated seedlings. Seedlings inoculated with the three *P. parasitica* isolates were kept separately in three growth chambers referred to as the Truro, Canning and PEI isolate growth chambers, respectively. The growth chambers were set at 14 hours light/ 10 hours darkness at 17ºC (Light intensities of Truro, PEI and Canning chambers are 90 µmol s^{-1} m^{-2}, 82 µmol s^{-1} m^{-2} and 70 µmol s^{-1} m^{-2}, respectively). RH was maintained of 60% - 70% RH inside each growth chamber by a Honeywell Cool Moisture Humidifier HCM-630. The RH was monitored by a Nexxtech^{TM} Indoor Thermo/Hygrometer. On the second day after inoculation, plastic bags were removed. On the sixth day, inoculated seedlings were put back into the misted plastic bags again to promote sporulation and taken out on the seventh day. On the eleventh day, the seedlings were again put into the misted plastic bags. On the twelfth day, they were taken out and the disease severity of each seedling was determined using a disease severity scale. This procedure was repeated 5 times for each isolate.

3.3.1.3 Disease Severity Scale

Table 3.1: Disease Severity Scale.

Class	Description
1	Small pinpoint to larger brown necrotic flecks on cotyledons. No sporulation
3	Only sparse sporulation on one side of the cotyledon
5	Dense white sporulation on one side of the cotyledon
7	Dense white sporulation on one side of the cotyledon and sparse to dense conidiophore on the other side
9	Dense white sporulation on one side of the cotyledon and sparse to dense conidiophore on the other side. In addition, sporulation can also be found on stem or true leaves

3.3.1.4 Data Analysis

The collected values of disease severity were used to calculate a disease index (DI) through the following equation:

$$DI = [\sum_{i=0}^{9} (i \times j)]/n \times 9$$

i=disease severity class, j=number plants/class, n=total number of plants (Ishii et al., 2001)

Then, the calculated DI from the isolates was analyzed separately using PROC Mixed procedure and Tukey test of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) at the 5% significant level.

3.3.1.5 A Two-Factor-Factorial Designed Field Trial

This trial was carried out in AAFC, Charlottetown, PEI in 2008 as the first year field fungicide experiment for our project. It was a two-factor-factorial design with camelina genotypes and fungicide treatments as the two factors. It was set up as four blocks. Within

each block, there were five plots treated with four fungicides and a control treatment. Furthermore, in each plot, there were 11 rows which were sown with 11 camelina genotypes, respectively. The disease severity was determined before the fungicide application and 1 week after the application. We first designed this experiment to determine both the disease reaction of 11 camelina genotypes to downy mildew and the efficacy of the tested fungicides on downy mildew control in field conditions. However, it appears that the fungicide application was too late. The disease progression halted, probably due to changes in environmental conditions and there was no significant effect of the fungicides. Hence, the only valuable data we obtained from this experiment is disease severity rating before the fungicide application. Moreover, the disease severity was determined at camelina's flowering stage. Therefore, it is also valuable to be compared with the disease severity evaluated from infected camelina seedlings in the PEI chamber.

3.3.2 Results

3.3.2.1 Canning Isolate

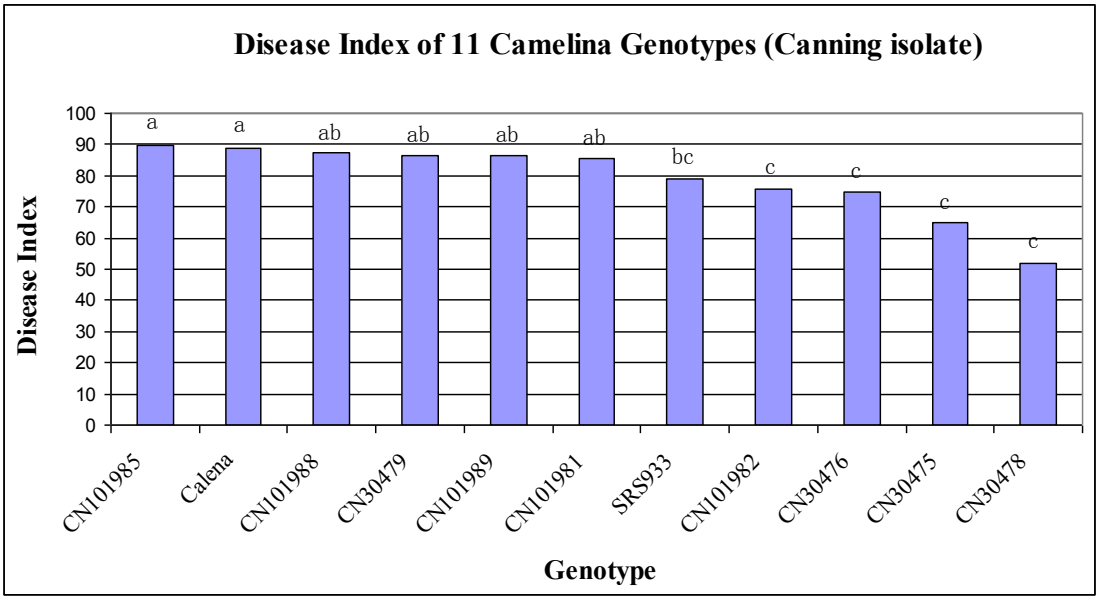

Figure 3.1: Downy mildew severity on 11 camelina genotypes inoculated with the Canning isolate. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

In Figure 3.1, CN101985, Calena, CN101988, CN30479, CN101989, CN101981 and were more susceptible, whereas CN101982, CN30476, CN30475 and CN30478 were more resistant. There was no significant difference among CN101988, CN30479, CN101989, CN101981 and SRS933.

3.3.2.2 Truro Isolate

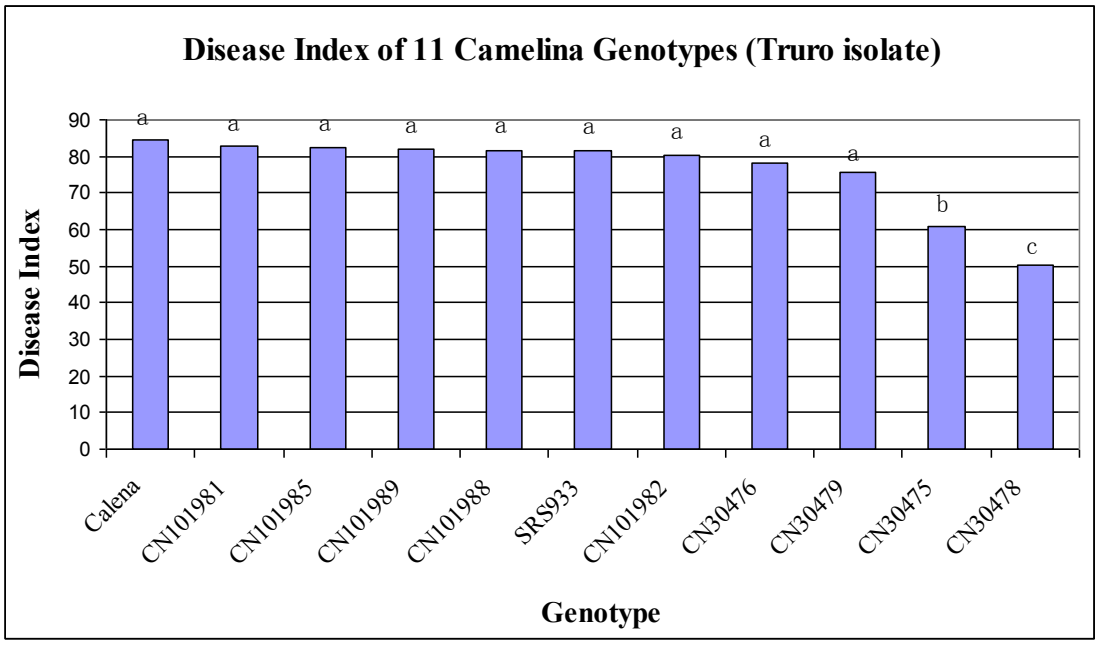

Figure 3.2: Downy mildew severity on 11 camelina genotypes inoculated with the Truro isolate. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

In Figure 3.2, Calena, CN101981, CN101985, CN101989, CN101988, SRS933, CN101982, CN30476 and CN30479 were the most susceptible genotypes with no significant difference among them. CN30478 was the most resistant.

3.3.2.3 PEI Isolate (In Growth Chamber)

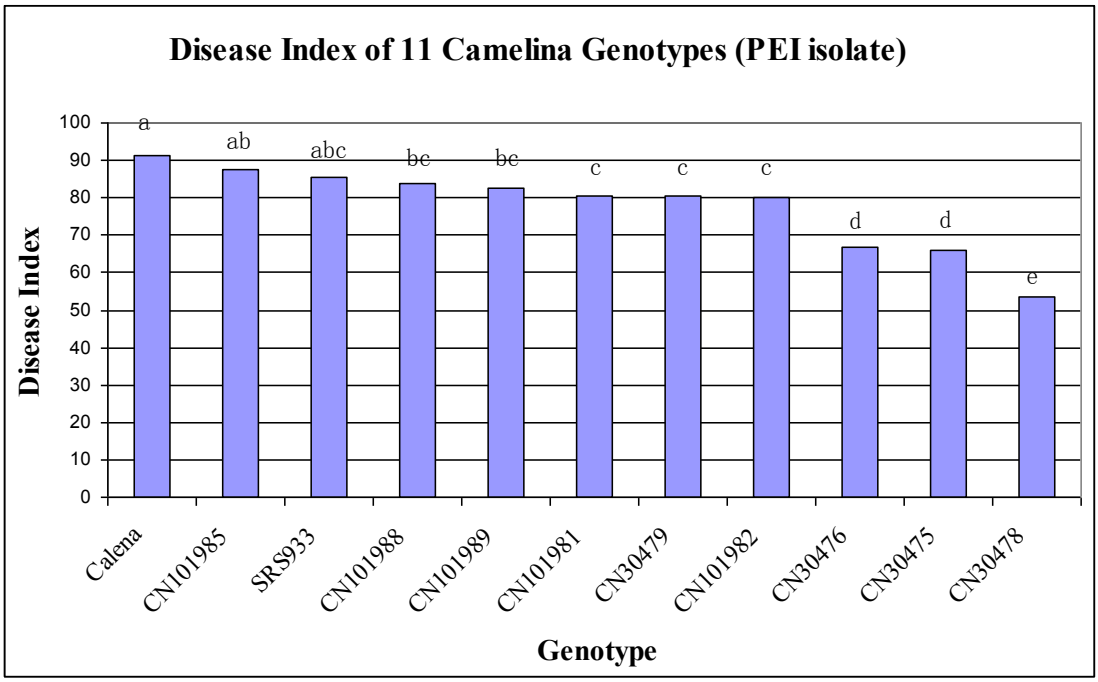

Figure 3.3: Downy mildew severity on 11 camelina genotypes inoculated with the PEI isolate. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

Figure 3.3 shows that Calena, CN101985 and SRS933 were the most susceptible genotypes, whereas CN30478 was the most resistant one. Further comments on this experiment can be found in the General Result 3.3.2.5.

3.3.2.4 PEI Isolate (In Field)

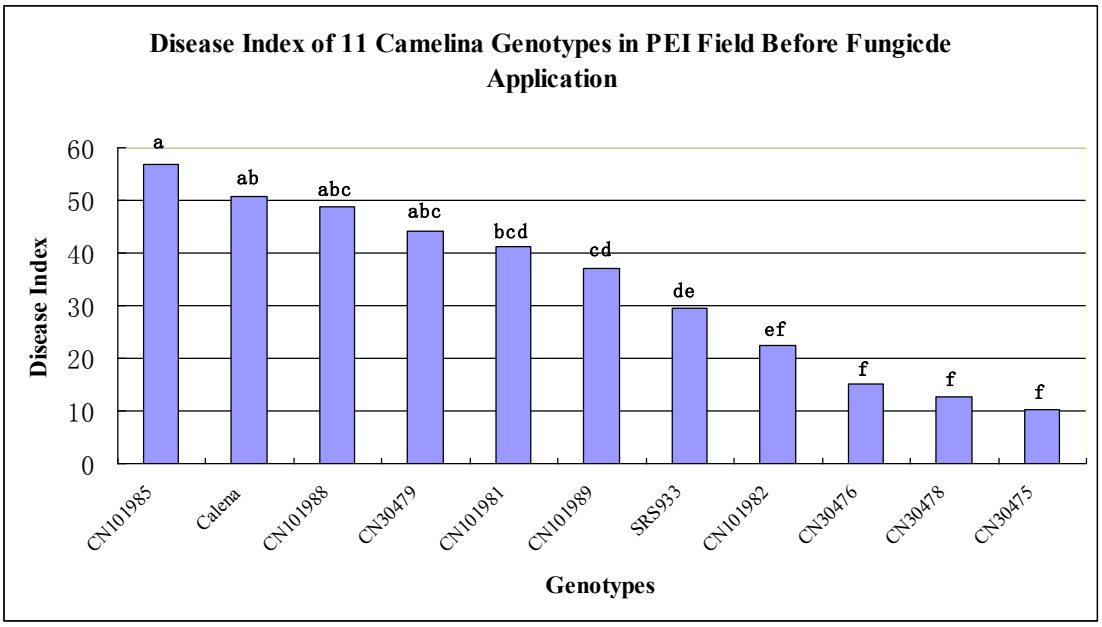

Figure 3.4: Downy mildew severity on 11 camelina genotypes inoculated with the PEI isolate in field before fungicide application in 2008. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

CN101985, Calena, CN101988 and CN30479 were the most susceptible genotypes, while CN101982, CN30476, CN30478 and CN30475 were the most resistant genotypes. The other genotypes were between these two extremes.

3.3.2.5 General Result

From the results of growth chamber experiments, Calena and CN101985 are the most susceptible genotypes, whereas CN30478 is the most resistant and CN30475 is the second most resistant genotype. Although the ranking of the remaining of the camelina genotypes varies a bit among the results, in general, they are at an intermediate level that attracts less interest than the most susceptible genotypes and the most resistant genotypes.

3.4 Experiment 2: Identification of Spore Suspension Concentration on Camelina Disease Resistance Performance

3.4.1 Purpose

In preliminary experiments, the PEI isolate exhibited a great aggressiveness by killing all camelina seedlings. With the inoculation by a high spore suspension concentration (70 000 spores/mL) of the PEI isolate, no seedlings survived 2 weeks. As a result, a much lower spore suspension concentration (10 000 spores/mL) was used to ensure the survival of inoculated seedlings under a successful infection. The purpose of this analysis was to determine whether there is a significant difference between inoculating camelina seedlings with either 10 000 spores/mL or 70 000 spores/mL using the isolates from Truro and Canning. The outcome of this experiment would help us to better understand the results of disease reaction of 11 camelina genotypes in Experiment 1.

3.4.2 Materials and Methods

3.4.2.1 Plant Preparation

Five camelina genotypes used were Calena, CN30475, CN30476, CN30478 and CN101985. All genotypes were seeded in multi-cell trays with four replications. Additionally, because there were two different concentrations of spore suspension used for each isolate, for each isolate, there were 40 randomized cells in total seeded with 5 camelina genotypes. Among the 40 cells, 20 cells were randomly selected and inoculated

with 10 000 spores/mL spore suspension and the other 20 cells were randomly inoculated with 70 000 spores/mL spore suspension. The seeding rate was 12 seeds per cell. All seeded multi-cell trays were kept in the Environmental Sciences' greenhouse of NSAC after seeding. Trays were watered daily until the sixth day after seeding. Before the inoculation, the number of seedlings was thinned to 7 seedlings per cell.

3.4.2.2 Inoculation

The inoculation method used in this experiment was the same as the one described in section 3.3.1.2.

3.4.2.3 Disease Severity Scale:

The disease severity scale used in this experiment was the same as the one mentioned in section 3.3.1.3.

3.4.2.4 Data Analysis:

This experiment was a two-factor factorial design with genotype and spore suspension concentration as the two factors.

The collected values of disease severity were used to calculate a disease index (DI) through the following equation:

$$DI = [\sum_{i=0}^{9} (i \times j)]/n \times 9$$

i=disease severity class, j=number plants/class, n=total number of plants (Ishii et al., 2001)

Then, the calculated DI from the isolates was analyzed separately using GLM procedure and LSMeans test of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) at the 5% significant level.

3.4.3 Results

3.4.3.1 First Disease Severity Readings (7 Days After Inoculation)

3.4.3.1.1 Truro Isolate

Table 3.2: P-values of factors and factor interaction of the Truro isolate experiment 7 days after inoculation.

Factors	Spore concentration	Genotypes	Spore concentration × Genotypes
P-value	0.0804	<0.0001	0.5520

Factors with P-value greater than 0.05 have no significant effect on plant disease index

In Table 3.2, the two spore concentrations had no significant effect on plant disease index, as well as the interaction between spore concentration and genotypes. However, there was a significant effect due to the camelina genotypes.

3.4.3.1.1 Canning Isolate

Table 3.3: P-values of factors and factor interaction of the Canning isolate experiment 7 days after inoculation.

Factors	Spore concentration	Genotypes	Spore concentration × Genotypes
P-value	0.1005	<0.0001	0.9283

Factors with P-value greater than 0.05 have no significant effect on plant disease index

From the analyzed result, the factor spore concentration and the interaction spore concentration × genotypes had no significant effect on plant disease index, whereas there was a significant difference among the camelina genotypes.

3.4.3.2 Second Disease Severity Readings (12 Days After Inoculation)

3.4.3.2.1 Truro isolate

Table 3.4: P-values of factors and factor interaction of the Truro isolate experiment 12 days after inoculation.

Factors	Spore concentration	Genotypes	Spore concentration × Genotypes
P-value	0.023	<0.0001	0.9381

Factors with P-value greater than 0.05 have no significant effect on plant disease index

From Table 3.4, we can see that both spore concentration and genotypes affected the plant disease index significantly, whereas the interaction between the two factors did not.

Table 3.5: Mean disease index of five camelina genotypes on two Truro isolate spore concentration 12 days after inoculation

Spore concentration (Spores/mL)	Mean Disease Index
10 000	0.802 b
70 000	0.8685 a

Numbers with different letters are significantly different

From Table 3.5, higher spore concentration contributed to significantly higher disease severity 12 days post inoculation when camelina seedlings were inoculated by the Truro isolate.

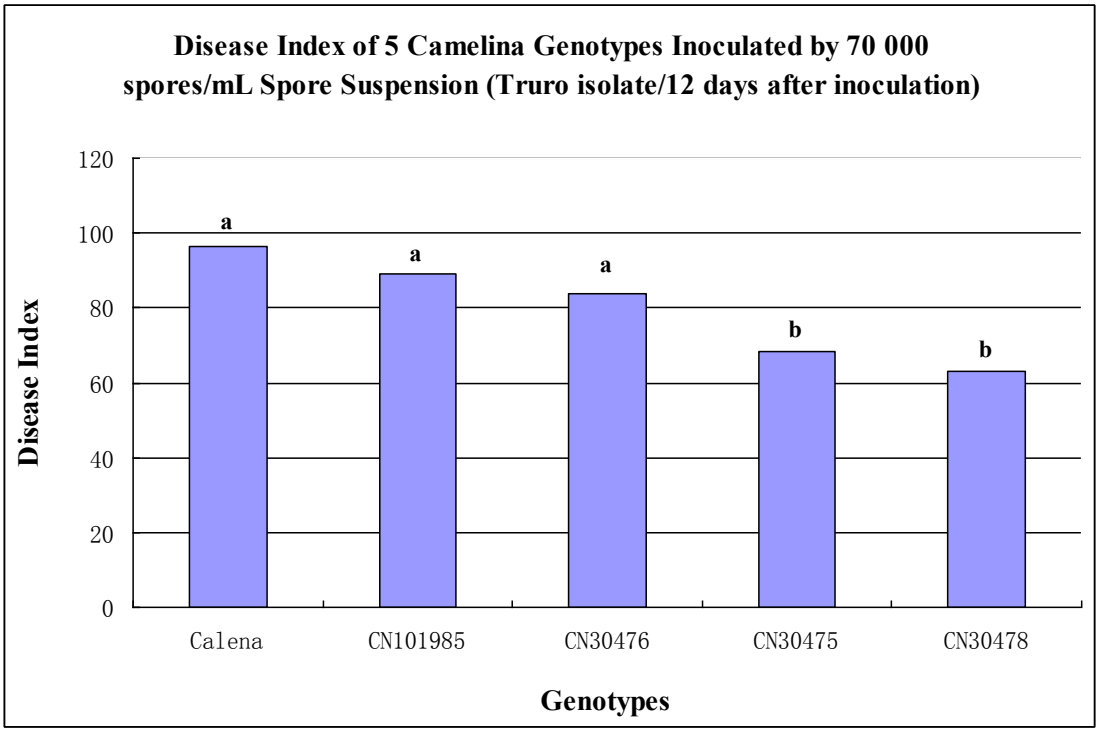

Figure 3.5: Downy mildew severity on five camelina genotypes inoculated by the Truro isolate (70 000 spores/mL) 12 days after inoculation. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

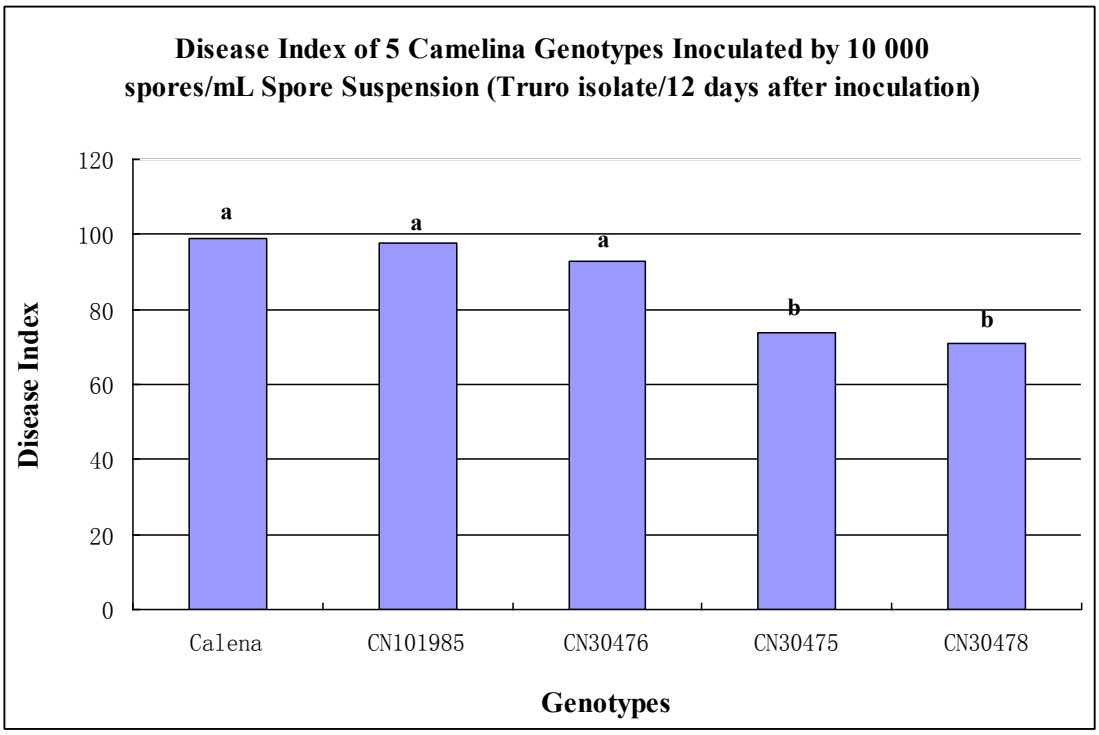

Figure 3.6: Downy mildew severity on five camelina genotypes inoculated by the Truro isolate (10 000 spores/mL) 12 days after inoculation. Disease severity was evaluated by DI calculation. DI's with same letters are not significantly different (P=0.05).

From Figure 3.5 and Figure 3.6, although DI varies, the rankings of the five genotypes are exactly the same, including the letter grouping.

3.4.3.2.2 Canning Isolate

Table 3.6: P-values of factors and factor interaction of the Canning isolate, 12 days after inoculation.

Factors	Spore concentration	Genotypes	Spore concentration × Genotypes
P-value	0.5449	<0.0001	0.5242

Factors with P-value greater than 0.05 have no significant effect on plant disease index

Table 3.6 indicates that spore concentration and the interaction between spore concentration and genotypes did not affect plant disease index significantly, whereas

significant difference existed among the genotypes.

3.5 Discussion

The first disease severity reading one week after inoculation in Experiment 2 (Table 3.2 and Table 3.3) indicated that different spore suspension concentrations of both isolates had no significant effect on the plant disease index. This suggests that it is possible to compare different camelina genotypes' disease resistance performance when they were inoculated with 70 000 spores/mL using the Truro and Canning isolates and 10 000 spores/mL for the PEI isolate in Experiment 1. The same was observed on seedlings inoculated by the Canning isolate in the second (12 days) disease severity reading (Table 3.6). In the second disease severity reading, for the seedlings inoculated by Truro isolate, both spore concentration and genotypes showed significant impact on disease severity (Table 3.4 and Table 3.5). However, the interaction between these two factors was not significant, i.e., although spore concentration affected disease severity significantly, it did not affect the ranking of the genotypes (Figure 3.5 and Figure 3.6). Therefore, disease resistance levels of camelina inoculated by 10 000 spores/mL and that inoculated by 70 000 spores/mL of the Truro isolate are comparable.

In general, the result of Experiment 2 suggested that we can still compare the results obtained from Experiment 1, although we used a different spore concentration for the PEI isolate. Moreover, since the disease resistance of 11 camelina genotypes was evaluated separately in terms of the three isolates, the results obtained from Experiment 1 can be

valuable for understanding the ability of each camelina genotype for downy mildew resistance.

In Experiment 1, CN30478 consistently displayed the best resistance to the three downy mildew isolates. CN30475 is the second most resistant genotype. On seedlings of these two genotypes, necrotic spots probably caused by hypersensitive reaction (HR) could be observed on infected cotyledons, implying the presence of a vertical resistance (Figure 3.7) in these two genotypes' defense system. In contrast, DI's of CN101985 and Calena were always the highest when they were infected by the three isolates, indicating their highest susceptibility to downy mildew. In addition, necrotic spots were not or rarely observed on infected cotyledons, suggesting the presence of horizontal resistance with low disease defense efficiency against the three isolates. Downy mildew resistance performance of the remaining genotypes indicated that those genotypes possess intermediate disease resistance. In the DI results obtained from the PEI growth chamber and PEI field (section 3.3.2.3 and section 3.3.2.4), although the genotype ranking differs, for the extremes, Calena, CN101988 and CN101985 are the most susceptible genotypes in both results, whereas CN30478, CN30476 and CN30475 are the most resistant. This indicates that, in general, camelina genotypes' disease resistance to the PEI isolate displayed at cotyledon stage is a good indicator of their disease resistance at the pre-flowering stage. Among the 11 genotypes, special attention needs to be paid to CN30476, CN101982 and SRS933. CN30476 was resistant to the Canning isolate (Figure 3.1) and moderately resistant to the PEI isolate (Figure 3.3). However, it was susceptible

to the Truro isolate (Figure 3.2). The performance of CN101982 was similar with that of CN30476. The only difference was that, compared with CN30476, CN101982 was a bit more susceptible to the PEI isolate (Figure 3.3). SRS933 was susceptible to the Truro and PEI isolates (Figure 3.2 and Figure 3.3), whereas it was resistant to the Canning isolate (Figure 3.1). It can therefore be inferred that the three isolates are different from one another. This evidence also indicates a vertical resistance was employed by CN30476, CN101982 and SRS933.

Figure 3.7 Necrotic spots probably caused by hypersensitive reaction on cotyledons

Seedlings of camelina genotypes at the cotyledon stage were tested for their resistance/susceptibility to downy mildew in this experiment. For further experiments, disease resistance needs to be evaluated at some other growth stages, such as the eight-leaf-stage and/or flowering stage. Furthermore, although disease resistance of 11

genotypes was evaluated in PEI field under natural infection, it should be repeated at various locations under natural conditions. Evaluations under field conditions are important because experiments under controlled environments are not always indicative of the real environment. The results collected from both field and growth chamber experiments will provide a whole picture on each genotype's downy mildew resistance performance. Then, the identified resistant genotypes will be valuable in future breeding programs. The present data is a good start to help the breeders.

3.6 References

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Chapter 4: Identification of the efficacy of four fungicides on downy mildew control on *Camelina sativa*

4.1 Abstract

Camelina (*Camelina sativa*) is characterized by its great plant disease resistance. In camelina fields, *Peronospora parasitica* (downy mildew) is the only disease that has been reported the most frequently. To date, no fungicide has been registered for downy mildew control in camelina. Therefore, four fungicides for downy mildew control, Dithane DG (mancozeb), Bravo 500 (chlorothalonil), Tanos 50 DF (famoxadone/cymoxanil) and Tattoo C (propamocarb/chlorothalonil) were chosen and identified for their efficacy of a single application for downy mildew control on four camelina genotypes when the disease first appears. The investigation consisted of a field fungicide experiment and a growth chamber fungicide experiment. The efficacy was evaluated by measuring their effects on disease severity and yield. The results suggest that none of these fungicides can be recommended for downy mildew control in camelina and selecting resistant camelina genotypes for cropping holds more promise as a method of downy mildew control.

4.2 Introduction

Camelina sativa (*C. sativa*) is an oilseed crop which belongs to the Brassicaceae family (Bonjean and Le Goffic, 1999). It has been shown that *C. sativa* has a great capacity for disease resistance so that it may not need much chemical protection during the cropping season (Zubr, 1997). However, some pathogens have still been observed in camelina fields: *Pseudomonas syringae* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Rhizoctonia solani* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Sclerotinia sclerotiorum* (Zubr, 1997; Séguin-Swartz et al, 2009), *Peronospora parasitica* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009), *Erysiphe* spp. (Föller *et al.*, 1998), *Albugo candida* (Föller *et al.*, 1998; Séguin-Swartz et al, 2009) and *Botrytis cinerea* (Zubr, 1997). Among these pathogens, *Peronospora parasitica* (downy mildew) is the most severe since it has been observed most frequently in the field (Vollmann *et al.*, 2001). *P. parasitica* prefers a cool environment which ranges from 8ºC to 24ºC to develop (Slusarenko and Schlaich, 2003). Oospores germinate and cause primary infection in the spring and secondary infection is by asexual mitospores (Slusarenko and Schlaich, 2003). To date, no fungicide has been registered for camelina (Anonymous, 2007a; McVay and Lamb, 2008). Gisi and Sierotzki (2008) suggested that chemical control is still the most effective way to control downy mildew in the field; however, Farnham *et al.* (2002) suggested that cropping a resistant variety is a long-term solution for downy mildew control.

Dithane DG (mancozeb), Bravo 500 (chlorothalonil), Tanos 50 DF

(famoxadone/cymoxanil) and Tattoo C (propamocarb/chlorothalonil) were the four fungicides chosen in this experiment. Among these four fungicides, both Dithane DG and Bravo 500 have been used for downy mildew control on some brassicas (Anonymous, 2001; Anonymous, 2006); whereas, Tanos 50 DF and Tattoo C have been used for downy mildew control on crops belonging to other non-brassicaceae families (Anonymous, 2007b; Anonymous, 2007c). These four fungicides were evaluated for efficacy on downy mildew control in camelina. Among these fungicides, Dithane DG and Bravo 500 are protectant fungicides that are applied prophylactically to the target crop to form a barrier on the crop surface against a pathogen's infection (Anonymous, 2001; Anonymous, 2006; Hewitt, 1998). Protectant fungicides are immobile and are not absorbed into plant tissues (Hewitt, 1998). Therefore, they stay on the plant surface and keep active in early stages of fungal infection (Hewitt, 1998). Because of their multi-site mode of action, they can protect the crop against a wide variety of leaf diseases (Hewitt, 1998). In contrast, both Tanos 50 DF and Tattoo C are systemic fungicides (Anonymous, 2007b; Anonymous, 2007c). Systemic fungicides can penetrate and be translocated within the plant (Hewitt, 1998). They tend to provide longer term disease control due to the fact that they are not weathered off plant surface after being absorbed (Hewitt, 1998). Therefore, they will still be effective after infection and this is so-called curative and eradicant activities of systemic fungicides (Hewitt, 1998). However, because they usually function only on one specific mechanism of the pathogen, they are easy to be overcome by a pathogen's evolution (Hewitt, 1998). Therefore, systemic fungicides are usually applied with

protectant fungicides; for instance, systemic chemicals in Tanos 50 DF and Tattoo C are cymoxanil and propamocarb, respectively, whereas the other two chemicals are protectant (Anonymous, 2007b; Anonymous, 2007c). Famoxadone in Tanos 50 DF inhibits the function of the ubiquinol cytochrome c oxido-reductase enzyme complex which catalyzes oxidative phosphorylation activity (Hewitt, 1998). The other chemical, cymoxanil, contained in Tanos 50 DF affects the hyphal growth stage (Hewitt, 1998). It was observed to inhibit nucleic acid and protein biosynthesis in *Phytophthora cinnamomi* and *Botrytis cinerea*. Propamocarb in Tattoo C is thought to affect membrane function of the pathogen; for instance, it causes efflux of cell constituents such as phosphate, carbohydrate and protein from *Pythium ultimum* (Hewitt, 1998). Both cymoxanil and propamocarb move upward through the xylem after being absorbed into the plant (Spencer-Phillips *et al.*, 2002; Grünwald *et al*., 2006).

The objective of this experiment was to evaluate the efficacy of the four selected fungicides in both field and growth chamber experiments. In all trials, the efficacy was measured by evaluation of disease severity; grain yield was also determined in field experiments as a quantitative measure of efficacy.

4.3 Hypothesis

1. Tanos 50 DF and Tattoo C will control downy mildew more effectively than Bravo 500 and Dithane DG in the field.

2. Tanos 50 DF and Tattoo C control downy mildew more effectively than Bravo 500

and Dithane DG in growth chamber experiments when fungicides are applied after inoculation.

4.4 Experiment 1: Field Fungicide Trials

4.4.1 Materials and Methods

4.4.1.1 Site Description and Treatments

This field fungicide experiment was conducted in 2009 to identify the efficacy of four fungicides on downy mildew control. The experiment was set up at three sites, including the Plumdale Farm of Nova Scotia Agricultural College (NSAC; Plumdale Trial), Truro, NS; Brookside Field of NSAC (Brookside Trial), Truro, NS and Lyndhurst Farms Ltd. (Canning Trial), Canning, NS. Herbicide Bonanza 400 Liquid (Trifluralin 1000 g/ha) was applied in Canning Trial on April 28th, 2009, whereas the Plumdale Trial and the Brookside Trial were treated with herbicide Treflan EC (PPI; Trifluralin 920 g/ha) on May 12th, 2009. Both Bonanza 400 and Treflan EC are effective on most annual grasses, such as barnyard grass (*Echinochola crusgalli* L.), crab grass (*Digitaria ischaemum)* and annual bluegrass (*Poa annua* L.; Anonymous, 2005a; Anonymous, 2005b). However, weeds were still a big problem in our trials. Weeds which were the largest problem at the Canning Trial were the ragweed (*Ambrosia artemisiifolia*), some lambs quarters (*Chenopodium album* L.) and wild buckwheat (*Polygonum convolvulus* L.). In the Plumdale and the Brookside trials, the largest weed problem was the wild

radish (*Raphanus raphanistrum* L.) and some lambs quarters (*D. ischaemum*). Potassium and phosphor content in all our trials was considered to be at high levels. In order to provide approximately 100 kg/ha nitrogen for our trials, fertilizer was applied before planting 190 kg/ha 18-5-5 at Canning and 370 kg/ha 14-14-14-10.19S at both the Plumdale Trial and the Brookside Trial. A second fertilizer application was done at Canning with 110 kg/ha of 46-0-0 on June 10th, 2009 and in both the Plumdale Trial and the Brookside Trial with 190 kg/ha 27-0-0 on June 26th, 2009. The experiment was designed as a randomized complete block design with four replicates. Each block consisted of 20 plots with 16 rows at 15 cm spacing. Eight middle rows were used for data collection to avoid edge effects. The area of each plot was 3 m × 2.75 m. Four camelina genotypes (three gene bank accessions and one cultivar), CN30476, CN30478, CN101985 and Calena (cv), were used in this experiment. Among these four genotypes, CN101985 and Calena were previously shown to be highly susceptible to downy mildew, whereas CN30478 was found to be the most resistant genotype against downy mildew (Chapter 3). CN30476 was previously found to be resistant to the Canning isolate and moderately resistant to the PEI isolate. However, it was susceptible to the Truro isolate. In 2008, the previous crop planted at the Plumdale, Brookside and Canning trial sites were winter cereal, soybean and camelina, respectively. The Canning Trial was seeded on April 30th, 2009, whereas both the Plumdale Trial and the Brookside Trial were seeded on May 13th, 2009. All trials were seeded using a double disc Hege 80 plot drill. The seeding rate at all sites was 500 seeds/m^2. Fungicides were applied at the first sign of disease. Hence,

fungicides were sprayed by a bicycle sprayer with TEEJET 8003 nozzles (the CO_2 pressure was set at 30 psi) on June 9th, 2009 for the Canning Trial. The same fungicide sprayer was used for the Brookside Trial and the Plumdale Trial on June 18th, 2009 and June 19th, 2009, respectively. The applied fungicide rate and water volume are described in Table 4.7.

4.4.1.2 Data Collection

Disease severity ratings and grain yield were recorded in this experiment. Disease severity was rated once a week until the third week after fungicide application in each trial. In each plot, only the middle eight rows were used for disease severity rating and harvesting. An "X" pattern was employed in each plot for disease severity rating (Figure 4.1). At each sampling point, five plants were evaluated for disease severity. Therefore, 50 plants in each plot were evaluated.

Mature camelina in the centre eight rows was harvested in each plot and the yield was weighed and analyzed for fungicide efficacy evaluation. The Canning Trial was harvested on August 14th, 2009; the Plumdale Trial was harvested on August 26th, 2009 and the Brookside Trial was harvested on September 9th, 2009.

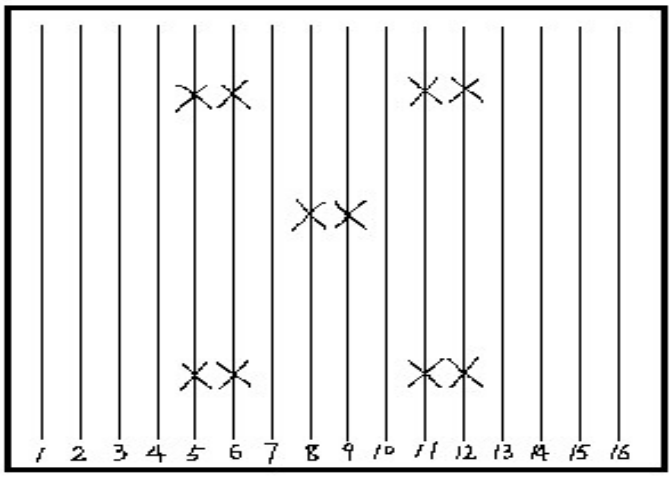

Figure 4.1: Sampling pattern in one plot. "X" in figure means sampling point. Five plants were evaluated at each sampling point.

4.4.1.3 Disease Severity Scale

The following scale (0-9) was used to evaluate the disease severity in the field:

Table 4.1: Disease Severity Scale

Class	Description
0	No sporulation or symptoms
1	1-20% of plant is infected
3	21-40% of plant is infected
5	41-60% of plant is infected
7	61-80% of plant is infected
9	81-100% of plant is infected

4.4.1.4 Data Analysis

This experiment was a two-factor factorial design and also a randomized complete block design with genotype and fungicide as the two factors.

4.4.1.4.1 Yield

Yield obtained from each trial was analyzed using PROC Mixed procedure and LSMeans test of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) at the 5% significant level.

4.4.1.4.2 Disease Index

The observed values of disease severity were used to calculate a disease index (DI) using the following equation:

$$DI = [\sum_{i=0}^{9} (i \times j)]/n \times 9$$

i=disease severity class, j=number plants/class, n=total number of plants (Ishii et al., 2001)

Then the calculated DI from the trials was analyzed separately using PROC Mixed procedure and LSMeans test of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) at the 5% significant level.

4.4.2 Results

4.4.2.1 Disease Severity Evaluation

Table 4.2: P-values of factors and factor interaction affecting disease severity in field fungicide experiment of the three sites.

Trial Site	Disease reading date	Factors		
		Genotype	Treatment	Genotype*Treatment
Canning Trial	first rating (17/06/1009)	<0.0001	0.1028	0.0328
	second rating (25/06/2009)	<0.0001	0.4775	0.9756
	third rating (03/07/2009)	<0.0001	0.9770	0.9798
Plumdale Trial	first rating (26/06/1009)	<0.0001	0.1448	0.0599
	second rating (02/07/2009)	<0.0226	0.5019	0.0169
	third rating (10/07/2009)	<0.0001	0.8296	0.7454
Brookside Trial	first rating (26/06/1009)	<0.0001	0.8967	0.4996
	second rating (02/07/2009)	<0.0001	0.6913	0.2524
	third rating (10/07/2009)	<0.0001	0.7231	0.9467

Factors with P-value greater than 0.05 are deemed to have no significant effect on plant disease index

From Table 4.2, we can find that the interaction of genotype and treatment

significantly affected disease rating in the first rating of the Canning Trial (details in Table 4.4) and the second rating of the Plumdale Trial. Because the highest disease index in the second rating of the Plumdale Trial was only 4.73% (maximum level was at 100%) which is considered to be too low to present the efficiency of the treatments, the data are not displayed. In the other ratings, genotypes consistently and significantly affected disease index (details in Table 4.3), whereas treatment and the interaction of genotype and treatment did not have a significant effect.

Table 4.3: Downy mildew severity on four camelina genotypes in three field fungicide trials. Disease severity was evaluated by DI calculation.

Trial Site	Disease reading date	Genotype			
		Calena	CN101985	CN30478	CN30476
Canning Trial	second rating (25/06/2009)	12.61a	10.59a	3.05b	0.89b
	third rating (03/07/2009)	5.52a	6.53a	1.26b	1.20b
Plumdale Trial	first rating (26/06/1009)	2.60a	2.32a	0.86ab	0.20b
	third rating (10/07/2009)	2.88a	4.36a	0.96b	0.27b
Brookside Trial	first rating (26/06/1009)	13.55a	9.93b	0.86c	0.23c
	second rating (02/07/2009)	8.08a	4.37b	1.21c	0.36c
	third rating (10/07/2009)	6.06a	5.06b	0.62c	0.32c

Numbers in the same row with the same letter are not significantly different at the 5% confidence level.

In Table 4.3, Calena, without exception, was the most susceptible genotype, whereas CN30476 and CN30478 were the most resistant. In the Canning and the Plumdale trials, CN101985 was one of the most susceptible genotypes, whereas it was moderately susceptible at Brookside.

Table 4.4: Ranking and letter grouping of interactions of Genotype*Treatment in the first rating of Canning Trial

Genotypes	Treatment	Disease Index (%)	Letter Grouping
Calena	Bravo 500	29.53	a
Calena	Check	28.83	a
Calena	Tattoo C	26.08	ab
Calena	Dithane DG	24.48	bc
Calena	Tanos 50 DF	22.85	bcd
CN101985	Check	21.83	cd
CN101985	Bravo 500	21.38	cd
CN101985	Dithane DG	21.35	cd
CN101985	Tanos 50 DF	19.40	de
CN101985	Tattoo C	16.20	e
CN30478	Tattoo C	5.23	f
CN30478	Bravo 500	5.18	f
CN30478	Tanos 50 DF	5.00	f
CN30478	Dithane DG	4.95	f
CN30478	Check	4.08	fg
CN30476	Dithane DG	3.73	fg
CN30476	Check	2.90	fg
CN30476	Tattoo C	2.83	fg
CN30476	Tanos 50 DF	2.20	fg
CN30476	Bravo 500	1.10	g

Numbers with the same letter are not significantly different.

In Table 4.4 above, the check plots for CN30478 and CN30476 and the fungicide treatments on CN30476 showed the best result on downy mildew control, whereas Calena treated with Bravo 500, no fungicide and Tattoo C showed the worst control on downy mildew.

4.4.2.2 Yield

Because of a severe weed problem found in the Plumdale and Brookside trials, replicate four at Plumdale and replicate three and replicate four at Brookside were not used in the final analysis. Seed yield analyses is shown in Table 4.5.

Table 4.5: P-value of factors and factor interaction affecting yield in field fungicide experiment of the three sites.

Trial Site	Factor		
	Genotype	Treatment	Genotype* Treatment
Canning Trial	<0.0001	0.7506	0.9384
Plumdale Trial	0.5816	0.5405	0.8159
Brookside Trial	0.0676	0.9455	0.8009

Factors with P-value greater than 0.05 are deemed to have no significant effect on plant disease index

In Table 4.5, among the factors and factor interactions in all three trials, only Genotype at Canning had a significant effect on yield (shown in Table 4.6).

Table 4.6: Result of yield in Canning Trial.

Genotype	Yield (kg/ha)	Letter grouping
CN30476	497.42	a
CN30478	428.03	b
Calena	297.85	c
CN101985	281.2	c

Numbers with the same letter are not significantly different at the 5% confidence level.

CN30476 was the highest yielding entry, while CN30478 was the second highest (Table 4.6); Calena and CN101985 were the lowest yielding entries. This is an interesting observation when it is associated with Table 4.3. The two resistant genotypes, CN30476 and CN30478, had significantly higher yield than the other two susceptible genotypes.

4.5 Experiment 2: Growth Chamber Fungicide Trials

4.5.1 Sub-experiment 1: Fungicide Application Before Inoculation

4.5.1.1 Plant Preparation

Calena was the only camelina genotype used in this experiment. It was sown into 25 15-cm diameter plastic pots. However, only 20 pots of the healthiest plants were selected to establish the trial which was set up as a 5 (treatments) × 4 (replications) layout. In each pot, 12 seeds were sown in Pro-mix, a commercial peat, vermiculite and perlite mix adjusted to pH 6.5 with lime. After seeding, all the pots were placed into a greenhouse set at 18 - 25ºC (day-night). Standard greenhouse sodium vapour lamps were set at 7 am/19

pm day/night regime. The light intensity varied from 140 – 880 μmol s^{-1} m^{-2} depending on the weather and time of day.

The pots placed in the greenhouse were watered thoroughly every day. After 2 weeks, the seedlings were approximately at the four leaf stage and the number of the seedlings per pot was thinned to seven and then approximately 2 weeks later thinned from seven to five. In the fifth week, 20 pots of the healthiest plants were selected for fungicide spray. In order to create four relatively uniform groups, 20 pots of plants were divided into four groups according to the condition of plant growth (the best; the second best; the third best and the fourth best growth condition). Therefore, in each group, there were five pots of plants which were randomly arranged into the five treatments, respectively. In other words, in each treatment, there were four pots serving as replications. Each pot was selected randomly from one class. Therefore, the four pots were from four different groups.

4.5.1.2 Fungicide Application

The four fungicides used in this experiment were applied at the highest recommended rate (Table 4.7). According to these rates and the surface area of the four pots (0.072 m^2) in each treatment, the amount of fungicide and water volume was determined. However, because there were only four pots in each treatment, the required amount of each fungicide was too small to spray accurately. Therefore, we sprayed a much larger area 0.5 m (the width of spray coverage provided by the single nozzle) x 4 m and pots were placed in the centre of this area as a 2 pots × 2 rows layout using a newly calculated amount of fungicide and water (Table 4.7).

A CO_2 pressurized sprayer with a single TZ 8002 VS nozzle was used to apply the fungicide. The CO_2 pressure was set at 30 psi . All the sprays were done outside.

Table 4.7: Parameters related to fungicide application for Growth Chamber Fungicide Study

Fungicide Names	Highest Recommended Application Rate	Water Volume Rate	The amount of undiluted fungicides	Applied Water Volume
Bravo 500	4.8 L/ha	225 L/ha	0.96 mL	45 mL
Dithane DG	3.25 kg/ha	225 L/ha	0.65 g	45 mL
Tanos 50 DF	840 g/ha	250 L/ha	0.168 g	50 mL
Tattoo C	2.7 L/ha	300 L/ha	0.54 ml	60 mL

After the fungicide application, all plants were placed into a growth chamber set at 17 ºC overnight. The next day, all plants were inoculated as described below.

4.5.1.3 Inoculation

In this experiment, the downy mildew isolate collected from Truro, NS was used to make a 70 000 spores/mL spore suspension for inoculation. One millilitre of the spore suspension was dropped onto leaves of the five plants in each pot using a 3 mL syringe with a hypodermic needle attached. After inoculation, plants were put into individually misted bags (180 mm × 75 mm × 510 mm) to create 100% relative humidity overnight. The next day, bags were removed and pots were placed into a growth chamber set at 14 hours light (incandescent and cool white fluorescent combination)/ 10 hours darkness with 17ºC and 60% - 70% relative humidity. RH was maintained by a Honeywell Cool

Moisture Humidifier HCM-630 and monitored by a Nexxtech™ Indoor Thermo/Hygrometer. The light intensity was 88 μmol s^{-1} m^{-2}. Plants were then put in misted bags overnight every 5 days, 3 times. Following that, plants were rated for disease severity.

4.5.2 Sub-experiment 2: Fungicide Application After Inoculation

The design of this experiment was the same as sub-experiment 1 except that the plants were inoculated 1 week before the fungicide application. The parameters related to fungicide application were as described in Table 4.7. After inoculation, plants were put into misted bags overnight and then placed into a growth chamber on the following day. On the sixth day after inoculation, plants were put back into misted bags overnight to promote sporulation and then, on the seventh day, fungicide treatments were applied. Then, the plants were put back into misted bags every 5 days from the sixth day until the 16^{th} day which was followed on the 17^{th} day by disease severity reading.

4.5.3 Disease Severity Scale

Disease severity of infected leaves and infected stems was evaluated separately using two different disease severity scales, as shown below. Then, a disease index of each pot was calculated.

4.5.3.1 Disease Severity Scale of Infected Leaves

Table 4.8: Disease severity scale for infected leaves

Class	Description
1	Small pinpoint to larger brown necrotic flecks on the leaf. No sporulation
3	Only sparse sporulation on one side of the leaf
5	Dense white sporulation on one side of the leaf or sparse sporulation on both sides of the leaf
7	Sparse to medium sporulation on a distorted leaf
9	Dense sporulation on a distorted leaf

4.5.3.2 Disease Severity Scale of Infected Stems

Table 4.9: Disease severity scale for infected stems

Class	Description
1	1-2 cm of the stem is sporulating
3	2-4 cm of the stem is sporulating
5	4-6 cm of the stem is sporulating
7	6-8 cm of the stem is sporulating
9	More than 8 cm of the stem is sporulating

The collected values of disease severity were used to calculate a disease index (DI) using the following equation:

$$DI = \{[\sum_{i=0}^{9} (i \times j)]/n_l \times 9\} \times (n_l/N_l) \times 100\% + \{[\sum_{a=0}^{9} (a \times b)]/n_s \times 9\} \times (n_s/N_s) \times 100\%$$

i=disease severity class of leaf, j=number infected leaves/class, n_l=total number of infected leaves, N_l=total number of leaves of each plot, a=disease severity class of stem, b= number infected stems/class, n_s=total number of infected stems and N_s=total number

of stems of each plot

Then, the calculated DI was analyzed separately using GLM and LSD of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

4.5.4 Results

4.5.4.1 Fungicide Application Before Inoculation

4.5.4.1.1 First Disease Index Rating (Because the original data did not fit a normal distribution and therefore they were transformed by log10 to satisfy a normal distribution)

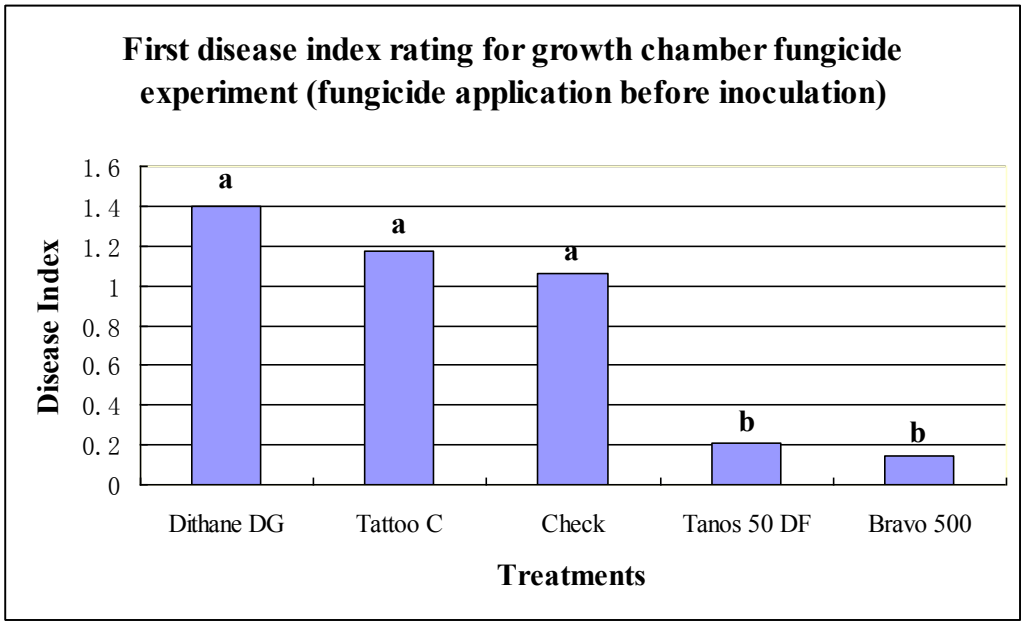

Figure 4.2: First disease index rating for growth chamber fungicide experiment (fungicide application before inoculation)

From Figure 4.2, we can see that Bravo 500 and Tanos 50 DF could significantly control downy mildew, whereas Dithane DG and Tattoo C could not.

4.5.4.1.2 Second Disease Index Reading (Because the original data did not fit a normal distribution and therefore they were transformed by log10 to satisfy a normal distribution)

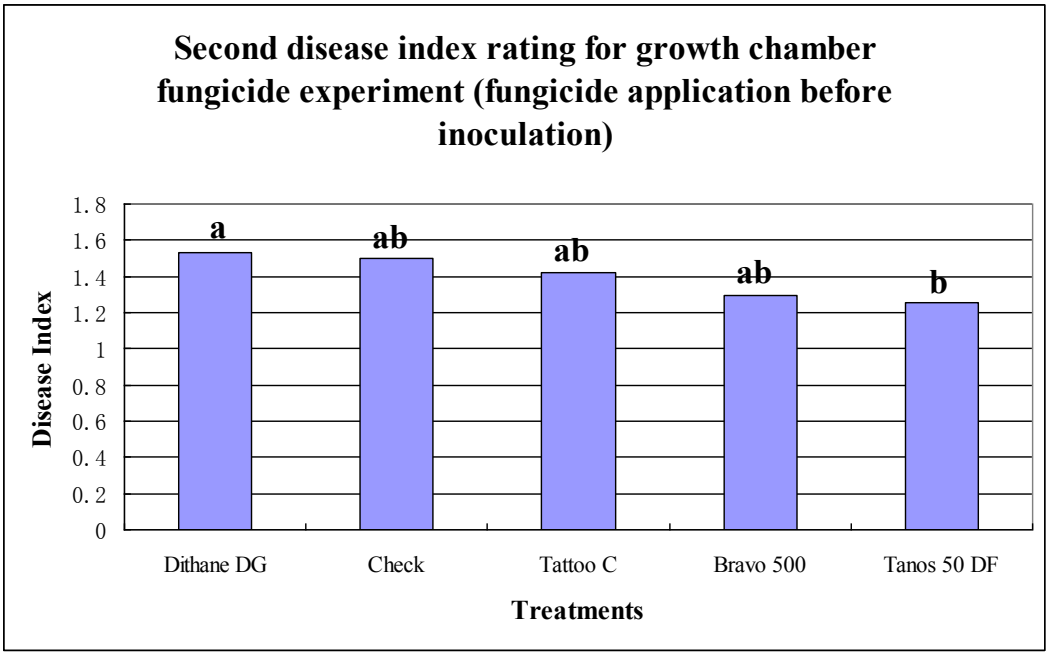

Figure 4.3: Second disease index reading for growth chamber fungicide experiment (fungicide application before inoculation)

In Figure 4.3, compared with the check, no fungicide significantly reduced the severity of downy mildew. In addition, Tanos 50 DF showed significantly difference from Dithane DG on disease severity.

4.5.4.2 Fungicide Application After Inoculation

4.5.4.2.1 First Disease Index Reading (Because the original data did not fit a normal distribution and therefore they were transformed by log10 to satisfy a normal distribution)

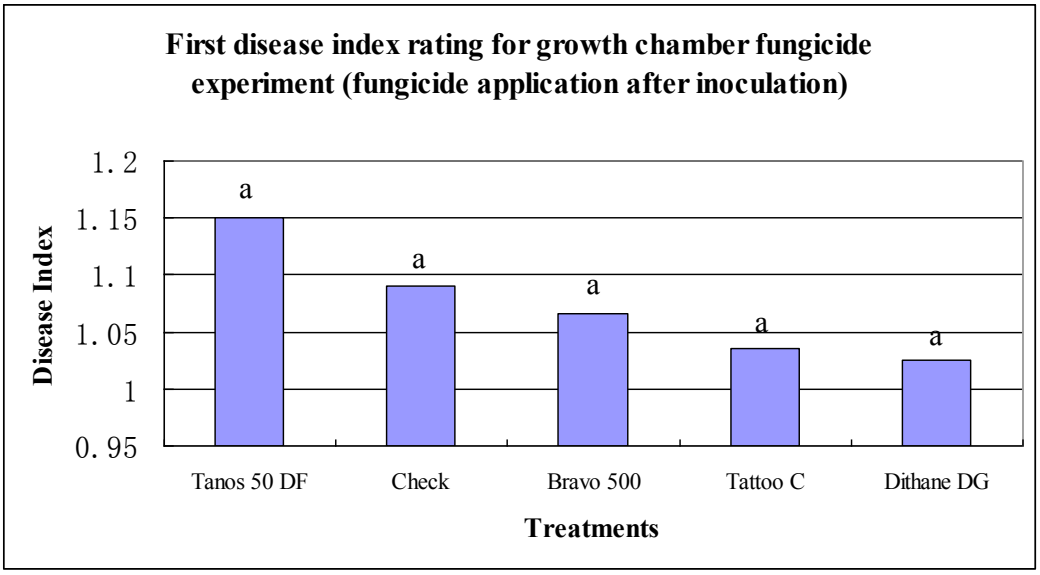

Figure 4.4: First disease index reading for growth chamber fungicide experiment (fungicide application after inoculation)

Figure 4.4 shows that no fungicide controlled downy mildew significantly.

4.5.4.2.2 Second Disease Index Reading

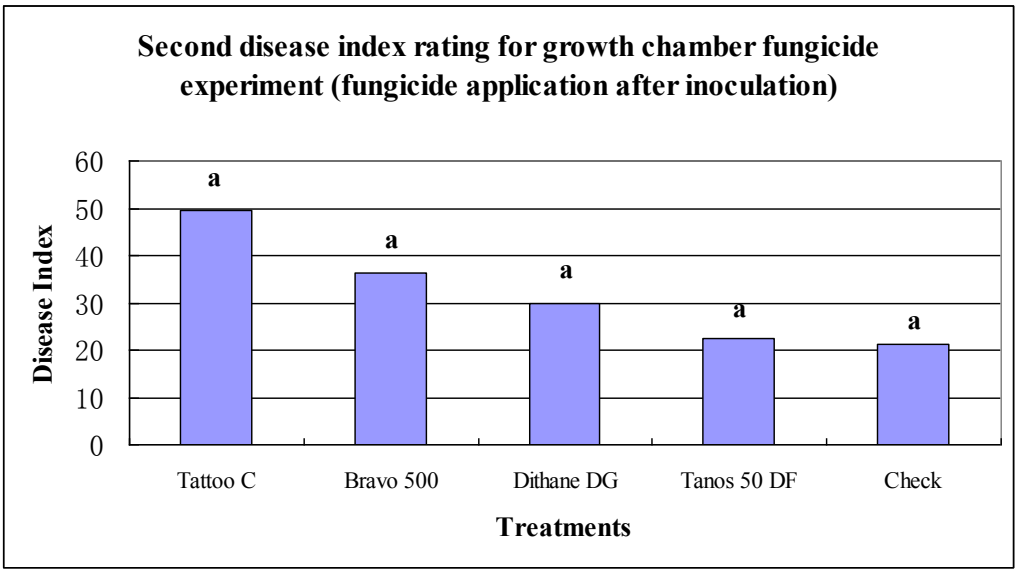

Figure 4.5: Second disease index reading for growth chamber fungicide experiment (fungicide application after inoculation)

Figure 4.5 shows that there was no significant effect of all four fungicides on downy mildew control.

4.6 Discussion

The results obtained from the field fungicide experiments showed that fungicides did not have a significant effect on controlling downy mildew except in the first disease severity rating at Canning (Table 4.4) and in the second rating at Plumdale. In the first disease severity rating at Canning, Dithane DG and Tattoo C were effective in controlling downy mildew on Calena, whereas Tattoo C showed the best downy mildew control on CN101985. Downy mildew was not controlled on CN30476 nor CN30478 by any of the

fungicides tested. In the second disease severity rating at Plumdale, Dithane DG controlled downy mildew on CN101985. Treatments were not effective on the other three camelina genotypes. Clearly, our observations were not consistent in identifying control of downy mildew.

Our field observations concur with that observed in the growth chamber experiments when they were applied 1 week after the inoculation. This may be because both Bravo 500 and Dithane DG are protectant fungicides so that they could not control the disease when downy mildew had already established itself on plants 1 week after the inoculation. It is a little surprising that both Tanos 50 DF and Tattoo C were not effective. Both of these two fungicides are systemic fungicides, which means that they are absorbed and then translocated in plants. Therefore, they should be able to inhibit a pathogens' spread or growth. On this basis we expected that both Tanos 50 DF and Tattoo C might be more effective than the other treatments.

In the growth chamber fungicide experiment, when the fungicides were sprayed before the inoculation, at the time of the first disease index rating, Bravo 500 and Tanos 50 DF effectively controlled downy mildew, whereas Dithane DG and Tattoo C did not. The second rating showed no significant differences among the treatments.

Because of the severe weed problem in both the Plumdale trial and the Brookside trial, yields were compromised. The result of these two trials is confounded by yield suppression from weeds. In Canning, which showed the least weed pressure, different genotypes had a significant effect on yield. Furthermore, resistant genotypes yielded

significantly more than susceptible genotypes. This suggests that disease might be the main contributing factor to yield loss.

In general, fungicides did not perform well. Even in some instances where significant differences attributable to the fungicides, or the interaction of genotypes and treatments, there was no fungicide consistently better than the others. Therefore, none of the fungicides tested can be recommended for downy mildew control in camelina. However, we found that genotypes differed markedly in their reaction to downy mildew. Table 4.4 shows that both CN30478 and CN30476 are much more resistant than Calena and CN101985. The DI of CN30478 and CN30476 is significantly lower than that of the other two genotypes. The high disease resistance of CN30476 found in the Truro trials is also interesting. Considering this genotype is susceptible to the Truro isolate at the cotyledon stage, there is a transition of its disease resistance from susceptibility to resistance during its development, which implies an adult plant resistance exists in this genotype. Observations from the fungicide experiment suggest that selecting resistant genotypes holds more promise as a method of downy mildew control than fungicide application.

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Chapter 5: Conclusion

Downy mildew (*Peronospora parasitica*) is an obligate parasite and therefore cannot exist without a host. In this project, *P. parasitica* was maintained on the susceptible camelina genotype cv. Calena. From preliminary experiments we found that, although *P. parasitica* prefers high relative humidity (RH) for its development, a continuously saturated environment tends to inhibit sporulation. Therefore, we alternated a high RH with a relatively low RH for disease maintenance. With this procedure, a susceptible host was inoculated and placed in an environment with approximately 100% RH overnight. The next day, the RH was reduced to approximately 70%. Thereafter, every five days after the inoculation, plants were placed into a near 100% RH environment to promote sporulation. Conidiophores bearing newly produced conidia could be found after the first sporulation promotion. These conidia were harvested and used in our project. Additional conidia were stored in a freezer at –20 ºC; viability was tested and it was determined that viability was good for at least one year.

The SEM observation revealed that *P. parasitica* germinated and finished penetration between 4 hours and 8 hours after inoculation. Each conidium produced one germ tube and one appressorium as initial infection structures on plant surface. On all our samples, no appressorium was found on the stomata which indicates a direct penetration by *P. parasitica*. Conidiophores with mature conidia attached to the tips were observed growing out through stomata 1 week after inoculation.

In order to identify camelina genotypes with superior resistance to *P. parasitica*, 11

camelina genotypes, CN30475, CN30476, CN30478, CN30479, CN101981, CN101982, CN101985, CN101988, CN101989, SRS933 and Calena were tested. Results obtained in this experiment showed that CN30478 was consistently the most resistant to all three *P. parasitica* isolates. CN30475 was the second most resistant genotype. On the other hand, Calena and CN101985 were the most susceptible genotypes. Among these 11 camelina genotypes, there were three interesting genotypes which are CN30476, CN101982 and SRS933. CN30476 was susceptible to the Truro isolate, moderately resistant to the PEI isolate and resistant to the Canning isolate. Similar performance was found with CN101981, although it showed more susceptibility to the PEI isolate than CN30476. SRS933 was susceptible to the Truro and PEI isolates, whereas it was resistant to the Canning isolate. We infer from this evidence that these isolates are different from one another. In addition, it also reveals the vertical resistance employed by CN30476, CN101982 and SRS933. The vertical resistance was also probably employed in CN30478 and CN30475 since there were necrotic spots which might be caused by a hypersensitive reaction found on infected cotyledons.

The results obtained from fungicide experiments showed that none of the four fungicides (Dithane DG, Bravo 500, Tanos 50 DF and Tattoo C) could significantly control downy mildew. Yield data from all three trials showed that fungicides had no significant impact on camelina yield. Although the yield might have been adversely affected by the severe weed problem, especially in the Plumdale and the Brookside trials, which might have masked any fungicide treatment effects, yields from the Canning trial

(had the least weed problem) also showed that fungicides did not significantly affect the yield. The other factor, genotypes, did not show a significant impact on the yield in the Plumdale and the Brookside trials. However, genotypes significantly affected the yield at Canning. CN30476 yielded the highest. CN30478 had the second highest yield. Both CN101985 and Calena yielded the lowest. This result is interesting because resistant genotypes, CN30476 and CN30478, yielded significantly more than the two most susceptible genotypes. This suggests a relationship between the disease severity and yield. A lighter disease severity contributes to a greater yield. Disease index, which was the other indicator measured in this experiment, showed that fungicides showed some impact in some experiments; i.e. the first disease severity rating of the Canning trial (Table 4.2), the second disease severity rating of the Plumdale Trial (Table 4.2) and the first disease severity rating of the growth chamber experiment when fungicides were applied before inoculation (Figure 4.2). However, there was no fungicide that showed sustained control. Furthermore, camelina genotypes (Calena, CN101985, CN30478 and CN30476) always showed significant impact on disease index in field fungicide experiment. In the field, compared with CN40378 and CN30476, both Calena and CN101985 were infected much more severely, i.e. the disease index of CN30478 and CN30476 was significantly lower than that of Calena and CN101985. Therefore, selecting resistant camelina genotypes is a preferred method for downy mildew control than chemical protection. In addition, CN30476 was very resistant to downy mildew in the Truro trials. Considering this genotype is susceptible to the Truro isolate at the cotyledon stage, it implies that there is

an adult plant resistance applied in this genotype which results in the transition of its disease reaction from susceptibility to resistance during its development.

In this project, we identified the resistant genotypes which can be used for further breeding research. In addition, at this time, selecting resistant genotypes was found to be more effective in controlling damage caused by downy mildew than the use of fungicides. However, in order to have a better understanding of the relationship between camelina genotypes and downy mildew, more experiments should be carried out. The 11 camelina genotypes evaluated in this project should also be identified for their adult plant resistance to downy mildew because a transition of disease resistance may exist. An example among the 11 genotypes is CN30476 which displayed susceptibility to the Truro isolate at the cotyledon stage, but was highly resistant at the adult stage in Truro. Therefore, the disease resistance performance of these 11 genotypes in the field is also valuable to know. Moreover, disease resistance performance in the field is more indicative than that in a growth chamber because camelina is a field-production crop. In further field experiments, however, the negative effects caused by weeds should be eliminated. Also, from a histological aspect, more SEM observations are needed to understand the timing of the differentiation of the penetration structures of *P. parasitica*. Moreover, fungal structures inside plant tissues should also be observed. The observation of intercellular hypha and haustorium will be helpful to understand the internal interaction between the host and the pathogen.

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